Submission for The degree of Philosophae Doctor (Ph.D.)


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Declaration
This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Statements
This thesis is the result of my own investigations, except where otherwise stated.

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Abstract

Here the use of the \textit{in vitro} spheroid confrontation model is carried out in such a way as to make use of confocal laser scanning microscopy on vital tissues. Its use in the observation of the invasion of stromal tissues by cancer tissues and the progression of this invasion dynamically was investigated. The employment of simple routine laboratory techniques and the adaptation of confocal microscopy for the dynamic investigation of human tissues and cancers during the invasion process was paramount. The spheroids for the confrontations were produced from re-aggregates of cells grown in monolayer culture stained with vital fluorescent dyes CMFDA and CMTMR. The confrontation method seemed appropriate for the examination of invasiveness as it enables the observation of the invasive process on a three dimensional structure which emulates the situation \textit{in vivo}. The use of the clinically significant parameter INVASLOG is described here and its limits have been elucidated. It has been demonstrated that the use of the INVASLOG enables an objective estimation of the invasive process in vital cultures. Autologous clones of mouse melanoma K-1735, of different invasive behaviour \textit{in vivo}, can be distinguished and that human melanomas A375 and A2058 can also be also be distinguished between in their behaviour. The investigation of the effect of retinoic acid has also demonstrated the usefulness of this method and the invaslog parameter in investigating invasion. Its use in the investigation of and its application to, diagnosis and prognosis of cancer should be investigated.
Introduction

The department of Medical physics and Biophysics at the Karl-Franzens University of Graz Austria is dedicated in its attempt to develop methods for the reduction of animal experiments in the medical sciences. Towards this attempt the method of investigating cancer invasiveness was looked at as it requires the use of laboratory animals which during the investigation many undergo unnecessary stress and pain.

The brief outline of this research project was to develop and modernise the method of determining and quantifying cancer invasiveness to increase its applicability and to adapt it for use with a confocal laser scanning microscope.

Here the *in vitro* spheroid confrontation model was made use of in such a way as to make use of confocal microscopy on vital tissues. This enabled the employment of simple routine laboratory techniques and the adaptation of this method for the dynamic investigation of human tissues and cancers during the invasion process.

The spheroid confrontation model has been used successfully for the investigation of various cellular processes *in vitro* and has been used by Prof. Mareel in a semi-quantitative manner up until the mid nineteen nineties\(^1\)\(^7\). In Graz, this method was developed further making invasion of the confronted spheroids quantitative through advanced image analysis in the living system and by comparison with the clinically estimated degree of invasion.
Cancer

Cancer is one of the areas of medical research where significant numbers of animals are used for the investigation of the processes involved and possible methods of treatment. Alternative methods of investigation that reduce or eliminate the need from animals is seen from many as being a desirable aim not only in medical research.

Cancer is a disease that affects most of us if not physically then as the result of relatives and/or friends being afflicted with this frequently lethal condition. Due to the nature of cancer and its manifold mechanisms it is necessary to find methods for the fast determination of therapeutic procedures suitable for each cancer on a patient to patient basis. Fast, because in many cases the speed of progression of the afflicting cancer or late diagnosis of it's presence results in a time deficit that can only be compensated for in that fast methods are used in determining the correct therapeutic form suitable for treating the patient effectively.

Origin of cancer

Cancer is the result of a heritable change in the afflicted cell's behaviour resulting in a deregulation of the control mechanisms responsible for growth, proliferation, cell contacts and motility. The change may be the result of chemical induction, transfer of energy from radiation sources or be virally induced and is a permanent change in the DNA which then results in a change in gene expression and regulation. A genotypic predisposition will substantially increase the probability of the occurrence of cancer such as can be seen in certain breast cancers. The result is growth that no longer responds to the usual cellular and intercellular control mechanisms. The cells resulting from the
division of the originating cell containing the heritable oncogenic change are therefore all potentially cancer forming. As the type of heritable change that occurs cannot at the moment be predicted or determined reliably, a method that can determine the desired therapeutic technique that will result in an inhibition in the development or in the cure of the cancer without the necessity to know the genetic cause and without involving painful or uncomfortable or even perhaps dangerous tests for the patient are desirable. This would also enable testing of a range of chemo-therapeutic or radio-therapeutic methods not possible morally or ethically on the patient enabling a patient specific treatment schedule tuned to that necessary for the particular cancer he/she has.

Cancer development

The resulting development of a tumour from potentially cancerous cells, is dependent on many factors, not least being the degree of contact and control the surrounding cells have upon them 47. These surrounding stromal cells may influence the development, growth and differentiation of the cancerous cells resulting in suppression or stimulation of the cancerous phenotype 48-50. The cells containing the heritable oncogenic change may also be recognised as being potentially dangerous by the immune system and result in their subsequent removal, or initiation of internal control mechanisms that result in apoptosis will result in the removal of such cells.

Once the barriers to tumour development have been overcome by the cancerous cells the next defence system that can be employed is the encapsulation of the cancerous cells inside a basal membrane 51,52. This would result from signalling with the surrounding tissue resulting in the development of the basal membrane 53-57. This does not occur in all cases. When the tumour
has reached a critical size less than a few cubic millimetres in diameter additional signals result in the development of a blood vessel system, (neo-angiogenesis) supplying the tumour with the nutrients necessary for growth.

Benign or malignant

Benign tumours are not much of a problem as their surgical removal or other less radical treatment usually results in a satisfactory cure of the problem, unless they are surgically in-operable and their growth presents a medical problem i.e. through induced pressure on neighbouring tissues resulting in mechanical disruption.

Malignant tumours are much more of a problem since their aggressive behaviour leads to the destruction of tissues invasion and metastasis which when essential leads rapidly to a pathological condition that in many cases may be terminal 58. If these cancers do not metastasise they can usually be effectively treated using a combination of surgery and chemo or radio-therapy. If they metastasise which is characterised by cell proliferation, by invasion into the surrounding tissue and by invrasion then treatment becomes rapidly more difficult and requires systemic treatment using very aggressive chemotherapy and/or whole body radiotherapy that is not only in both cases uncomfortable for the patient but also carries significant health risks with it e.g. the risk of cancer induction by radiation, the use of retinoids has a significant health risk as they tend to be teratogenic 61 or in the case of doxorubicin 62 lead to heart failure63-65.
**Therapy**

One of the most important rules in the treatment of cancer is *early diagnosis* in order to be able to start treatment before the metastatic process has begun. Secondly the determination of the degree of metastasis, in that the lymph nodes in the immediate area are investigated for signs of cells originating from the primary tumour. This is done as 60% of metastasising cancers infiltrate the body using the rapid transport provided by the circulatory system and can be subsequently found at very large distances from the original primary tumour. The lymph nodes are good indicators for the dissemination of such cells as they tend to act as collection points where the cells initially accumulate.\(^ {66-79}\)

**Cancer types**

The treatment is dependent to a large degree on the primary tumour's origin as the behaviour of the ensuing cancer can be deduced to a large extent from the tissue from which it originates as this has an effect on the degree to which the cell from which the cancer develops is capable of regressing to an embryonic state i.e. its state of differentiation.

Cancers are grouped by their tissue of primary origin and there are well established grading systems of cancer progression\(^ {80}\). The cell types or tissues with the highest regenerative potential are those that have experienced a minimum of differentiation and are the source of new tissues and also have the highest potential for generating cancerous cells.

The classification of the ensuing cancer into the TNM (Tumour lymph Node and Metastasis) grouping system enables a unified treatment of cancer by physicians worldwide.
Here is a typical TNM classification scheme for breast cancer taken from the internet link shown below:-


**TNM Classification 1/3**

The most widely used classification for breast carcinomas is the TNM classification(2). T,N and M categories are assessed by the combination of physical examination and imaging such as mammography.

**T Categories**

Primary Tumour (T) Definitions for classifying the primary tumour (T) are the same for clinical and for pathologic classification. The telescoping method of classification can be applied. If the measurement is made by physical examination, the examiner will use the major headings (T1, T2 or T3). If other measurements, such as mammographic or pathologic, are used, the examiner can use the telescoped subsets of T1.

TX – Primary tumour cannot be assessed

T0 – No evidence of primary tumour

Tis – Carcinoma in situ: intraductal carcinoma or lobular carcinoma in situ, or Paget's disease of the nipple with no tumour

T1 – Tumour ≤2 cm in greatest dimension

T1a ≤0.5 cm in greatest dimension

T1b >0.5 cm to 1 cm in greatest dimension

T1c >1 cm to 2 cm in greatest dimension

T2 – Tumour >2 cm and ≤5 cm in greatest dimension

T3 – Tumour >5 cm in greatest dimension

T4 – Tumour of any size with direct extension to chest wall or skin

T4a Extension to chest wall

T4b Oedema (including peau d'orange), or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast

T4c Both T4a and T4b above

T4d Inflammatory carcinoma

1 Paget’s disease associated with a tumour is classified according to the size of the tumour

**TNM Classification 2/3**

**N Categories**

NX – Regional lymph nodes cannot be assessed

N0 – No regional lymph nodes metastasis

N1 – Metastasis to movable ipsilateral axillary lymph node(s)

N2 – Metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures

N3 – Metastasis to ipsilateral internal mammary lymph node(s)
Pathologic Classification (pN)2
pNX – Regional lymph nodes cannot be assessed (e.g. previously removed, or not removed for pathologic study)
pNO – No regional lymph node metastasis
pN1 – Metastasis to moveable ipsilateral axillary lymph node(s)
pN2 – Metastasis to ipsilateral axillary lymph nodes that are fixed to one another or to other structures
pN3 – Metastasis to ipsilateral internal mammary lymph node(s)

M Categories
MX – Presence of distant metastasis cannot be assessed
M0 – No distant metastasis
M1 (scf) – Metastasis only to ipsilateral supraclavicular lymph nodes
M1 – Distant metastasis

TNM Classification 3/3
UICC(2) Stage Grouping

Classification
Stage T N M
Stage 0 Tis N0 M0
Stage 1 T1 N0 M0
Stage IIA T0 N1 M0
T1 N1 M0
T2 N0 M0
Stage IIB T2 N1 M0
T3 N0 M0
Stage II A T0 N2 M0
T1 N2 M0
T2 N2 M0
T3 N1 M0
T3 N2 M0
Stage IIIB T4 Any N M0
Any T N3 M0
Stage IV Any T Any N M1

Comments
- Pathological staging is preferable, as it:
  - is more accurate for tumour size,
  - is especially relevant for N stage, and
  - relates better to treatment strategies
- Avoid a mixture of clinical and pathologic staging.
TNM classification
Is as above grouped into three main categories,

1. the tumour primary origin and size,

2. if it has reached the lymph nodes and

3. if it has become metastatic.

With each step exact descriptions of the staging has been evaluated for many primary tumours and their ensuing staging schemes each dependent on the physical and histological evidence that a tumour presents. These schemes have been developed clinically to aid the treatment of cancer in a standardised manner hopefully maximising the chances of success.

Treatment

The degree and type of therapy is dependent on the above TNM classification. This method has been used over many years for determining the aggressiveness and the possible receptiveness of particular cancers to treatment. Although it does not produce accurate results due to the inherent inaccuracies in the methods used, the diagnostic data are presented and a treatment technique is chosen depending on the most successful technique for treating these results and not for the cancer that the patient has, as this has not been determined and cannot be reliably determined, using the TNM technique. The cancer type that a patient has is dependent on his own genotype and thus is different for each of us. The typing of the cancer can then only be a near assumption based on the statistical data produced for a particular TNM category.
Radio-therapy

This is the attempt to kill the tumour cells selectively either:-

- through their physical position in the patients body and selectively administering a lethal dose of radiation with its associated side effects to the afflicted areas whereby the geometry of the afflicted area must be precisely defined so that as little of the surrounding healthy tissue as possible is damaged but making sure that all of the tumour has been successfully treated.\textsuperscript{81-86}

- Or whole body treatment where the tumour has been previously treated to be more sensitive to radiation than the surrounding tissues or as in the case of bone marrow transplants it is desired that the treated tissue is completely destroyed and that it is then replaced with that from a donor which is particularly effective for cancers that affect the patient systemically such as some forms of leukaemia.\textsuperscript{87}

- Or in the form of radio-immuno-therapy where, radioactive immuno-globulins, or drugs designed to attach to specific targets, are administered where then they reach lethal concentrations of radioactivity locally situated at the target tumour as well as other sites associated with the target antigen.\textsuperscript{85,88-92}

- Or local injection of radioactive sources to administer lethal doses locally.\textsuperscript{93-95}
Chemo-therapy

Cancers that cannot be localised or treated with radiation therapy reliably are treated using chemotherapeutic agents, very often cytostatic agents that rely on the speed of growth of the tumour for it to be more susceptible to the treatment agents than the surrounding tissues. This strategy does not help in the treatment of slow growing cancers or quiescent cells with metastatic potential. The side effects for the patient are well known, hair loss, a constant feeling of wanting to be sick, pain in addition to that caused by the tumour. In addition it is well known that organ failure often pursues chemotherapy.

Very often a mixture of radio and chemo therapy is used to make sure the cancer is cured but this leads to, in very many cases, detrimental sides effects or even death of the patient in therapy.
**Metastasis**

All of the above treatment methods have very depressing success rates in the treatment of metastatic tumours despite the fact that the chemotherapy form of treatment would be ideal for systemically treating patients for potentially metastatic cells. 106-116

Research into cancer also concentrates on the aspect of the metastatic processes involved, methods of inhibiting it and inhibition of the establishment of further metastases. In order to achieve these aims a complete understanding of the metastatic processes is necessary.

The stage or degree of metastasis is one of the criteria by which treatment and therapeutic strategies are assessed and pursued. This is necessary as the size of the primary tumour when it is initially detected is usually such that metastasis has already taken place (if it is a metastatic form of cancer). A treatment can only be effective in such cases if the metastatic process can be stopped preventing further metastatic naevae from developing and the already present metastatic centres can then be eliminated or suppressed from growth and proliferation.

**Metastatic stages**

Metastasis can be divided into ten processes.

**Detachment** from the primary tumour. Here it is not clear what exactly induces the separation of cells from the primary tumour but it can be an active or passive process i.e.

active migration away from the tumour through the induction or activation of the processes necessary for motility where the cell detaches from the ECM (extra
cellular matrix) involving disruption of the ECM connections and connections to the surrounding tissue followed by destruction of the surrounding ECM so that the cell may leave the position in which it was fixed, or passive movement in that the cell involved does not actively detach but due to physical processes around it e.g. the rupture of a blood vessel due to the growth of the tumour or a haematoma in the area which will lead to the washing away of cells to other sites, or the use of surgical or medical intervention where the tumour is investigated and the instruments used free potentially metastatic cells from the tumour (biopsy), or through other incidental mechanical activities such as injuries, or simply pressure in the area. This will of course have additionally biochemical as well as physical consequences for the tumour and the cells of which it is composed.

Infiltration into the surrounding tissue. Once the cells have been freed from the surrounding cells and the associated ECM the next consequence is the infiltration of the now surrounding tissue. This is a requirement for reaching vessels or parts of the body suitable for the development of metastatic naevae. This may be unnecessary in some cases where the development of blood vessels (neo-angiogenesis) in and through the primary tumour aid the transport and facilitate extra-vasion through the stimulated reorganisation of the tissue with the associated hormonal etc. signalling, without the necessity for infiltration of the surrounding tissues. The process of angiogenesis also supplies the tumour with the required nutrients and oxygen required for its, in comparison to the surrounding tissue, accelerated growth.

Intra-vasion into vessel (either blood or lymph). This may or may not be a necessary step or even sought by the metastatic cells. It involves among other
events the break down of the ECM and basal membrane protecting the vessel walls and passage through the vessel wall into the circulatory system. This may be due to chemotaxis or haptotaxis or due the continuing effect of the factors that lead to detachment of the cell from the tissue earlier. This is a consequent part of the “seed and soil theory” where the metastatic activity of the cells is directed so that a particular region must be reached where then suitable conditions for growth can be found and the development of the metastatic naevae can continue uninterrupted. This assumes an active and organised metastasis. In the case of passive transport it may well lead to the distribution of the metastatic cells to areas where their growth may be hindered. This will of course lead to a reduction in their speed of growth and also their response to chemotherapeutic agents that make use of the speed of proliferation for their effectiveness. Here a reduction in growth rate means an increase in survival for the metastatic cells.

Transport. The entry into the blood or lymphatic systems enables the rapid dispersion of cells and is energetically advantageous, to other parts of the body. Along these systems although it also exposes these cells to direct contact and rapid response of the immune system it is only a matter of time when the dispersion of the cells will be successful. This passive transport is very effective at reaching all parts of the body within a few minutes.

Attachment to the vessel wall. This is considered by many to be significant when considering the metastatic process and several theories have been proposed to explain some of the observed phenomena.

Selective attachment to the vessel wall and then extra-vasation controlled by receptors on the cell surface may lead to an increased probability of cancer
metastasis in particular organs or tissues. This does not detract from the fact that many metastases are found where the physical size of the vessel does not allow the transported cell to pass through it thus blocking the vessel concerned and now physically inducing the cell that now blocks the vessel to leave it.

Extra-vasion out of the vessel (either blood or lymph). Either due to physical constraint or chemotaxis induction through proximity to the source of a chemo-attractant. The cell attaches to the vessel wall and leaves as it entered infiltrating the neighbouring tissues. The so-called stationary motility of cancer cells is most likely the key factor for finding a gap in the endothelium for extravasation.

Infiltration into the surrounding tissue. Movement through the tissue to a site where further movement is unnecessary or not possible. These may be found in the form of reaching the source of the chemo-attractant, remaining next to the extra-vased vessel in order to remain near a nutrient source which might infer the absence of a chemo-attractant or the presence of an impenetrable barrier for the cell concerned.

Quiescence. When the site for development of the metastasis has been reached the cell will switch from motility to growth (by unknown factors) might quiesce immediately or after a few divisions and act “normally” for many years. This hinders treatment drastically.

Growth and establishment of the metastatic tumour. Either with or without a period of sesation the metastatic cell now at its site for development divides and proliferates producing a secondary tumour with the same potential as the primary tumour. The secondary tumours are often more dangerous than the
primary for the patient due to the selective effect for success means that successful metastases are more unlikely to be recognised by the patients immune system and the successful treatment of the primary tumour leaving the metastasis behind may have also lead to the selection of resistant metastatic clones as secondary tumours.

**Neo-angiogenesis.** After reaching the critical size of about 100 to 150μm neo-angiogenesis is stimulated and the surrounding host tissues co-operate in the organisation and construction of the necessary vessels. This form of interaction with the surrounding tissues optimizes the conditions for the secondary tumour making it potentially lethal for the patient.

The order of events as described above may not be followed exactly and not all stages may be executed but in general the establishment of metastases follow this scheme 117-125
Figure 1 Role of proteinases in the metastatic process. Reprinted from 126

The traditional view of the functions of proteinases involves proteinases in matrix degradation occurring during local invasion, intravasation and extravasation. Additional steps of the metastatic process apparently regulated by proteinases.
**Host tissue cancer cell interaction.**

Although our knowledge of the metastatic process in general seems to be complete the actual signalling between the cells and the processes involved at the biochemical level both extra-, inter- and intra-cellularly are unclear but advances are being rapidly made in the area of ECM (extra cellular matrix) break down and reconstruction as well as the role of the stromal cells and their interaction with the cancer cells in these processes (Figure 2). It has been demonstrated that the stromal cells have a significant role in the development of the metastases and some strategies exist that attempt to make use of this potential in order to inhibit the further growth and development of the metastatic centre.\textsuperscript{127-129}

*Figure 2 Host cell cancer interaction printed from \textsuperscript{130}.*
Proteases

The use of specific proteases by the cells demonstrates the very controlled way in which the ECM can be removed and replaced in synchronised action so that the desired direction of movement can be attained. Here the role of metallo-proteases is, in their controllability and specificity, essential to the invasive process as the use of metallo-protease inhibitors has demonstrated.  

Cellular connections

Connections between cells can have a direct effect upon the behaviour of both the cancer cells as well as the stromal cells surrounding them and can be used as a possible chemotherapeutic method for stimulating the differentiation of cancer cells. The possible role of gap junctions is not well understood.

Motility

The movement of cells is a developmental necessity in higher organisms. This leads in the form of contractile movements to when collectively organised by many cells, the movement of the whole organism. Also individual cells are able to change their relative position in the matrix of surrounding cells enabling the reorganisation of structures. This then can lead to organisational changes in tissues so fundamental that the original tissue ceases to exist. A dramatic form of this would be the metamorphosis found in many insects and metamorphic changes found in many other species and genera.

In *Homo sapiens*, the motility of cells either active or passive, is essential to the survival of the organism as a whole, either in the role of blood or lymph in supplying gases, nutrients and other essential functions to the tissues through passive movement enforced by the pumping actions of heart and other muscle...
tissues or in the role of cells not confined to the blood system as part of the
defence and maintenance system as well as many other cell types with quite
distinct functions which are actively transported by their own actions.

As seen above, the motility of cancer cells is an important factor in the
development of metastasis and it has been shown in our and other laboratories
previously that a complete inhibition of the motile phenotype through disruption
of the cytoskeleton leads to an inability to be invasive and therefore inhibits the
formation of metastases. \(^{7,148-165}\)

In order to study motility, many methods are available, two dimensional studies
of cell movement and spheroid outgrowth have previously been made for the
study of the effects of many environmental and chemical constraints on the
directional motility of cells \textit{in vitro}. \(^{166-172}\) Single cell motility has also been used
in the determination of the effects of ECM components and fragments on the
motility of individual cells. \(^{173-175}\)
Models of invasion

A suitable model for the simulation of the microenvironment that leads to an in vitro system that emulates the system in vivo is not simple. Attempts at investigating invasion at the two dimensional level have shown some success but have also been shown to have significant pitfalls when the processes to be studied require an environment where the third dimension is required. The third dimension is a prerequisite for any invasion study with cancer cells, because in vivo it is a three dimensional process.

It is a well known fact that cells behave differently when surrounded by other cells of either the same type where contact signalling processes are intact or where the contact to the surrounding cells is interrupted or the surrounding cells are of another type or even from another organism.\textsuperscript{176,177}

Tissue culture

The in vitro culture of tissues is necessary for the observation of cells and tissues at the macro and microscopic level. The development of tissue culture has made it an essential tool in the observation of cellular behaviour in vitro. This gives us an insight into the complex events and processes that occur in vivo without the necessity of subjecting the donor of the tissues to unnecessary pain or discomfort. Tissue culture has enabled the reduction of animal experiments (Bjerkvig 1992 6784 /id) and assisted not only in the development of drugs for specific targets, treatments and therapies, but also enabled the development of techniques such as in vitro fertilisation and cloning, therefore the development of this powerful tool for the observation of biological processes has also enabled significant steps to be made in medicine, not least the
development of techniques for the investigation of cancer and its treatment and therapy.

Animal experiments
The use of tissue culture has made it possible to reduce or even replace animal experiments (such as that in Figure 3) in some areas of research so that the number of animals used for experiments in these areas have been reduced but new areas of research have made it necessary to use even more animals in total per year. Routinely many animals are used in the study of invasion in which the cell or spheroid suspensions are injected into animals and their following distribution in the animal after it has been culled is then investigated. This leads not only to the death of large numbers of animals but also to their potential suffering during the metastatic process and the painful development of the metastatic tumours. The in vivo situation is so complex that it cannot be simulated totally in vitro at the moment making such experiments necessary in order to exclude the unforeseeable events that take place before administering prospective drugs, treatments or therapies to human test subjects. But the in vivo tests have also the disadvantage that all the individual steps of the metastatic cascade cannot be observed or measured, only the final outcome (number of metastases) can be evaluated.

Intravital microscopy
This involves the use of animals for the investigation of the processes involved in invasion for a review see Nature Reviews: Cancer Dec 2003 178 and Figure 3
Figure 3 In vivo video-microscopy method.

The animal is placed on an inverted microscope platform, and the exposed intact organ is visualized through a cover-slip using x 10 to x 100 objectives. Oblique trans-illumination using a fiber-optic light guide and/or episcopic fluorescence illumination with appropriate filter blocks is employed for observing fluorescently labelled cells. The images are viewed with a video-camera on a monitor and recorded on SVHS tapes and/or sent to a computer image capture system for further analysis. The technique is equally applicable for observing chick CAM, mouse liver, or other organs. Reprinted from
The Boyden chamber

For the investigation of invasion in vitro, a simple method is widely used i.e. the Boyden chamber\textsuperscript{134,180-184}. It was developed in 1962 and it's variations including the use of, multiple chambers, placental membranes, as well as reconstituted basal membranes (Matrigel) for their studies\textsuperscript{185} have taken a hold in invasion investigations and become a standard method for testing the invasive properties of cell lines.

There are several problems in the use of this system when comparing it to the in vitro situation. The method usually requires the use of a chemo-attractant in order to induce the cells to invade, this leads to artificially high and constant concentration gradients of chemo-attractant over very long times and distances\textsuperscript{126} and may lead to results that are not representative of the in vivo situation i.e. the response signalling is not present making the interpretation of the cause of the cells movements a question. This means that a fast sequence of interactive signals with the target tissue, \textsuperscript{186-188} required for progression or inhibition of the cancer is not possible and may due to the high concentrations required and due to the long distances involved induce otherwise quiescent cells to become invasive and as it has been shown that the host tissue has a very definite role to play in the invasive process (see Figure 2) the results are questionable.

3D-cultures

The development of methods to examine cultures of three dimensional structures such as tissue fragments or whole organs was the beginning of tissue culture \textsuperscript{189} and is increasingly significant in relationship to the interaction of tissues and cells with each other. It enables observation under controlled
conditions and by making use of molecular biological techniques controlled interaction such as signalling or electrophysiological effects etc., can be studied at the microscopic and macroscopic levels. This has led to the observation of behavioural differences of cells and tissues when in three dimensional culture as compared to mono-layer culture techniques.

Spheroids
The culture of tissue fragments in stirrer culture as spheroids or the re-aggregation of mono-layer cultures to spheroids for further examination has proved invaluable in our understanding of many of the processes involved in invasion of cancer cells into host tissues and the behaviour of isolated tissues in culture. This can be seen in the study of cells in spheroid culture as compared to the more usual mono-layer culture. The spheroids produced from re-aggregates have been shown to exhibit structures similar to those found in vivo and in the case of mouse breast cells the development extends to the point where they develop lumen and excrete milk. The use of equipment that maintains the re-aggregates in suspension culture has been developed and this has even been extended to studies in micro-gravity on the space shuttle and is projected for future research on the ISS (international space station), where the medical problems associated with micro-gravity are pronounced during long term exposure to environments with reduced gravity. Many questions arise out of the specific problem of motion in micro-gravity.

Confrontation cultures
In order to study changes at the microscopic scale the confrontation of small tissue fragments grown in culture with subsequent histological examination has given insight into the interaction of tissues with each other. In this particular
study we are interested in the information related to the interaction of cells that are cancerous and metastatic with stromal cells. The confrontation method seemed appropriate for the examination of invasiveness as it enables the observation of the invasive process on a three dimensional structure which emulates the situation *in vivo*, initial studies were done in 1977 by Mareel\textsuperscript{193} which showed that some cells are capable of invading the confronting spheroid. Although some factors are missing from the *in vivo* situation (e.g. the immune system) *in vitro* it has been shown that confrontation studies emulate the *in vivo* situation very well to the point where organ culture can be pursued.
Microscopy

In order to examine spheroids and confrontation cultures, histological sections of confrontation cultures have initially been used. Although this has resulted in several advances in our understanding of the invasive process, dynamic observation of the processes involved would drastically improve the speed and efficacy with which the results can be obtained. A method for obtaining optical sections from vital tissues minimising the disturbing influences on the tissues to be examined was searched for and laser scanning confocal microscopy offers the best possibilities for optical sectioning.

Confocal laser scanning microscopy

The use of a confocal laser scanning microscope enables dynamic observation in optical sections of the confrontation cultures without the necessity for fixation and physical sectioning with a microtome. Fixation can lead to distortion of the results if not carried out correctly and interpretation of the results can be difficult and may lead to interpretations that when studied in this dynamic system may lead to completely misleading results.

The confocal microscope has not only excellent resolution in the xy plane but by the use of new advances in light microscopy has a high resolution in the z plane. The principle of confocal microscopy has been known and sporadically used for over 40 years. It has recently become more interesting to the scientific community as it enables the use of relatively simple techniques for optically sectioning tissues with high resolution. The use of normal light microscopy would not enable optical sectioning with adequate resolution in the z plane at the required magnification and object depth. For a complete study of the
physics and problems with confocal microscopes see “Handbook of Biological
Confocal Microscopy” \(^{194}\)

Figure 4 Depth discrimination in laser scanning confocal microscopy
1 collimating lens 2 objective 3 specimen out of focal plane 4 specimen in focal plane 5 one sided mirror 6 detector 7 filter, the black rectangle is the pinhole. reprinted from\(^{194}\).

Laser scanning

The use of laser scanning in combination with confocal microscopy has increased the resolution of light microscopy through the reduction of chromatic aberrations in using monochromatic coherent light for the excitation of fluorescent markers and chromatically corrected optics for the collection of the emitted signals and the use of pinholes.
Time lapse microscopy

Making use of fluorescent markers, it is possible to analyse to a very great extent the digitally stored images. Three dimensional reconstructions and quantitative analysis of the resulting data allows the investigation of processes spatially, with the addition of time-lapse imaging it is also possible to follow these processes dynamically. The latter being a significant advance over the original methods of taking histological sections that require the fixation of the cells, meaning that only a single point in time could be observed for each specimen.

A suitable incubation technique had to be found where the cells would not be disturbed during the periods of observation where the induction of aberrant effects may ensue. This involves continuous incubation during the period of observation. This would require incubation on the microscope stage. A micro-incubator was developed in the laboratory to enable temperature and gas control to an accuracy of 0.1°C for periods of up to eight days or longer where the medium is changed.

Fluorescent markers are used to follow the cells during the experiment and are chosen here so that they did not influence the cells by their presence through photo-toxic or cyto-toxic effects and such that they did not require complex or long loading techniques, and where they were unspecific for the cell type to be stained. Once in the cell they were retained by the cell in question, were long lived and resistant to photo-bleaching effects. Here the cell tracker dyes from Molecular probes were used CMFDA (5-chloromethylfluoresciendiacetate) and CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl)amino)tetramethylrhodamine)
fluroscein and rhodamine derivates that make use of the available FITC and TRITC filter sets from the microscope.
Quantification

In order to attain reliable quantitative results, a criterion for the assessment of invasion had to be found that is quantifiable and is representative for the degree of invasiveness in vivo and has clinical significance.

Several methods are in use where most of them rely upon area measurements made from histological sections or subjective assessments of the degree of invasion without an objective parameter for the degree of invasion$^{195,196}$. The parameters that have thus been used for measuring the invasiveness of cancers in the confrontation system were insufficient as they did not reflect the clinically significant parameters for assessing invasion in vivo. A study was conducted to elucidate the factors involved in assessing invasion from histological sections, from all the possible parameters that can be collected quantitatively making use of digital images of the histological sections of confrontation cultures$^{197}$. In parallel these sections were assessed for the invasion stage on a scale of 1 to 10 by 5 experienced clinical pathologists skilled in estimating the degree of cancer invasion from patient biopsies. The sections were also analysed digitally and all the collected parameters which included changes in area, perimeter, intensity, size, etc. were correlated with the pathologists estimation of invasion and a significant correlation was found between the stromal area and perimeter in comparison to the estimation of invasion. The parameter derived has been called INVASLOG and is described in detail below. The INVASLOG parameter is mathematically dimensionless. Although this is mathematically the case, there are certain physical restrictions to the use of the formula and they will be elucidated in the section on image analysis.
Figure 5 *Melanoma cell invasion in vitro correlation with STRCSTR.*

Correlation of the measuring parameter STRCSTR (stromal contour/stromal area). The x-axis shows the ranking of invasion by five independent observers, the y-axis the ranking provided by the parameter STRCSTR. Spearman's rank correlation test: $r = 1$, $t > 18.000$, $p < 0.0001$. reprinted from[196]
Figure 6 Melanoma cell invasion in vitro Correlation with STRCONT.

Correlation of the measuring parameter STRCONT (Stroma contour). The x-axis shows the ranking obtained by five independent observers, the y-axis the ranking provided by the parameter STRCONT. Spearman's rank correlation rest: $r = 0.903$, $t = 5.945$. $p < 0.0005$. reprinted from 195
Figure 7 Melanorna cell invasion in vitro correlation with GVSDREL.

Correlation of the measuring parameter GVSDREL (relative grey value). The x-axis shows the ranking obtained by five independent observers, the y-axis the ranking provided by the parameter GVSDREL Spearman's rank correlation test: $r = -0.903$, $t = 5.945$. $p < 0.005$.

reprinted from $^{195}$
Aims

The main aims of this study can be seen below as being

- The transfer of the histochemical technique of estimating the Invaslog to a portable method for the investigation of the Invaslog with optical sectioning making use of the confocal laser scanning technology.

- The limits of the invaslog estimation should be demonstrated and the difficulties involved with estimating the invaslog with digitised images described and if possible numerically defined.

- The long term observation of the invasive process should be investigated enabling an insight into the events connected with it.

- To refine the technique to make it possible to replace some animal experiments for the investigation of the invasive behaviour of cancer cells.

- The screening of available cell lines for their invasive activity.

- The effect of chemical treatment on invasiveness.

- The effect of radioactivity on invasiveness.
Methods and Results

Cell lines in summary

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<th>Symbol</th>
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<td>(M2)</td>
<td>Mouse cutaneous melanoma</td>
</tr>
<tr>
<td>K1735-cl16</td>
<td>(cl16)</td>
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<td>K1735-cl23</td>
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<tr>
<td>A2058</td>
<td>(A2058)</td>
<td>Human cutaneous melanoma</td>
</tr>
</tbody>
</table>

The above cell lines were provided courtesy of Dr. I.J. Fidler Anderson Medical Center Houston Texas

A375           | (A375-SM)| Human cutaneous melanoma |

From the ECACC (European Cell culture collection)

HF her         | (HF-her) | Human fibroblasts 5 year old male |

Donated by Dr. Pfragner Karl-Franzens University Graz

Embryonic chick heart cells (ECH) from 10 day old fertilised eggs
**Description of the cell lines**

**A-375**

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<td>Cell Line Description</td>
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<td>Morphology</td>
<td>Epithelial</td>
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<td>Passage Number</td>
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<td>Sub Culture Routine</td>
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<td>Country</td>
<td>USA</td>
</tr>
<tr>
<td>References</td>
<td>J Nat Cancer Inst 1973;51:1417</td>
</tr>
</tbody>
</table>

**Additional Literature Report**

Cell characteristics:
1) expresses ganglioside GM2 (2), high levels of TGF-beta 3 mRNA (8), heparanase antigens (15).
2) expresses low levels of alpha-6-integrin, in spite of a high level of mRNA (10).
3) produces Manganese superoxide dismutase (MnSOD), a mitochondrial protein enzyme, in response to interferon-gamma and TNF (11).
4) produces subunits of both placental and pituitary gonadotropins (51).
5) is capable of preventing the cytotoxic effects of chloro-ethylyng agents (37).

Variants:
1) A375-C6, an IL-1 sensitive cell line (7, 13, 18).
2) A375-C5, an IL-1 resistant cell line (18).
3) A375-B, a cell line resistant to lysis mediated by TNF and IL-1 (27).
4) an highly sensitive, specific, IL-1 sensitive cell line. This subclone does not respond to other cytokines (25).
5) a variant with high heparanase activities (38).

Applications:
1) Study of gene expression induced by IL-1, regulation and mechanism of action (1, 7, 13).
2) Study of antiproliferative action of IL-1 (14, 18, 23, 29,32, 41), interferon-alpha (9, 12, 43, 44), TNF (18).
3) Study of oncostatin M: isolation and characterization (33), cell binding (5), anti-tumour activity (16).
4) Study on the mechanism involved in cell tumour cytotoxicity: regulation and mechanism of action (10, 12, 21, 26, 27, 35,36, 39-44, 48).
5) Establishment of IL-1 assays (20, 25), cytotoxicity tests (22).
6) Identification, isolation and characterization of proteins (11, 15, 17, 24, 50); antibody production (24, 31); identification of tumour derived factors (49).
7) Screening of antineoplastic agents (2, 19, 26).

Bibliography:
Table 1 ECACC description of cell line A-375
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<th>ATCC Number:</th>
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<td>Medium &amp; Serum:</td>
<td>See Propagation</td>
<td>Growth Properties: adherent</td>
<td></td>
<td></td>
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<tr>
<td>Organism:</td>
<td>Homo sapiens (human)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue:</td>
<td>skin; malignant melanoma</td>
<td></td>
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<tr>
<td>Permits/Forms:</td>
<td>In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please <a href="#">click here</a> for information regarding the specific requirements for shipment to your location.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tumorigenic:** yes, in immunosuppressed mice

**DNA Profile (STR):**
- Amelogenin: X
- CSF1PO: 11,12
- D13S317: 11,114
- D16S539: 9
- D5S818: 12
- D7S820: 9
- TH01: 8
- TP0X: 8,10
- vWA: 16,17

**Age:** 54 years

**Gender:** from female organism(s)

**Propagation:** ATCC medium: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%; fetal bovine serum, 10%

**Temperature:** 37.0 C

**Subculturing:** Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks.

**Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended

**Medium Renewal:** Every 2 to 3 days

**Freeze Medium:** culture medium 95%; DMSO, 5%

**Related Products:**
- Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC No: 30-2002
- Recommended serum - ATCC No: 30-2020
- Parental cell line - ATCC No: CRL-1872

**References:**
Table 2

American Type Culture Collection (ATCC) Description of the cell line A-375

A-2058

A2058 (human, skin, melanoma, metastatic)

ECACC 91100402
Morphology: Epithelial
Human caucasian metastatic melanoma
Depositor: Dr M Simpkins, Sir William Dunn School of Pathology, Oxford, UK
No restrictions. Patent: None Specified By Depositor
Properties: Receptors: Nerve growth factor (NGF), Laminin

Available in the following LABORATORY:

- CAMR Centre for Applied Microbiology & Research (ECACC, Salisbury, Wiltshire)
  EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 10% Fetal Bovine Serum (FBS). Split confluent cultures 1:3 to 1:6 i.e. seeding at 2-4x10,000 cells/cm² using 0.25% trypsin or trypsin/EDTA; 5% CO2, 37°C.
  Hazard: CX
  This cell line has been isolated from a lymph node metastasis from a 43 year old male patient with malignant melanoma. Due to their highly invasive properties, the cells can be used as a source of cellular invasion associated proteins e.g. collagen type IV collagenase, tissue inhibitor of metalloproteinase-2 and autocrine motility factor.

- Further information
  Research council deposit: Yes
  Biomed_1 Cell characteristics:

  1) expresses IGF I receptor (12), a 68 kDa type IV collagenase/gelatinase (9).
  2) secretes autocrine motility factor, AMF (6, 12).

Variants:

  1) HT168 with low liver metastatic capacity (4).
  2) HT168-M1 with high liver metastatic capacity (4).

Applications:

  1) Study of mechanism of melanoma cell adhesion (2), cell transformation (5).
  2) Study on cell movement and metastatic activity: role of AMF (6), type IV collagenase (7, 10, 11), insulin (6, 15), IGF-I and IGF-II (6, 15), thrombospondin (16).
  3) Purification and characterisation of proteins (13, 17, 18). 4) Studies of protein degradation by melanoma cells: regulation of enzymatic activity (1, 8), ELISA (3).

Bibliographic references:


**Table 3 ECACC description of the cell line A-2058**

<table>
<thead>
<tr>
<th>Cell Lines</th>
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<tr>
<td><strong>Tissue:</strong></td>
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<tr>
<td><strong>Permits/Forms:</strong></td>
</tr>
</tbody>
</table>

This material is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims.

**Related Cell Culture Products**

| Comments: | This cell line is highly invasive and provides a source of cellular invasion associated proteins (such as the 72000 dalton type IV collagenase. Tissue inhibitor of metalloproteinase-2 [TIMP-2], autocrine motility factor and the 67000 dalton laminin receptor. |
| Receptors: | nerve growth factor (NGF) |
| Tumorigenic: | Yes, tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10(7) cells. |
| DNA Profile (STR): | Amelogenin: X,Y  
CSF1PO: 10,11  
D13S317: 13,14  
D16S539: 9,13  
D5S818: 9,12  
D7S820: 11  
THO1: 7,9  
TPOX: 8  
vWA: 14,18 |
| Age: | 43 years |
| Gender: | from male organism(s) |
| Ethnicity: | Caucasian |
| Propagation: | ATCC medium: Dulbecco’s modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%; fetal bovine serum, 10%  
Temperature: 37.0 C |
| Subculturing: | 1. Remove and discard culture medium.  
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contain trypsin inhibitor.  
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).  
Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.  
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.  
5. Add appropriate aliquots of the cell suspension to new culture vessels.  
6. Incubate cultures at 37°C. |
| Subcultivation Ratio: | A subcultivation ratio of 1:6 to 1:12 is recommended |
| Medium Renewal: | Every 2 to 3 days |
| Freeze Medium: | culture medium 95%; DMSO, 5% |
| Related Products: | Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC No: 10-2002  
recommended serum - ATCC No: 10-2020 |

Table 4 ATCC description of the cell line A-2058
K-1735 and its clones

K-1735 M2

Mouse amelanocytic melanoma

Highly metastatic derived from K-1735 a UV induced skin melanoma on a mouse. K1735, an ultraviolet-light-induced murine melanoma arising in a mouse strain (C3H).

K-1735 cl23 and K1735 cl16

Are both derived from K-1735 and are less invasive in animal experiments. The cl23 cell line is moderately less invasive than the M2 line and the cl16 is weakly invasive. Refs \(^{196-207}\)
**Culture media**

Cells were cultured in mono-layers in 75cm² tissue culture flasks at 37°C and 95% humidity in Dulbecco’s Modified Eagles Medium (DMEM) with 10% Foetal calves serum (FCS) glutamine and Antibiotics.

**Soft Agar**

10ml Krebs-Ringer solution with 20mg Bacto-Difco Agar were autoclaved 121°C 15mins and 20ml Sterile filtered DMEM was added and poured into petri dishes or moulds under aseptic conditions for further use when set.

**Trypsin EDTA solution**

Sterile filtered 0.5g Porcine Trypsin, 0.2g EDTA per 100ml Hanks Balanced Salt Solution (Sigma chemicals T3924) stored at –20°C

**FCS Foetal calf serum**

Gibco FBS Cat N° 10270-106 Lot N° 40F5120K, stored at –20°C

**DMEM Dulbecco’s modified Eagle’s medium (DMEM)**

It was purchased as liquid medium from Sigma Aldrich with high glucose and phenol red without L-glutamine and without antibiotics. (Cat N° D 6546) In a 500ml flask of liquid DMEM 10ml glutamine stock and 6ml Penicillin and Streptomycin solution was added.

**Antibiotic stock solution**

Penicillin and streptomycin 100x stock solution from Sigma Aldrich cat N° 9 4333 10000 Units penicillin and 10mg Streptomycin per ml.

**Glutamine stock solution**

200mM Glutamine from Sigma Aldrich cat N° G 7513
HBSS Hank’s buffered saline solution
Sigma Aldrich cat N° (H 8264)

Krebs-Ringer solution
Sigma Aldrich cat N° k 4002 supplemented with 1800mg/L glucose. 9.5g/L powder with 1.26g/L sodium bicarbonate.

**Software**
KS300 from Zeiss, image recording and evaluation of motility.

ImageJ from NIH, for image evaluation and data collection and INVASLOG measurements.

Windows 2000™, operating system for ImageJ and KS300

Windows NT 3.5™ operating system for the PC controlling the CLSM microscope

Leica CLSM TCS software control software for the image collection and recording from the CLSM TCS microscope.
ECH mono-layer cell cultures

Preparation of the embryos

For the preparation of a single 75cm² culture flask of cells ten, 10 day old, fertilised eggs were required. They were swabbed with alcohol and in a lamina flow cabinet under aseptic conditions opened and the embryo removed Figure 8.

The head was immediately severed from the embryo and the heart surgically removed from the cadaver Figure 9. This involved opening the thorax with a sharp sterile scalpel holding the embryo in place with a tweezers. The internal organs are removed from the thorax with a gentle pressure using the blunt side of the scalpel thus scooping the internal organs out of the thorax among which the heart may be found. If it is not seen moving then it may be stimulated to do so by touching it with the scalpel thus making it easier to identify for the lesser experienced investigator. Before transfer to HBSS the unwanted attachments to the heart material were removed under a binocular dissecting microscope.

Heart preparation

The chick hearts (Figure 10) were collected in cold (4°C) sterile filtered (0.22µm Millipore filter) Hank’s buffered saline (HBSS) and washed to remove as much blood as possible. The hearts were cut in two to aid removal of the remaining blood cells.

The hearts were then removed from the HBSS to a petri dish where they were cut into small fragments using sterile surgical dissection scissors and as little fluid as possible. This prevents the cells from drying out but to aid cutting in keeping the suspension as concentrated as possible. Under a binocular
dissection microscope that had a calibrated ocular micrometer the size of the fragments was observed. The fragments were cut repeatedly until optically an average size of about 100µm was determined using the calibrated ocular micrometer using the conversion table found in Table 5.

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<th>Magnification</th>
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<tr>
<td>Size in µm</td>
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<tr>
<td>400</td>
<td>6.6</td>
</tr>
<tr>
<td>200</td>
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<td>100</td>
<td>1.64</td>
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<tr>
<td>50</td>
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</table>

*Table 5 Calibration table for the ocular micrometer of the binocular microscope for spheroid selection*

Single cell suspension of heart cells

5ml of warm (37°C) trypsin EDTA was added to the fragment suspension and transferred to a 50ml sterile plastic test tube (from the firm FALCON) and incubated at 37°C for 10 minutes with gentle occasional shaking.

On inspection of the incubated cell and fragment suspension it was decided whether or not a further 10 minutes was necessary so that the cells could be satisfactorily separated from each other providing a single cell suspension without severe loss of vitality of the cells before continuing.

The next step was to add 20ml of DMEM with 10% FCS to stop the trypsin reaction and using a sterile 10ml pipette with a gentle up and down motion of the fragment suspension through the pipette the cells were mechanically separated from each other through the thus produced shear forces to produce a cell suspension.
The cell suspension was centrifuged at 1500rpm for 5 minutes in a Beckman Centrifuge seen in Figure 19(f). After centrifuging the supernatant was aspirated and the pellet carefully resuspended in 10ml DMEM with 10%FCS

This cell suspension was then placed in a 75cm² incubation flask and incubated overnight at 37°C, 95% relative humidity and 5%CO₂. The next day the medium was changed for fresh DMEM with 10%FCS in order to remove the unattached, dead and blood cells from the flask.

Figure 8 Asceptic opening of the egg and removal of the embryo. Reprinted from\textsuperscript{208}
Figure 9 Removal of the head

Figure 10 The removal of the heart
Monolayer cell culture of cell lines

The cell lines other than the embryonic chick heart cells were cultured in 25cm² culture flasks in DMEM with 10% FCS until almost confluent. Passaging was carried out for all cell lines just before confluence to maximize the cell numbers at harvest. The medium was removed and the cells washed with 10ml of sterile HBSS. 1 to 2 ml of trypsin EDTA solution was used to cleave the cells from the attachment points in the flask. With constant gentle rocking it could be observed when the cell monolayer entered suspension. The cells were then taken up in 10ml DMEM with 10%FCS for transfer and dilution to further flasks for growth. When spheroids were to be prepared the cells were transferred to 75cm² flasks for growth to near confluence.
**Stains**

Vital dyes were used to stain either the cytoplasm or the membrane for the subsequent image analysis.

**CMFDA and CMTMR**

Molecular Probes' CellTracker reagents such as CMFDA CellTracker Green or CMTMR Cell Tracker Orange are fluorescent chloromethyl derivatives that freely diffuse through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes undergo what is believed to be a glutathione S-transferase-mediated reaction to produce membrane-impermeant glutathione-fluorescent dye adducts (Figure 11), although experiments suggest that they may also react with other intracellular components.

Regardless of the mechanism, many cell types loaded with the CellTracker probes are both fluorescent and viable for at least 24 hours after loading and often through several cell divisions. Here, experiments have been conducted that have been successfully carried out over up to 5 days. Most other cell-permeant fluorescent dyes, including the acetoxymethyl (AM) esters of calcein and BCECF are retained in viable cells for no more than a few hours at physiological temperatures.
Figure 11 Thiol reaction of CMFDA upon entry into the cell to be stained producing a charged fluorescent product

Figure 12 Structure of CMFDA
(5-chloromethylfluorescein diacetate)

Figure 13 Structure of CMTMR
(5-(and-6)-(((4-chloromethyl) benzoyl)amino)tetramethylrhodamine)
Figure 14 CMFDA and CMTMR spectra
Dil and DiO

Carbocyanines are among the most strongly absorbing dyes known and have proven to be useful tools in several different areas of research. Carbocyanines with short alkyl tails attached to the imine nitrogens are employed both as membrane-potential sensors and as organelle stains for mitochondria and the endoplasmic reticulum. Those with longer alkyl tails (≥12 carbons) have an overall lipophilic character that makes them useful for neuronal tracing, long-term labelling of cells in culture and non-covalent labelling of lipoproteins. This section describes the use and properties of dialkylcarbocyanines as general-purpose probes of membrane structure and dynamics.

Dil, DiO

The most widely used carbocyanine membrane probes have been the octadecyl (C_{18}) indocarbocyanines and oxacarbocyanines, often referred to by the generic acronyms Dil Figure 15 and DiO Figure 17, or more specifically as DillC_{18(3)} and DIOC_{18(3)} (the subscript is the number of carbon atoms in each alkyl tail, and the bracketed numeral is the number of carbon atoms in the bridge between the indoline or benzoxazole ring systems).
Figure 15 Structure of FAST DiO

3,3'-dilinoleyloxacarbocyanine perchlorate

Figure 16 Spectrum of FAST DiO

Figure 17 Structure of FAST Dil

Figure 18 Spectra of Dil
**Vital staining of mono-layer cell cultures**

Staining with CMFDA or CMTMR

A 20mM stock solution in anhydrous dimethylsulphoxide (DMSO) was prepared individually for both CMFDA (carboxymethyl-fluorescien diacetate) and CMTMR (carboxymethyl-transmethyl-rhodamine) stains from Molecular Probes and added singly to mono-layer cultures of cells to a final concentration of 20μM to obtain the desired fluorescent staining. The cultures were then incubated for two hours and finally washed with DMEM 10% FCS.

Staining with Dil or DiO

DiO and Dil from Molecular Probes are membrane intercalating stains and require a much longer staining period i.e. 24 hours. A 20mM stock solution was prepared individually for both stains and added singly to mono-layer cultures of cells at a final concentration of 20μM and incubated for 24 hours. The cells were then washed with DMEM 10% FCS.

The staining process was carried out in the dark or under very low light conditions due to the photosensitivity of the stain and to reduce the stress on the cells due to the photo-activity of the stains.
**Multicellular spheroids**

Multicellular spheroids were prepared from the stained monolayer cultures for the ensuing confrontation experiments.

**Spheroid preparation.**

Spheroids were prepared by removing the stained cells from the 75cm² culture flask in which they were stained by aspirating the growth medium and washing the cells gently with HBSS solution.

The cells were then treated with trypsin EDTA 1ml at 37°C until the cells detached from the flask bottom.

10ml DMEM with 10%FCS and antibiotics was then added and pipetted up and down to mechanically separate the cells from each other to a single cell suspension.

The cell suspension was then transferred to a 9cm diameter sterile Greiner plastic vented petri dish. The cells have a severely reduced ability to attach to these petri dishes and so preferentially attach to each other producing re-aggregates of cells that when large enough round up to form spheroids. Alternatively the cells when they did not adequately form spheroids on the petri dish base were poured onto “soft agar” (0.75%agar w/v) that had been used to cover the base of the petri dish. This ensured that the cells could not attach to the base and promotes the re-aggregation of the cells to spheroids.

The petri dish was then incubated from 24 to 48 hrs depending on the re-aggregation rate and size of the spheroids desired. Several factors affect these parameters, one being the foetal calf serum and it’s preparation where the
enzymatic and hormonal content plays a role, and secondly the state and density of the cells when transferred to the re-aggregation plate.

**Spheroid selection.**

A binocular dissection microscope (Figure 19a) was used in a horizontal laminar flow cabinet to prevent contamination of the cultures during the selection process.

A capillary micro-pipettor (Transferpettor) which consists of a glass capillary pipette with a metal piston for positive fluid control, was used to select the spheroids and an ocular micrometer for the measurement of the spheroid diameter prior to selection (Figure 19b,c,d). Rapid suction of 2\(\mu\)l of medium surrounding the spheroid with the micro-pipettor drew the selected spheroid into the capillary of the micro-pipettor enabling its removal for further processing.
**Confrontation culture**

Two stained spheroids, one stained green fluorescent (usually non-cancerous cells) and the other either unstained or stained red fluorescent (usually cancer cells) were selected and placed in contact with each other and co-cultured on soft agar 0.75% w/v (the supporting agar matrix concentration was chosen so that the outgrowth of the spheroids on the agar was prevented) under standard incubation conditions 5%CO₂, 95% humidity on DMEM with 10% FCS either in a petri dish in a laboratory incubator or in the micro incubator as required.
**Incubation chamber**

The incubation chamber is a construction developed in co-operation with F-Tech in Graz Austria, the chamber was developed to enable its use in the observation of cells directly on the microscope stage under standard controlled growth conditions.

**Micro incubation chamber construction**

The micro-incubation chamber seen in Figure 20 and Figure 21 and in cross section in Figure 22 and Figure 23 consists of a control unit seen in Figure 26 d that mixes compressed CO₂ with sterile filtered air to a mixture that consists of 5% CO₂ The gas mixture is then transferred to the chamber via silicon tubing. The control unit Omron 5CK also includes control electronics for remote control of the temperature of the chamber. The temperature is regulated in the chamber in that the administered gas on entry to the chamber passes through a circular passage (Figure 21 (3)) around the cultivation chamber (Figure 21 (5)) where a heating element heats the gas to a temperature that upon passage through the cultivation chamber heats the incubation chamber and the medium to 37°C. In order to maintain control of the temperature in the incubation area with adequate accuracy a temperature probe Minco PB100 was cast into the silicon seal surrounding the incubation area (see Figure 22) and calibrated using a calibration coverslip which was a coverslip that had a temperature sensor NiCr/Ni type K +/- 0.15°C at 37°C placed through it so that the temperature could be directly measured inside the chamber.
Figure 20 Incubation chamber assembly
A and B lower chamber seal
C and D Heating element with gas control
E and F Upper seal and insulating coverslip
Figure 21 3D assembly diagram of the Incubation chamber

(1) the sealing ring
(2) The insulated covering glass
(3) The heating and control element
(4) The lower coverslip
(5) The lower sealing ring and insulating air cushion controller
Confocal microscopy

Figure 22 Cross section of the incubation chamber used for the dynamic investigation of inversion using...
Figure 23 Modification of the incubation chamber enabling observation during growth without attachment to the support

The objective used must be dry to avoid heat loss through the objective.
**Agar confrontation form**

The agar used to prevent attachment of the cells to the coverslip was formed in a special mould in order to enable the agar to be cast such that small pits with a depth that are at a controlled distance from the coverslip are formed so that the long distance objective can scan the confrontation pairs in these pits without loosing it out of the range of focus of the objective.

**Casting mould**

In order to enable the growth of spheroid confrontation cultures within the chamber the glass coverslip had to be coated in such a way that the cells were unable to attach to the coverslip and were not more than 1mm from the bottom coverslip. A mould was constructed that enabled the casting of a soft agar pellet with indentations that were 1 to 1.5mm at their deepest point from the supporting coverslip. The cultures that were to be observed were placed in the indentations such that they were as close to the bottom as possible.

![Diagram of Casting Ring, Indent Stiff, and Mould]

*Figure 24 Cross section of the agar moulding chamber for use in casting the soft agar support for confrontation cultures.*

**Agar casting**

30ml of soft agar was prepared as described above and an aliquot poured into the sterile mould. The mould was placed in a sterile container and cooled in a refrigerator for 20 minutes to aid removal of the agar.
When set, the excess agar was cut away by sliding a sterile coverslip over the mould. The coverslip was then removed and the casting ring removed.

The mould was inverted above a sterile coverslip supported on a sterile tube about 5cm in length. The inverted mould was placed as near as possible to the coverslip without the agar touching it. A sterile scalpel was then used to gently lever the set agar support from the mould so that it gently falls onto the coverslip.

The incubation chamber is then assembled around the coverslip carrying the agar support. 2ml of growth medium is then aseptically added to the incubation chamber and sealed for further use.
Confocal microscopy

The Confocal Laser Scanning Microscope from Leica (Type TCS NT, Heidelberg, Germany) Figure 25 was used for optical sectioning of the specimen.

Figure 25 Confocal laser Scanning Microscope (CLSM)
This confocal laser scanning microscope is one of the type with a scanning laser beam for the lateral (xy scan) information and with a moveable object stage for the axial (z-scan) information. Two pinholes (one in the excitation path and the other one in the emission path) are positioned at the conjugated points of the optical path (confocal) and therefore eliminate the out of focus signal.

![Microscope Images](image1.jpg)  
*Figure 26 CLSM stage (a) with adapter (b) and with incubation chamber (c) and the control unit (d)*

For scans making use of the incubation chamber a special stage was constructed to replace the piezo stage (see Figure 26) for scans in the z axis as the chamber was too heavy for the piezo scan mechanism. For z scans the E-stage of the microscope was used where the objective stage and not the sample stage is moved for the z-scans.
The stained specimens were excited simultaneously through a 10 X 0.3NA objective with the laser wavelengths 488 and 568 nm. Sections have been recorded with an intersectional axial distance of 1 to 50 μm.

The pinhole in the emission path has been set in such a manner, that the theoretical maximum of the resolution (lateral 0.65 μm, axial 4.77 μm) has been approximately reached. A total image size of 500 μm X 500 μm has been obtained with 512 X 512 pixels and a zoom factor of two.

Alternatively for scans that required the long distance objective the 20 X 0.4 NA objective without the zoom was used. That result in the same magnification and field size.

Dry objectives were used to prevent a thermal contact with the coverslip that would have acted as a heat sink and prevent reliable temperature control.

In Figure 27 an Argon Krypton Laser (Type Omnichrome Series 43) in Multi Mode operation was used for the exciting illumination light.

This light passed a short-pass filter SP590, so only light with a shorter wavelength than 590 nm (mainly 488 and 568 nm) was reflected by the double dichroic mirror (DD488/568).

After passing the mirror and the objective (Leica 10 x 0.3 NA, Heidelberg, Germany), the light was focused on several planes of the stained specimen. Transmitted excitation light was measured with the Photomultiplier PMT.T.

Fluorescence emitted from the stained specimen was detected with the same objective (epi-fluorescence), reflected by the mirror, transmitted by the double dichroic mirror and passed through the pinhole. The dichroic mirror RSP 580
reflected the green part of the emission spectrum (the main emission from the dye), and the band pass BP530/30 eliminated scattered light of the laser.

![Diagram of illumination path and filter settings of the confocal laser scanning microscope](image)

Figure 27. *Illumination path and filter settings of the confocal laser scanning microscope*

The Photomultiplier PMT.1 detected the light intensity of the fluorescence light. The second detection path (mirror, LP 590, PMT.2) was used to detect the fluorescence in the red part of the emission and PMTT was examined only for control purposes.

For observation of the invasion process the prepared Incubation chamber containing the confrontation pairs of spheroids was placed on the microscope stage and attached to the control unit.

Because the stage can act as a heat sink the chamber was calibrated with the stage and the stage was thermically isolated from the chamber as far as was possible. The thermal isolation consisted of setting the chamber on three points
of contact that were used for levelling the chamber and reducing them to very small points of contact with the stage.

In Figure 28 in the upper panel, two images can be seen representing the fluorescence signals from each of the fluorescence channels and the lower panel shows the transmission signal. All three channels were recorded for the image analysis process. The channels were examined separately and cross talk or spill over between the channels digitally corrected.
Flourescence images (side length = 512 pixels = 500\mu m the images are square)

Transmission image of the above spheroid pair (side length = 512 pixels = 500\mu m, the images are square)

*Figure 28 Transmission and fluorescence images of an early (4h) confrontation showing the degree of staining*
**Choice of objective**

For initial experiments where single time points were to be observed a simple observation chamber was built where an agar support for observation was unnecessary. Here the usual objectives 10x and 40x could be used and the data collected for a single time point.

For experiments using the agar support in the incubation chamber a long distance objective was necessary to enable penetration through the cover-slip and agar support with enough room to be able to scan through the confrontation pairs, this was carried out with a long distance 20x objective.

<table>
<thead>
<tr>
<th>Magnification.</th>
<th>NA</th>
<th>Oil or dry</th>
<th>Compensation</th>
<th>Resolution xy Axis</th>
<th>Resolution z axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>0.3</td>
<td>dry</td>
<td>No</td>
<td>650.67nm</td>
<td>4700nm</td>
</tr>
<tr>
<td>40x</td>
<td>1</td>
<td>Oil</td>
<td>Yes</td>
<td>195.2nm</td>
<td>584.15nm</td>
</tr>
<tr>
<td>20 x Long</td>
<td>0.4</td>
<td>Dry</td>
<td>Yes</td>
<td>630nm</td>
<td>4700nm</td>
</tr>
</tbody>
</table>

*Table 6 comparison of the physical data for the objectives used for confocal microscopy (source Zeiss).*

Here (Table 6) it can be quite plainly seen that the z resolution is significantly worse than that in the xy plane. This is also seen to get worse at various depths in Figure 31.

The lateral resolution of a single infinitely small point for an ideal objective is given by the Airy disc in Formula 1 where \( \lambda \) is the wavelength of the light used, \( \eta \) is the refractive index of the medium and \( \alpha \) half the angle of the collected rays from the object point.
\[ d_{\text{Airy}} = \frac{1.22 \lambda}{\eta \sin \alpha} \]

Formula 1 Airy disc

*Figure 29 Airy disc*

The Airy disc is generated through destructive interferences seen in figures Figure 29 and Figure 30.
Figure 30 Airy disc generation
The Rayleigh criterion

The Rayleigh criterion sets the limit for the smallest resolvable distance $d$ between two points as being half the airy disc distance i.e.

$$r_{Airy} = \frac{1.22 \lambda}{2NA}$$

**Formula 2 Rayleigh criterion**

NA is the numerical aperture of the objective used

![Figure 31 Change in z-resolution of a 40x 1.3NA oil immersion objective with increasing depth of penetration into water along the z axis](image)

Point spread function

The point spread function is defined as the "distribution of irradiance in the focal region i.e. the size an shape of the illuminating spot. The maximum irradiance is defined as
\[ I_p = \frac{\pi}{2} PT \left( \frac{\eta}{\lambda} \right)^2 \left( 1 - \cos \alpha \right) \left( 3 + \cos \alpha \right) \]

\( P \) = input power in Watts,

\( T \) = transmission factor of the objective,

\( \alpha = \sin^{-1} \left( \frac{NA}{\eta} \right) \) where \( \eta \) is the refractive index of the immersion medium,

\( NA \) = the numerical aperture of the lens

*Formula 3 The maximum irradiance.*

The full width of the irradiance distribution measured at one half the maximum irradiance value is defined below for unpolarized light.

Axially \[ w = \frac{0.51\lambda}{\eta \sin \alpha} \]

Laterally \[ w = \frac{0.44\lambda}{\eta \sin^2(\alpha/2)} \]

*Formula 4 The full width of the irradiance distribution at one half the maximum irradiance value.*

For a 10x objective with a NA of 0.45 is the conventional lateral resolution 0.68 \( \mu m \) and in confocal microscopy the same lens has an lateral resolution of 0.48\( \mu m \)
Pinhole

The use of pinholes increases the resolution significantly axially as opposed to a normal optical microscope. For fluorescence imaging the resolution can be \( \approx \sqrt{2} \) greater than with conventional microscopy\(^\text{194}\).

Axial resolution with depth

The axial resolution is more complex as can be seen in Figure 31 as the depth or distance from the objective plays a role as does the medium in which it is to be found.

![Figure 32 Z-resolution of objectives](image)
The z-resolution of the objectives used was tested using the reflection mode of the CLSM and the reflective surface of a microscope slide. In z-scan mode a relative intensity graph plotted against scan depth as above can be produced and shows the value of the z-resolution that was given by the company to be ½ the width of the curve at 50% of the maximal intensity as seen in Figure 32.

It can be very misleading in that some companies express the resolution as the total width of the peak at 50% of the peak height and so it is necessary to check the data thoroughly to ensure the correct objectives are used.

Laser interaction with the specimen

![Diagram](image)

*Figure 33 Diagrammatic representation of the interaction between an incident light beam and a specimen.\(^{194}\) \(I_0\) Intensity of illuminating light source \(I_{REF}\) Intensity of Reflected emission \(I_{FLU}\) Intensity of fluorescence \(I_s\) Intensity of scattered light \(n_x\) are the interfaces. Reprinted from\(^{194}\)*
The shaded space in the Figure 33 represents the volume (voxel) which is imaged by the confocal microscope to form an image pixel. This is not a cube as might be perceived from the depiction of voxels but a rather more complex polygon. Reflection results from media interfaces (e.g., \( n_1 \) and \( n_2 \) interfaces), and their intensity is a function of the difference in refractive indices between the interfacing media. \( I_s \) intensity of scattered light; \( I_{\text{TRANS}} \) intensity of transmitted light; \( I_{\text{FLU}} \) intensity of fluorescence; \( I_{\text{REF}} \) intensity of reflected emission, and incident intensity \( I_o \). In the case here simple interfaces are used for clarity but in the case of spheroids in the actual situation is more complex.

The effect of section depth on signal intensity
The decrease in fluorescence seen when scanning objects are not at the surface but where the focal plane is to be found inside the object under examination can be expressed as a function of the observation depth. Although it is here called "absorption" it is in fact the sum of effects that result in a decrease in the measured signal intensity seen (see Figure 33). This may be due to absorption, refraction, diffraction etc. of the laser which in turn decreases the intensity of excitation light reaching a particular depth therefore limiting the amount of fluorescence that can be emitted if the concentration of fluorophor is such that the excitation light intensity is the limiting factor and similar effects on the return path of the emitted photons to the photo-multiplier tubes. The emitted light may then also be absorbed refracted diffracted etc thus resulting in a reduction of the signal intensity proportional to the depth of observation in the specimen.

One might think that the solution lies in the fact that with a constant concentration of fluorophor that one should increase the laser intensity with
increasing depth in order to ensure the fluorophor is saturated and the signal is then fluorophor concentration dependent and depth independent. This idea sounds fine from the point of view of the physics, but is totally unsuitable in reality due to other effects caused by high laser intensities such as phototoxicity, bleaching dimer formation etc.

The depth at which the section is taken in the object under observation has a detrimental effect not only on the point spread function but also on the signal intensity.
Depth dependence of the relative fluorescence of a stained spheroid
(K1735M2 stained with CMFDA 20μM)

![Graph showing relative fluorescence vs. depth of the slice (μm).]

Schematic orientation of the 2D-slices:

![Schematic diagram of spheroid cross-sections at different depths (0μm to 150μm).]

Figure 34 The effect of section depth on the relative fluorescence.
Serial sections

Figure 35 Serial sections of a single large spheroid stained with CMFDA. Here it can be seen that with increasing depth the signal intensity decreases (steps are 7.5 μm).
Figure 35 shows serial sections of a large spheroid, the sections are numbered 1 to 16 representing increasing section depth. The sections were taken from a uniformly stained spheroid of K 1735 M2 cells of about 400μm in diameter and shows the signal intensity decrease with depth. Here it can be seen that with increasing depth the signal intensity in the middle of the spheroid relative to that at the edge depreciates.

![Effect of object depth on signal intensity](image)

**Figure 36 3D reconstruction of the signal intensity**

along a z section of the spheroid passing through the centre of the spheroid demonstrating signal loss with object depth
In Figure 36 a 3D reconstruction of the signal intensity values from a section in the z-plane through the centre of the spheroid is shown where it can be seen that the signal depreciates rapidly in the middle of the spheroid but is still high at the edges.

In Figure 37 a plot of the decrease in signal intensity in the middle of the spheroid as opposed to that at the edge demonstrated that the loss in signal intensity was not due to the long scan distances through the agar support but due to signal loss in the spheroid itself.
Figure 37: The relationship of depth with signal intensity by a large sphere.

Regression equation:

- Linear regression: $y = -0.0069x + 0.9612$
- Exponential regression: $y = 1.4717e^{-0.077x}$
Self Shadowing

The effect shown in Figure 35 is sometimes referred to as self shadowing, this effect varies from specimen type to type and from microscope to microscope as well as the way in which the microscope is adjusted. In order to determine the exact effect on the microscope used in the normal setting a large spheroid that had been uniformly stained was chosen and scanned. The ensuing data is presented in 2D and 3D representations.

The relative intensity of the outer pixels of each section decreases linearly with depth whereas a pixel chosen near the middle of the spheroid decreases in intensity exponentially \((y = 1.4717e^{-0.0372x})\) see Figure 37. This would suggest that the Beer-Lambert law for absorption could be used here but the are many factors to be considered not only the absorption of the exciting light but also that of the emitted light and various other effects that influence the detection of an emitted photon. It does though suggest that the predominant effect here is the absorption of the excitation light by the fluorophor.

The loss of signal intensity in thick specimens is in many cases a problem when a quantitative assay is to be used as the values required are usually based on signal intensities. The use of methods where the signal intensity does not play a significant role are preferable. As in Figure 37 the signal intensity decreases exponentially with depth. This is to be expected as the whole cell has been stained and so the effect of absorption overweighs the other effects in signal reduction such as diffraction etc to such a degree that the Beer-Lambert law can be safely applied although the intensity reduction obtained cannot by definition be an absorption value but in this case a signal intensity depreciation
co-efficient. In order to increase the depth at which the signals can be measured and taking into account that the absorption is due to the stain then the concentration of stain used should be decreased so that the desired intensity of emission at the desired depth can be reached. This would also require a high level of laser intensity which is detrimental to the cells and so a concentration is required where the depth can be reached that is required and the stain intensity is high enough that at low laser levels measurements may be made over long periods. This also requires that the stains are stable bleaching resistant.

Bleaching

Exposure of fluorescent dyes to intense light can result in bleaching effects that can in this case be detrimental to the investigation as can be seen in Figure 38.

Figure 38 Photobleaching

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The fluorescence intensity reduction can be for many quantitative measurements a problem when precautions are not taken especially when absolute values are required. In the case of the measurement of the INVASLOG this is not a problem as long as the signal does not diminish to such an extent that it cannot be determined from the inherent noise in the system. Sometimes bleaching may be advantageous as in the use of bleachable dyes for the observation of physiological effects with the FRET technique.

The effect of prolonged exposure of the stain CMFDA to the laser scanning under experimental conditions was tested using repeated scans of the same section. A single spheroid is placed in an observation chamber in HBSS and was scanned at a single level several times and the results presented in Figure 39.

Neighbouring pixels were examined demonstrating the variation from scan to scan as well as the tendency to signal loss through stain loss from bleaching effects. Figure 39 shows the effect of repeated scans on neighbouring pixels.

Despite the fact that each value was determined using 4 times averaging a large variation of single pixels from scan to scan can be seen but not in the relationship of the pixels to each other per scan series, this can be due to variations in laser intensity that is due to the nature of the laser technology used and not to bleaching effects.
Figure 39 The effect of repeated scanning of the sample spheroid.

The numbers one to eight are repeated scans of neighbouring pixels along a line passing through the centre of the spheroid in the horizontal plane and each bar represents the resulting signal intensity from each scan series represented on a scale from 0 to 255 in the gray scale image. 4x averaging was used so that each result represents 4 scans.
A general tendency toward a reduction in the fluorescence intensity could not be significantly determined due to the variation between scans being higher than the loss detected but a general tendency to a reduced signal can be seen. Although a slow bleaching could be measured after 1000 scans (data not shown) in comparison to other stains used for confocal microscopy this is significantly more stable and then suitable for long term time-lapse observations where many scans are necessary. This is also supported from the producing company Molecular Probes in that the stain was developed to be stable over long periods.
Cyto-toxicity

It was measured as the effect on the motility of the cells using directional motility analysis of the cells in two dimensional migration assays (see page 164) before and after staining. No noticeable difference was seen when the cells were stained or unstained in their motility.

Photo-toxicity

This is where the photons and their imparted energy on the surrounding masses on their path through matter causes effects that may influence the chemistry around and lead to unwanted effects that may lead to toxic processes. One of these can be the production of reactive radicals or molecules through the uptake of the energy. It has been assumed that little photo-toxicity takes place as the motility of the cells in directional assays under high light intensities was unaffected stained or unstained.
Dye transfer between stained cells and gap junctional properties

I proposed that the cytosolic stains used CMFDA and CMTMR, may pass through cellular gap-junctions. The stains have a molecular size that is physically small enough in comparison to the size of the gap in the gap junction to enable them to pass through it. Although the stains are charged the gap junctions are not voltage gated enabling the transfer of charged and uncharged molecules from cell to cell.

The Gap junction

The gap junction protein is both evolutionarily very conservative and present in both the chick heart cells as well as the confronted melanoma cells and it may well under suitable conditions produce functional gap junctions and as such the possibility of transfer of the dyes may exist. The consequence would be the loss of definition or marking of the cells, thus requiring the use of other more toxic dyes that stain the membranes.

Dye transfer test

In Figure 40 the cells were stained firstly as a mono-layer with CMFDA (green) then the cells were then re-aggregated to spheroids and stained with CMTMR (red). The areas where both stains are present (co-localised) appears yellow due to the way RGB images digitise colours in that the red and the green channel contain signals that numerically result in the colour yellow.

This shows that the stain cannot penetrate deeply into the re-aggregated spheroid as only the first two layers of cells have been stained whereas if transfer had occurred between the cells i.e. gap junctional communication exists, it should be seen to be uniformly transferred through the spheroids. For information on the time constants concerned and the diffusion characteristics of
gap junctions $^{210,211}$. This does not conclusively demonstrate that diffusion through the gap junctions does not occur as the penetration of the stain may have been hindered otherwise. Two further experiments were devised to demonstrate that the stain does not pass to other cells through the gap junction.

![Image](image1)

Figure 40 Double staining of the same tissue before with CMFDA and after spheroid formation with CMTMR illustrating the lack of penetration of the spheroid by the stain after spheroid formation. Upper left Channel 1 (CMFDA fluorescence), upper right Channel 2 (CMTMR fluorescence), lower left transmission channel, lower right overlay of all three channels from CLSM microscope.
In Figure 41 The dye DiO (red) was used as it is a lipophilic stain that selectively stains membranes. The chick heart cells were stained with it and the confronting A375 SM cells were stained using the cytosolic stain CMFDA (green). On examination the Green cell in the middle of the image contains a vesicle that has been stained red. No other cells showed that they contained the green cytosolic stain. This suggest transfer of the red stain through membrane ingestion but does not show transfer of the soluble cytosolic stain into the red cells.

Figure 41 Dye gap junction transfer test
Figure 42 Gap junctional communication shown by dye transfer

Fluorescence pictures of cells 1 min after microinjection. The injected cells are marked with an asterisk.

a Melan-a cells cultivated in the presence of TPA and cholera toxin: the dye has spread to neighbouring cells.

b Melan-a cells cultivated in SF-j: no dye transfer to neighbouring cells.

c K1735-M2: the dye remained in the injected cell.

d K1735-M2, phase contrast micrograph corresponding to c: the cells are actually in contact. Scale bar = 40 μm.

reprinted from

In Figure 42 The gap junctional communication was tested using Lucifer yellow a dye that passes through gap junctions. This experiment was carried out here earlier by colleagues and was confirmed in a repeat of this experiment (results not shown). Melan-a cells are a cell line that produces melanin a and SF-j is a serum free medium, please refer to the citation for further details.

Image analysis
The confrontation cultures were scanned using the optimised dynamic range for the photomultipliers set at the middle of the examinable depth of the confrontation i.e. the examinable depth found between the start of the specimen to the point at which the fluorescence signal dissipated to a level where it was no longer measurable at amplification voltages of the photomultipliers where autofluorescence did not play a role and the signal to noise ratio was acceptable. Single time point observations were made using 16 sections from the cover slip to a depth where the signal was too diffuse or too small to be accurate. A section at about one third of the diameter of the spheroid pair was chosen for examination and analysed using the program KS300 Imaging system Release 3.0 from Zeiss and a program macro that was developed for the elimination of cross-talk between the channels and the calculation of the area and perimeter of each of the stained cell types automatically. Alternatively the program ImageJ from NIH (National Institute of Health, USA) was also used to confirm initial results. This enabled the calculation of the INVASLOG a parameter for the measurement of the clinically significant invasion of the host (stromal) tissue.

Cross-talk correction

Cross-talk is where the emitted light from the fluorescent stain is detected by both photomultipliers see Figure 43, Figure 44 and Figure 45.

The spectra of the stains used overlap in such a way that the FITC filter configuration is unable to separate the two emission spectra totally. This can be interpreted as being co-localisation but this has been demonstrated earlier as unlikely as the transfer of the stain to other cells does not occur and thus co-localisation also does not occur. Then the apparent localisation of both stains
at the same place spatially is only a question of the resolution in each of the three dimensions making it difficult to separate them and thus they appear apparently as co-localised stains.

![Fluorescence emission spectra](image)

**Figure 43** Emission spectra of fluorescein (FL), tetramethylrhodamine (TMR) and tetrarhodamine (TR) illustrating the spectra overlap that results in cross-talk (data from Molecular Probes)
The spectra were supplied by Molecular Probes.

Figure 44: Excitation and emission spectra of CMFDA and CMTRMR and the detection channels used.

[Diagram showing excitation and emission spectra with wavelengths in nm and intensity in arbitrary units.]
Figure 45 Cross talk or spill over between FITC channels $^{194}$

Using the assumption that the stain cannot be transferred to other unstained or otherwise stained cells, then only one stain must be present at a particular point in a section. When a thin enough section is made i.e. when the section is thin enough that only a single cell is observed at any one point in the section then only a single stain should be observed. Therefore at each point if stained so that each stain emits an equal signal intensity to its respective detector the signal observed at the second detector for the opposing stain will be less.

The signal intensity for green when detected in the red channel must be less than that of the red signal detected in the red channel and vice versa. Therefore
it is possible to determine the origin of the signal using a simple arithmetic algorithm to determine the correct channel for detection and eliminate the erroneous signal.

Image analysis with cross-talk correction

Image analysis was carried out using the analysis software KS300 (Zeiss, Munich, Germany) and a personal computer (Pentium 200MHz).

To compensate for the cross-talk artefacts the primary images were enhanced by histogram stretching (making use of the full 256 grey values 8bit resolution), 3 x 3 median filtered and then subtracted from each other. To avoid negative grey values, an offset of 128 was added to each new grey value calculated from the subtraction algorithm and grey values > 255 were set to 255, grey values <0 were set to 0. The totally black background (grey value = 0) in both the primary images would appear in the difference image with the grey value 128. Dual thresholding of the difference image at levels > 128 and < 128 gives two binary images, which are used as masks and applied to the primary grey level images. This enables the separation of the signals into two “clean” signal images.

Overall these steps represent a binary decision for each single image pixel, i.e. to which channel the image pixel belongs without losing the grey value information. Relevant for this decision is the grey level difference of the spatially equivalent image pixels of both the primary images. Each individual image pixel is related to that channel in which the higher grey level was detected.

From these cross-talk corrected images of the specimen under study, the area and the dimensionless negative logarithm of the form factor INVASLOG = - log
[form factor] = -\log [(4 \pi \times \text{area}) / (\text{perimeter})^2] was calculated. Any deviation of the shape of the specimen or additional cross-talk alter this parameter. Furthermore, in this case the parameter INVASLOG is additionally used as a parameter for estimating the degree of invasion of host tissue by cancer.²¹⁶,²¹⁴-²¹⁷

Reliability of the cross-talk correction algorithm

For the evaluation of the quality and reliability of this subtraction method for compensating for cross-talk, we tested the algorithm with artificially increased cross-talk. Starting from pairs of images showing practically negligible cross-talk, we added 10, 20, 30% etc. up to 100% of the grey value of the image from the FITC channel to the images from the TRITC channel and vice versa. Therefore, 11 images were created for each channel, including the original image. From these 22 images, 121 different image pairs were constructed in each case with a different, but defined additional cross-talk value. Each image pair has been fed to the cross-talk reduction algorithm described above and the parameter area and INVASLOG, which act as quality parameters, were calculated.
Figure 46 The cross-talk algorithm is based on a simple arithmetic comparison of the channel signals.

The grey levels from the images in Figure 46 of the TRITC channel (Ch2) are subtracted from the grey levels of the FITC channel (Ch1) and a constant of 128 is added to each value. Totally black areas in image Ch1 and image Ch2 (grey value = 0) would get the exact value 128 in the difference image. If the grey value of the image Ch1 is greater/less than the value of the image Ch2, the calculated grey value would be greater/less than 128. Thresholding values
greater and less than 128, gives the cross-talk corrected binary images, which are further used as masks for the grey level images.

This algorithm is based on the assumption that the stain is not transferred between cells and so both stains are not present in a single cell at the same time. It has been investigated to what level the cross-talk algorithm is able to discriminate correctly between cross-talk and signal.
Figure 47 Cross talk correction experiment

Deviation of the parameter INVASLOG on varying artificial additional cross-talk values, reflecting the cross-talk compensation capability. The primary image pair (see Figure 50) resulted in an INVASLOG value of 0.2 for the TRITC channel, equivalent to an approximately circular shape. (a) 3-D surface chart with cross-talk values from 0 to 100% for both the channels; (b) the average values and the standard deviations of the parameter INVASLOG for FITC to TRITC cross-talk from 0 to 70% (10% steps) in relation to the cross-talk from TRITC to FITC from 0 to 70%.
Figure 48 Cross talk correction experiment 2

The primary image pair (Figure 51) resulted in this case in an INVASLOG value of 0.59 for the TRITC channel, equivalent to a noncircular shape. (a) 3-D surface chart with cross-talk values from 0 to 100% for both the channels; (b) the average values and the standard deviations of the parameter INVASLOG for FITC to TRITC cross-talk from 0 to 70% (10% steps) in relation to the crosstalk from TRITC to FITC from 0 to 70%.
Figure 49 Cross talk experiment 3

The primary image pair (see Figure 52) resulted in an INVASLOG value of 0.89 for the TRITC channel, equivalent to a very irregular shape. (a) 3-D surface chart with cross-talk values from 0 to 100% for both the channels; (b) the average values and the standard deviations of the parameter INVASLOG for FITC to TRITC cross-talk from 0 to 70% (10% steps) in relation to the cross-talk from TRITC to FITC from 0 to 70%.
Figure 50 Cross talk simulation experiment 1.

The image columns "Grey Ch1" and "Grey Ch2" are the grey value images of the FITC (cancer) channel or TRITC (host) channel, respectively. Descending these columns, the images with artificially increased cross-talk values of 0, 40 and 80% can be seen. The column "Detected Ch2" is the image processed and the cross-talk compensated grey level image of the TRITC channel. The column "Detected Binary" is an overlay of the image processed and the cross-talk compensated binary images from both the channels. The columns "Detected Area Ch2" and "INVASLOG Ch2" are two quantitative parameters, respectively, for the TRITC channel. The primary image pair shows objects with approximately circular shape.
Figure 51 Cross talk simulation experiment 2

Figure 52 Cross talk simulation experiment 3
Digital image analysis

**IMAGE ANALYSIS**

- 3x3 Median Filter
  - Grey Level Picture
- Histogram Normalisation
- Thresholding
  - Binary Picture
- Close, Opening (Erode, Dilation)
- Area, Perimeter
  - Mathematic
- Parameter (i.e., INVASLOG)

*Figure 53 Schematic drawing of the process of image analysis*

Here (Figure 53) a rough outline of the image processing for the calculation of the INVASLOG is shown. In Figure 54 the process is show on hand from actual images during image processing. The cross talk correction and the binary image production which the results in several closing and opening operations to remove erroneous signals. The results are binary images from which the INVASLOG may be calculated.
Figure 54 Digital Image analysis
Optimal pixel size for image analysis

There are limitations to the accuracy of any analysis and that is determined by the accuracy of the measurement system. Here the limits are determined by the pixel size relative to the object to be examined in combination with the attainable optical resolution of the microscopic technique used.

It is necessary to over-sample with pixels so that an adequate representation of the image can be analysed without loss of resolution. This is especially necessary as the INVASLOG parameter for the estimation of invasion requires determination of the circularity of the object using the form factor

\[ \text{formfactor} = \frac{4 \cdot \pi \cdot \text{area}}{\text{perimeter}^2} \]

Formula 5 Form factor

This is especially difficult as pixels are inherently square by definition but the area of detection does not necessarily have to be square for a particular pixel. The estimation of the circularity of an object using squares poses a problem when the pixel size relative to the object to be measured nears the ratio of 1 i.e. the object is shown as a single pixel, therefore it is desirable to have as high a ratio as possible (object size/pixel size) in order to attain as accurate a reflection of the degree of circularity as possible. This is again restricted by the optical resolution of the microscope and the wavelength of light used.
Figure 55 Relationship of the form factor to the image size length in pixels determined for circles from Figure 54.
Circles and pixels

The form factor, Formula 5, is a measure of the ratio of the perimeter to the area with respect to a perfect circle. A test image of 1024 pixels square was constructed containing a circle almost filling the image. The resolution of the picture was artificially reduced by half, this was repeated several times until the length of the side of the image was reduced to 8 pixels see Figure 56. In order to correct for size differences in the values produced from each of the images each image was then corrected to have side length of 1024 pixels and the area and perimeter of a circle in the image was measured again using ImageJ. The results of the investigation were plotted as the form factor against the pixel resolution used Figure 55.

It was found that the value depends not only on the resolution but also on the position of the object in question, the effect can be seen by under sampling where the degree of accuracy is reflected by the number of samples taken. Theoretically, an infinite number of samples would be necessary but practically it can be seen that the difference in the form factor does not change drastically for objects that almost fill the 512 pixel square. The position of the object in Figure 56 appears to change when the resolution is 32 or below and this is reflected in an unexpected change in the form factor in Figure 55.
Figure 56 resolution reduction.

The numbers represent the number of pixels of one side of the square used to generate the picture. A positional shift appears to occur at pixel side length 32 where this aberration has an effect on the form factor can be seen in Figure 55.
Pixels and the INVASLOG

The size of an object relative to the size of the pixels used to determine its form determines the accuracy with which the form can be determined in Figure 57, aberrations such as positional changes due to the pixilation can also be seen.

![Diagrams illustrating pixilation of form](image)

*Figure 57* Pixilation of form

Depending on where the circular object is to be found the pixels that are detected are either one or four and all combinations between depending on the centre of the radius in relation to the pixel pattern. As the position of the object cannot be foreseen the question arises "How reliable are pixels for determining the form of an object?" As seen above, positional and form aberrations and artefacts can be seen when the pixel resolution of the image is reduced. Here we can deduce that a particular pixel resolution is required in order to resolve
object in the field of view. From Table 6 it can be seen that with the 10x objective an optical resolution of 650nm can be reached. In order to obtain the maximum resolution with the pixels an over sampling of two pixel to 650nm would be required. In our experiments the images are 512x512 pixels in size which is a real size of 500x500μm. This results in one pixel every 0.977μm and an under sampling of 1:0.67. According to the Rayleigh criterion is the optical resolution of the lens in use 1220nm at a wavelength of 600nm which is in this case 1:1.2 this does not reach the Shannon criterion of 1:2 but is nearer than that purported from the company selling us the objectives. The Shannon criterion also known as the Nyquist criterion, a general "rule" for measurement of frequencies, stating that the measurement (sampling) frequency must be at least twice the maximum frequency to be measured. This results in optical data loss through under sampling or inadequate over sampling and raises the following question:

How much resolution is enough?

In Figure 58 the parameter INVASLOG was determined for a theoretical figure that has the form of a single pixel. Additional pixels are then added sequentially to one side of the initial pixel resulting in a rectangular object and demonstrates the change in INVASLOG with an increasing departure from the circular form. In this way the INVASLOG for a particular number of pixels is maximised for a single object. This demonstrates the maximum reachable INVASLOG for an object that has a fixed number of pixels. Since the INVASLOG is a parameter that measures then degree of departure from the idealised circle the values shown here reflect the accuracy with which the INVASLOG can determine the
departure of a line from a circle and that being dependent upon the number of pixels forming the line.

In Figure 59 the results from Figure 58 are shown on a logarithmic scale demonstrating a linear correlation for objects with an x/y ratio above 10. This would mean that objects that are linear and above a pixel length of 10 have an INVASLOG that can be adequately determined.

Figure 60 shows the change in INVASLOG not only with increasing x/y ratio but also if the object number is increased. An exponential increase in object number leads to a linear increase in the INVASLOG. This is due to the departure of the linear object from that of a square where the thickness of the object in pixels plays a role and the ability to differentiate between a line and a circle diminishes.

Looking more closely one sees that at object x/y ratios of between 1 and 10 (Figure 61) a linear relationship exists to the INVASLOG whereas above 10 (Figure 62) and exponential relationship exists.
with the x/y pixel ratio along the x axis of the graph.

Figure 38 Dependance of the INVASLOG parameter on a rectangular object.

Single linear
Figure 59 AS Figure 58 but using a log scale along the x-axis

Change in Invaslog with Length.
The same length figure 60 Effect of object form and number on INVASLOG as figure 58 but with addition separate objects of
Figure 61: at low xy ratios the relationship to the INVASLOG is linear.
Figure 62. The effect of object form and number on INVASLOG at high values of INVASLOG is exponential.
Limits of INVASLOG in determining invasiveness

Object form

The determination of the parameter INVASLOG is dependent on an accurate representation of the area and perimeter of the stromal spheroid. One of the problems at low magnification is the resolution capacity of the digital image analysis i.e. the ability to determine the form of the object.

In Figure 66 several different idealised geometric forms and their number have been compared mathematically and the effect they have on the determination of the INVASLOG. As can be seen a logarithmic increase in the number of objects results in a linear increase in the INVASLOG, independent of the form. The form determines the initial value of INVASLOG, and the number of objects the resulting value.

A change in the regular geometric form changes the initial value of the INVASLOG as would be expected, the triangular form having a large effect. Increasing the number of objects logarithmically results in a linear change in the INVASLOG seen. A single square object has almost the same value as a semicircle and also increases in INVASLOG parallel to the semicircle on increasing object number. Linearization of a square to a rectangle by increasing the length of one of the sides also increases the INVASLOG.

In Figure 63 the change in the INVASLOG for a theoretical circle is shown where the dependence of the INVASLOG on the pixilation of the image can be seen. The integer values for the calculated area and perimeter of idealised circles was taken to simulate the pixilation effect on the calculation of the INVASLOG.
Figure 64 Shows the change in INVASLOG with the size of the object concerned. Here objects with the same area but differing form have been examined. The triangular form as a regular shape rapidly attains its typical INVASLOG value a little above 0.2. With all other figures (except the linear one pixel wide rectangle) they do not pass a value typical for its form (Figure 65).

Although at low values, due to pixilation, the values of INVASLOG depart from the expected. This would suggest that the INVASLOG represents the degree of linearization of single objects because values over 0.2 are only seen here in objects that are stretched along a particular axis departing from the regular geometric form.

Number of objects

The INVASLOG is determined from the sum of the areas and the perimeters of the objects found in an image. Since single objects attain maximally if they are regular geometric objects a value of 0.2 or less. What happens when the number of objects increases? The effect on the INVASLOG can be seen in Figure 66 the same data are shown in Figure 67 but scaled logarithmically, this shows that the form except for rectangular or irregular forms does not have much of an effect but their number changes the INVASLOG substantially and linearly when considering the $\log_{10}$ of their number.

The INVASLOG of these objects is interesting, but what is the situation in reality and can these data be applied such that more information may be attained than that at first glance?
Figure 63. The change on the INVASION for a hypothetical circle the diameter of which is plotted along the x-axis.
Respect to their geometry.

Figure 64 For simple regular geometric objects with the same area the INVERSE changes with
Figure 65 Maximum expected INVLASLOG for a single polygon.
The effect for a square, rectangular, hexagonal, or various lengths of rectangular objects and their number on I/NVSA TLOG are compared here. Several geometric shapes were tested and compared to theoretical determined I/NVSA TLOG.

Figure 66 Here several geometric shapes were tested and compared to theoretically determined I/NVSA TLOG.
Figure 67 as Figure 66 but scaled logarithmically

Number of objects

Inverselog and object number

\[ R_2 = 1 \]
\[ \text{Hexagon: } y = 0.435ln(x) + 0.0402 \]
\[ R_2 = 1 \]
\[ \text{Triangle: } y = 0.4347ln(x) + 0.2171 \]
\[ \text{Linear 100: } y = 0.4326ln(x) + 1.5991 \]
\[ R_2 = 1 \]
**Invading object size and Host Spheroid size**

The effect of object size and number when invading a circular object has been simulated and is demonstrated in Figure 68 and Figure 69. Taking into consideration how the INVASLOG is measured i.e. it is measured as the sum of the perimeter and the sum of the area found in the fluorescence channel representing the stroma or host material, variations in the change in the perimeter and area that simulate that found under normal experimental conditions were calculated.

In Figure 68 the effect on an idealised target object is illustrated. The results have been calculated using a mathematical model of initial constant target size to invader size relationship and then the relationship of the invading object (the green invading cells) and the host (red target) object (the host cell spheroid) was varied and the simulation repeated resulting in Figure 69. Two possible forms of invasion were taken into account i.e. destructive invasion being the destruction of the stroma on entry of the invading cells into the stromal spheroid or passive invasion in that the invading cells do not destroy the stroma but merely push it to one side.

**Destructive invasion.**

Assuming that for mathematical purposes the invasion seen is a result of destruction of the target material. This would result in an increase in the perimeter with loss of the associated area from the host occupied by the invading particle. Assuming also that the invader is circular in form (this of course is not the real case but for simulation purposes here adequate) a change in the INVASLOG would be seen as in Figure 69 (solid lines) where the ratio in diameter of the host spheroid to that of the invading particle can be
Figure 68 Simulation of invasion Note that the final volume in destructive invasion is smaller than that resulting from passive invasion.
In Figure 69 it can be seen that where the invading object has a size that is less than one tenth of the host spheroid no difference can be seen between destructive and passive invasion.

Although this may be seen as a disadvantage, the behaviour of the INVASLOG does not change when the particle size of the invader is less than 10% of the object to be invaded see Figure 69. This makes the INVASLOG here interesting as a parameter as in the system used for confocal microscopy the size of the cells is about 10μm in diameter and the target tissue used (spheroid) is between 100 and 120μm in diameter. Although the difference between destructive invasion or passive invasion cannot here be directly determined from the INVASLOG. Time has not been taken into consideration here and certainly plays a role in determining the invasive behaviour of the cells involved.

The model presented here does not take growth or proliferation into account.
The effect on the INAASLOG solid lines are destructive invasion dotted passively Invasion.

Figure 69: Destructive or passive Invasion a comparison of the invading object size to the host object size and relationship host diameter cm.

![Graph showing the relationship between host diameter cm and invasion size](image)
c1d/c08/c09/c07/c19/c16/c09/c19/c01/c08/c04/c46/c10/c18/c19/c01/c07/c06/c3d/c10/c01/c06/c09/c18/c0c/c10/c16/c07/c06/c09/c12/c01/c09/c03/c05/c04/c10/c0c/c1a/c11/c10/c07/c19/c0c/c03/c18/c19/c06/c22/c10/c01/c06/c09/c22/c16/c07/c06/c08/c09/c1c

Figure 70. Descriptive inversion: Increasing object number of constant size (destructive inversion)

INVASTLOG is calculated and taken into account. An initial size of the stream is taken as being 150 pixels in

The theoretical limits when the form of the stream from which the

Diameter
Figure 71: Passive invasion. Increasing object number of constant size and change in INVASLOG.
Figure 72: Here the change in the INVASTLOG can be seen where the sum of the area of the individual linear segments remains constant, but the number of objects increases.

Constant total area increasing object number (object size = area/number of objects)
or passive invasion of a host of 150 pixels diameter by objects of 15 pixels in diameter.

blue the change in EVASIOG with increasing numbers of linear objects of constant size and in red Descriptive measuring circles with pixels where the centre of the circle is ideally placed for measurement. The rest are in EVASIOG found when examining perfect circles. The EVASIOG predicted shows the discrepancy when Figure 73 The theoretical limits of the EVASIOG. The line EVASIOG theoretical represents the

![Graph showing object number vs. object diameter with lines representing different scenarios.](image-url)
Figure 74 as in Figure 73 with the addition of real data from confrontation experiments with K1735-M2

K1735 M2 against CH day 3
check heat on day 3 of the conjunction.

Figure 7.5 as in Figure 7.3 with the addition of real data from conjunction experiments with A375-SM against

A375-SM against CH day 3
Figure 76 as in Figure 73 with the addition of real data from confrontation experiments with all data from confrontation of KS1735-M2 or A1775-570 or A2058 or Chick Heart against Chick Heart on day 2 and 3 of the experiment.
Object size and number

In Figure 70 The effect of destructive invasion on a host object of 150 pixels in diameter of objects of varying diameter and size is shown.

In Figure 71 The effect of passive invasion on a host object of 150 pixels in diameter of objects of varying diameter and size is shown.

In Figure 72 The change in the maximum INVASLOG for linear objects with constant area sum i.e. a single linear object of e.g. 4096 pixels in area is subdivided successively into ever smaller objects but with in total a constant area.

In Figure 73 The various limits of the INVASLOG are shown.

In Figure 74 and Figure 75 as well as Figure 76 an alternative approach was used to elucidate some of the properties of the INVASLOG parameter. Here the same information as that in Figure 69 was plotted as a function of the object number.

For the value of INVASLOG reachable during the invasion of the host sphere by circular objects, the maximum reachable INVASLOG calculable for the object to host ratio of 150:16 and was plotted against the expected values of INVASLOG for increasing object number.

The maximum INVASLOG was plotted for objects having in sum a constant area and increasing object number i.e. 256 objects of one pixel in size or one object of 256 pixels. These data were plotted against the object number.

These data were also plotted along with actual experimental data from four experimental runs using chick heart, K1735 M2, A375 SM, A2058 and two
different time points of 48 hours and 72 hours. This amounted to 720 data points plotted as a scatter plot on this figure. It can be seen that the data points do not cross below the 16:150 object to host spheroid ratio and it must then be assumed that the ratio of sizes approximately equal 16:150. Which is not surprising when the host spheroid is between 100 and 120 µm in diameter and the single cells between 10 and 15µm This data needs further analysis and may reveal some other useful aspects of this research. Further investigations should make use of Fluorescence Assisted Cell Sorting (FACS) in order to corroborate these data.
**Specimen orientation**

The degree of invasion as estimated by the INVASLOG requires that the confrontation pair is sectioned along an axis parallel to the confrontation axis in order to obtain results that are comparable to each other where the degree of variation has been minimised. A rotation about this axis should have little effect but a rotation about an axis departing from this orientation will result in increasing values of INVASLOG therefore producing erroneously high results. The section chosen should be as near to the axis of the spheroid confrontation pair as possible.

Because the confrontation is symmetrical about this axis the question arises, how does the INVASLOG vary with section depth when sections are made parallel to the axis of confrontation? In Figure 77 the mean values seen at a particular section number is shown. The INVASLOG is as always calculated from the host tissue spheroid. In Figure 78 the variation seen in a single experiment has been shown to illustrate variations in the section depth and the INVASLOG at a particular depth.
Normalisation of INVASLOG Against expected values

The INVASLOG is limited by the size of the pixel relative to the object being examined and the total size of the observation window. If the object being examined is totally contained within the field of view then the INVASLOG is only limited by the total number of pixels covered by the object or objects being examined i.e. assuming that all the pixels detected in the channel for the stromal spheroid are linearised to form a single object with the length equivalent to the number of pixels then a maximum value for the INVASLOG for that object can be determined.

e.g. a 512x512 pixel square contains 262144 pixels in order to attain the maximum INVASLOG only half of them may be used 131072 pixels. This results in an area of 131072 and a perimeter of 2 x pixels + 2 = 262146 but when the line is tilted to 45° the perimeter is pixels x 4 there fore it follows.

\[ \text{INVASLOG}_{\text{field max}} = -\log_{10} \left( \frac{4 \cdot \pi \cdot \text{pixels}}{\left( \text{pixels} \cdot 4 \right)^2} \right) \]

Formula 8 Maximum attainable invaslog for a given field of view. Pixels=total pixels from the field of view/2

The maximum attainable INVASLOG can be read from Figure 64 where it can be seen that low numbers of pixels lead to misleading results in determining the INVASLOG. Objects with a size less than 10 pixels in diameter lead to aberrant results and with this in mind objects with an area of less than 70 pixels are not taken into account.
Figure 77: Change in INVASION with section depth after 48 hours confrontation with an embryonic chick heart cell spheroid.
Figure 7.1 INVASLOG variation with section depth data for a single experiment on day 2.
on day 2 (xx-2) and day 3 (xx-3).

Figure 79: In-vitro growth with section number (depth) for various cell lines confronted with embryonic chick heart
Figure 80 Invasiorge for comparisons on day 2 and day 3 normalized to Invasioge Max

[Graph showing data with various lines and markers, detailing comparisons on day 2 and day 3 normalized to Invasioge Max]
Figure 81: Invasive for conformation on day 2 and then normalized to the

value for KN735 M2
normalised and adjusted to t max

<table>
<thead>
<tr>
<th>Cell line</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2058</td>
<td>16</td>
</tr>
<tr>
<td>A375SM</td>
<td>6</td>
</tr>
<tr>
<td>CH</td>
<td>28</td>
</tr>
<tr>
<td>K1735M2</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 7 N values for the figure Figure 77 and Figure 78

\[ \text{INVASLOG}_{\text{max}} = -\log_{10}\left(\frac{4 \cdot \pi \cdot \text{area}_{\text{det}}}{(2 \cdot \text{area}_{\text{det}} + 2)^2}\right) \]

Formula 9 INVASLOG<sub>max</sub>

\[ \text{Area}_{\text{det}} = \text{Area detected} \]

For the normalisation of the data, as the size of the surviving spheroid is unknown and its relation to the size of the invading cells is also unknown but can be estimated from initial values, a simplified method of normalisation was used i.e. the INVASLOG<sub>max</sub> was determined and the calculated INVASLOG expressed as a fraction of the INVASLOG<sub>max</sub>. This makes use of the detected area and the INVASLOG calculated for that area. As can be seen in Figure 80 this increases the range of section depths where the INVASLOG may be measured successfully and decreases the need to measure exactly in the middle of the confrontation that may well be difficult due to absorption effects.
Observation of invasion

Here in Figure 82 the development of the invasion process with time can be seen. These are images of different spheroid pairs at varying time points after confrontation. In Figure 83 the associated image analysis from the images in Figure 82 is shown. The inherent variation between spheroid pairs also presents a statistically relevant problem when carrying out studies in this fashion. A sufficient number of spheroid confrontations need to be examined whereby the ideal time point for the examination may vary from cancer to cancer.

In Figure 83 the images from Figure 82 have been analysed and the images produced by the analysis process are shown with the resulting values for the INVASLOG calculation.

In Figure 84 the 16 serial sections from the 44 hour confrontation pair is shown where the signal intensity loss with depth can be seen.
K 1735-M2 stained with CMTMR (Red)  
Embryonal Chick heart Stained with  
CMFDA (Green)

Figure 82 Initial development of the invasion process with time
Figure 83 Gray scale and binary images of the invasion process
Figure 84: serial optical sections of the confrontation seen in Figure 86.
From the serial sections in Figure 84 the 3D reconstruction in Figure 86 and Figure 87 was possible. Red are the chick heart cells and green are the K-1735 M2 mouse melanoma cells.

Figure 85 Surface reconstruction of early confrontation

Here in Figure 85 it can be seen that after 24 hours of confrontation some chick heart cells appear at the opposing side of the confrontation pair showing that not only the cancer cells are motile. Due to the single time point method used here it is not possible to know what route the cells took to reach their present position. It may have been around the spheroid or through it.
Three dimensional reconstruction of a confrontation pair from a series of optical sections

Figure 86 3D-reconstruction of a confrontation between an embryonic chick heart multicellular spheroid with a K-1735 M2 multicellular spheroid. Green are the chick heart cells, red are the K-1735 M2 mouse melanoma cells. The black areas represent areas where both signals were detected.
Figure 87 3D-reconstruction from serial sections of a confrontation between K-1735 M2 and embryonic chick heart multicellular spheroids

In the panels on the left are the signals detected from the chick heart cells and in the panels on the right those from the K-1735 M2 cells. The top panels are the same as the lower ones except that a rectangular cut into the spheroids has been made to show that the K-1735 M2 cells have surrounded the central core of chick heart cells.
Comparison of the histological with the confocal method.

<table>
<thead>
<tr>
<th>Transmission</th>
<th>Fluorescence</th>
<th>Image analysis</th>
</tr>
</thead>
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<td><img src="18h.png" alt="Image" /></td>
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<td><img src="38h.png" alt="Image" /></td>
<td><img src="38h.png" alt="Image" /></td>
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<tr>
<td><img src="105h.png" alt="Image" /></td>
<td><img src="105h.png" alt="Image" /></td>
<td><img src="105h.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Histological sections**

| 12h | ![Image](12h.png) | ![Image](12h.png) |
| 144h | ![Image](144h.png) | ![Image](144h.png) |

*Figure 88 Comparison of histology with confocal microscopy*²¹⁵
The earlier methods for the investigation of invasion required the use of histological methods, see Figure 88, including fixation and staining with immuno-histochemical techniques. This carried with it several difficulties and disadvantages, the fixation can lead to shrinkage effects, the section made must pass through the axis of confrontation and in order to assure this the spheroids must be of sufficient size so that the orientation through the use of differently sized spheroids may be deduced and then oriented for sectioning appropriately. The size of the spheroids were 400 and 200 μm for the stromal and cancer spheroids respectively. With respect to necrosis this is not only a very large risk at this size of spheroid but almost definite. Neo-angiogenesis is also to be expected at sizes above 150μm and so the choice of spheroids above this size carries with it the risk of observing effects which are in situ significantly influenced by neo-angiogenesis and do not longer reflect the invasive process per se. The numbers to the left of the panels in Figure 88 are the hours of confrontation.
**Motility analysis**

A single spheroid was selected from cultures prepared as above and washed in Hank’s buffered saline solution. The washed spheroid was placed on a coverslip on the bottom of a well of a 12 well tissue culture plate. The coverslip was pre-coated with selected extracellular matrix components. In order to elucidate the directed motility after initial growth the outgrowth from the spheroid is measured by microscopical examination and the determination of the circle diameter that encloses all cells present. The spheroids are cultured for a further time period and a second circle diameter determined. The difference represents the directed motility as difference in the diameters / time. See Figure 89.

Extreme caution had to be used in selection of the spheroids as small differences in the spheroid diameter had an effect on the initial velocities and so the 24hr to 48hr circle diameters were taken also to reduce this initial aberrant effect.
Figure 89: Directional migration evaluation of directional migration on glass.

- A375-SM
- A375-SM + HF-HER
- HF-HER

96 hours 72 hours 48 hours 24 hours
Figure S9: Histogram of the mortality of K1735 M2 and chicken heart cell lines on various extra-cellular matrix components measured using spheroid outgrowth.

Mortality on Glass and ECM components.
As can be seen in Figure 90 the motility observed using the directional motility method of observation shows the chick heart cells that were tested had a higher degree of motility than the cancer cells that are highly invasive in invasion assays *in vitro* and *in vivo*. This suggests that motility and invasion are separate functions. The reaction to treatment with All-trans Retinoic Acid (RA) reversed the observed motility (the motility was calculated as the difference between the 24 hour values and the 48 hour value of the circle diameter and is expressed as μm per 24 hours) and in general the motility of the k1735 cells on the various surfaces demonstrates a more "normal" pattern. It has been demonstrated earlier by our group\(^{222}\) that complete inhibition of motility completely inhibits invasion but the incomplete inhibition of motility does not hinder invasion. It has also been shown that membrane intercalating substances also changes the behaviour of the cells\(^{223,224}\)
Correlation of motility with invasion.

Figure 91 The main observation made during the process of invasion schematically drawn for comparison as observed during confrontation experiments.
Figure 92 Forms of invasion observed during experiments

The typical forms presented during invasion experiments can be seen in Figure 91 and Figure 92 where the panels in Figure 91 1 to 4a represent that commonly seen in highly motile metastatic cancers. The panels 1 to 4b represents a confrontation of chick heart cells with a spheroid of the same cells type stained differently i.e. one green and one red. This is made possible due to the use of the fluorescent makers that do not rely on a particular attribute of the cells for marking them as with immuno-histochemical means. The (a) series seems to reflect a higher motility of the one cell line to the other. The (b) series seems to show contact inhibition. The (b) form was almost always seen in chick heart versus chick heart, the (a) form was almost always seen when cancer cells were confronted with chick heart where the cancer cells surrounded the
chick heart cells. This raises the question, why? when the chick heart cells are apparently more motile, see Figure 90. It may well be the result of the tissue interaction on a signalling basis where the stromal tissue may well be involved in stimulating the cancer tissue to move. Alternatively the cancer cells were also confronted with themselves and exhibited identical behaviour as the chick heart suggesting that some interaction between the two cell types must take place to stimulate the invasive process whereas the incorporation of both spheroids to form one was unaffected. This would seem to suggest that the measure of invasion for glioma spheroids needs looking into and that the parameter used by Wild-Bode and others reflects only the speed of incorporation of the spheroids to a single spheroid i.e. the cell motility and not the invasiveness.127,225
The effect of retinoic acid on invasion

Retinoic acid is used in the treatment of cancer and has been used to investigate the processes involved in invasion\textsuperscript{216,226-232}

Here in Figure 93 the effect of using 1\(\mu\)M all trans retinoic acid (RA) on the invasion process has been studied. Control values of confrontations without (RA) were used and the difference plotted at 24 hours and 72 hours.

In the first 24 hours there seems to be an increase which then changes to a decrease in invasion after 72 hours, the time dependence of this process is certainly an important factor in treatment.

The reason for the increase may be an increased rate of movement and passive invasion leading to a new and differentiated structure that is then quiescent later or due to a delayed effect of the retinoic acid that may initially result in a higher degree of invasion.
Figure 93. The effect of 14M retinoic acid on the invasiveness of melanoma cell lines relative to control values.
The invasiveness of melanoma clones and fibroblasts.

Experiment 11 was evaluated twice both using automated and manual techniques for determining the INVASLOG. This was done in order to elucidate whether or not the automated methods produce reliable results. As can be seen in Figure 94 the standard deviation is very large and would seem to make the results unusable. The small n values result in large standard deviations with little reliability although the median value in comparison to that of controls (chick heart versus chick heart) compares well see Figure 95. In Figure 95 two experiments with each at least 20 spheroid pairs were carried out to illustrate the differences between the clones and the effectiveness of the INVASLOG parameter. The values were normalised to the median value obtained for K 1735-M2 in each of the respective experiments. When the results are normalised the results for Chick heart (CH) confronted with CH as well as the other results compare well when using the median values.

In Figure 95 the differences between the K 1735 clones can be well seen. Additional experiments have shown that the highly invasive human clone A375-SM here shows an unexpectedly low value for a highly metastatic and invasive cutaneous melanoma cell line. Further experiments using the two highly invasive human cutaneous melanoma cell lines A 375-SM and A 2058 Figure 96 has shown that the highly invasive mouse melanoma K 1735-M2 and A 2058 exhibit a similar level of invasiveness. This is what would be expected from a system where the invasiveness seen in vivo should be reflected in the in vitro results. The results shown here compare well with the literature.
Figure 4: Manual comparison of values of INVASION with standard error bars representing one standard error of the mean from the sample.
Figure 95 Comparison of the invasiveness of various cancerous clones.
Figure 96 Comparison of the invasiveness of two human melanoma clones A2058 and A375-SM after 48 hours with respect to K1735M2 and chick heart confrontations the numbers in the legend represent experiment numbers with automatic evaluation. The 3T3 cells are mouse fibroblasts as a control value for normal invasive (infiltrating) cells.

Additional experiments (Figure 96) have shown that the highly invasive human clone A375-SM here repeatedly exhibits an unexpectedly low value of INVASLOG. Although its response to Retinoic acid is exactly that which is to be expected. Further experiments using both A 375-SM and the highly invasive human melanoma cell line A 2078 Figure 96 has shown that the highly invasive mouse melanoma K 1735-M2 and A 2058 exhibit the same level of invasiveness.
**Dynamic observation of spheroid confrontation**

Time dependence of INVASLOG

As can be seen in Table 8 the INVASLOG increases with time. It would of course then be interesting to know the rate of increase and its behaviour during the invasive process.

<table>
<thead>
<tr>
<th>Hours after confrontation</th>
<th>Area in arbitrary units</th>
<th>Perimeter in arbitrary units</th>
<th>INVASLOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>7599</td>
<td>1644</td>
<td>0.454</td>
</tr>
<tr>
<td>38</td>
<td>34500</td>
<td>18525</td>
<td>0.898</td>
</tr>
<tr>
<td>105</td>
<td>27065</td>
<td>2347</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Table 8 The parameter INVASLOG (-log ((A x PI x area)/perimeter^2 )) has been calculated here from Figure 88*

Initial experiments to observe the invasive process dynamically have produced the results seen in Figure 97 and Figure 98. The initial section taken from the confrontation pair was placed such that the signal intensities were sufficient and the expected change in the spheroid diameter should not interfere with the signal.
Dynamic observation of the changes in INVASLOG.

The following Figure 97 and Figure 98 illustrate the change in the INVASLOG with time during a confrontation experiment. In Figure 97 from the time of confrontation the confrontation pair were imaged every 10 minutes until the available virtual RAM of the computer was full. (Unfortunately the software is programmed so as to enable fast physiology imaging (fast being relative to the operating system and control software as well as the speed at which the microscope can scan) but with the slow processor that it has there is a limit i.e. the available virtual RAM making longer observation a problem). The number of images that were recordable was here about 133 images.

The staining was changed so that the host cells were green and the cancer cells were red to demonstrate that the behaviour of the cells was stain independent i.e. the invasive behaviour was not differentially influenced by the stain used.

In Figure 97 a rapid rise in the invaslog with time can be seen. This rise is due to the absorption effect reducing the signal received and thus not being detectable then in the middle of the specimen. This is a known factor as discussed earlier and the section depth was then so adjusted that the complete data could be collected in Figure 98 and in Figure 99 the data collected in Figure 97 have been corrected using a perfect circle to cover the area of missing signal.

In Figure 98 after adjusting the section depth to one where the absorption of the signal was no longer a problem, a series of images with only 3 minutes between images was taken to demonstrate that little difference can be seen between 3 minute and 10 min images. 130 images were recorded here.
Spherical daimeter

Figure 97 Time lapse observation of the invasive process day 0 to 1. A section depth of approximately 1/4 the

Minutes from start/10

Day 0 to 1
Figure 98: Time lapse observation dependency of IVASLOG day 1 to 2 at section depth of 1/3 of the spherical diameter.
Through the use of additional circular objects to compensate for the loss of signal, Figure 99 corrects for the erroneous INVASTLOG data that was caused by absorption of the excitation light.
Time lapse images

The signal loss seen in Figure 100 due to absorption of the excitation laser light causes the INVASLOG to increase rapidly as can be seen in Figure 97 calculated from the same data. In Figure 99 an attempt at correcting this aberration has been made in that the area of lost signal was covered with a perfect circle placed such that as much of the signal loss area as possible was covered but keeping the circle as small as possible. As shown in Figure 63 single object of simple geometric form change the expected INVASLOG little and a circular form should result in no change. The resulting reduction in the invaslog compares well with the results with the absorption artefact when the invaslog is reduced by 0.85.

The numbers in Figure 100 are the image number in the sequence, between each image ten minutes had elapsed, this means the image number multiplied by ten is the time at which the image was taken after the start of the experiment. As can be seen in Figure 103 the stromal (in this case green) cells also play a role in invasion, on contact with the cancer spheroid the stromal spheroid also moves and rearranges itself not only on a cellular basis, i.e. each cell not only acting for itself but also as a whole. The restructuring that occurs in spheroid re-aggregates is well known and is used e.g. in stem cell culture and can aid in re-establishing the "normal" behaviour of the cells in continued laboratory culture. The dynamics of this may well shed light on the mechanics of the invasive process.
Figure 100 Video series of the fluorescence signals during invasion.

Confrontation of embryonic chick heart (green) with K1735 M2 cells (red). Day 0 to 1 The numbers are the minutes after confrontation divided by 10.
Figure 101 The fluorescence and transmission signals. The white bar top right is 100μm long.
Figure 102 the confrontation in Figure 101 27 hours after initial confrontation. The white bar top right is 100µm long
Figure 103 as in Figure 101 with an ellipse showing the initial from of the host spheroid illustrating the large movement of the stroma during invasion
The relationship of the INVASLOG to the fractal dimension

![Graph showing the relationship between INVASLOG and fractal dimension.]

Time between images is 3 mins, examination depth 40μm, from 28 hours after initial contact.

Figure 104 Time dependent changes in INVASLOG calculated from Figure 102 and the associated fractal dimension during confrontation of K1735 M2 and Chick heart multi-cellular spheroids. Day 1 to 2^{215,233}
The connection between the INVASLOG and the fractal dimension cannot be founded here but an indicator can be seen in Figure 104 where it can be seen that both the INVASLOG and the fractal dimension correlate relatively well although a dedicated investigation would be necessary to determine the dependence and to what degree they depend on each other.
Results Summary

Minimum pixel numbers required

The minimum number of pixels in area needs to be at least 70 to determine the INVASLOG for a single object. Where several objects are concerned the inclusion of objects with an individual area below 70 pixels will also lead to aberrant determination of the INVASLOG. In order to increase the sensitivity of the method the ratio of host spheroid diameter to invading cells should be increased i.e. the size of the host spheroid should be as large as possible.

Maximum expected INVASLOG

The maximum expected INVASLOG can be expressed as being the INVASLOG calculated from 50% of the pixels contained in the captured images with their respective area and perimeter.

\[
\text{INVASLOG}_{\text{field max}} = -\log_{10} \left( \frac{4 \cdot \pi \cdot \text{pixels}}{(\text{pixels} \cdot 4)^2} \right)
\]

\[
\text{INVASLOG}_{\text{max}} = -\log_{10} \left( \frac{4 \cdot \pi \cdot \text{area}_{\text{det}}}{(2 \cdot \text{area}_{\text{det}} + 2)^2} \right)
\]

Formula 10 Maximum expected INVASLOG is with
\( \text{INVASLOG}_{\text{field max}} \) from multiple objects covering the complete field of view where pixels = the total number of pixels in the field of view/2 and \( \text{INVASLOG}_{\text{max}} \) for an assumed single object where \( \text{area}_{\text{det}} \) is their number of pixels detected for an object.
Geometric form and INVASLOG
The geometric form has little effect on the INVASLOG where regular polygons are concerned. Where irregular polygons with concave forms are concerned this has a significant effect on the INVASLOG. As seen in several figures the linearization of the objects has a significant effect on the values of the INVASLOG. This would suggest that the form is significant but also the degree to which the host has been divided by the invading cells also increases the INVASLOG. The clinical significance for the estimation of invasion seems to be the degree of invagination and disruption of the host tissues. This would qualitatively seem to be obvious but here it is used as a quantitative parameter and enables a deeper insight into the decisions protocols used by a pathologist in the estimation of invasion, according to their long lasting experience in grading the invasiveness of cancers.

The fractal dimension and INVASLOG connection
The fractal dimension is a theoretical parameter for measuring the degree to which the outline of an object from an image represents a two dimensional or one dimensional object. For special regular geometric forms with repeating patterns the generalised fractal dimensions are known and the theoretical values have been elucidated. These values can only be reached where the image is infinitely large and the pixels infinitely small. This presents us with a small problem. The use of the fractal dimension would enable the use of a more intricate mathematical investigation of cancer invasiveness and should be less vulnerable to errors than the INVASLOG. The relationship of the fractal dimension to the INVASLOG cannot be overseen as they both determine the linearization of the objects found. The required resolution for the determination
of the fractal dimension has been determined and resides at an image size of at least 512 x 512 pixels\textsuperscript{234}. Therefore the determination of the fractal dimension in this experimental setup is feasible but its usefulness has as yet not been determined and so further investigation is necessary. The use of the fractal dimension on the dynamic study of the invasion process would enable insights into the entropy of the invasion processes and shed light in a quantitative matter on a natural dynamic process.\textsuperscript{221,235}

**Dynamics of invasion**

The study of the dynamics of invasion will help in shedding more light on the processes involved. The three dimensional structure of this investigation system emulates the *in vivo* situation but without the later influence of the immune system and angiogenesis. Here it has been shown that it is possible to view the invasive process dynamically in an *in vitro* three dimensional model and elucidate continuously a clinically significant parameter the INVASLOG.

**Role of stroma in invasion**

As can be seen in Figure 103 the stroma not plays biochemically a role but physically as well. The analysis of video data show that the stroma is extremely active even when the form has settled to approximately a sphere the cells within move and reorganise themselves in the spheroid. This is known from larger spheroids but is shown here for the first time dynamically. In data shown here in Figure 92 the activity of the re-aggregated spheroids when confronted with cells of the same cell type do not actively invade. This is true also for the highly invasive cell lines (data not shown here) illustrating some form of communication between the cells.
**Biomechanics of invasion**

The activity and biomechanics of cell movement are relatively well known but how the cells act in the spheroid environment and the activity found in apparently stable spheroids leads to questions as to the biomechanics involved and the processes that lead to their control with also the question what role does this play in invasion. Although it has been shown here that motility in the stromal spheroid may also contribute to the invasive activity further experiments would be necessary to elucidate conclusively the role the stroma plays in the mechanical activity during invasion.

**Cell line invasiveness**

It has been shown that the highly invasive human melanoma cell line A375 SM exhibits a low degree of invasiveness compared to K1735 M2 and A2058 whereas all are in animal experiments highly invasive. On investigation of this phenomena it was noted that A2058 produces a growth hormone constitutively whereas A375 SM does not. Initial experiments suggest that after two days of confrontation the invasion rate of A375 increases but decisive data was not available at this time.

**Apoptosis and invasion**

Unfortunately I was unable to investigate the role of apoptosis as suitable intravital fluorescent markers were not available.
Conclusions

The method presented here provides a reliable and proven method for the investigation of the early invasive processes and those involved in the establishment of metastatic tumours *in vitro*.

It has also been shown that the data collected has clinical relevance and the parameter used (INVASLOG) can be successfully used in the investigation of invasion *in vitro*.

One of the most significant advances is the possibility of observing the invasive process directly without the need for *in vivo* experiments where animals may be unduly stressed for the attainment of initial data where the *in vitro* method here can successfully replace such experiments.

The use of fluorescent probes in marking the cell cytoplasm enables the confrontation of the same cell type with itself and thus a value for invasion that is independent of the effects found when a non-invasive cell type is used and the spheroids merely change their form due to convergence of the two spheroids to another.

One of the restrictions of the fluorescent stains are their inherent half-life leading to loss of signal. A method that would change this situation and enable constant study would be cells containing GFP (green fluorescent protein) this would then enable long time observation without signal loss. The coupling of a second fluorescent protein that fluoresces only when for instance apoptosis takes place would make this an important and interesting tool for the investigation of the role of apoptosis in invasion.
Since it has been shown that the differences between clones of known varying invasiveness, as well as differences between clones classified as being highly invasive, it is worth investigating the reliability as well as its effectiveness in evaluating cancer therapies. A method for the statistical analysis that makes use of the expected values should be developed so that the data collected becomes more reliable and repeatable.

A recent publication has shown that invasiveness increases when treated with sub-lethal doses of radiation. Although in this case the methods for the investigation of the results, particularly in the spheroid confrontations are questionable as to their relevance for estimating invasion and may only be the result of the increased motility of the cells induced by the sub-lethal radiotoxic effects, as found in our experiments with retinoic acid where an apparent induction takes place initially but then the retinoic acid becomes effective in reducing the rate of invasion. These findings though, are significant for the treatment of patients with glioma and additional work on melanoma would be of significant interest.

The studies presented here have clearly shown that the dynamic process of early invasion of cells of a primary tumour into the three dimensional structure of normal tissue can be studied continuously with high precision. Quantification of this process is possible in excellent correlation to clinical data of experienced pathologists. Also cancer cell motility, a key factor of invasion and metastasis can be quantified and monitored continuously. In animal experiments no information can ever be obtained of the invasion process from the primary tumour (mostly artificially injected cells) or of cancer cell motility in the body or of cancer cell tissue interactions or of establishment of early metastasis. All
these early steps of the metastatic cascade cannot be studied or measured in vivo. Hence, these methods are superior in replacing animal experiments and in studying early stages of cancer in vitro.

**Diagnostic assays**

Three dimensional systems for the investigation of invasion have been used over a number of years (see introduction). There are a number of reviews by Mareel et al\(^{236}\) on molecular considerations, Friedl et al\(^{237}\) on escape mechanisms, Corcoran et al\(^{238}\) on applications in investigating brain tumours, Kim et al\(^{239}\) on applications in investigating breast cancer and Brooks et al\(^{240}\) on applications in metastasis research. The popularity of this method varies with the research aims as the technique is considerably more difficult to use than the commercially available kits for the investigation of invasion that make use of modifications of the Boyden Chamber (Millipore and Chemicon and TECAN amongst others) and is relatively fast compared to the 3D techniques.

The Boyden Chamber is a simple method for the investigation of cell penetration of various barriers either stimulated or unstimulated by a chemical or chemicals placed on one side of the barrier. The barrier may be either (i) an artificial membrane with holes of a specific size that may or may not be coated with a layer of a basal membrane components and/or components or alternatively with extra-cellular matrix components as found in the commercially available MATRIGEL (a product of Becton Dickinson), (ii) a cellular barrier formed by cells grown across the supporting membrane or (iii) the membrane itself may be formed from a placental membrane. In order for the evaluation to be automated fluorescence methods have been adapted to provide a semi-quantitative method of estimating the degree of invasion. The degree of
invasion or migration is estimated as a percentage of the applied cells that successfully cross the barrier.

The microenvironment of the 3D spheroid confrontation model is histotypically nearer to the in vivo situation than that of the Boyden Chamber or its variants or monolayer cultures\textsuperscript{241-243}. Other media such as soft agar are also used to demonstrate the attachment independent behaviour \textsuperscript{244} and in some cases shows a correlation with invasiveness (as measured by the Boyden Chamber) \textsuperscript{245} or the use of collagen gels \textsuperscript{246} for investigating capillary morphogenesis or 3D collagen sponges \textsuperscript{247} 3D scaffolds \textsuperscript{248-253}. In the review of Friedl et al\textsuperscript{237} a difference is made between amoeboid movement as can be seen by single cells traversing a surface in motility studies (see fig 89) or the mesenchymal migration found in tissues or in the 3D spheroid confrontation model. A combination of both mechanisms seems appropriate for the events found in vivo. In the 3D confrontation model presented here both mechanisms can be seen in the speed at which the invading spheroid surrounds the host spheroid probably using the amoeboid movement system and then as the host spheroid or the invading cell is surrounded the mesenchymal mechanism is used.

For diagnostic assays several methods are used that are faster than the culture methods. Blood samples for the testing of specific markers for estimating the cancer type and treatment before and after surgery \textsuperscript{254,255} the use of tissue microarrays for gene expression studies in the tissue biopsies \textsuperscript{256}. These tests can be completed in hours or one or two days. The use of such tests requires knowledge of the cancer to be investigated and the patient to be treated in order to determine an appropriate treatment program. The use of the 3D culture technique presented here requires at least a period of two weeks (under
ideal conditions) from the initial sample but would enable the investigation of the reaction of the patients own tissues to the chemo/radiotherapy. This at the moment can only be predicted but not observed before treatment begins.

**Future considerations**

The use of this method for the standardised screening of chemicals and drugs for their effectiveness in inhibiting invasion.

The use of this method for the screening of primary tumours for their invasiveness and their reaction to chemical and radioactive treatment with respect to the known patient history. This could help in the adaptation of treatment protocols to maximise their effectiveness with the reduction of side effects for the patient.

Using this as a diagnostic method the possibility of investigating patient biopsies and testing treatment methods on the patients own cancer tissue would also simplify the search for a suitable treatment regime for the patient concerned.
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Appendix

Publications to date
The influence of edge detection algorithms on the estimation of the fractal dimension of binary digital images

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The boundary of a fractal object, represented in a two-dimensional space, is theoretically a line with an infinitely small width. In digital images this boundary or contour is limited to the pixel resolution of the image and the width of the line commonly depends on the edge detection algorithm used. The Minkowski dimension was evaluated by using three different edge detection algorithms (Sobel, Roberts, and Laplace operator). Three of these operators were investigated because they are widely used and because their edge detection result is very distinct concerning the line width. Very common fractals (Sierpinski carpet and Koch island) were investigated as well as the binary images from a cancer invasion assay taken with a confocal laser scanning microscope. The fractal dimension is directly proportional to the width of the contour line and the fact, that in practice very often the investigated objects are fractals only within a limited resolution range is considered too.

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I. INTRODUCTION

The determination of the fractal dimension of two-dimensional digital images of objects is inherently restricted to a limited range of orders of magnitude, the pixel size of the image being the lower limit and the size of the image itself being the upper limit. Furthermore, the fractal of a two-dimensional object is defined as being its contour or boundary which is infinitesimally small. When the boundary of an object is detected by edge detection algorithms, the resulting line is always of a finite thickness. Because of this limitation it is only possible to make an estimate of the fractal dimension, this is due to the resolution limit making it impossible to resolve the fractal dimension exactly. Moreover, many experimental fractals cannot be considered as geometric fractals, because the self-similarity is not valid over an infinite range of order of magnitudes.

All these aspects lead to the problem that the practical estimation of the absolute value of the fractal dimension could be inaccurate and the values derived lack reliability. As a consequence the estimation is often restricted to relative values, for instance if the fractal dimension is different for pathological tissues in comparison to healthy tissues. To address this widespread practical and interdisciplinary widespread problem, three edge detection algorithms, commonly used for image processing tasks were applied to two sets of artificially generated fractal images and to one set of experimental images. Each set of fractal images contained six images with a varying range of self similarity, representing the practical condition that the experimental objects are very often not self similar over many orders of magnitude.

II. METHODS

A. Edge detection

The Sobel, Roberts, and Laplace operators were used to detect the edge (contour, boundary) of objects in the binary images. More specialized algorithms such as Canny, derivatives of the Gaussian kernel or smart edge linking methods are not common at all and are mainly used for gray value images and for extracting the edges directly from the gray value image. Here the edges are detected from binary images, either the binary images of common fractals (Sierpinski carpet, Koch island) or the binarized images of the cancer invasion images. As the binary images represent the object, the object identification is not carried out by the edge detection algorithm but by the binarization step, especially for the cancer invasion images. Edge detection of binary images is done well by these three operators and there is an advantage in using these operators as they are widely used and are implemented in many image processing software packages.

The edge detection operators were approximated by convolution of the images with following kernels:
All these kernels have the same property of summing up the neighboring pixels, weighted by the corresponding integer numbers of the kernel and are subtracted from the central pixel, which is weighted with the corresponding integer number of the kernel too. Consequently, in a region of uniform pixel values the result of these kernels will be the value of zero. If there is a discontinuity such as a point, a line or edge the convolution of these kernel give nonzero values and for the special case of binary images the value will be one (or 255 in an image with 8bit gray level representation). There are a variety of differences in the characteristics and constraints of each method such as a difference in noise dependence, orientation of the edge detection, etc. It was obvious that the Sobel operator would lead to the thickest edge (contour) compared to the Roberts or Laplace operator. The contour obtained by the Sobel operator was about 3 pixels in width compared to the contours created with the Roberts and Laplace operator which were about 2 pixels and 1 pixel wide, respectively.

Mainly the thickness of the contour influenced the values of the fractal dimension. These three different operators with their distinct contour widths were, therefore, chosen for investigation. Despite the fact that the Laplace operator leads to the thinnest contour line, it is in practice avoided, because of its relatively strong noise dependence and furthermore it leads very often to interrupted contour lines.

### B. Construction of the image sets

Three sets of images (each 512×512 pixels) were investigated, comprising of a set of Sierpinski carpets alternatively constructed with circles, a set of alternative Koch islands and a set of images taken from a cancer invasion study.

The first image of the Sierpinski carpet set was only the central circle, the second image consisted of this central circle and eight smaller circles around and so on up to six images [see Fig. 1(a) for the first three images]. The images were initially constructed with discs rather than lines and later on the edge detection algorithms produced circles.

The first image of the alternative Koch island was constructed with a four sided filled polygon, where each element (generator) of the polygon consisted of 3 sublines with $\Theta = 2\pi/4$, $\Phi$ being the angle between the first and the second subline and between the second and the third subline. By exchanging each subline with this generator up to six images [see Fig. 1(b) for the first three images] with increasing "sub details" were constructed (for details see the book by Mandelbrot$^3$). Both image sets, the Sierpinski carpet and the

![Fig. 1](https://example.com/fig1.png)

**FIG. 1.** Example images of the three sets of digital images. (a) Image #1, #2, #3 out of the set of six Sierpinski carpets constructed with circles instead of rectangles. (b) Image #1, #2, #3 out of the set of the four sided polygon. (c) Example image of an in vitro cancer spheroid (light gray) invading into a host spheroid (dark gray). The image was taken with a laser scanning microscope using double fluorescence labeling.

Koch islands contained six images numbered from 1 up to 6 and represented the fractal objects with an increasing range of self similarity. Nevertheless it is clear that the first few images of the sets and especially the first image of each set were not fractals but rather ordinary one dimensional objects.

### C. Images of cancer invasion

The experimental set of images consisted of 16 images of an in vitro cancer invasion model described in detail elsewhere.$^4$-$^9$ Isolated cancer cells and isolated chick heart cells were labeled with fluorescence dyes and cultivated to form spheroids. Cancer spheroids were confronted with chick heart spheroids and the course of invasion was measured by taking digital images with a confocal laser scanning microscope (Leica, TCS NT, Heidelberg, Germany). Both fluorophores were excited simultaneously with an ArKr-laser ($\lambda = 488$ and 568 nm). The fluorescence light was detected with a standard fluorescein–rhodamine (FTTC/TRITC) filter arrangement using a 530/30 nm bandpass filter and a 590 nm longpass filter. A $10\times0.3$ NA objective and a zoom factor of 2 was used and the 512×512 pixel images had an image size of 500 $\mu$m×500 $\mu$m. At this magnification a single pixel has 0.977 $\mu$m side length and a single cell typically has a size of 10–15 $\mu$m. This "pixel resolution" was in practice sufficient to calculate the estimate of the fractal dimension and was near the theoretically obtainable optical maximum for the apparatus used. Several optical sections (up to 16) were recorded as tif files from the Leica system.
These files were not converted into other formats to circumvent any degradation by converting algorithms (especially data compressing algorithms).

An example image is shown in Fig. 1(c). The light gray value of Fig. 1(c) represents the two-dimensional area of a K1735M2 mouse melanoma spheroid after invading into the host spheroid (dark gray value) for a duration of two days. Prior experiments showed that the boundary of the cancer area could be interpreted as a fractal and that there is a dependence of the fractal dimension with respect to the invasion process.1,12

D. Determination of the fractal dimension

The determination of the fractal dimension of an object represented by a two-dimensional image is characterized by finding the boundary of this object and by further steps, where only the boundary of the object is represented in the form of a curve in a two-dimensional space. As this curve is treated as a fractal, the length of the curve is dependent on the resolution of the measuring device. Let us divide the curve into separated line segments; then the length of a curve L is defined as

\[ L = N(r)r, \]

where \( N(r) \) is the number of line segments and \( r \) is the resolution or the length of these line segments. The common relation between the number of line segments \( N(r) \) and the fractal dimension \( D \) is

\[ N(r) = r^{-D}. \]

Therefore, the length \( L \) of the curve is

\[ L = r^{1-D}. \]

By using dilation and the concept of Minkowski for the definition of the length of a curve, it is possible to determine the fractal dimension \( D_m \), the Minkowski or Minkowski–Bouligand dimension.15

Minkowski introduced very basic definitions for the length of a curve and for the surface of an area by following descriptions.17,18 The integral \( \int f(x_1, x_2, \ldots, x_n) \) of a manifold is for \( n=3 \) well defined as the volume of an object in a continuous space. The foundation or the definition of the length of a curve or the surface of an area are more difficult to give in comparison to the term of the volume.

If \( C \) is a curve in a three-dimensional space, then each point on the curve can have a sphere projected around it with radius \( r \), \( r \) being constant and positive. All the points within the projected spheres along the curve represent a set of points with a distance \( \leq r \), where \( V(r) \) represents the resulting volume of this set. The length \( L \) of the curve \( C \) can then be defined as being

\[ V(r)/\pi r^2 \] as \( r \to 0 \)

\[ L = \lim_{r \to 0} \frac{V(r)}{\pi r^2}. \]

Let \( A \) be an area. Again the construction of a region with spheres centered in \( A \) of a distance \( \leq r \) defines a volume \( V(r) \). Then it is possible to define the surface of the area \( A \) to be \( V(r)/2r \) as \( r \to 0 \)

\[ A = \lim_{r \to 0} \frac{V(r)}{2r}. \]

By using arbitrary convex objects instead of spheres, Minkowski generalized this concept and gained very fundamental characterizations of volume and surface. Similar to the concept of Minkowski in three dimensions it is possible to find the length of this boundary or curve in two dimensions. The spheres are replaced by circles with radius \( r \) and similarly there is a region within a distance \( \leq r \) of the curve which defines an area \( A(r) \). The length of the curve \( L \) is then

\[ L = \lim_{r \to 0} A(r). \]

As Eq. (6) holds true for the limit \( r \to 0 \), Eqs. (6) and (9) for the length of a curve can be combined

\[ \lim_{r \to 0} r^{1-D} = \lim_{r \to 0} \frac{A(r)}{2r}. \]

Logarithmic expansion gives

\[ \log_{10} \left( \lim_{r \to 0} r^{1-D} \right) = \log_{10} \left( \lim_{r \to 0} \frac{A(r)}{2r} \right). \]

and further

\[ D = \lim_{r \to 0} \frac{\log_{10} A(r)}{\log_{10} 2r} = \lim_{r \to 0} \frac{\log_{10} 2r}{\log_{10} A(r)}. \]

Using the rule of L'Hôpital, the last term is

\[ \log_{10} 2r = \lim_{r \to 0} \frac{(\log_{10} e)/r}{\log_{10} A(r)} = 1 \] and \( D \) is then

\[ D = D_m = 2 - \lim_{r \to 0} \frac{\log_{10} A(r)}{\log_{10} r}, \]

which is the Minkowski or Minkowski–Bouligand dimension \( D_m \). The Minkowski dimension \( D_m \) was actually examined by dilating the (boundary) images with discs of increasing radii. Each point of the image is enlarged (dilated) by discs of radius \( r \) and for each radius \( r \) the sets of all points of the image are the corresponding areas \( A(r) \). The slope \( k_D \) of a double logarithmic plot of the resulting area \( A(r) \) (the number of pixels of the dilated boundary) in relation to the radii gives the Minkowski dimension

\[ D_m = 2 - \lim_{r \to 0} \frac{\log_{10} A(r)}{\log_{10} r} = 2 - k_D. \]

The discs were approximated by using rectangles with side length \( 2\pi + 1 \) (\( \pi_{\min} = 0, \pi_{\max} = 20 \)), measured actually in pixels.

The corresponding radius was approximated with

\[ r = \sqrt{(2\pi + 1)^2 / \pi}. \]
where \((2n+1)^2\) is the area of the rectangle and \(r\) is the radius of a disc with the same area.

III. RESULTS

Figure 2 shows a typical double logarithmic plot in the range of \(n = 0, 1, 2, ..., 17\). The linear regression line was calculated from \(n = 4, 5, 6, ..., 17\) and linearly extrapolated to the range \(n = 0, 1, 2, 3\). Although the distribution of all points is very linear, a closer visual inspection reveals that the slope for the smaller radii is different from the slope of the remaining radii. It turned out that for most of the images examined for this study there was a more or less prominent change of the slope at roughly \(n = 4\). Furthermore it turned out that the results differed strongly if small radii \((n \leq 4)\) were included or if a range consisting mainly of these small radii was chosen. These differences can be seen in Fig. 3, where the values for the fractal dimension \(D_m\) are shown for the set of Sierpinski carpets with the different edge detection algorithms as parameters. Figure 3(a) shows the values with \(n = 0, 1, 2, ..., 9\) and Fig. 3(b) shows the values with \(n = 5, 6, 7, ..., 12\). The theoretical fractal dimension of the Sierpinski carpet is 1.8928 and, therefore, it is obvious that the curves should start at a dimension value of one and then grow to the theoretical value as the self-similarity of the images increases in this order. Using the small radii for the regression line [Fig. 3(a)] leads to a very poor estimation of the theoretical fractal dimension, even for the images (#4 and #5) with a high degree of self-similarity. Furthermore the value for the one-dimensional object (image #1) is incorrectly overestimated using the Sobel operator. Using the larger radii for the regression [Fig. 3(b)] leads to an improved estimation of the theoretical fractal dimension value from image #3 onwards, and furthermore the overestimation of the Sobel operator for the one or nearly one dimensional objects is less prominent, also the differences between the distinct edge detection algorithms are less prominent. For both ranges of radii, the differences between the Roberts operator and the Laplace operators are less compared to that of the Sobel operator and show very often similar values and where they differ, the values of the Roberts operator are higher than the values of the Laplace operator.

Equivalent to Fig. 3, Fig. 4 shows this comparison for another object, the four-sided polygon with a theoretical fractal dimension of 1.3562. In this case where the theoretical fractal dimension is closer to one, the fractal values gained with small radii [Fig. 4(a)] are very uncertain especially for the Sobel operator starting with a value of 1.2 for the one dimensional object. Again there is a clear improvement when larger radii were used for the linear regression [Fig. 4(b)]. The differences between the edge detection algorithms are smaller and there is again the improved but slightly overestimated dimension obtained by the Sobel operator.

Figure 5 summarizes the results of the experimental images and compares both ranges of radii and the different edge detection operators. The estimated values for the fractal dimension \(D_m\) given in Fig. 5 are the mean and standard deviation of 16 images of an assay of K1735M2 mouse melanoma cancer spheroids invading into host spheroids. The difference for the range of radii is quite similar to the previous cases, as there is a smaller difference between the distinct edge detection operators for the large radii, also the Sobel operator results always in the highest value followed by the Roberts operator and the Laplace operator.

Figures 3–5 show two possible ranges of radii, other ranges were chosen and tested too. When the small range of radii was limited to even smaller values such as \(n = 0, 1, 2, ..., 5\) or less, the estimation of the theoretical values.

FIG. 2. Example of a double logarithmic plot for radii \(n = 0, 1, 2, ..., 17\) and a linear regression from \(n = 4, 5, 6, ..., 17\), extrapolated to \(n = 0\).

FIG. 3. Fractal dimension \(D_m\) of the Sierpinski carpet image set (#1 to #6). The parameter "Original" represents the images constructed "per se" with lines, the parameters "Sobel," "Roberts," and "Laplace" represent the images constructed firstly with filled objects and then afterwards processed with the respective edge detection algorithms to obtain the boundary lines. (a) Values derived with radii \(n = 0, 1, 2, ..., 9\) for the linear regression of the double logarithmic plot; (b) with radii \(n = 5, 6, 7, ..., 12\).
worsened. Expanding the larger range of radii to the range, e.g., \( n = 3,4,5,...,20 \) did not alter the results markedly. It turned out that excluding the range of radii smaller than 4 and expanding the range of radii to at least 10 or higher led to consistent and nearly equivalent results.

**IV. CONCLUSION**

First, the results presented show a difference in the absolute value of the estimation of the fractal dimension \( D_m \) depending on the actual edge detection algorithm used. According to the thickness of the contour line, the estimated value increased. This is a consistent result because thicker lines are more space filling and therefore the Sobel operator leading to the thickest line of about three pixels led to the highest dimension values. This led especially in the one-dimensional case to a 20% overestimation, whereas in the case of fractal or nearly fractal objects (images 5 or 6 in Figs. 3 and 4) the overestimation was not so prominent or even very small, thus practically negligible (images 5 and 6 in Figs. 3(b) and 4(b)). Both the Roberts and the Laplace algorithm led to a very good estimate of one-dimensional objects but tend to underestimate the values for fractal objects, especially where the small radii were chosen. With larger radii, roughly about \( n = 4 \) and at least up to \( n = 10 \) the absolute differences between the distinct algorithms were smaller.

Secondly, in this case, when the differences between the methods were small, the estimation of the theoretical fractal dimension was found to be the best and this for all three operators. Only for the object with a low fractal dimension (polygon) the Sobel operator slightly overestimated the real value.

From these results it is deduced that for experimental images of objects, the estimation of the fractal dimension could be processed with all three edge detection operators and the range of radii for the linear regression should be chosen, so that the differences between the results would be minimized. Then the value of the Roberts operator should give the estimation of the fractal dimension with a higher reliability. This procedure of finding the optimal range of radii could be implemented automatically and could give very promising results, but before that it must be tested further with other fractals and under other circumstances ensuring its reliability. For the experimental example presented here a value of about 1.26 for the K1775M2 carcinoma spheroid should be a very reliable value.

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How much resolution is enough?
Influence of downscaling the pixel resolution of
digital images on the generalised dimensions

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Abstract

Fractals are very often illustrated by digital images, this is a consequence of the widespread availability of electronic and computational equipment. These images are always limited by their pixel resolution. Subsequently the determination of dimensions for such images is restricted practically because for their calculation the range of scales is not infinite. Downscaling the pixel resolution of images to get images with reduced resolution worsens the achievable absolute computational results but surprisingly the changes in these values and especially the changes of the courses of the calculated generalised dimensions give very promising results. This is due to the qualitative nature of these changes with respect to pixel downscaling. This opens the possibility of determining the quality of the estimation of the fractal dimensions of any image with any particular pixel resolution. Furthermore the pattern of changes itself caused by downscaling is different between the individual fractals. Several geometrical fractals (Sierpinski gasket, a fern, a Menger gasket and a modified Menger gasket with circles) as well as an example of a measured fractal, the digital images of cancer invasion studies in vitro were investigated to study the influence of downscaling the pixel resolution of digital images on the estimation of fractal dimensions.

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1. Introduction

It is well known and mathematically well defined that the calculation of the fractal dimensions is only correct towards the mathematical limit of zero lengths.

As the digital image representations of fractals are principally limited to the pixel resolution and size of the image, exact calculations are not generally possible and therefore the determination of the dimensions is restricted to an estimation. If fractals are represented by pixels of a digital image the single pixel serves as the lower limit and the number of pixels for one side length of the image serves as the upper limit. The formulae for the determination of dimensions could be

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correspondingly adapted [1,2]. Besides this restriction there are many successful examples of interpreting digital images of objects as fractals and the estimation of dimensions gave very revealing information [3–10]. The degree of exactness or "goodness" of the estimate could be increased if the number of pixels for the representation was increased. Beside very special cases and applications, an image resolution of 512 × 512 or 1024 × 1024 pixels per digital image is today very common, for instance, in the field of microscopy, where when considering scanning techniques a huge amount of computer and data storage capacity has to be available in order to be able to handle a long series of images. Increasing the number of pixels would therefore increase the need for computer time and capacity dramatically and to impracticable heights and the calculations of the fractal dimensions would require prolonged computations too. From the practical viewpoint the restriction to the pixel size of a digital image cannot be avoided even when future computer capabilities will surpass those of today by several magnitudes. There is now and will be in the future the question, when fractals are represented by digital images, whether the actual pixel resolution is enough for a reliable estimate of the fractal dimensions.

This study will demonstrate a method for enlightening this question by downscaling the pixel resolution of the digital image under investigation. Downscaling leads to images with reduced resolution and therefore the determination of the dimensions will be reduced until the calculated values are meaningless. If the downscaling procedure changes the values only slightly it is proposed that there is the possibility to make useful statements about the actual image. Calculating the estimated generalised dimensions of downscaled images increases our knowledge of the effects of downscaling in comparison to single fractal dimensions such as the capacity dimension because there is in addition to a shift of the absolute values a pronounced dependence of the course or distribution of the generalised dimensions. These changes describe qualitatively the connections between the dimension calculation and the actual pixel resolution.

2. Methods

2.1. Fractals generated by an IFS

Several fractals were generated by an deterministic iterated function system (IFS) [11]. The Sierpinski gasket, a fern, a Menger gasket and a modified Menger gasket constructed with circles instead of squares were each implemented with the appropriate coefficients for the IFS. The Menger gasket is the two-dimensional analogue of the three-dimensional Menger sponge and is also designated as the Sierpinski carpet [12]. Two-dimensional digital images of these fractals were iteratively constructed with a resolution of 1024 × 1024 pixels. The iterations were stopped as the fine details of the fractals approached the single pixel resolution. Further iterations would cause for some fractals artificial effects like melting together of lines, caused by the limited resolution. This effect was more pronounced with the Menger gasket fractals in comparison to the other fractals chosen for this study. The actual number of iterates was 11 for the Sierpinski gasket, 15 for the fern and 5 for both Menger gaskets.

This images with 1024 × 1024 pixels were used for downscaling the resolution described below.

2.2. Generation of fractals with different initial pixel resolutions

For additional investigation and comparing the downscaled images the fractals generated by the IFS (Fern, Sierpinski, Menger gasket and modified Menger gasket) were not only generated with a pixel resolution of 1024 × 1024 but also with 512 × 512, 128 × 128, ..., 32 × 32 pixels. Despite that the iterations (especially for images with lower pixel numbers) could have been stopped prior, each of these images were iterated as often as the corresponding 1024 × 1024 pixel image had been iterated.

2.3. Experimental fractals

As an example of a fractal derived from in vitro, we took two-dimensional images of cancer spheroids. The methods of cultivating and aggregation of K1735/M2 mouse melanoma spheroids for the investigation of
cancer invasion, especially the double labelling with fluorescence dyes and the use of a confocal laser scanning microscope for the non-invasive capturing of digital images were described elsewhere [13,14]. Shortly, K1735/M2 mouse melanoma spheroids were confronted with embryonic chick heart spheroids and the invasion process was imaged with a confocal laser scanning microscope (Leica, TCS NT, Heidelberg, Germany). The digital images with a pixel resolution of 512 × 512 were corrected for cross-talk signals and binarised [15]. Cross-talk occurred because the emission spectra of the fluorescent dyes unavoidably overlapped and the separation capabilities of the dichroic mirrors and filters of the microscope were not perfect. Therefore, some light emitted from a specific dye is unavoidably collected by the photosensitive corresponding to the other dye. This resulted in images of the cancer spheroid with some information (normally with reduced grey values) from the host spheroid and vice versa. The cross-talk correction by means of image processing techniques is described in detail in Ref. [15], which eliminated the cross-talk signals very well and revealed clearly separated images of the cancer spheroids and the host spheroids, respectively.

The boundary images of the binary images were generated by the application of the Roberts operator. Nineteen images from the second day of invasion were investigated. Recent studies, where the boundary of these spheroid images were treated as fractals were very successful [16,17]. In addition the boundary of the spheroids have been shown to be fractal, the estimated fractal dimension was a very good quantitative measure of the cancer invasiveness.

2.4. Downscaling of images

In order to investigate the relation between the actual pixel resolution of the fractal image and the generalised dimensions we generated from initial images subsequent images with, respectively, halved numbers of pixels per side length. This downscaling or "resizing" of the images was done using the standard method of neighbourhood averaging. As neighbourhood averaging of binary images resulted in grey value images, the interpolation was followed by conversion to binary images, where all grey values equal to zero were interpreted as being background and all grey values greater than zero were interpreted as corresponding to the actual object of the image.

Images with less than 32 × 32 pixels were not investigated. The initial images for the fractals generated with the IFS (Fern, Sierpinski, Menger gasket and modified Menger gasket) were the images with 1024 × 1024 pixels and for the cancer images the original 512 × 512 pixel images. Therefore from the fractals generated with the IFS we got downscaled images with 1024 × 1024, 512 × 512, 256 × 256, ..., 32 × 32 pixels and from the experimental cancer spheroid images we got downscaled images with 512 × 512, 256 × 256, ..., 32 × 32 pixels. With this procedure the individual fractals were preserved in their geometrical proportionality but the details got increasingly lost by reducing the number of pixels. For each image out of a downsampling sequence the generalised dimensions were calculated and graphically plotted in one plot.

2.5. Summary of the image generation

For clearance, a short summary of the distinct image sequences generated for this study is given here.

For each fractal generated with the IFS (Fern, Sierpinski, Menger gasket and modified Menger gasket), two series of images with 1024 × 1024, 512 × 512, ..., 32 × 32 pixels were constructed. The first series was generated by downsampling a 1024 × 1024 image and the second series was generated by applying the IFS algorithm itself to construct images with differing pixel resolutions.

For each image of the cancer spheroid only the series 512 × 512, 256 × 256, ..., 32 × 32 generated by downsampling the original 512 × 512 images were constructed.

2.6. Generalised dimensions

The generalised dimensions \( D_q \), introduced for strange attractors and for \( q \geq 0 \) by Hentschel and Procaccia [18], are in the meantime widely applied to investigate measured time series but could be used to characterise fractal images too. Expansion
for $q < 0$ gives theoretically a decreasing sigmoid function [19–21]. The generalised dimensions $D_q$ are mathematically defined by

$$D_q = \frac{1}{q-1} \lim_{\varepsilon \to 0} \frac{\log C_\varepsilon(q)}{\log \varepsilon},$$

where $q$ could be in the range of $-\infty, \ldots, 0, \ldots, +\infty$ and $\varepsilon$ the side length of a $d$-dimensional cube $\varepsilon^d$. $C_\varepsilon(q)$ is the generalised correlation integral and can be written as

$$C_\varepsilon(q) = \frac{1}{N} \sum_i \left( \frac{1}{N} \sum_j \theta(\varepsilon - |\tilde{X}_i - \tilde{X}_j|) \right)^{q-1},$$

where $\theta$ is the Heaviside step function, $N$ the number of points of the fractal, and $\tilde{X}_i, \tilde{X}_j$ the individual points of the fractal. The correlation integral ($q = 2$) could be simply explained as the sum of pairs of points which have a distance $|\tilde{X}_i - \tilde{X}_j|$ less than $\varepsilon$.

For $q = 1$ the corresponding dimension $D_1$ is defined by

$$D_1 = \lim_{\varepsilon \to 0} \frac{C_\varepsilon(1)}{\log \varepsilon}$$

with

$$C_\varepsilon(1) = \frac{1}{N} \sum_i \log \left( \frac{1}{N} \sum_j \theta(\varepsilon - |\tilde{X}_i - \tilde{X}_j|) \right).$$

As $q$ is in the range of $-\infty, \ldots, 0, \ldots, +\infty$ there are an infinite number of different dimensions but only integer values for $q \geq 0$ may have a physical meaning. $D_0$ is the similarity dimension, $D_1$ the information dimension and $D_2$ the mentioned correlation dimension. In expansion of this, the dimensions $D_3, D_4, \ldots$ are associated with correlation integrals of triplets, quadruplets $\ldots$ of points of the fractal. Theoretically $D_q < D_q'$ for $q > q'$.

For the digital two-dimensional images used for this study the generalised dimensions were estimated by counting for each object pixel the number of neighbouring object pixels within the distance $\varepsilon$. $N$ was the total number of object pixels and $\varepsilon$ was also interpreted in pixels. The sums as defined in the formulas above were calculated and from the slopes of the graphs of the sums versus $\log \varepsilon$ the dimensions for $q = -20$ up to 20 were estimated. The range of $\varepsilon$ was chosen to be $\varepsilon = 2n + 1$ with $n = 0, 1, 2, 3, \ldots, 8$, reflecting the main linear part of the double logarithmic plot. The actual range of $\varepsilon$ is crucial, because the calculated spectrum of the values of $D_q$ depends on it. It is possible to create for an object under investigation a spectrum which is closer to the theoretical falling sigmoid function. But this setting is very special for each object and there is no commonly applicable rule for choosing this range. Furthermore, for this study the changes of the spectrum during downscaling of the images were the main interest and not the appearance of the initial spectrum itself. Therefore the range of $\varepsilon$ was held constant for all the objects under investigation.

3. Results

The uniform scales of each of the plots (Figs. 1–4) which will be presented in this section were chosen to emphasise the differences between the distinct fractals. The values of $D_q$ are shown in each figure in the range of 0–2 and therefore especially the values for the Sierpinski gasket (Fig. 1(b)) are very close together compared to the other investigated fractals. As a first and common result it must be stated that the graphs of the generalised dimensions $D_q$ mostly did not show the theoretic decreasing sigmoid function as mentioned in the method section. But this finding is not in disagreement with the literature, where similar results have also been shown [22–24].

Fig. 1(a) shows the generalised dimensions $D_q$ of the fern for the subsequent images generated by downscaling the 1024 × 1024 pixel image. The values of $D_q$ from the first two images (1024 × 1024, 512 × 512) are nearly indistinguishable for $q \geq 0$ and show a slight deviation for $q < 0$. The $D_q$ values for the 256 × 256 image have minor deviations when compared to the 1024 × 1024 image but this throughout the whole range of $q$'s. The values for the small images (128 × 128, 64 × 64, 32 × 32) are very different for $q < 0$ but there is still a conformity for $q \geq 0$. The values for the Sierpinski gasket (Fig. 1(b)) show the highest overall conformity for all $q$'s when compared to the fern and even to the other fractals presented in the
Fig. 1. Generalised dimensions $D_q$ of a fern (a) and the Sierpinski gasket (b) for the range of $q = -20$ up to 20. The legend shows the pixel resolutions of the subsequent images generated by downscaling the 1024 $\times$ 1024 pixel image.

The following figures and is in accordance with the monofractality of the Sierpinski gasket. Furthermore the values of the first three images (1024 $\times$ 1024, 512 $\times$ 512 and 128 $\times$ 128) are very close together. In Fig. 2 are the values and distributions of the generalised dimensions for the Menger gaskets shown. Compared to the fern and the Sierpinski gasket the values for the Menger gaskets have less conformity especially the modified Menger gasket constructed with circles (Fig. 2(b)). Nevertheless there are some correspondences. In Fig. 1(a) the values for the 1024 $\times$ 1024 image and for the 512 $\times$ 512 image show a very similar course despite of a nearly constant shift of the absolute values. Furthermore the values for the 256 $\times$ 256 image are very close to the values from the 512 $\times$ 512 image. The values for the images with less than 256 $\times$ 256 pixels show no more than a real correspondence even not in the course. Although the values for the modified Menger gasket in Fig. 2(b) have a pronounced variation there still is a very obvious conformity of the course of the values of the images 1024 $\times$ 1024 and 512 $\times$ 512. Fig. 3 shows the average values for the experimental fractal, the K1753/M2 mouse melanoma spheroids (only the light grey area of the insertion). The dark grey area represents the chick heart spheroids (host). The variation of the values is about in the range as the modified Menger gasket (Fig. 2(b)) and once again there is
a very evident correspondence of the course of the values of the first two images, in this case the images with 512 × 512 and 256 × 256 pixels. As in the case of the fractals generated with the IFS the values for the generalised dimensions $D_q$ of images with less pixel numbers deviate very strong and furthermore the courses of the values are very distinct. Fig. 4(a) shows the values for $D_q$ of the fern of subsequent images generated by pixel reduction of the image with 1024 × 1024 pixels and is actually the same figure as Fig. 1(a). Here it is shown again as an example for demonstrating the extraordinary conformity with the values of $D_q$ derived from images generated with different pixel resolutions by the IFS itself (Fig. 4(b)).

There is nearly no difference of the values for all images except a slight deviation of the 32 × 32 image for $q < 0$. This result was not exclusively an outcome with the fern, each fractal chosen for this study showed exactly this behaviour. Therefore, there is no possibility to distinguish between images constructed with the IFS with a given pixel resolution and an image which has been downscaled from an image with more pixel resolution. As a further example of demonstrating this conformity, in Fig. 5 are the images with a resolution of 512 × 512 and 256 × 256 pixels of the modified Menger gasket shown. The images in Fig. 5(a) are the downscaled images and Fig. 5(b) shows the images after five iterations of the IFS and done for each resolution separately. Obviously there are no recognisable differences. The difference images
of Fig. 1(a) and (b), shown in Fig. 5(c) support that there are really only small deviations of single pixels which are subjectively invisible. The independence of the method of generating these images implies accordingly that any digital image taken from any fractal object with a given pixel resolution could be interpreted as being downscaled from an image with a higher pixel resolution. Furthermore this implies that outgoing from any image with a given pixel resolution it is possible to create a downscaled image which could be interpreted as an image taken from the object with viewer pixel resolution.

4. Discussion

There are mainly two important consequences of this study, first the fact that downsampling of any digital image of a fractal could serve as a tool for the determination of whether the actual pixel resolution was practically enough and second the fact that different fractals show not only a different course of the generalised dimensions but show also a different change of the course of the generalised dimensions in dependence of downsampling.

4.1. Downsampling for determination if the pixel resolution is enough

Downsampling of digital images from a high pixel resolution to a limited resolution led to different changes of the course of the generalised dimensions. For each fractal under investigation at least one downsampling step did not change the principal course of the values. Fractals, produced with the IFS with few iterations (Menger gaskets in Fig. 2) show larger deviations of the values by further downsampling in comparison to fractals produced with more iterations (Fern and Sierpinski gasket in Fig. 1). In the case of the Menger gaskets only eight iterations for a 1024 × 1024 image led to scales of one pixel in comparison to 11 iterations for the Sierpinski gasket. Therefore, we conclude that the downsampling has more effect for images of fractals which are represented by a lower number of scales. This is further in accordance with the pronounced variations of the values for the Menger gaskets. In the case of the investigated experimental image (cancer spheroid in Fig. 3) one step of downsampling did not change the course of the values, only a minor shift of the absolute values appeared. Considering that instead of an 1024 × 1024 image a 512 × 512 image was the ini-
Fig. 4. Generalised dimensions $D_q$ of a fern for the range of $q = -20$ up to 20. The legend shows the pixel resolutions of the images. (a) $D_q$ for the image sequence generated by downscaling the 1024 x 1024 pixel image. (b) $D_q$ for the image sequence generated with different pixel resolutions with the IFS.

stantial image in this case, these changes are less than that for the Menger gaskets. For all fractals generated with the IFS the first step of downscaling had the smallest effect and therefore we estimate with strong evidence that a hypothetical image of the cancer spheroid with 1024 x 1024 or even more pixels would not change the course and even the absolute values of the generalised dimensions very much. The assumption that the image of the cancer spheroid with 512 x 512 pixels could be treated as a downscaled image (and therefore that there would be the same changes when downscaling a 1024 x 1024 pixel image to a 512 x 512 pixel image) is deduced from the fact that for all IFS fractals the downscaled images are nearly the same as the images from which they were constructed and iterated with different initial pixel resolutions (Figs. 4 and 5). Therefore, we conclude and want to state that for our presented experimental fractal (cancer spheroid) it is not necessary to force the pixel resolution to the highest possible values. As a high pixel resolution is adequate for a representative imaging of the fractal under consideration, there are many drawbacks as, for instance, a limited amount of storage capacity and an increased amount of image processing time if large image series are to be treated. Nevertheless the direct proof with high pixelated images
of the images with the highest pixel resolution of 1024 × 1024 there were nevertheless different courses for the different fractals. This alone could serve as a tool for distinguishing between fractals. The fern showed an increasing sigmoid function (Fig. 1(a)), the Sierpinski gasket and the Menger gasket showed a very moderate first increasing and then a decreasing function (Figs. 1(b) and 2(a)) whereas the modified Menger gaskets with circles and the experimental cancer spheroid showed a first increasing and then a slightly decreasing function (Figs. 2(b) and 3). The individual circles of the latter Menger gasket could serve for this correspondence as the cancer object itself is a very “round” object but nevertheless they are very different looking objects. Furthermore the change of course of the generalised dimensions due to downscaling varies from fractal to fractal in a very special manner. There appears a very unique pattern for the individual fractals. The values for the fern “switch” for $q < 0$ and are very close together for $q \geq 0$, the values for the Sierpinski gasket are very close together for the whole range, whereas the values for the Menger gaskets as well as the cancer spheroid were increasingly scattered. Again the strongest correspondence of the cancer spheroid is with the modified Menger gasket, despite that there are again distinct differences.

Downscaling digital images of objects and interpreting the variations of the generalised dimensions could serve as a tool for discriminating fractals, especially if the determination between different biological states of one kind of object or fractal is of interest.

Although our findings are empirically based, the presented results strengthen the fact that the proposed methods for determining the variations of the values of the generalised dimensions during downscaling are well suited to answer the question if the actual pixel resolution of any digital image is enough. This method is applicable to any fractal under consideration and could be used for other research fields too. Nevertheless further confirmations with other fractals and the implementation of a theoretical frame would be the future task to determine more intensively the power of the proposed method.

Fig. 5. Example of a 512 × 512 and a 256 × 256 image of the modified Menger gasket. (a) Images generated by downscaling the 1024 × 1024 pixel image. (b) Images generated with different pixel resolutions with the IFS and iterated as much as the 1024 × 1024 pixel image which is not shown. (c) Difference image of (a) and (b).

is for our case undone and would be a task for future examination when such images are available.

4.2. Change of the course of the generalised dimensions during downscaling

The course of the generalised dimensions of the fractals under investigation were mostly not a decreasing sigmoid function. Comparing the courses
Acknowledgements

K1735/M2 mouse melanoma cells were kindly provided by Dr. I.J. Fidler, Inst. f. Cell Biology, M.D. Anderson Hospital, Houston, Texas.

References

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Fractal dimension of K1735 mouse melanoma clones and spheroid invasion in vitro

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Abstract An in vitro tumour-host confrontation method to investigate the invasion behaviour of cancer has been applied to K1735 mouse melanomas. Fluorescently labelled spheroids of cancer cells and host cells were confronted and the temporal course of cancer invasion into the host was investigated using confocal laser scanning microscopy. To improve the quantitative data of this method, the boundary images of the fluorescently labelled confrontation pairs were treated as fractals. The physical and mathematical framework for determination of the fractal capacity dimension is widely used in biology and medicine and has proved to be a very useful tool for describing the cancer invasion process. The fractal capacity dimension determination was carried out by dilation of the binary boundaries of the objects, which were treated as an estimate of the Minkowski-Bouligand dimension. The fractal dimension correlated well with the degree of invasion of the K1735-M2 clone. Control experiments, with host-host confrontations and various K1735 clones with reduced invasiveness, support these results.

Keywords Fractal geometry · Confocal laser scanning microscope · Cancer invasion · Metastasis · Image processing

Introduction

Invasion of cancer into the surrounding tissues is one of the major mechanisms by which cancer is able to form metastases at various locations in the human body. As the rehabilitation and survival chances of a cancer patient are correlated in the case of metastatic cancers to the state and the number of secondary tumours, further insights into the behaviour of cancer during invasion has a significant impact on the fight against cancer. Recent observations (Ahamer et al. 2001) using the spheroid confrontation technique for investigation of cancer invasion has led to the conclusion that application of this physical method for determining the fractal capacity dimension can support this fight, if more thoroughly investigated.

Fractal related work on cancer has been mostly successful in determining the mammographic density of breast carcinoma (Byng et al. 1997), MCF-7 breast cancer cells (Losa et al. 1998), leukaemic cells (Losa et al. 1992; Losa and Nonnenmacher 1996), pigmented skin lesions (Cross et al. 1995), malignant melanoma (Claridge et al. 1992), benign and malignant breast epithelial cell nuclei (Einstein et al. 1998), mastopathic and mammary cancer tissue (Mattfeldt 1997), dysplastic lesions of cervix uteri (Sedivy et al. 1999), gallbladder adenocarcinomas (Waliszewski 1999), retinoid-induced differentiation of cancer cells (Waliszewski et al. 1999, 2000) and quantification of nuclear pleomorphism using an asymptotic fractal model (Landini and Rippin 1996); it has also been used for the study of the role of fractals, chaos and cancer (Sedivy and Mader 1997).

In comparison to single cells or monolayers, spheroids are well suited for the investigation of invasion in vitro (Bjerkvig 1992). Here dual fluorescent staining is used, as previously described (DeVaney et al. 1997; Ahamer et al. 1998), in order to observe the invasion optically and make possible the digital image analysis of the resulting spheroid confrontations. Analysis of the degree of invasion is carried out making use of fractal analytic and image analysis techniques.

Optical sections of the confronted spheroid pairs are digitally recorded and processed in order to obtain binary images. Dilating the binary images of the boundaries enables the implementation of a double logarithmic plot of the object areas as a function of the dilation disk radii. With the value of the slope of these
plots, the fractal capacity dimension is calculated and serves as an estimate of the Minkowski-Bouligand dimension. The results of this dimension estimate are compared to the quantitative parameter INVASLOG, which has been proven in prior work to be a quantitative parameter well suited to describe the invasiveness of the considered confrontation cultures in vitro (Smolle et al. 1992).

Materials and methods

Spheroids, staining and confrontation

K1735-M2, K1735-C116 and K1735-C123 mouse melanoma cells (kindly provided by Dr. I.J. Fidler, Institute of Cell Biology, MD Anderson Hospital, Houston, Texas) and spheroids from freshly trypsinized embryonal chick heart cells were reaggregated to spheroids as described previously (Marcel and Meyvisch 1981; DevaneY et al. 1997). Before reaggregation the mouse melanoma cell monolayer was stained with the fluorescent dye CMFDA (chloromethylfluorescein diacetate) or Dil (DILCO8[3]) and the chick heart cell monolayer was stained with the fluorescent dye CMTMR (tetramethylrhodamine derivative) or DiO [DIOCA8[3]]. With a stain concentration of 10 µM and a staining duration of 2 h, these dyes are appropriate for staining live cells and are stable for up to 5 days. All dyes were purchased from Molecular Probes Europe (Leiden, The Netherlands).

After reaggregation to a size of about 50 µm, the mouse melanoma spheroids were mechanically confronted (touching one another) with the chick spheroids, forming cancer-host spheroid pairs. These pairs were incubated in a microincubation chamber at 37 °C, 95% RH and 5% CO2.

Confocal laser scanning microscope

The confronted cancer-host spheroid pair images were recorded using a confocal laser scanning microscope (Leica, TCS NT, Heidelberg, Germany). A 10×0.3 NA objective and a zoom factor of 2.1 was used and the 512x512 pixel images had an image size of 500 µm x 500 µm. A single pixel has, at this magnification, 0.977 µm side length; a single cell has, typically for K1735, a size of 10–15 µm. This “pixel resolution” was in practice sufficient to calculate the estimate of the fractal capacity dimension, as can be seen in the double logarithmic plot in Fig. 1, and was near the theoretically optimal optical maximum for the apparatus used. Several optical sections (up to 16) were recorded as tif files from the Leica system. These files were not converted into other formats to circumvent any degradation by converting algorithms (especially data compressing algorithms). Both fluorophores were excited simultaneously with an ArKr laser (λ = 488 nm and 568 nm). The fluorescence light was detected with a standard fluoresceine/rhodamine (FITC/TRITC) filter arrangement using a 530/30 nm bandpass filter and a 590 nm longpass filter.

Image processing

Several image processing steps were carried out before calculating the fractal capacity dimension from a single optical section of each of the spheroid confrontation pairs. The software KS300 (Zeiss, München, Germany) was used to eliminate cross-talk signals, which resulted from an overlap of the fluorescence spectra. This step has been previously published in detail (Ahammer et al. 1999).

Binary images (representing the cross-talk eliminated regions) were generated for calculating the quantitative invasion parameter INVASLOG which was calculated by:

\[
\text{INVASLOG} = -\log(\text{Formfactor})
\]  

with:

\[
\text{Formfactor} = \frac{4\pi \times \text{area}}{\text{perimeter}^2}
\]

The parameter INVASLOG was calculated for the host as well as for the cancer region. Previous investigations (Smolle et al., 1990, 1992) included subjective judgments by trained pathologists and showed especially that the parameter INVASLOG for the host region is able to quantify the invasiveness of the cancer spheroid.

Boundary images for determination of the fractal dimension were generated from the binary images using the Roberts operator. This operator is an edge detection function with a 2x2 matrix. From this matrix the differences of the diagonal elements are taken and the root of the sum of the squared differences gives the new value for the matrix. The results with the Roberts operator were compared with other edge detection operators, such as the Laplacian or the Sobel operator, which both use a 3x3 matrix. In comparison to the Roberts operator, the Laplacian operator led to very thin edges which were often broken and artificially segmented; the Sobel operator, on the other hand, led to very thick edges which tended to smooth out fine details.

Fractal dimension

To calculate the fractal capacity dimension (Mandelbrot 1982; Barnsley 1993; Liebovitch 1998), the boundaries of the images of both cancer and host regions were used. Several methods have been investigated in a prior publication (Ahammer et al. 2001), including box counting, the sausage method and a local method (Stoyan and Stoyan 1994), as well as the influence of subsequent image processing steps, such as median filtering, a combination of binary closing and opening and a special hole filling and eroding step and also the influence of noise. For the investigations here, solely the sausage method without any additional image processing steps was implemented.

The capacity dimension yielded by the sausage method, Dc, is based on the Minkowski cover, which was further examined by Bouligand (1929) using dilation. Outgoing from a set A in R² the set of all points A(ε) with:

\[
A(\varepsilon) = \{ f : f \in B_\varepsilon(x), x \in A \}
\]
is the Minkowski cover of the set \( A \), where \( B_\varepsilon (x) \) is the structuring or dilation element and is a disk of radius \( \varepsilon \) located around the point defined by \( x \). Increasing/decreasing the radius \( \varepsilon \) will increase/decrease the set \( A(\varepsilon) \). Simply, the set \( A(\varepsilon) \) is the area of the dilated set \( A \). Taking the infinitesimal limit \( \varepsilon \to 0 \), the Minkowski-Bouligand dimension or sausage dimension \( D_s \) from a set \( A \) can be calculated by:

\[
D_s = D_s(A) = \lim_{\varepsilon \to 0} \left( 2 - \frac{\ln A(\varepsilon)}{\ln \varepsilon} \right)
\]  

(4)

All the capacity dimensions should theoretically give the same value; \( D_s \) is one of these. Despite the mathematical differences in their determination, the practical implementation of different algorithms such as dilation, box counting or local methods prevent the dimensions from reaching the same value. When determining \( D_s \) from pixelated images the limit \( \varepsilon \to 0 \) cannot be reached because the minimal \( \varepsilon \) is equal to the pixel size of the image. This non-zero pixel size is furthermore the reason why the dilation element \( B_\varepsilon (x) \) cannot be determined exactly. Implementing a practical approach, boxes with pixel sizes of 1x1, 3x3, 5x5, ... 17x17 were used to approximate the disks. The corresponding approximated radius \( \varepsilon \) in pixels was calculated by:

\[
\varepsilon = \frac{B_\varepsilon}{\pi}
\]  

(5)

where \( B_\varepsilon \) denotes the approximated dilation element area in pixel².

By varying \( \varepsilon \) the corresponding applied dilation element (box) results in a varying area \( A(\varepsilon) \) of the object. \( A(\varepsilon) \) denotes the approximated area of the diluted object, again in pixel². The slope \( k_s \) of a double logarithmic plot of \( A(\varepsilon) \) versus \( \varepsilon \) gives the estimated fractal capacity dimension, \( D_s \).

---

**Results**

In order to evaluate quantitatively the invasiveness of the K1735 mouse melanomas, making use of the fractal capacity dimension \( D_s \), binary fluorescence signal images of the spheroid confrontation pair were investigated. Figure 1 shows a sample double logarithmic plot for the determination of \( D_s \). The scaling window for the determination of the slope \( k_s \) was set to \((2n + 1) \times (2n + 1)\) pixels with \( n = 0, 1, 2, 3 \ldots 8 \). To verify that the invasiveness and no other factors would be quantized by \( D_s \), additional control experiments with host-host confrontations and various K1735 clones were investigated.
The values of $D_3$ not only increased during the subjectively visible process of invasion but were beyond that compared to the parameter INVASLOG of the host spheroid, which served for this study as a standard value giving the standard quantitative values. The qualitative process of the invasion is depicted exemplarily in Fig. 2. As a single pixel is far smaller in size than a single cell, the two-dimensional section of the three-dimensional spheroid shows areas which are not directly correlated with multiples of the single cell size, as fractions of single cells may be present. Beginning with day 0 (Fig. 2), shortly (about 4 h) after the initial confrontation, both spheroids are structurally well separated but touching. Only one cancer cell (little dark grey area in the light grey region) has invaded the host spheroid. On the following days (Fig. 2, days 1, 2, 3) an increasing state of invasion with increasing clefing of the spheroids and increasing irregular forms could be observed. The quantitative results of this behaviour are shown in Fig. 3 for K1735-M2 mouse melanomas. First, the value of the fractal dimension $D_3$ shows a dependence on the state of invasion for both the cancer and the host regions (Fig. 3a). Second, this increase of $D_3$ is in accordance with the increase of the standard parameter INVASLOG for the host region. The INVASLOG values for the cancer were in this case equally accordant.

Quantitatively all the parameters correlated very well, with a failure probability $P < 0.001$. The actual values of the linear correlation coefficient can be seen in Table 1.

Confronting a host spheroid with another host spheroid instead of creating a cancer-host pair shows and verifies further whether this increase in the $D_3$ or INVASLOG values is really dependent on the invasiveness of the cancer. Qualitative inspections of such confrontations showed subjectively and clearly that there was no distinct invasion (images not shown). Although both spheroids were attached, they remained distinct. Nevertheless, the shape of the spheroids had increasing irregularities at the boundaries. Figure 4 shows that for both methods (Fig. 4a and Fig. 4b) no recognizable increase in the parameters during the course of the invasion is detectable.

![Fig. 3 Fractal capacity dimension $D_3$ (a) and the quantitative parameter INVASLOG (b) of K1735-M2 mouse melanoma-host confrontations up to 5 days. The $n$ numbers in parentheses denote the number of experiments](image)

![Fig. 4 Fractal capacity dimension $D_3$ (a) and the quantitative parameter INVASLOG (b) of control host-host confrontations in dependence on the confrontation duration from 1 to 3 days. The $n$ numbers in parentheses denote the number of experiments](image)

<table>
<thead>
<tr>
<th></th>
<th>$D_3$</th>
<th>INVASLOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1735M2</td>
<td>0.732</td>
<td>0.598</td>
</tr>
<tr>
<td>Host</td>
<td>0.632</td>
<td>0.786</td>
</tr>
</tbody>
</table>
in Fig. 3, it is clear that the increase of $D_s$ accords well with the state of invasion. The values of the linear correlation coefficients in the table show that both parameters $D_s$ and INVASLOG highly (within $P < 0.001$) correlate with the course of invasion. The values for $D_s$ are, for both spheroids (K1735-M2 and host), closer together than the values for INVASLOG. The high value of the parameter INVASLOG with the host spheroid confirms the prior work of the reliability of this parameter as a quantitative indicator of the invasiveness (Smolle et al. 1990, 1992). As the correlation coefficients for $D_s$ are evenly very high, the indication that the fractal capacity dimension could serve as a quantitative invasiveness parameter is strongly supported.

Evaluation of host-host confrontations showed only low values for both parameters, confirming further that the fractal dimension $D_s$ could serve as a quantitative parameter for the invasion process. $D_s$ probably quantifies the overall dynamic state of the object observed and this state is, despite the absence of invasion, definitely not zero because only living specimens were investigated. The overall dynamic state of the living objects can be assumed to vary with time as seen in the cancer host confrontations (Ahammer et al. 2001), but only small changes would be expected in the control experiments. This results in the observed fluctuations of the quantitative parameters seen in Fig. 4.

Finally, the results gained by investigations of various clones corroborate and harden the importance of $D_s$ because again the accordance with the parameter INVASLOG is obvious. As $D_s$ presents similar values for the host as well as for the cancer (except for a small absolute difference) and these values show exactly the same dependence on the experimental conditions, it can be assumed that both values are equally suitable for the quantitative description of cancer invasion.

In order to significantly increase the relevance of the presented methods and data with regard to in vivo applications, it would be necessary to calculate $D_s$ under various pharmaceutical conditions or under heat or ionizing radiation treatment. This could demonstrate further the properties of the fractal capacity dimension in relation to the invasiveness of cancer. This would enable the replacement of some animal experiments that are used for the determination of the quantitative data of invasion under various forms of chemo- or radiotherapy that is not only painful but also distressing for the animals involved.

Acknowledgements K1735-M2, K1735-C116 and K1735-C123 mouse melanoma cells were kindly provided by Dr. I.J. Fidler, Institute for Cell Biology, M.D. Anderson Hospital, Houston, Texas.

References

cent microscopy and fluorescent probes. Plenum Press, New York, pp 231–254


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4D imaging of invasion using Confocal Laser Scanning Microscopy and a 3D in vitro spheroid confrontation model of invasion.

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Key words: Living cells, cancer, melanoma, invasion, confocal microscopy, microincubator, vital stain, INVASLOG, confrontation of spheroids.

Previsouly in order to make comparative studies of invasion in confrontation studies a fixed time point was required for the classical histological method where the confronted spheroids were harvested and the degree of invasion was determined. Unfortunately the processes involved in attaining the then observed situation are lost, and only a static image remains from which the dynamics of the situation can only be inferred.

We have modified the spheroid confrontation method for the investigation of invasiveness so that it is possible to monitor the invasive process dynamically. The modified confrontation technique incorporates the use of fluorescent vital stains (CMFDA and CMTMR) [1,2] and long time observation of up to 5 days using a confocal laser scanning microscope (CLSM) with time dependent multichannel recording of the invasion process.

The confrontation used was that of embryonic chick heart multicellular spheroids with multicellular spheroids of K1755-M2, a highly invasive mouse melanoma cell line kindly donated from Dr. Fredrick Houston (Texas). The degree of invasion was determined using the parameter INVASLOG that has been established as a good measure of the degree of invasion as assessed by pathologists [3]. For continuous observation an incubation chamber Fig. 1 is required on the microscope stage which enables temperature and gas control as well as preventing the confronted spheroids from attachment to the coverslip which would lead to their migration from the spheroid pair. Fig. 2 shows a typical spheroid pair harvested at various periods during the process of invasion.

Fig. 3 shows the initial increase in invaselog on confrontation with embryonic chick heart cells.

Further studies are planned where the influence of therapeutic practices on the invasion process are planned.

References:
FRACTAL DIMENSION FOR A CANCER INVASION MODEL

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Abstract
A long-established investigation method for in vitro cancer invasion using cancer cell spheroid and normal cell spheroid confrontation for the quantitative determination of invasion was expanded to make use of fractal methods. The box-counting method, the sausage method and a local method were used in comparison with image pre-processing steps such as median filtering, closing and opening, or hole filling and scrapping, and artificially added noise images. It was possible to examine the potential role of the fractal capacity dimension for this invasion model. Several aspects such as the absolute value and variance of the different methods were taken into account and compared to well-established quantitative parameters. The fractal approach led to very promising results which improved the determination and examination of the invasion process of cancer.

1. INTRODUCTION
The ability of a primary cancer to generate metastases is determined mainly by the motility and the invasiveness of the primary cancer cells. These processes can be studied in vitro by a spheroid confrontation model. Cancer spheroids in vitro are, unlike single cells or monolayers, three-dimensional objects, and behave therefore similarly in many aspects to cancer in vivo and are considered to be a cytophilfunctional analogue of the pre-vascular stage of in vivo tumour development.1–4 Confronting a cancer spheroid with a host spheroid1,5–7 in vitro is a very powerful model for the investigation of the invasion process. The incorporation of quantitative invasion parameters lead to an objective determination of invasiveness and has the potential ability to supplement the subjective determination of the trained pathologist.

Outgoing from the well-established and objective invasion parameter Invaslog5,9 and the implementation of a confocal laser scanning method,10,11 we
expanded the above model by the addition of fractal analysis of long-term measurements of the invasion process.

The determination of fractal dimensions in the biomedical field has been especially successful in cancer-related work that has been carried out for determining the mammographic density of breast carcinoma, MCF-7 breast cancer cells, benign and malignant breast epithelial cell nuclei, mastopathic and mammary cancer tissue, dysplastic lesions of cervix uteri, gallbladder adenocarcinomas, retinoid-induced differentiation of cancer cells, malignant epithelial lesions of the mouth, and has also been used for the study of the role of fractals, chaos and cancer.

The box-counting method, the sausage method and a local method have been implemented for the calculation or estimation of the fractal capacity dimension. This enables the direct comparison of the detectivity of the fractal dimension with well-established parameters.

Method-dependent differences will also be considered. The variability of the fractal dimension in a sequence of images taken from a confronted cancer/host pair was investigated. For this reason, image pre-processing such as median filtering, a combination of closing and opening, and hole filling and scarping were done. Images where noise has been added were generated and compared with the pre-treated and the original images. This comparison leads to the conclusion, that the fractal capacity dimension could serve as a quantitative parameter for the invasion of cancer. In general, the superior capability of the fractal capacity dimension to differentiate the cancer tissue from the host tissue makes the fractal approach a very promising tool for examining the invasion process.

2. MATERIALS AND METHODS

2.1 Biological Methods

K1735-M2 mouse melanoma spheroids stained with the fluorescent Cell Tracker dye CMTMR (tetramethylrhodamine derivative, Molecular Probes, Leiden, The Netherlands) were confronted with embryonic chick heart spheroids (host) stained with the fluorescent Cell Tracker dye CMFDA (chloromethylfluorescein diacetate, Molecular Probes, Leiden, The Netherlands) as described previously. The confronted spheroid pairs were incubated in a microscope stage compatible microincubator (F-Tech, Graz, Austria) for the complete microscopic image detection period of up to four days. The microincubator enabled a temperature and CO₂-regulated atmosphere for microscopic measurements under optimal biological conditions.

2.2 Confocal Microscope and Image Pre-processing

Optical sectioning of the specimen was carried out with a confocal laser scanning microscope (Leica, TCS NT, Heidelberg, Germany). For a review of confocal laser scanning techniques in biology see Refs. 21-23. Both fluorescent dyes were excited simultaneously through a 20 x 0.4 NA objective with the laser wavelength of 488 and 568 nm and a theoretical optical resolution of 0.63 μm lateral and 4.7 μm axial. In an epifluorescence setup, the fluorescence of channel 1 (CMFDA dye) has been detected with a photomultiplier after it passed a bandpass filter 530/30 nm. The fluorescence of the channel 2 (CMTMR dye) was detected simultaneously with the second photomultiplier after passing a longpass filter of 590 nm. Images of a single optical section of the specimen where recorded and stored every three or ten minutes for a duration of about six up to 22 hours. Therefore an image sequence of 128 up to 134 images was generated, with 512 x 512 pixels and a zoom factor of one — an image size of 500 μm x 500 μm was obtained.

Image pre-processing of the above image sequence was carried out using the analysis software KS300 (Zeiss, Munich, Germany) on a Windows NT platform. Figure 1 shows the principal steps of the image processing for an image pair from this sequence, which was carried out for the complete sequence and is described as follows.

Cross-talk compensation and image segmentation using image subtraction was carried out as described in Ref. 11. Here, it is outlined as being the original grey level image of channel 2 (cancer) subtracted from the grey level image of channel 1 (host). Both images were recorded with an intensity quantization of 256 grey levels, and therefore adding the half of this depth, the value 128 to each pixel of the subtraction image results in an image where all grey levels from zero up to 127 belong to channel 2, and all grey levels from 129 up to 256 belong to channel 1. By thresholding this image with the
Fig. 1 Image pair #100 from a sequence of 128 images of a cancer/host spheroid confrontation, recorded with a confocal laser scanning microscope. Channel 1 (Ch1) measured the fluorescence light of the stained host spheroid and channel 2 (Ch2) measured the fluorescence light of the alternatively stained cancer spheroid. The middle column shows the combined images of the cancer and host spheroids as well as representing the biological situation of the confrontation. Clearly visible is the advanced invasion process: clefted invasion front and islands of cancer cells isolated in the host region. Cross-talk correction and segmentation was carried out as described in the method section. The binary images were edge filtered with the Roberts operator to generate contour images and one of the image processing steps (median, closing/opening, hole filling/scraping or additional noise) was applied before creating contour images. These contour images were used for the determination of the fractal dimension.
value 128, two binary images were generated which served at the same time as masks for segmentation of the original grey level images of channels 1 and 2, respectively.

The quantitative parameters: Area, Perimeter, Formfactor, Invaslog and the number of objects (Count) of the binary images with Formfactor = 4π Area/Perimeter² and Invaslog = −log (Formfactor) of both channels were calculated with KS300. The parameter Area stands for the sum of the areas of all individual objects of one image and is therefore simply the sum of all pixels of the image. The parameter Perimeter stands for the sum of all individual perimeters of all individual objects in the image and was calculated correspondingly. The parameter Count stands for the number of objects in the image and is simply the sum of objects (each having a different area and perimeter) of one image.

The value of Invaslog has been specially proven to be a strong quantitative measure for the invasiveness in the cancer/host spheroid model,8,9 and is therefore the reference value for the fractal analysis.

Outgoing from the original cross-talk corrected binary image sequence, the following image sequences were generated with KS300 (see Fig. 1):

(i) Box-counting method with box sizes of 1 × 1, 2 × 2, 4 × 4 ..., up to 512 × 512 pixels. The number of counted pixels dependent on the pixel size was double logarithmically plotted, and the negative value of the slope k_B of the first-order regression line was taken as the fractal capacity dimension estimate (D_B = −k_B).

The regression was restricted to box sizes of up to 16 × 16 pixels because we observed for some images (including test images, lines, circles, etc.), especially above box sizes of 32 × 32 pixels, deviations from the straight line. Furthermore, this restriction slightly increased the degree of accuracy in the estimation of the fractal dimension for images with known values of the fractal dimension.

(ii) Sausage method or boundary dilation method which is very closely related to the Minkowski dimension. The images were dilated with circles of increasing diameter. As an approximation for a circle, we once again used boxes with pixel sizes of 1 × 1, 3 × 3, 5 × 5, ..., 17 × 17. The corresponding approximated radius r in pixels was calculated by

\[ r = (A/\pi)^{1/2} \]

where A denotes the area in pixels.²

The slope k_S of the regression line of the double logarithmic plot of the counted pixels with respect to the radii gives with

\[ D_S = 2 - k_S \]

the estimated fractal capacity dimension D_S. Curve end correction was not implemented because the images (binary images of 2D slices of cancer or host spheroids) were closed curves.

(iii) Local dimension which is related to the local Hausdorff dimension. Contrary to the sausage method where the whole image is dilated, the local approach applies the dilation concept to a single point (pixel) out of the image. Originally a statistical approach to pick several image points, we picked all individual image points (image pixels) and dilated it separately with circles as described for the sausage method. Then the calculated averages of the counted image pixels for every radius double logarithmically plotted in dependence of the radius gave again the estimate of the fractal capacity dimension D_L,

\[ D_L = k_L \]

where k_L stands for the slope of the regression line.

2.3 Fractal Analysis

To estimate the fractal or capacity dimension of the objects, we implemented three methods²⁵,²⁶ with IDL 5.3:
The calculation time for the local method is directly proportional to the total number of image pixels. A rough estimate for the images concerned in this study gave an average value of about 24 seconds per image. For comparison, the box-counting algorithm needed about 20 seconds per image and the sausage method only two seconds per image.

In order to evaluate the ability to discriminate between different specimens using the above methods, the confidence intervals (p = 0.05) of the differences between the means were calculated and normalized, that the differences between the means had the value one. Higher/lower values of the normalized confidence intervals were therefore associated with lower/higher discrimination ability.

3. RESULTS

Estimating the fractal capacity dimension D with three different methods and the large set of pre-treated image sequences (median filtering, closing opening, fill scrap and added noise) yielded a large amount of information which, if D is an appropriate quantitative measure of the dynamic behavior of the cancer invasion spheroid model, will be significant in estimating invasion.

Section 3.1 shows the quantitative evaluation of this process with the well-established "classical" parameters for our case. Section 3.2 shows the results established with different methods for the estimation of the fractal capacity dimension, and Secs. 3.3 and 3.4 are concerned with the influence of pre-processing the images and adding noise.

3.1 Dynamic Behavior

Figure 1 shows one sample image pair out of a sequence of 128 images recorded every three minutes. The invasion process was in this case in an advanced state. Nevertheless, the temporal behavior in this time domain was not very fast. Subjective inspection of the image sequence (e.g. with a slide show procedure) showed only minor movements of parts of the tissues between the neighboring images, and clearly visible changes in the form of the specimen were not observed, whereas a significant change is seen between the first and the last image. Therefore, all these images of this sequence are able to be subjectively classified as having a high grade of similarity. An evaluation of this sequence with a quantitative parameter such as Invaslog is shown in Fig. 2. After an initial increase, the value is roughly constant and not equal for each of the channels. The normalized confidence interval has the value ± 0.185 (p = 0.05).

![Figure 2](image-url)

**Fig. 2** Calculated value of the parameter Invaslog depending on the image number of a sequence of 128 images measured every three minutes. The calculations were made from binary images. Ch1 (channel 1) represents the host spheroid and Ch2 (channel 2) the cancer spheroid. The normalized confidence interval of the difference of the mean values of Ch1 and Ch2 was: ± 0.185 (p = 0.05).
The mean values and the standard deviations of \textit{Invaslog} in comparison to the \textit{Formfactor}, \textit{Area}, \textit{Perimeter}, and number of pixels (\textit{Count}) are plotted in Fig. 3. All values are normalized to the channel 1. The different values for \textit{Area} and \textit{Perimeter} differentiate very well between the two objects but do not have the relevance to represent any dynamic behavior of the specimen.

3.2 Fractal Dimensions

Outgoing from the results of Sec. 3.1, the estimates of the fractal capacity dimension $D_B$, $D_S$, $D_L$ were calculated using the same image sequence. Figure 4 shows the characteristic plots for the box-counting method (a), the sausage method (b), and the local method (c), for the sample image pair of Fig. 1. Figure 5 shows the results of the fractal capacity dimension determination for the whole image sequence. Additionally, the outer contour of the combination of both host and cancer together has been evaluated. An example of such a combined image is shown in Fig. 1. All three methods show an increased channel separation capability in comparison to the parameter \textit{Invaslog} (see Figs. 2 and 5). The normalized confidence intervals for channels 1 and 2 are: box count method: $\pm 0.0809$ ($p = 0.05$), sausage method: $\pm 0.0476$ ($p = 0.05$), and for the local method: $\pm 0.0564$ ($p = 0.05$). Furthermore, there are differences between the methods which can be very clearly seen. The value of the standard deviation of the box-counting method is the highest one ($\pm 0.02$) in comparison to the values of the sausage method and local method, which are nearly equivalent ($\pm 0.0116$ and $\pm 0.0126$). The value for the fractal capacity dimension of the combined object is for the box-counting method and the sausage method — roughly the same value as for the corresponding channel 2 (cancer). Only the local method shows a value in between the two channels. The absolute values for the fractal capacity dimension are the lowest for the sausage method and the highest for the local method, whereas the difference between the sausage method and the box-counting method is less than the difference between

![Fig. 3](image-url)  

\textbf{Fig. 3} Overview of quantitative parameters for channels 1 and 2. The average values are normalized to the value of channel 1 because the absolute values of each of the parameters would be far too different as to plot in one graph (\textit{Invaslog}: 1–1.3, \textit{Formfactor}: 0.01–0.03, \textit{Area}: 3000–4000, etc.).
Fig. 4 Double logarithmic count/size plots for the sample image pair of Fig. 1 for the box-counting method (a), the sausage method (b) and the local method (c). The linear regression lines for both channel 1 (host) and channel 2 (cancer) are shown and the values for the slopes are listed in the legends correspondingly. The linear regression lines for the box-counting method were restricted to box sizes of up to 16 × 16 pixels in order to improve the detection of the absolute values for the fractal capacity dimension $D_B$. 
the local method and the box-counting method. Figure 5(d) summarizes in graphical form the differences of these methods for the different channels and the combination of both channels.

3.3 Influence of Image Processing

In order to further evaluate the results presented in Fig. 5, several image processing steps were done before the calculation of the fractal capacity dimension. Commonly used algorithms like median filtering, a combination of closing and opening, and hole filling combined with scrapping of objects, each with three steps of strength were considered. Figure 6(a) shows the results for median filtering, Fig. 6(b) for closing-opening, and Fig. 6(c) for the hole filling and scrapping pre-treatment. Each pre-treatment caused a decrease of the average value of the absolute fractal capacity dimension which was calculated in each case using the sausage method. This decrease is mainly and not surprisingly an effect of averaging the original object. Whereas a median filtering with size $3 \times 3$ [Fig. 6(a)] decreases the value only moderately, the decrease for one step of closing and opening [Fig. 6(b)] is larger. The difference in these decreasing effects is enlarged by increasing the degree of pre-treatment (e.g. median filtered with a $7 \times 7$ operator compared to applying three times the closing and opening procedure). The hole filling and scrapping pre-treatment [Fig. 6(c)] shows a different behavior. The scrap step is especially dependent on the size distribution of individual objects in an image. In our case, the increase of the scrap value from 200 to 300 pixels did not cause a relevant decrease of the fractal capacity dimension. This indicates that there are more single objects with a size of up to 100 pixels (which contribute to the value of the fractal capacity dimension) than singular objects with a size of up to 200 or 300 pixels. All pre-treatment methods show, that the effect of the treatment is principally the same for each channel and for the combined image. There is only a deviation when applying higher degrees of pre-treatment (Fig. 6(a), median $7 \times 7$ and especially Fig. 6(b), three times closing and opening).

Despite decreasing the fractal capacity dimension $D$, it is very important to realize that the standard
Fig. 5 Overview of the estimated fractal capacity dimension $D$ for the box-counting method (a), the sausage method (b) and the local method (c). The image sequence was the same as for the determination of the parameter Invaslog in Fig. 2. Ch1 represents the host spheroid, Ch2 the cancer spheroid, and Comb represents the results for the combined image of channels 1 and 2. (d) summarises the results for the different methods.
Fig. 5. (Continued)
Fig. 6  Average fractal capacity dimension $D_s$, calculated with the sausage method, and standard deviation for channel 1 (host), channel 2 (cancer) and combination of both. (a) to (c) show the influence of pre-treatment of the binary images. (a): median filtering — 3 $\times$ 3, 5 $\times$ 5, and 7 $\times$ 7. (b): a combination of a binary closing and opening — one time, two times, three times. (c): a combination of hole filling and object scrapping — scrap size $\leq$ 100, $\leq$ 200 and $\leq$ 300 pixels. (d) shows the influence of artificial added noise. One image pair was picked out of the sequence of 128 images, and respectively 100 different images were generated with signal to noise ratio 100, 10 and 1.
Fig. 6. (Continued)
Fig. 7 Fractal capacity dimension $D_B$, $D_S$ and $D_L$ of the Henon attractor (a), theoretical dimension: 1.26 and the Koch curve (b), theoretical dimension: 1.2618 in dependence of noise and for different calculation methods. SN100, SN10 and SN1 stands for a signal to noise ratio of 100, 10 and 1, respectively. The images are for example with SN10 [Henon, (a)] and with SN1 [Koch, (b)].

deviation for all the pre-treatment procedures and degrees did not, or only marginally, change its value. Therefore given an original image sequence, the pre-treatment is able to change the average absolute value but not the standard deviation and therefore decreases the separation possibility for the fractal approach. This result is shown in Fig. 6 for the sausage method and it also holds true for the box-counting method and the local method (not shown). Hence, there is a strong evidence that the higher value of the standard deviation for the box-counting method is method inherent and not caused by a forced dependence on noise or something else. The strong tendency for the standard deviation to remain constant under "filtering conditions" or "averaging conditions" raises the question, that these variations may be caused by biological variations of the specimen during observation. To verify this issue further, we investigated artificially noise added images. The results are described in Fig. 6(d) and in the next section.

3.4 Influence of Noise

The previous section showed the influence of image processing techniques on the determination of the fractal capacity dimension. Median filtering is especially used as a standard for filtering noise from an image without smoothing the image too much. As
the variation of the value of the fractal capacity dimension from image to image did not decrease with median filtering, we concluded that these variations are not caused by artefacts such as noise. Therefore we investigated this issue the other way around and contaminated images with additional noise, which can be seen for two test figures in Figs. 7(a) and (b). In Fig. 6(d), the results of added noise to the images of the specimen are described. Decreasing the signal to noise ratio markedly decreased the absolute value of the fractal capacity dimension, whereas the standard deviation with values of ± 0.0045 did not change very much. The standard deviation was 2.5 times smaller than the standard deviation found in the original image sequence. To compare these results with commonly known fractals, noise added images of the Henon attractor and the Koch curve were investigated. As can be seen in Figs. 7(a) and (b) for the sausage method the results are quite the same — decreasing average value and approximately constant and very low standard deviation with decreasing signal to noise ratio.

3.5 Comparison with well-known fractals

The calculations for the images of Fig. 7 were carried out with all three fractal dimension calculation methods. A very low standard deviation and a decrease in the average value of the fractal capacity dimension were common to all three methods but the influence of noise was less for the local method. Furthermore, the objects were fractals with known fractal dimension (Henon: 1.26, Koch: 1.261826). The box-counting method and the sausage method yielded values very close to the theoretical values (see Fig. 7, original) whereas the local method with values of about 1.4 over-estimated the theoretical values.

4. DISCUSSION

Fractal analysis of a cancer spheroid invasion model has been done and compared to well-established quantitative parameters. Especially the parameter Invaslog has been proven, in prior work, to be a direct measure of the invasiveness of cancer as evaluated by experienced pathologists.8 Important for such a parameter is the ability to differentiate between different specimens. Implementing for the first time, a setup where the invasion process can be observed continuously over several hours up to days, the time-dependent behavior of invasion can be determined. The fractal approach for such a dynamic system has been investigated with this work and has lead to very promising results.

Comparing the data for the parameter Invaslog (Figs. 2 and 3 — Invaslog) and the data with the fractal methods, (Fig. 5) it is clear that the discrimination ability of the fractal methods is higher. The normalized confidence intervals of the mean differences for the fractal methods were on an average three times smaller than compared to the value for the parameter Invaslog. The sausage method leads, with 3.88 times smaller values for the confidence intervals. Due to this fact and the higher standard deviation of the box-counting method, the sausage method and the local method appear preferable. Several image pre-processing steps, including median filtering, closing-opening and hole filling-scraping changed the variability (standard deviation) of the results only marginally (see Fig. 6). This is in accordance with the case of additional noise (Figs 6(d) and 7), which caused a very small variability, but a marked decrease of the absolute value. Therefore, these variations have so far been proven as biological variations in the image sequence. Figure 8 orders the different values of the standard deviation in three groups. Firstly, the highest values were gained by the box-counting method, independently of image pre-processing steps. The second group with lower values summarizes both the sausage method and the local method and is again mainly independent of image pre-processing. The third group is the group with the lowest values and represents the values gained for additional noise whereas the local method has the lowest value.

The local method appears to be able to discriminate better between the specimen [see Fig. 5(c)] because the value of the fractal capacity dimension of the combined image was calculated to be between the values of channel 1 (host) and channel 2 (cancer). In addition to that, the influence of noise (Figs. 7 and 8) was less pronounced for the local method. Otherwise, the absolute value of the local method was in every case, including the values of known fractal dimensions, higher than the two other methods (Figs. 5(d) and 7). The box-counting and the sausage method corresponded very well to the absolute values of the known fractals (Fig. 7).
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K1735-M2 mouse melanoma cells were kindly provided by Dr. I. J. Fidler, Institute for Cell Biology, M.D., Anderson Hospital, Houston Texas.

REFERENCES


Summarizing the results, the sausage method appears to be preferable for the determination of the fractal capacity dimension applied to the dynamic cancer spheroid invasion model. Nevertheless, this method should be further examined in the future, by applying and changing biological relevant conditions like pharmaceutical treatment (invasion or motility inhibitors or stimulators) or treatment with ionizing radiation. Furthermore, the local method should be further investigated by methods for correcting the high absolute value. Apart from the absolute value, the discrimination capability and the noise resistivity were superior. Local methods expanded to fractal spectra determinations can give principally more information of the fractal appearance of the specimen under investigation. Especially for the presented in vitro cancer invasion model, the temporal change in the boundary (between cancer and host) and of the boundary of the spheroids to the incubation medium would be an interesting field for future examination.
Ersatz- und Ergänzungsmethoden zu Tierversuchen

Herausgegeben von
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Die Motilität von menschlichen und Mausmelanomzelllinien und embryonalen Hühnerherzzellen unter Einfluß von 1µM all-trans-Retinsäure

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Zusammenfassung


Es konnte durch diese Experimente gezeigt werden, daß die Geschwindigkeit mit der Zellen aus dem Spähroid auswandern auf verschiedenen extrazellulären Matrixkomponenten unterschiedlich groß ist und daß die Invasivität nicht immer mit der gerichteten Motilität korreliert.

Unter Einfluß von 1µM RA konnte auf Collagen I beschichtetem Glas bei den Mausmelanomzellen eine Zunahme des Kreisdurchmessers gegenüber den Kontrollwerten gemessen werden, wohingegen die embryonalen Hühnerherzzellen einen geringeren Kreisdurchmesser gegenüber den Kontrollwerten aufwiesen.
Summary

Directional migration of human melanoma cells, murine melanoma cells and embryonic chick heart cells under influence of 1µM all-trans retinoic acid

Tumour-host interaction and tumour interaction with the extracellular matrix plays a crucial role in invasiveness and determines cancer metastasis. To get insight in tumour-host interactions a reliable 3D confrontation assay could be performed where tumour spheroids will be confronted with chick embryonic heart spheroids. After 24h the infiltration of tumour cells into the chick embryonic heart spheroid can be evaluated using an image analysis computer program. A reduction of tumour cell invasion can be seen under the influence of all-trans retinoic acid (RA).

Under the influence of RA spheroids were used to investigate the interaction of tumor cells with extracellular matrix concerning directional migration. Reaggregated spheroids consisting of tumour cells and chick embryonic heart cells were explanted on collagen or laminin coated surfaces. In evaluating the diameter change emerging from cells leaving the multicell spheroid after 24, 48 and 72 hours a quantification of directed motility can be made. We investigated cells with metastatic potential from human and murine origin and chick embryonic heart cells with this *in vitro* method of directional motility.

It could be shown that the directional migration depends on the coverslip coating and invasiveness is not strictly correlated with directional migration of the tumour cells. 1 µM all-trans Retinoic acid could increase the diameters in the case of murine melanoma cells whereas embryonic chick heart cells had decreased diameters on collagen Type I coated surfaces.

1. Einleitung

2. Material und Methode

2.1. Zelllinien


Von 10 Tage alten Hühnerembryonen wurde das Herz entnommen und mechanisch zerkleinert. Nach erfolgter Behandlung mit 0,25%igem Trypsin bei 37 Grad Celsius wurde die Zellsuspension mit einem Filter (100 μm Maschenweite) von restlichen Gewebeteilen befreit (PELZMANN B., 1990).

Beide Tumorzelllinien als auch die embryonalen Hühnerherzzellen wurden in DMEM mit 10% fütalem Kälberserum unter Standard Zellkulturbedingungen kultiviert. Für die Bestimmung der gerichteten Motilität wurde hitzeinaktiviertes fütales Kälberserum verwendet (1h bei 56 Grad Celsius).

2.2. Beschichtung mit Laminin und Collagen I


2.3. Sphäroide


2.4. Gerichtete Migration

Sphäroide wurden in 24 Multiwellplatten ausgebucht und nach 24h und 48h wurde der sich durch auswandernde Zellen ergebende Kreisdurchmesser mittels Computersoftware (CorelDraw, Canada) ermittelt. Die das Sphäroid verlassenden Zellen wurden über ein invertiertes Mikroskop (Zeiss, D-Oberkochen) mittels CCD-Kamera (Sony, Japan) auf einen S-VHS-Videorecorder (Panasonic Inc., Japan) aufgenommen und der Film anschließend digitalisiert (Screen Maschine) und in Bitmapbildern umgewandelt. Es wurde der gerade noch alle Zellen umfassende Kreis über das Bitmapbild gelegt und so der Kreisdurchmesser ermittelt. Diese Methode wurde in (BRÄUNER T., 1987) beschrieben, welcher auch zeigen konnte, daß der Kreisdurchmesser über 5 Tage linear zunimmt. Es wurde der 48h Kreisdurchmesser vom 24h Wert abgezogen, um etwaige Unregelmäßigkeiten in der Sphäroidgröße zu umgehen. Der Einsatz von Cycloheximid als Syntheshemmer für endogenes Fibronectin bzw. anderen
Komponenten der extrazellulären Matrix wurden bewußt vermieden, denn diese Substanz findet auch bei bisherigen gerichteten Motilitätsstudien und Invasivitätsstudien keinen Einsatz und wird ausschließlich bei Adhäsionsstudien verwendet.

2.5. Zellfärbung mit Fluoreszenzfarbstoffen

Celltracker Green Vitalfarbstoff (CMFDA, Molecular Probes, Eugene Oregon USA) wurde für die Färbung der Tumorzellen und Celltracker Orange (CMTMR, Molecular Probes, Eugene Oregon USA) wurde für die Färbung der embryonalen Hühnerherzzellen verwendet. Die Farbstoffe wurden in DMSO gelöst und anschließend wurden 10 μl zu 10ml Medium gegeben, um bei 10μM 2h lang zu färben.

In einem zusätzlichen Experiment konnte gezeigt werden, daß die Vitalfarbstoffe keinen signifikanten Einfluß (p < 0,05) auf die gerichtete Motilität von K1735-M2 und embryonalen Hühnerherzzellen auf Glas, Laminin und Collagen I haben.

2.6. RA-Behandlung der Zellen bzw. Sphäroide

72h vor Ausbringen und auch während der Versuche wurde all-trans Retinsäure (RA) in einer Konzentration von 1μM dem Medium zugesetzt.

RA wurde in 100% Ethanol gelöst und 10μl davon wurden 10ml Medium zugesetzt, um eine Konzentration von 1μM zu erhalten. Alle Arbeiten mit RA wurden in abgedunkeltem Licht oder bei Rotlicht durchgeführt. Die humanen Mausmelanomzellen wurden nicht unter Einfluß von RA untersucht.

2.7. Statistische Tests

Statistische Tests wurden durchgeführt mit Microcal Origin 5.0 unter zur Hilfenahme des t-Tests für Wertepaare (Paired t-Test).

3. Ergebnisse

3.1. Ergebnisse aus dem gerichteten Motilitätsversuch ohne RA

Die embryonalen Hühnerherzzellen zeigten auf Laminin und Collagen I im Vergleich zu den Mausmelanomzellen einen signifikant (p < 0,05) größeren relativen Kreisdurchmesser (48h Wert minus 24h Wert) (siehe Abb. 1). Die embryonalen Hühnerherzzellen bildeten auf Glas den geringsten, auf Collagen I und Laminin vergleichbar relativ große Kreisdurchmesser. Die Mausmelanomzellen hatten auf verschiedenen Komponenten der extrazellulären Matrix unterschiedlich große relative Kreisdurchmesser und zeigten auf Glas den geringsten relativen Kreisdurchmesser gefolgt von Collagen Typ I und Laminin. Die humanen Melanomzellen zeigten ein sehr geringes Migrationsvermögen und so mußten die 72h Werte von den 48h Werten abgezogen werden, um die Daten auszuwerten. Auf Laminin zeigten die humanen Melanomzellen über einen Beobachtungszeitraum von 72h keine Migration.

3.2. Ergebnisse aus dem gerichteten Motilitätsversuch unter Einfluß von 1μM RA

Die embryonalen Hühnerherzzellen zeigten unter RA-Einfluß auf allen getesteten extrazellulären Matrixkomponenten geringere relative Kreisdurchmesser als die Kontrollwerte ohne RA, wobei besonders auf Collagen I und Laminin die relativen Kreisdurchmesser signifikant (p < 0,05) zurückgingen. Die Mausmelanomzellen bildeten unter Einfluß von RA auf Glas und Collagen I signifikant (p < 0,05) größere relative Kreisdurchmesser als die Kontrollwerte ohne RA. Auffällig ist der beträchtliche Anstieg des rel. Kreisdurchmessers auf Glas und Collagen I.
4. Diskussion


4.1.

Embryonale Hühnerherzzenellen wurden von COUCHMAN (COUCHMAN J. and REES D., 1979) bezüglich Motilität untersucht. Sie zeigen bis 48h einen sehr motilen Phänotyp und sind durch wenige Retraktionsercheinungen nach Kontakten mit anderen Zellen gekennzeichnet, weiterhin zeigen sie in dieser Phase kaum Proliferation. 48h nach Ausbringen zeigen sie eine geringere Motilität, Synthese von endogenem Fibronectin und erhöhte Zelladhäsionskontakte ohne jedoch die Zellfläche zu ändern. Da die embryonalen Hühnerherzzenellen in der vorliegenden Migrationsstudie nur 48h beobachtet wurden, konnte man auf allen getesteten Oberflächen eine hohe Migration resultierend in großen relativen Kreisdurchmesser beobachten. Embryonale Hühnerherzzenellen zeigten nicht nur auf Laminin, sondern auch auf Glas, welches keinerlei Wachstums- bzw. motivierenderen Einfluß durch Haptotaxis ausübte, einen sehr motilen Phänotyp.


4.2.


Der Rückgang der relativen Kreisdurchmesser ist größtenteils auf erniedrigte Motilität zurückzuführen. Durch den Rückgang der relativen Kreisdurchmesser auf Laminin und Collagen I im Vergleich zu Glas konnte man annehmen, daß die motilitätssteigernde Wirkung von Collagen I und Laminin auf embryonale Hühnerherzzellen unter RA nicht mehr so stark zu tragen kommt, weil man es unter RA-Einfluß mit differenzierteren Hühnerherzzellen zu tun hat.


Aufgrund der vielfachen Effekte von RA auf Tumorzellen sind zusätzliche Untersuchungen der Zellausbreitung, der stationären Motilität und der Zelladhäsion notwendig, um die vorliegenden Ergebnisse vollständig erklären zu können.

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Literatur

Bertram J.S., Role of gap junctional cell/cell communication in the control of proliferation and neoplastic transformation, Radiation Research, 123, 252-6, 1990
Boxberger H.-J. and Pawelitz N., Influences of various substrata on morphology, motility and invasiveness of rat tumour cells. II. Studies on the highly metastatic variant BSp 73 ASML, Anticancer Research, 10, 1265-74, 1990
Engebraten O., Schwachenwald R., Valen H., Bierwig R. et al., Effects of high and low single dose irradiation on glioma spheroid invasion into normal rat brain tissue in vitro, Anticancer Research, 12, 1501-6, 1992


Hossain M.Z. and Bertram J.S., Retinooids suppress proliferation, induce cell spreading, and up-regulate expression in postconfluent 10T1/2 cells: implications for the role of gap junctional communication, Cell Growth and Differentiation, 5, 1253-61, 1994

Hossain M.Z., Wilkens L.R., Mejia P.P., Loewenstein W.R. et al., Enhancement of gap junctional communication by retinooids correlates with their ability to inhibit neoplastic transformation, Carcinogenesis, 10, 1743-8, 1989


Kozloskis J.M., Hart J.R., Fidler J.J., Hanna N., A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice, Journal of the National Cancer Institute, 72, 913-5, 1984


Lotan R., Amos B., Watanabe H., Raza A., Suppression of Melanoma cell motility factor receptor expression by retinoic acid, Cancer Research, 52, 4878-84, 1992


Pellmann B., Isolierung und Kultivierung embryonaler Hühnerherzszellen, Diplomarbeit, 1990

Ranson M., Posey S., Mason R.S., Extracellular matrix modulates the function of human melanocytes but not melanoma cells, Journal of Cellular Physiology, 136, 281-8, 1988

Siletti S. and Raza A., Regulation of autocrine motility factor receptor expression in tumor cell locomotion and metastasis, Current Topical in Microbiology and Immunology, 213, 137-69, 1996


Talmadge J.E. and Fidler I.J., Enhanced metastatic potential of tumor cells harvested from spontaneous metastases of heterogeneous murine tumors, Journal of the National Cancer Institute, 69, 975-80, 1982


Weilung H.-O., Speiss E., Pawelec N., In vitro motility of Bsp 73 rat tumor cells with different metastatic potential, Invasion and Metastasis, 6, 257-69, 1986


Ersatz- und Ergänzungsmethoden zu Tierversuchen

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In vitro-Tests zur Untersuchung der Invasivität und der Metastasierung von Tumoren

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Zusammenfassung


Summary

In vitro assays to study invasion and metastasis of cancer

Because of problems in evaluating and observing the metastatic cascade using animal experiments, many different in vitro models for investigating metastasis and tumour cell invasiveness have been developed over the last 15 years. MAREEL was one of the first who has been successful in developing an in vitro invasion assay using chick embryonic heart spheroids (MAREEL M. et al., 1981). KALEBIC published that one has a distinct advantage in using in vitro invasion assays for the analysis of individual factors involved in the formation of metastatic cell colonies (KALEBIC T., 1988). In vitro invasion and cell motility assays involving animal experiments provides insufficient data for the investigation of each of the individual steps that result in the metastatic cascade. Using in vitro methods one can under controlled conditions elucidate the precise factors involved in each step of the cascade. Influences exerted through chemo-therapeutic agents can be exactly determined, enabling investigation of the causes and effects resulting from the metastatic process. In this paper different in vitro assays and their role
in the investigation of the metastatic steps will be discussed. Further, we want to show the advantages of *in vitro* assays as opposed to the animal models, which is in the most cases the nude mouse model.

Tabelle 1. Eine kleine Auswahl an Experimenten an verschiedenen Tiergattungen, die in den letzten 3 Jahren bezüglich Invasivität bzw. Metastasierung durchgeführt wurden

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<td>Nacktmäuse</td>
<td>humanes Dickdarmkrebsegewebe</td>
<td>heterotrophe Transplantation</td>
<td>Bestimmung von Lymphknoten-, Leber und Lymphphgefäßmetastasen</td>
<td>(Togo S. et al., 1995)</td>
</tr>
<tr>
<td>Nacktmäuse</td>
<td>NIH 3T3 Fibroblasten und stark beeinträchtigte Zellen</td>
<td>intravaginal</td>
<td>Lungenmetastasen</td>
<td>(Takiguchi Y. et al., 1995)</td>
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<td>M21 menschliche Melanomzellen</td>
<td>subkutan</td>
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<td>(Mitani F. et al., 1995)</td>
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<td>(Usuki S. et al., 1995)</td>
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<td>menschliche Pankreaskarzinomzellen</td>
<td>orthopädische Transplantation</td>
<td>Überlebenszeit der Maus und Nachweis verschiedener Metastasen</td>
<td>(Anders et al., 1996)</td>
</tr>
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<td>orthopädische Implantation</td>
<td>spontane Metastasen in Lymphknoten, Leber und Lunge</td>
<td>(Sun F.X. et al., 1996)</td>
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<td>Weiterkultivieren der Lebermetastasen</td>
<td>(Yasoshima T. et al., 1996)</td>
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<td>Thymuslose Nacktmäuse</td>
<td>TYS &quot;adenoid squamous carcinoma cell line&quot;</td>
<td>Transplantation</td>
<td>Abmessung der Tumorgröße</td>
<td>(Sato M. et al., 1995)</td>
</tr>
<tr>
<td>Thymuslose Nacktmäuse</td>
<td>menschliche MDA-MB-435 Brusttumorzellen</td>
<td>intraperitoneal</td>
<td>Abzählung der Lungenmetastasen</td>
<td>(Sledge G.W. et al., 1995)</td>
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1. Invasion, Metastasierung und Motilität


2. In vitro-Methoden

2.1. In vitro-Methoden zur Untersuchung der Zellmotilität

Hierbei werden die Spuren, die Zellen auf einem mit kolloidalem Gold beschichteten Deckglas hinterlassen, untersucht.

Tumorphädroide mit einem Durchmesser von ca. 200μm werden in eine 24-well Gewebekulturplatte gebracht und alle 24h wird mit Hilfe eines Invertmikroskops der Radius, der sich ergibt, wenn Tumorzellen von einem zentral gelegenen Sphäroid auswandern, untersucht.

Eine spezielle Mikroinkubationskammer erlaubt es, mit Hilfe eines inversen Mikroskops die stationäre und translokative Tumorphämodilität zu quantifizieren. Mit Hilfe kompziierter digitaler Bildverarbeitungsalgorithmen können Membranbewegungen, Änderungen am intrazelullären Organellentransport und die Zellwanderung von Einzelzellen über das Deckglas quantifiziert werden.

2.2. In vitro-Methoden zur Untersuchung der Invasivität

Bei diesen Invasionstests wird meist rekonstruierte Basalmembran-Matrigel auf Microporefilter aufgebracht und in eine Boydenkammer gebracht. Dieses System erlaubt zum einen den Einsatz von verschiedenen chemotaktischen Faktoren und zum anderen eine genaue und reproduzierbare Quantifizierung der durch die Membran wandernden Tumorzellen auf der Unterseite des Filters. Mit diesem in vitro-Invasions assay ist es möglich, die Teilschritte (2) und (5) der metastatischen Kaskade zu untersuchen. Natürliche Basalmembranen wie z.B. die CAM
von Hühnerembryonen werden für die Invasivitäts- und die Metastasierungsuntersuchung von Tumorzellen verwendet (OSSOWSKI L. and REICH E., 1980). Hierbei werden Zellen auf die CAM eines 10 Tage alten Hühnerembryos aufgebracht und nach einer bestimmten Zeit die Anzahl der Lungenmetastasen im Embryo bestimmt.


Bei diesen in vitro-Experimenten werden Tumorzellen nach deren Invasion in verschiedenste Organfragmente histologisch untersucht. Eine Variante ist das Vermessen der Abnahme an Radioaktivität von radioaktivenen Tumorzellen nach der Invasion in Gewebosphäroide. Diese in vitro-Methode erlaubt es, die metastatischen Teilschritte (1), (2) und (5) zu quantifizieren.


Diese Experimente sind denen von Punkt 2.2.1. sehr ähnlich, nur daß zusätzlich noch eine Endothel- oder Epithelzellachichte von den Tumorzysten durchwändert wird. Es ist mit diesem in vitro-Modell möglich, das Eindringen in Blutgefäße und damit die metastatischen Teilschritte (2) und (3) zu untersuchen.

2.2.4. 3D-Konfrontation mit embryonalen Hühnerherzgewebemodell (MAREEL M.M. et al., 1979; SMOLE J. et al., 1990; MATTANA N. et al; 1991; HELIGE C. et al., 1993)

Bei diesem Experiment werden embryonale Hühnerherzspäroide mit Tumorzellsphäroiden konfrontiert. Da die Tumorzysten oder Herzzellen mit einem Vitalfarbstoff gefärbt sind, kann man die Invasivität von Tumorzysten mit einem Laserscanningmikroskop (DEVANEY T.T. et al., 1996) verfolgen oder man fertigt histologische Gewebeschnitte an. Dieses in vitro-Modell erlaubt nicht nur die genaue Quantifizierung der Invasivität von Tumorzysten, sondern ermöglicht auch die Einstufung in klinische Invasionsstadien und simuliert damit die metastatischen Teilschritte (1), (2) und (5).

2.2.5. Intravitale Videomikroskopie zur Beobachtung der frühen Phase in der Metastasierung (GROOM A.C. et al., 1996)


Da das Hostgewebe bei der Invasion eine ebenso wichtige Rolle spielt wie die Tumorzysten selbst, wurde eine große Anzahl von verschiedenen Invasionssubstraten in Zusammenhang mit in vitro-Methoden verwendet, um verschiedenste Tumorzelllinien und Patiententumore zu untersuchen: Mono- oder Multilayer von Muskelzellen, Endothelzellen, Herzorganfragmente, Lungengewebe, Knorpel, Gehirn, ganze Organstücke von Venen oder Blasen, CAM und

3. Diskussion

In vielen Invasivitäts- und Metastasierungsstudien an Tiermodellen werden immunsupprimierte oder thymuslose Tiere mit einem teilweise oder gänzlich fehlenden Immunsystem verwendet, um zu verhindern, daß die speziesfremden Tumorzellen nicht abgestoßen werden. Da auch bei allen mit Ausnahme von 2.2.5 angeführten in vitro-Modellen das Immunsystem nicht berücksichtigt wird, gelten daher sowohl für in vitro- als auch in vivo-Modelle vergleichbare immunologische Randbedingungen.


Literatur

ALBRECHT-BUEHLER G., The phagokinetic tracks of 3T3 cells, Cell, 11, 395-404, 1977
DEVANEY T.T. et al., In vitro evaluation of cancer cell invasiveness by confocal laserscan techniques, 1996
DUNHAM W.B. and TSAO C.S., Intradermal transplantation of hepatoma-cells in guinea-pigs - A model of early cancer, Oncology Reports, 1, 949-51, 1994
FINK-PUCHES R., HELGIC C., KERL H., SMOLE J. et al., Inhibition of melanoma cell directional migration in vitro through different cellular targets, Experimental Dermatology, 2, 17-24, 1993
HIROSHI S., MORIMOTO I., TANIGUCHI Y., MORI H., Multiple metastases of mammary-carcinoma cell-lines isolated from feral mouse, Cancer Letters, 92, 83-6, 1995
JUN S.H., THOMPSON E.W., GOTTARDIS M., TORRI J. et al., Laminin adhesion-selected primary human colon-cancer cells are more tumorigenic than the parental and nonadherent cells, International Journal of Oncology, 4, 55-60, 1994
KALEBIC T., WILLIAMS J., KAO-SHAN C.-S., KRAVITZ B. et al., A novel method for selection of invasive tumor cells: derivation and characterization of highly metastatic K1735 melanoma cell lines based on in vitro and in vivo invasive capacity, Clinical and Experimental Metastasis, 6, 301-18, 1988


KUBOTA T., Metastatic models of human melanoma xenografted in the nude-mouse - The importance of orthotopic transplantation, Journal of Cellular Biochemistry, 1, 8 1994

LINGES-HAN C., Biomedical laboratory animal use in the United States 1973-1995 [Abstract], Alternatives to Laboratory Animals, 24, 206 1996

LOYD R.D., ANGUS W., TAYLOR G.N., THURMAN G.B. et al., Occurrence of metastases in beagles with skeletal malignancies induced by internal irradiation, Health Physics, 66, 293-9, 1994


MARCEL M.M., DE BRUYN G.K., VANDESANDEN F., DRAGONETTI C., Immunohistochemical study of embryonic chick heart invaded by malignant cells in three-dimensional culture, Invasion and Metastasis, 1, 195-204, 1981


OSSOWSKI L. and REICH E., Experimental model for quantitative study of metastasis, Cancer Research, 70, 2300-9, 1980

PAWLEITZ N., PAKU S., WERLING H-O., SPESS E., Experimental approaches to problems of invasion and metastasis, Anticancer Research, 6, 119-28, 1986


SATO M., HARADA K., BANDO T., SHIRAKAMI T. et al., Characteristics of Antitumour-activity of 3,4-Dihydro-6-(4-[3,4-Dimethoxybenzoyl]-1-Piperazinyl)-2(1H)-Quinolone (Venarinine) against a human adenoid squamous carcinoma-Forming cell-Line grown in athymic nude-mice, Cancer Letters, 91, 1-9, 1995


SLEDGE G.W., QULALI M., GOULET R., BONE E.A. et al., Effect of matrix metalloproteinase inhibitor batimastat on breast-cancer regrowth and metastasis in athymic mice, Journal of the National Cancer Institute, 87, 1546-50, 1995


STORME G. and MARCEL M.M., Effects of anticancer agents on directional migration of malignant C3H-mouse fibroplastic cells in vitro, Cancer Research, 40, 943-8, 1980
TERRANOVA V.P., HUJANEN E.S., LOEB D.M., MARTIN G.R. et al., Use of reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells, Proceedings of the National Academy of Sciences USA, 83, 465-9, 1986
TOGO S., SHIMADA H., KUBOTA T., MOOSSA A.R. et al., Host organ specifically determines cancer progression, Cancer Research, 55, 681-4, 1995
USHIO S., IWAKI K., TANAI M., OHTA T. et al., Metastasis-promotin activity of a novel molecule, AG 234-5, derived from mycoplasma, and the complete nucleotide-sequence, Microbiology and Immunology, 39, 393-400, 1995
VERSCHUEREN H. and VAN LAREBEKE N., A new model for the quantitative analysis of cell movements in vitro: definition of a shape change factor, Cytometry, 5, 557-61, 1984
VLODARKSY I., SCHIRMACHER V., ARIAV Y., PURS Z., Lymphoma cell interaction with cultured vascular endothelial cells and with the subendothelial basal lamina: attachment, invasion and morphological appearance, Invasion Metastasis, 3, 81-97, 1983
YASOSHIMA T., DENNO R., KAWAGUCHI S., SATO N. et al., Establishment and characterization of human gastric carcinomas lines with high metastatic potential in the liver - Changes in integrin expression associated with the ability to metastasize in the liver of nude-mice, Japanese Journal of Cancer Research, 87, 153-60, 1996
ZAMORA P.O., DANIELSON K., HOSICK H.L., Invasion of endothelial cell monolayers on collagen gels by cells from mammary tumor spheroids, Cancer Research, 40, 4631-9, 1980
ZETTER B.R., Migration of capillary endothelial cells is stimulated by tumour-derived factors, Nature, 285, 41-3, 1980
Cross-talk reduction in confocal images of dual fluorescence labelled cell spheroids

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Abstract

Dual fluorescence labelling is an advanced method to separate two individual specimens in a biological system using confocal microscopy. An inherent problem of this method is fluorescence channel cross-talk, which causes problems for the exact spatial determination and separation of the specimens. Using a parallel fluorescence detection and an image processing technique, based on an image subtraction method, we have developed a very straightforward method for correcting the dual channel fluorescence images. We successfully applied this method to a 3-dimensional cancer spheroid invasion assay and controlled the cross-talk compensation efficiency by a quality parameter. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Confocal microscopy; Subtraction; Image processing; Image enhancement; Cancer; Confrontation

1. Introduction

With the increasing use and development of confocal microscopy in the 3-dimensional (3-D) analysis of biological structures, it has lead to an increased use of multiple fluorophore detection systems (Sheppard and Cogswell, 1990; Wilson, 1990; Pawley, 1995). The latest developments being in the direction of three or even four channel systems (Carlsson and Liljeborg, 1997). In most cases, multiple labelling causes imperfect fluorescence separation of specimens because the fluorescence spectra of fluorophores overlap (Mossberg and Ericsson, 1990; Carlsson and Mossberg, 1992), hence, cross-talk between fluorescence channels can occur. Sequential detection of each channel or parallel detection in combination with image processing techniques can drastically reduce channel cross-talk and help in the improvement of the image quality. With sequential detection (Mossberg and Ericsson, 1990) cross-talk can be reduced, through the spectral optimisation of the excitation wavelengths and switching between these optimised wavelengths. The disadvantages are that the detection of the images takes longer and that misalignments of the system from image-to-image are a potential source of errors. Furthermore, movements of living specimens result in additional errors and for that reason the observation of dynamic processes are more difficult to attain. Parallel detection of images is realised with fixed single- or multiple frequency excitation, several detectors and with fluorophore dependent dichroic mirrors and bandpass filters for the fluorescence light paths. Advantageous is the relatively short time for the image to be taken, reducing misalignments through movement or other means and therefore dynamic processes can be measured with a higher degree of precision. In most cases the fixed excitation is not the optimal choice concerning cross-talk and therefore the spectral separation of the fluorophore would be worse. Through technical improvements e.g. the modulation of the laser in combination with lock-in amplifiers (Carlsson et al., 1994) a reduction of cross-talk is possible. Furthermore, if in addition to the spectral information also the life-time behaviour of the fluorophores is taken into account, up to four stains can be detected simultaneously and with sufficient accuracy (signal-to-noise ratio) (Carlsson and Liljeborg, 1997). Apart from technical improvements, the image processing techniques are able to reduce cross-talk. The simplest system needs a priori knowledge of the degree of cross-talk, resulting in most cases in unreasonable results because the degree of cross-talk varies from measurement-to-measurement. Sophisticated methods allow the reduction of cross-talk for each individual measurement under the actual conditions. Image subtraction with a suitable coefficient, representing the degree of cross-talk (Carlsson and Mossberg, 1992), image subtraction with noise

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considerations (Vassy et al., 1990) or with specially equalised total intensities (Axner et al., 1991) have been developed and very successfully applied. A more sophisticated method using local image correlation with enhanced colour look up tables and pixel fluorograms overcomes not only cross-talk, but also photobleaching and specimen movements (Demandoix and Davoust, 1997). Some of these methods were previously applied in flow cytometry systems (e.g. Loken et al., 1977). The practical implementation of the image processing techniques for cross-talk compensation requires especially in relation to biological or medical questions, firstly robustness and the possibility of automation without subjective interactions and secondly the exact knowledge of the limit of the cross-talk compensating ability. Furthermore, the limits of the individual methods have not been satisfactorily determined.

Our approach for overcoming cross-talk with dual labelling, which includes the above stated requirements of robustness and automatedness uses image subtraction combined with a local logical intensity comparison. Assuming that the cross-talk grey value is never greater than the grey value of the channel to be detected, each pixel is examined in order to determine to which channel it belongs correctly. The binary image that results is placed as a mask over the original grey value image resulting in an image where only those grey values appear which spatially correspond to the mask. Image pixels that lie outside the mask are identified as cross-talk and are therefore eliminated (grey value is set to 0). All the image pixels, that lie inside the mask are regarded as belonging to the respective channel and the original grey values of these pixels are not changed. Artificial variation of additional cross-talk leading to increasingly worsening conditions (excessive cross-talk) was simulated and the consequences with respect to the measurement results were investigated. Furthermore, a slightly modified form of the commonly used “form factor” (Russ, 1995) named INVASLOG (Smolle et al., 1990, 1992; DeVaney et al., 1997; Ahammer et al., 1998) served as a quality factor for the cross-talk compensation capability. The form factor is a very common shape descriptor representing the “roundness” of an object. For an ideal circular shape the form factor is one and the INVASLOG is zero. Deviations from the circular shape are represented in values less than one for the form factor and in values greater than zero for the parameter INVASLOG. Considering the connected or side-by-side preparations cross-talk signals lead to artificial deviations of the shape. The cross-talk compensation method presented was applied to images of confocal slices of a 3-D multi-cellular spheroid cancer invasion model. Three primary image pairs with different specimen shapes were chosen to evaluate the shape dependency of the algorithm. A fluorescein-derivative stained cancer spheroid is confronted (mechanically placed in contact) with a rhodamine-derivative stained host spheroid (of chick heart cells) and temporarily monitored. The progress of invasion is investigated using the same quantitative parameter INVASLOG, where it is dependent on an exact spatial separation of cancer- from host-areas. Essentially the form and shape of the resulting host tissue area represents the invasiveness of cancer. With the help of the cross-talk compensation method it was possible to construct a quantitative system for evaluating automatically and without subjective evaluation criteria a huge amount of images in a very short time with high precision.

2. Materials and methods

2.1. Confocal microscope

The Confocal Laser Scanning Microscope from Leica (Type TCS NT, Heidelberg, Germany) was used for optical sectioning of the specimen. This confocal microscope was one of the type with a scanning laser beam for the lateral information and with a moveable object stage for the axial information. Two pinholes (one in the excitation path and the other one in the emission path) were positioned at the conjugated points in the optical path (confocal) and therefore eliminated the out of focus signal. The stained specimens have been excited simultaneously through a 10×
0.3NA objective with the laser wavelengths 488 and 568 nm. Sixteen sections have been recorded with an intersectional axial distance of 5–50 μm. The pinhole in the emission path has been set in such a manner, that the theoretical maximum of the resolution (lateral 0.65 μm, axial 4.77 μm) has been approximately reached. A total image size of 500 μm × 500 μm has been obtained with 512 × 512 pixels and a zoom factor of two. The fluorescence of the dye CMFDA (chloromethylfluorescein diacetate) has been detected with a photomultiplier after passing a bandpass filter of 530/30 nm (FITC channel). The fluorescence of the dye CMTMR (tetramethylrhodamine derivative) was detected with the second photomultiplier after passing a longpass filter of 590 nm (TRITC channel).

2.2. Image processing

Image analysis was carried out using the analysis software KS300 (Zeiss, München, Germany) and a personal computer (Pentium 200). To compensate for the cross-talk artefacts the primary images were enhanced by histogram stretching (making use of the full 256 grey values), 3 x 3
median filtered and then subtracted from each other (Fig. 1). To avoid negative grey values, an offset of 128 was added to each new grey value calculated from the subtraction algorithm and grey values > 255 were set to 255, grey values < 0 were set to 0. The totally black background (grey value = 0) in both the primary images would appear in the difference image with the grey value 128. Dual thresholding of the difference image at levels > 128 and < 128 gives two binary images, which are used as masks applied to the primary grey level images. Overall these steps represent a binary decision for each single image pixel, i.e. to which channel the image pixel belongs without loosing the grey value information. Relevant for this decision is the grey level difference of the spatial equivalent image pixels of both the primary images. Each individual image pixel is related to that channel in which the higher grey level was detected.

From these cross-talk corrected images of the specimen under study, the area and the dimensionless negative logarithm of the form factor (Russ, 1995).

\[ \text{INVASLOG} = - \log \left( \text{form factor} \right) = - \log \left( \frac{4\pi \text{area}}{(\text{perimeter}^2)} \right) \]

were carried out. Any deviation of the shape of the specimen or additional cross-talk alter this parameter. Furthermore, in our case the parameter INVASLOG is
Fig. 4. The primary image pair (see Fig. 7) resulted in an INVASLOG value of 0.89 for the TRITC channel, equivalent to a very irregular shape. (a) 3-D surface chart with cross-talk values from 0 to 100% for both the channels; (b) the average values and the standard deviations of the parameter INVASLOG for FITC to TRITC cross-talk from 0 to 70% (10% steps) in dependence on the cross-talk from TRITC to FITC from 0 to 70%.

Additionally used as a parameter for estimating the degree of invasion of host tissue by cancer (Smolle et al., 1990, 1992; DeVaney et al., 1997; Ahammer et al., 1998).

For the evaluation of the quality and reliability of this subtraction method for compensating for cross-talk, we tested the algorithm with artificially increased cross-talk. Outgoing from pairs of images showing practically negligible cross-talk, we added 10, 20, 30% etc. up to 100% of the grey value of the image from the FITC channel to the images from the TRITC channel and vice versa. Therefore, 11 images were created for each channel, including the original image. From these 22 images, we were able to construct 121 different image pairs in each case with a different, but defined additional cross-talk value. Each image pair has been fed to the cross-talk reduction algorithm described above and the parameter area and INVASLOG, which act as quality parameters, were calculated.

2.3. Spheroids, fluorescent dyes and staining

K1781-M2 mouse melanoma cells (kindly provided by Dr. J. Fidler, Inst. of Cell Biology, MD Anderson Hospital, Houston, Texas) in culture were stained and reaggregated to spheroids in a petri dish by the method of Mareel and
Meyvisch (1981), modified by DeVaney et al. (1997). Embryonal chick heart fragments were dissected from a 9 Day old embryo, trypsinised to form single cells, stained and reaggregated to spheroids by the method of Marcel and Meyvisch (1981). The K1735-M2 cells were stained with the fluorescent Cell Tracker Green dye, CMFDA (chloromethylfluorescein diacetate, Molecular Probes, Leiden, The Netherlands) with a concentration of 10 μM for 2 h. The embryonic heart cells were stained with the Cell Tracker Orange dye CMTMR (tetramethylrhodamine derivative, Molecular Probes, Leiden, The Netherlands) and also with a concentration of 10 μM for 2 h.

3. Results

Performing our spheroid experiments, we have observed, depending on the actual measurement conditions (e.g. fluorophore concentrations) varying cross-talk from practically negligible values up to the very bad values of about 100% (unpublished results). By implementing the image processing technique for cross-talk compensation described earlier, we evaluated the limits of the method with additional cross-talk and with the compensation quality parameter INVASLOG. Fig. 2(a) shows in a 3-D surface chart the calculated values for the parameter INVASLOG on varying additional cross-talk values from an original pair of images with negligible cross-talk taken for about 4 h after confrontation of the spheroid pair. There is a very wide range of cross-talk, where the compensation method yields very satisfactory results. This range can be seen as a more or less flat plane in the 3-D surface grid. The spikes in the grid at cross-talk values above 80% represent large deviations in the calculated value of the parameter INVASLOG. In this range (cross-talk > 80%) it becomes physically impossible to compensate for cross-talk, because the cross-talk grey values approximate the grey values of the signal to be detected. The actual value of the INVASLOG of the host (TRITC channel, Ch2) without cross-talk was 0.2 (both cross-talk values = 0 in the chart), reflecting an object with approximately circular shape or in our case with a very low level of the progress of invasion at the beginning of the confrontation. Outgoing from this value, the values obtained with additional cross-talk from up to 70% in both the channels resulted in an overall average value for INVASLOG of 0.18 ± 0.0214 (12%). It is worth noting that without cross-talk compensation the very low standard deviation of 12% would be excessively increased. Fig. 2(b) shows the individual average values and standard deviations, if one image is fixed to a selected cross-talk value and the other image varies from 0 to 70% of cross-talk.

The same evaluations were done with another pair of images from spheroids 1 day after the beginning of confrontation (Fig. 3). The value for INVASLOG for the originally detected images without additional cross-talk was in this case 0.59, reflecting an object with more deviation from the circular shape and in our case an increased progression of invasion. The overall average value for INVASLOG with additional cross-talk up to 70% was 0.63 ± 0.087 (14%). A third pair of images (see Fig. 4) resulted in a value of 0.89 without additional cross-talk and in an average value of 0.83 ± 0.0676 (8%) with up to 70% of additional cross-talk.
In accordance with Figs. 2(b), 3(b) and 4(b), the parameter INVASLOG decreased slightly and the standard deviations increased with increasing cross-talk. Nevertheless neither the standard deviations nor the deviation of the average values were greater than the differences of the values of INVASLOG (0.2, 0.59 and 0.89) of the different original image pairs.

Figs. 5–7 (according to Figs. 2–4, respectively) show the original image pairs and the images with 40 and 80% additional cross-talk for the three cases mentioned above. Each figure includes the image processed and cross-talk compensated TRITC channel (Detected Ch2) and the overlay of the detected binary images of both the channels. Additionally the evaluated values for the INVASLOG and the detected area of Ch2 are shown.

### 4. Discussion

Using the very powerful dual labelling technique, the physically inherent cross-talk between the fluorescence channels has to be considered. This artefact is especially increased if different stain concentrations are present. For example, if measurements are made under conditions in which the fluorescence light intensity of one channel is very low and that from the other channel is high, increasing
the amplification (by applying higher voltages to the photomultiplier) would, without noise considerations, compensate for less light intensity, but the drawback is that the fluorescence light from the overlapping part of the spectrum of the second fluorophore would be amplified with the same amount resulting in enlarged cross-talk. Simple grey value thresholding segmentation algorithms are not applicable, because they cannot distinguish between the different signals making up the whole grey level. The implementation of our developed subtraction algorithm enables the compensation even under a very high cross-talk. The fluorescence light intensity of the cross-talk signal from the erroneous fluorophore has to be in any case less than the light intensity coming from the fluorophore to be detected. Furthermore, the fluorophores must be spatially separated in the specimen. Spatial distributions of the fluorophores in the sub-solution range would be detected in that the fluorophore which results in the higher light intensity in the corresponding image would be determined as being the proper signal. Additionally, local shifts of the fluorescence spectrum would contribute to worse results, because such shifts would alter the local light intensities and therefore the local grey levels. Moreover there must be a negligible misalignment caused by spherical aberrations or misalignments of the optical components in the optical path and the image intensity should be linearly proportional to the dye concentration without interactions such as nonradiative energy transfer or reabsorption. These requirements have to be fulfilled even for the other cross-talk compensation methods mentioned in the introduction section and are therefore not a drawback even for the presented method.

A slight modification of the algorithm enables the detection of the co-localised areas. If the cross-talk signal values do not overlap the co-localised signal values, a logical operator is used to generate a binary mask, representing the co-localised areas. This mask is used to remove these areas prior to the cross-talk reduction steps. Three masks are generated, two of them being one for each channel and the third representing the co-localised areas.

The quantitative evaluation of the cross-talk reduction algorithm showed clearly (see Figs. 2(a)–4(a)), that only very excessive cross-talk in the range of 80–100% in both the channels causes intolerable deviations from the original value of the investigated quality parameter (INVASLOG).

In a very wide range of at least up to 70% of cross-talk, the algorithm deviates only with about ±11% from the original value. Furthermore, this deviation is reduced if less cross-talk is present (as can be seen in Figs. 2(b)–4(b)). The differences between the shapes of the specimen (approximately circular to very irregular shapes) did not affect significantly the compensation capability, representing a wide applicability. The slight decrease in the value of the INVASLOG in dependence on the cross-talk can be explained with the tendency of the cross-talk signals to average the border of the original objects and therefore makes the objects more "round".

Qualitatively even the images with 80% of cross-talk in both the channels, which would represent a situation at very bad condition, could be processed very well (see Figs. 5–7) and this reflects the power of the presented algorithm. The method is not limited to the evaluation of the form factor serving as a quality factor. There are a lot of possible quantitative parameters such as area, perimeter etc. or other shape descriptors e.g. elongation, compactness etc. Once the limits of the cross-talk compensation method have been determined, subtraction combined with a logical operator which is robust will enable the system to be used for automated analysis of fluorescence images in the biological or biomedical field.

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References


INVESTIGATION OF CANCER INVASION USING CELL TRACKER DYSES AND A CONFOCAL LASER SCANNING MICROSCOPE

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INTRODUCTION

Besides the proliferation of the primary tumour, the invasion of cancer in host tissue is a crucial and important step involved in the lethal behaviour of cancer (Mareel M.M. et al. 1991). Our *in vitro* cancer invasion assay uses the Cell Tracker dyes CellTracker Green (CMFDA) and CellTracker Orange (CMTMR), both from Molecular Probes (Leiden, The Netherlands). Therefore it was possible to detect both, the stained tumourous cells and the stained host cells (stroma) in multicell- spheroid preparations separately by means of a confocal laser scanning microscope. The Cell Tracker dyes are membrane permeable and can be used as a cell tracer over a long period. The confocal laser scanning technique enables the non-invasive sectioning of the living specimen producing a 2D picture containing information, comparable to or better than a histological slice. By performing the invasion assay over period of several days, the invasion of the cancer into the host preparation could be quantitatively analysed with image processing techniques. Invasion parameters were generated, e.g. INVASLOG (Smolle et al. 1992), which corresponded to the degree of invasiveness of different cancer types.

METHODS

Spheroids

K1735-M2 mouse melanoma cells (kindly provided by Dr. I.J. Fidler, Inst. f. Cell Biology, M.D. Anderson Hospital, Houston Texas) in culture were stained and reaggregated to spheroids in a petri dish by the method of Mareel and Meyvisch 1981, modified by DeVaney et al. 1997.

Embryonal chick heart fragments were dissected from a 9 day old embryo, trypsinised to form single cells, stained and reaggregated to spheroids by the method of Mareel and Meyvisch 1981.
Fluorescent dyes and staining

Beside the spectral behaviour, phototoxicity or the quantum yield, the property of long term investigation is essential for the confrontation assay. Therefore membrane permeable Cell Tracker dyes forming a stable membrane impermeable glutathione - dye product in the cytosol are very suitable for this case. We tried several fluorescent dyes with different excitation and emission properties with various staining concentrations and staining durations (unpublished results). Following protocol was seen to be the proper solution for our purpose.

K1735-M2 cells were stained with the fluorescent Cell Tracker Green dye, CMFDA (chloromethylfluorescin diacetate, Molecular Probes, Leiden, The Netherlands) with a concentration of 10μM for 2 hours. The embryonic heart cells were stained with the Cell Tracker Orange dye CMTMR (tetramethylrhodamine derivative, Molecular Probes, Leiden, The Netherlands) with the same staining conditions as with the Cell Tracker Green dye.

Confocal Microscopy

The Confocal Laser Scanning Microscope from Leica (Type TCS NT, Heidelberg, Germany) was used for optical sectioning of the specimen. This confocal microscope was one of the type with a scanning laser beam for the lateral information and with a moveable object stage for the axial information. Two pinholes (one in the excitation path and the other one in the emission path) were positioned at conjugated points in the optical path (confocal) and therefore eliminated the out of focus signal. The principal specifications and settings can be seen in the schematic diagram in Figure 1.

![Confocal Microscope Diagram](image)

Figure 1. The stained specimens have been excited simultaneously through a 10x 0.3NA objective with the laser wavelengths 488nm and 568nm. 16 sections have been recorded with an intersectional axial distance of 5-30μm. The pinhole in the emission path has been set in such a manner, that the theoretical maximum of the resolution (lateral 0.65μm, axial 4.77μm) has been approximately reached. A total image size of 500μm x 500μm has been obtained with 512 x 512 pixels and a zoom factor of 2. The fluorescence of the dye CMFDA has been detected with the photomultiplier PMT.1 after passing a bandpass filter of 530nm/30nm. The fluorescence of the dye CMTMR has been detected with the second photomultiplier PMT.2 after passing a longpass filter of 590nm.
Image Processing

Image analysis has been carried out using the analysis software UTIImage Tool 1.27 (developed at the University of Texas Health Science Center at San Antonio, Texas). After dimension calibration, histogram normalisation, median filtering, generation of a binary image and several closing and opening operations, the mathematical operations such as area evaluation, perimeter measurements etc. have been carried out. The dimensionless logarithm of the formfactor $\text{INVASLOG} = \log \left( \frac{\text{perimeter of stroma}^2}{4\pi \text{area of stroma}} \right)$ from the binary picture of the stroma was carried out and has been used as a parameter for the progression of the invasion (Smolle et al. 1990).

RESULTS

The experiments performed so far have shown, that using double staining with the dyes Cell Tracker Green (CMFDA) and Cell Tracker Orange (CMTMR) and the CLSM technique an invasion assay could be built up, where the specimens could be monitored for a time period of up to 3-4 days. The fluorescence intensity has been high enough to reach high signal to noise ratios, which are very important for the quality of the image processing results. For the invasion assay only 1 to 2 total illuminations per invasion day are necessary. A number of 6 total illuminations (1 total illumination means 10 sections and each section measured 4 times = 64 illuminations) for K1755-M2 melanoma spheroids stained with CMFDA led to a bleaching of about 10% of the initial fluorescence and therefore the bleaching is not a crucial point. Figure 2 shows typical results gained so far.

![Figure 2: Several pictures after 4h, 28h and 48h of invasion assay](image)

- a: Grey value pictures of the fluorescence intensity of the Cell Tracker dye CMFDA, corresponding to the K1755-M2 mouse melanoma
- b: Grey value pictures of the fluorescence intensity of the Cell Tracker dye CMTMR, corresponding to the host preparation (embryonal chick heart)
- c: Binary pictures of the overlapped grey level pictures. The light grey value correspond to the host preparation (b) the mean grey value to the melanoma (a) and the dark grey value to the region were both dyes gave a signal.
The grey level and binary pictures after different times of the initial contact represent one of the 16 slices of the whole measurement. The information of all 16 slices has been used for 3D visualisation and is not shown in this paper. Figure 2c shows the results of the image processing algorithm described in the method section. Very interesting are the dark grey values, which correspond to areas, where both dyes gave a detectable signal.

The calculation of the parameter INVASLOG was possible and corresponded to the progression of the invasion (see Table).

**Table. Correspondence of the parameter INVASLOG with the progression of invasion.**

<table>
<thead>
<tr>
<th>Time after initial contact [h]</th>
<th>Area of stroma [arbitrary units]</th>
<th>Perimeter of stroma [arbitrary units]</th>
<th>INVASLOG [no dim.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>75990</td>
<td>1644</td>
<td>0.454</td>
</tr>
<tr>
<td>38</td>
<td>34500</td>
<td>1852.5</td>
<td>0.898</td>
</tr>
<tr>
<td>105</td>
<td>27095</td>
<td>2347</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Performing the invasion assay with this fluorescence and CLSM technique enabled the generation of the relevant data in a very short time period, comparable to other techniques used so far (e.g. Helige et al. 1993). Especially the time consuming sectioning by means of histological procedures could be avoided and the use of animals in the metastatic invasion research could be reduced through the use of this accurate and clinically significant method (Smolle et al. 1990). Future prospects are the examination of the influence of pharmaceutical and chemotherapeutical reagents or radiation (UV or ionising radiation, including X-rays and light ions) to the invasion of cancer.

**REFERENCES**


Vorträge

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DOPPELFURORESZENZMARKIERTE KONFOKALE
MIKROSKOPIE UND DIGITALE BILDVERARBEITUNG ZUR
REDUKTION DES ÜBERSPRECHENS

H. Ahammer, T.T.J. DeVaney, M. Hartbauer, H.A. Tritthart


Zusammenfassung


Einleitung


Da sich die Emissionspektren der beiden Farbstoffe, wenn auch nur im geringen Ausmaß bei optimierter Kombination, überschneiden, muß das Übersprechen (CrossTalk) der beiden Kanäle berücksichtigt werden. Insbesondere ist das der Fall, wenn nicht nur schöne Bilder aufgenommen werden sollen, sondern diese Bilder in weiterer Folge automatisiert quantitativ ausgewertet werden sollen. Aus diesen Gründen wurde von uns mittels digitaler Bildverarbeitung das Übersprechen reduziert und zusätzlich mit künstlich erhöhtem Übersprechen die Auswirkung auf die Messergebnisse untersucht. Die Genauigkeit des Verfahrens wurde bis an seine Grenzen ausgetestet, wodurch genau ermittelt werden konnte, welchen Einfluß das Über-

 sprechen auf die Auswertung des Invasionsparameters hatte.

Methode

Die Krebszelllinien (K1755/M2, A375/SM- Krebs bzw. isolierte embryonale Hühnerherzzenlen als Modell für das gesunde Gewebe(Host)) wurden mit geeigneten Vital-Fluoreszenzfarbstoffen (CMFDA bzw. CMTMR oder DiI bzw. DiO, Molecular Probes) getrennt gefärbt (ca. 10μM, 2 Stunden) und davon wurden jeweils in einer Kultur Sphäroiden (Ø 50-200μm) gebildet (DeVaney et al. 1997). Nach Konfrontation eines Krebsphäroids mit einem Histonphäroid konnte der Verlauf der Invasion mit einem konfokalen Laser-Scanning-Mikroskop (Leica TCS NT, Objektiv 10x NA0.3, Heidelberg) schrittweise und gleichzeitig in zwei Emissionsbereichen (entsprechend der beiden Farbstoffe, FITC bzw. TRITC Bereich) vermesset werden. Die optische Auflösung lag nicht wesentlich über dem theoretischen Minimum. Jeweils 2 digitalisierte Bilder (jeweils 512×512 Pixel), pro Schnitt (16 Schnitte insgesamt, maximale Schnitttiefe ca. 50-100μm) wurden auf ein Magneto-Optisches Laufwerk (230MB) abgespeichert und mit einer Bildverarbeitungssoftware (KS300, Zeiss) auf einem PC (Pentium 200) quantitativ ausgewertet.


An den korrigierten Bildern wurden quantitative Parameter wie z.B.: Fläche, mittlerer Grauwert etc. und der Invasionsparameter INVASLOG (Logarithmus des Formfaktors), der im wesentlichen die Abweichung von der Kreisform angibt, bestimmt. Der Parameter INVASLOG stellt ein quantitatives Maß für die Invasivität dar (Smolle et al. 1990 und 1992).

**Ergebnisse**

In Abb.2 sind die Ergebnisse exemplarisch an einer Konfrontation von einem K1755 Sphäroid (Krebs) mit einem Hühnerherzcellspäroid (Host) nach einer Konfrontationszeit von ca. 4 Stunden gezeigt. Bedingt durch die kurze Konfrontationszeit war in diesem Fall die Invasion noch nicht fortgeschritten, sondern zeigt den Fall der Kontaktaufnahme beider Sphäride. Beide Sphäride zeigten eine relativ hohe und ungefähr gleiche Fluoreszenzintensität, weshalb in beiden Bildern praktisch kein Übersprechen ersichtlich ist (Abb2.: „Grey Ch1“ und „Grey Ch2“ bei zusätzlichem „Crosstalk 0%“). Beide Objekte konnten daher sehr gut durch die digitale Bildverarbeitung detektiert und quantitativ ausgewertet werden (für Abb2 wurden „Detected Ch2“, „Detected Binary“, „Detected Area Ch2“ und der Invasionsparameter „INVASLOG Ch2“ ausgewählt). Zum ursprünglichen Bildpaar wurden jeweils 40% bzw. 80% des Grauwertes des jeweils anderen Kanals künstlich addiert, wodurch ein künstlich erhöhtes Übersprechen simuliert wurde. Die wiederum ausgewerteten Bilder und Parameter sind in den entsprechenden Rubriken in der Abb.2 dargestellt. In beiden Fällen konnte das sehr hohe Übersprechen eliminiert werden, wobei die Detektion der Objekte kaum beeinflußt wurde, wie an den quantitativen Meßwerten zu erkennen ist.

**Diskussion**


Die Anwendung dieser bildanalytischen Methode ist nicht auf die konfokale Mikroskopie beschränkt und kann daher auch für die herkömmliche Zweikanal Fluoreszenzmikroskopie verwendet werden.
Abb. 2. Die Bilderspalt „Grey Ch1“ bzw. „Grey Ch2“ sind Grauwertbilder des FITC (Krebs) bzw. TRITC (Host)-Emissionskanals mit 0% bzw. 40% und 80% künstlich erhöhtem Übersprechen (Cross Talk). Die Spalten „Detected Ch2“ bzw. „Detected Binary“ sind die jeweils von der digitalen Bildverarbeitung erkannten Objekte für den TRITC-Kanal und die Spalten „Detected Area Ch2“ bzw. „INVASLOG Ch2“ sind zwei ausgewählte quantitative Parameter für jeweils den TRITC-Kanal.

Literatur


Adresse

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Bestimmung der Invasivität von Krebsphäroiden mittels konfokalem Laser Scanning Mikroskop

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Reference List


   Research 6, 419-436 (1986).

3. Mareel,M., Bracke,M. & van Roy,F. Cancer metastasis: negative regulation by an invasion- 

4. Bracke,M.E. et al. Confrontation of an invasive (MO4) and a noninvasive (MDCK) cell line with 
   embryonic chick heart fragments in serum-free culture media. In Vitro Cell Dev. Biol. 22, 508- 
   514 (1986).


   embryonic chick heart invaded by malignant cells in three-dimensional culture. Invasion and 

7. Mareel,M.K. & Meyvisch,C. Invasion of malignant cells in vivo and in vitro: similarities and 

8. Shen,Y., Vogel,I. & Kalthoff,H. [Comparative study of metastasis-associated characteristics of 
   (2000).

   Neurosurg. 53, 808-815 (1980).

10. Leskovar,P. & Bielmeier,J. Treatment of solid tumors should obligatorily be combined with the 
    in vivo codepletion of tumor-protecting, CD8(+)HLA-DR(+)suppressor T cells by alloreactive 
    donor T cells whose preprogrammed cell death allows a high GvL-effect before GvHD can be 
    established. Results of animal experiments, including more than 6000 mice. PFLUG. ARCH. 

11. Wild,C.P. & Kleihues,P. Etiology of cancer in humans and animals. Exp. Toxicol. Pathol. 48, 
    85-100 (1998).

12. Yambe,T. et al. Fluctuations of the sympathetic nerve discharges in animals without natural 

13. Vainio,H. & Cardis,E. Estimating human cancer risk from the results of animal experiments: 

    to the liver, and the roles of proteinases and adhesion molecules: new concepts from in vivo 


17. Mirvish,S.S. Studies on experimental animals involving surgical procedures and/or 


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