CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: http://orca.cf.ac.uk/56862/

This is the author’s version of a work that was submitted to / accepted for publication.

Citation for final published version:

Thomas, Christopher P., Clark, Stephen Robert, Hammond, Victoria Jayne, Aldrovandi, Maceler, Collins, Peter William and O'Donnell, Valerie Bridget 2014. Identification and quantification of aminophospholipid molecular species on the surface of apoptotic and activated cells. Nature Protocols 9 (1) , pp. 51-63. 10.1038/nprot.2013.163 file

Publishers page: http://dx.doi.org/10.1038/nprot.2013.163
<http://dx.doi.org/10.1038/nprot.2013.163>

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher’s version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.

information services
gwasanaethau gwybodaeth

Identification and quantitation of aminophospholipid molecular species on the surface of apoptotic and activated cells.

Christopher P. Thomas[†1], Stephen.R. Clark[†1], Victoria J. Hammond[1], Maceler Aldrovandi[1], Peter W. Collins[1], and Valerie B. O'Donnell[1*]

Affiliations: [1]Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK.

thomascp@cardiff.ac.uk, clarksr2@cardiff.ac.uk, hammondrj@cardiff.ac.uk, collinspw@cardiff.ac.uk, o-donnellvb@cardiff.ac.uk

*To whom correspondence should be addressed: Valerie B O'Donnell, Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK. Phone: 00442920687313, Fax: 00442920687303, Email: o-donnellvb@cardiff.ac.uk

[†]These authors contributed equally to the manuscript.

Abstract

This protocol measures externalization of aminophospholipids (APL) to the outside of the plasma membrane using mass spectrometry (MS). APL externalization occurs in numerous events, and is relevant for transplant medicine, immunity and cancer. In this protocol, externalized APL are chemically modified using a cell impermeable reagent (sulfo-NHS-biotin), then isolated using a liquid:liquid extraction, and quantified using reverse phase liquid chromatography tandem MS, against in-house generated standards. This protocol describes a complimentary method to existing assays that are not quantitative, e.g. annexin V flow cytometry~~. Herein, the protocol is presented in full, including approach, methodologies and troubleshooting. The method~~ and is applicable to the study of membrane reorganization in all cell types during apoptosis (e.g. during development, cancer, psychiatric disorders and other conditions), ageing, vesiculation and cell division). ~~, and~~ The procedure takes approximately 2-4 days, including generation of standards).

Ontology:

Biological sciences / Cell biology / Cell death / Apoptosis

Biological sciences / Biological techniques / Spectroscopy / Mass spectrometry

Categories:

Cell Biology; Spectroscopy

Keywords: aminophospholipid, aminophospholipid externalization, aminophospholipid quantitation, aminophospholipid quantification, apoptosis, cell death, mass spectrometry, LC/MS, membrane reorganization,

INTRODUCTION

Development of the protocol. The external leaflet of the plasma membrane is comprised of predominantly phosphatidylcholine (PC) and sphingomyelin with the aminophospholipids (APL), phosphatidylethanolamine (PE) and phosphatidylserine (PS) facing the inside (*1*). This asymmetry is maintained in healthy cells, but lost during cell ageing, apoptosis and immune cell activation, when APL translocate to the outside in order to aid coagulation factor and complement binding (*2, 3*). APL externalization is also important for transplant medicine (e.g. platelet transfusion) and cancer. Up to now, its measurement has been indirect, typically using annexin V as a flow cytometry indicator, and it has been difficult to determine to either the specific APL molecular species, or amounts externalized. This has hampered studies into their mechanism of action in important cellular processes including coagulation and cell clearance.

Comment [DB1]: Should be superscript throughout, but copyedit can fix.

PE and PS are families of lipids that are structurally related in that they contain a glycerol backbone, phosphate, and two fatty acids, but with differing headgroups, serine (PS) or ethanolamine (PE). Furthermore, the fatty acids can be either saturated or unsaturated, unoxidized or oxidized and be of varying length hydrocarbon chains. Thus, both PE and PS comprise families of several different lipids. The function of phospholipid fatty acid substitutions and ether vs acyl bonds in health and disease has not been widely investigated at least in part because of the difficulties in measuring their externalization directly. To address this, we developed a tandem mass spectrometry method that allows determination of both PE and PS externalization, and also reports on both specific APL molecular species trafficked, their amounts and % total cellular PS/PE pool. The APL species externalized by human platelets upon

Comment [DB2]: I assume that the reference here is “number 4”?

activation have been determined using this assay and are shown (Figure 1) (*4*). The assay was also recently applied to human neutrophils (Figure 2).

This assay represents a step-change from annexin V flow cytometry in terms of characterizing APL externalization by activated/apoptotic cells. Knowing the specific molecular species and amounts that are externalized can allow mechanistic studies of how phospholipids participate in key cellular processes to be undertaken. Using the approach, we found that platelets specifically externalized five PE and two PS species, and that the platelet specific isoforms were the most potent at promoting tissue factor dependent thrombin generation, a critical component of physiological hemostasis (*4*). The results allowed us to pose a novel paradigm regarding the role of APL fatty acids in regulating coagulation.

Comment [DB3]: Please rephrase to clarify what you mean by this?

Comparison with other methods Up to now, measurement of APL externalization has been determined using approaches that include (i) addition of fluorescent phospholipids to cells, (ii) annexin V flow cytometry and (iii) additional protein indicators including lactadherin and cinnamycin. The most widely used, annexin V, recognizes APL on the surface and is used as a non-quantitative probe. It is generally described as detecting PS, although several annexins are known to bind both PS and PE (*5-11*). None of these methods are quantitative. Furthermore, none allow identification of the specific molecular species of APL that externalize on cell activation/apoptosis/ageing. A small number of studies have used the UV visible, cell-impermeable reagent trinitrobenzenesulfonic acid (TNBS) for UV detection following derivatization. However, most of these did not identify molecular species externalized, and when this was undertaken, it required HPLC purification followed by saponification and GC/MS to identify the fatty acid side chains, a highly laborious process that results in significant losses (*12-*

14). Furthermore, TNBS derivatization was undertaken at 4° C, and UV detection would not be sufficiently sensitive for use on small amounts of primary human tissue, such as used herein. Thus, reliable and robust methods for monitoring and quantifying APL exposure routinely are currently not available. We acknowledge that for routine monitoring of APL externalization, some investigators may prefer to use Annexin V as it is faster, more convenient, and doesn't require as high a level of specialist expertise as the method outlined herein. Thus, the methods should be considered complimentary, providing distinct information that could be utilized in different ways to further our understanding of the biology of APL externalization on mammalian cells.

Experimental design.

The approach described is based on the use of the cell impermeable reagent, EZ-link sulfo-NHS-Biotin (SNB). This biotinylates primary amines, and has been extensively used for proteomics of extracellular facing proteins on intact cells (*15*). In a similar way, it can be used to derivatize external facing APL primary amine headgroups (NH_2 groups), leading to a mass shift of 226 amu. Thus, external APLs are distinguished from internal forms through a change in chemical structure that is then measured using a sensitive and specific LC-MS/MS method (Figure 3).

Optimising the approach for other cell types: When working with cells other than those described herein, the specific lipids to monitor using the assay must be determined by characterizing the PE and PS in the specific cells of interest. This is described in the ~~protocol~~procedure (steps….). In the case of some mammalian cells, it is anticipated that there will be a high proportion of unsaturated and plasmalogen (PE) species as for platelets and neutrophils. Further details of this process is given in the online Supplementary Data section for (*4*).

Formatted: Font: Bold

Comment [DB4]: Please replace "this process" with words that make it clear what you are referring to (at present ambiguous as to whether it is a biological or an experimental process). Obviously happy to refer this supplementary data, but consider carefully whether any more information needs to be brought forward to this protocol.
Given that this supp info is quite important to the protocol, please add the URL for easy navigation.

First, the predominant PE and PS molecular species contained in the cells of interest are characterized using MS scanning approaches. This requires generation of a total cellular lipid extract which is then scanned for PE and PS using MS. PS is identified by the neutral loss of 87 amu in negative ion mode, in crude lipid extracts. Specifically, PS is detected by analysing for molecules that lose the characteristic headgroup of 87 amu on collision-induced-dissociation. However, PE needs to be purified from other phospholipid classes before MS scanning, due to the presence of isobaric and interfering ions. To do this, we analyze crude cell lipid on normal phase HPLC, which separates based on headgroup, monitoring at 205 nm (based on unsaturated fatty acids).

A further issue when working with other cell types is the need to prevent membrane changes that might result in APL exposure in the outer plasma membrane artifactually, for example during cellular dissociation (collagenase digestion when processing tissue samples), or during flow-assisted cell sorting using antibodies that might directly activate cells.

Generating appropriate primary and internal standards Next, a series of biotinylated primary and internal standards that correspond to these are synthesized and standard curves generated. Our assay uses biotinylated analogs of 1,2-dimyristoyl-sn-glycero-3-PS (DMPS) and 1,2-dimyristoyl-sn-glycero-3-PE (DMPE) as internal standards since these are low/absent in mammalian cells. Primary biotinylated lipids for the molecular species to be quantified are also required. Where a specific lipid is not commercially available, we used the most closely related for quantitation. A lipid with a plasmalogen bond between the sn2 fatty acid and the glycerol backbone, for example, would be used for a commercially unavailable plasmalogen species e.g. 16:0p/20:4-PE. Similarly a lipid with a diacyl bond in this position is used for unavailable diacyl species. See Figure 1 for structures.

Formatted: Font: Bold

Overview of the procedure: Cells (platelets and neutrophils are described herein) are isolated, activated appropriately, then external APL species labelled using the reagent. Last, the lipids are extracted from the samples, and analyzed for their biotinylated forms using LC-MS/MS. Quantitation is achieved using the standard curves. Separately, the % of the total APL pool externalized can be determined by comparison with samples where total APL has been biotinylated using a cell-permeable analog, NHS-biotin.

Optimizable parameters: When the method was first established, we undertook extensive optimization of all steps involved, including varying incubation time for labeling and quenching, and varying concentrations of both biotinylation reagent and lysine. Extraction efficiencies were also determined for a number of solvent-solvent methods, and HPLC separation of both PE-biotin (PE-B) and PS-biotin (PS-B) species compared across a range of reverse phase columns and mobile phases. The detailed characterization is presented in the online Supplementary Data section for (*4*). The conditions presented here will be transferable to other laboratories, although it will be essential to conduct tuning on all standards for MS detection on individual instruments, since ionization potentials, temperature, etc will differ across platforms. We include a flow diagram that demonstrates the steps involved and order of work, including timelines and options (Figure 4).

Applications of the method.

In this protocol, we demonstrate how the method can be used to quantify PE and PS externalization to the external side of the platelet and neutrophil plasma membrane, as examples. The method is widely applicable to mechanistic studies on phospholipid translocation, e.g. the study of flippase, floppase, and scramblases in several cell types, as well as characterization of membrane lipid behaviour in additional important events where phospholipid translocation is

Formatted: Font: Bold

Comment [DB5]: I am sure that there is a better in-line heading that could be used here… Please have a look, and edit as appropriate

Formatted: Font: Bold

Formatted: Font: Bold

known to occur, such as apoptosis, ageing, vesiculation, cell division and cell activation. Apoptosis occurs during many cellular events, including development, cancer, psychiatric disorders, indicating wide potential utility of the assay. The method could be adapted to HPLC-fluorescence or absorbance detection, by utilizing an amine-reactive fluorophore or chromophore in place of SNB, several of which are commercially available for protein-labeling studies from Pierce. As this method involves the use of mass spectrometry, technical competence in this technology, and access to a tandem instrument is required. Most labs should be able to access this through either core-facilities or collaborators, if it is not available in-house. Alternatively, we would collaborate with those wishing to use the assay, either to advise on set-up, provide analytical standards, or to run samples on our instrument in Cardiff.

Limitations.

Currently, the most widely-used method for determining APL exposure is Annexin V-FITC flow cytometry. As described in the introduction, Annexin V is a non-quantitative probe that only reports on whether a cell is positive or negative for APL exposure, but provides no information on specific molecular species, or their amounts. The primary disadvantages of the new LC-MS/MS assay *versus* annexin V are: (i) inability of the new assay to distinguish individual cells that externalize APLs vs from those that don't and (ii) inability to study cells while they are suspended in lipid- or protein-containing solutions (e.g. plasma, or serum-containing tissue culture medium). Last, this method takes longer than annexin-based methods and requires a considerable degree of technical training and access to more expensive mass spectrometry equipment. A MS expert would easily set up the method, but a more junior researcher might require some support. We would be very happy to give advice, provide biotinylated standards,

or to analyse samples for researchers unable to access the technology in their own institutions, as required.

MATERIALS.

CAUTION. Working with organic solvents Extraction of lipids with solvents must be performed in glass and not plastic, using a fume hood and with appropriate personal protective equipment. Due to the possible contamination of glassware with highly concentrated lipid extracts during this procedure, we dispose of glass tubes after single use.

Reagents

EZ-Link Sulfo-N-hydroxysuccinimide (NHS)-Biotin (SNB): Thermo Scientific

N-hydroxysuccinimide (NHS)-biotin (NB): Thermo Scientific

~~Solvents: chloroform, methanol, hexane, isopropanol, water, acetic acid: Fisher Scientific (Massachusetts, USA). Use only HPLC grade solvents.~~

~~Phospholipid standards (all from Avanti Polar Lipids (Alabaster, Alabama, USA):~~

~~1,2-dimyristoyl-sn-glycero-3-PS (DMPS; 14:0/14:0-PS) Catalog no. 840033~~

~~1,2-dioleoyl-*sn*-glycero-3-PS (DOPS; 18:1a/18:1-PS) Catalog no. 840035~~

~~1-stearoyl-2-oleoyl-sn-glycero-3-PS (SOPS; 18:0a/18:1-PS) Catalog no. 840039~~

~~1-stearoyl-2-arachidonoyl-sn-glycero-PS (SAPS; 18:0a/20:4-PS) Catalog no. 840064~~

~~1,2-dimyristoyl-sn-glycero-3-PE (DMPE; 14:0/14:0-PE) Catalog no. 850745~~

~~1-(1Z-stearoyl)-2-arachidonoyl-sn-glycero-3-PE (SpAPE; 18:0p/20:4-PE) (plasmalogen, vinyl ether linked lipid denoted by p following sn1 fatty acid) Catalog no. 852804C~~

~~1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (SAPE; 18:0a/20:4-PE) Catalog no. 850804~~

~~Brain or egg PE (Avanti Polar Lipids 840022, 840021)~~

Lysine: ~~,~~ Sigma Aldrich (Missouri, USA)

calcium chloride: ~~,~~ Sigma Aldrich (Missouri, USA)

Comment [DB6]: Please add an item for the cells to be analysed. You can refer to the Introduction for further information, but make sure that you have mentioned the basic requirements – number of cells per sample; how they should be obtained / maintained; media / buffers to avoid etc.

Comment [DB7]: Meaningful to add catalog numbers? I assume not… given that you have provided these for some but not others…

I have re-arranged the list to make the sub subheadings meaningful.

Formatted: German (Germany)

ammonium acetate: Sigma Aldrich (Missouri, USA)

triethylamine: Sigma Aldrich (Missouri, USA)

dimethylsulfoxide (DMSO): Sigma Aldrich (Missouri, USA).

Solvents

chloroform: Fisher Scientific (Massachusetts, USA)

methanol: Fisher Scientific (Massachusetts, USA)

hexane: Fisher Scientific (Massachusetts, USA)

isopropanol: Fisher Scientific (Massachusetts, USA)

water

acetic acid: Fisher Scientific (Massachusetts, USA). Use only HPLC grade solvents.

Phospholipid standards (all from Avanti Polar Lipids (Alabaster, Alabama, USA):

- 1,2-dimyristoyl-sn-glycero-3-PS (DMPS; 14:0/14:0-PS) Catalog no. 840033
- 1,2-dioleoyl-*sn*-glycero-3-PS (DOPS; 18:1a/18:1-PS) Catalog no. 840035
- 1-stearoyl-2-oleoyl-sn-glycero-3-PS (SOPS; 18:0a/18:1-PS) Catalog no. 840039
- 1-stearoyl-2-arachidonoyl-sn-glycero-PS (SAPS; 18:0a/20:4-PS) Catalog no. 840064
- 1,2-dimyristoyl-sn-glycero-3-PE (DMPE; 14:0/14:0-PE) Catalog no. 850745
- 1-(1Z-stearoyl)-2-arachidonoyl-sn-glycero-3-PE (SpAPE; 18:0p/20:4-PE) (plasmalogen, vinyl ether linked lipid denoted by p following sn1 fatty acid) Catalog no. 852804C
- 1-stearoyl-2-arachidonoyl-sn-glycero-3-PE (SAPE; 18:0a/20:4-PE) Catalog no. 850804

Brain or egg PE (Avanti Polar Lipids 840022, 840021)

Equipment

~~HPLC Columns: Ascentis C18, 5 μm, 150 × 2.1 mm (Sigma-Aldrich)~~

~~Luna C18, 3 μm, 150 × 2 mm (Phenomenex, Torrance, CA)~~

Formatted: Font: Bold

Formatted: Font: Bold

Formatted: Spanish (International Sort)

Formatted: Spanish (International Sort)

Comment [DB8]: I have re-arranged the list to make the sub subheadings meaningful.

Discovery C18, 5 µm, 250 x 4.6 mm (Supelco Analytical; Sigma-Aldrich)

Spherisorb S5W, 5 µm 150 × 4.6 mm column (Waters, Herts, UK)

Mass spectrometer: AB Sciex 4000 Q-Trap *<CRITICAL> Note: The use of this protocol is not limited to the AB Sciex 4000 Q-Trap, and other mass spectrometers can be used. Other instruments that could be used for this protocol could include: AB Sciex 3000, 5500 and 6500 Q-Traps, as well as equivalent tandem MS instruments from Waters, Bruker, Agilent and Thermofisher.*

HPLC System: Shimadzu DGU 14A degasser

SIL-HTc Autosampler

LC-10AD *VP* µ Binary Pump System

An analytical or micro weigh balance, e.g. Mettler Toledo **XP2U**

Vacuum Evaporation System: Labconco RapidVap *Note:,<CRITICAL> if not available, a Dryblock connected to nitrogen or argon stream can be used instead.*

HPLC System:

Formatted: Font: Bold

Shimadzu DGU 14A degasser

SIL-HTc Autosampler

LC-10AD *VP* µ Binary Pump System

HPLC Columns:

Formatted: Font: Bold

Ascentis C18, 5 µm, 150 × 2.1 mm (Sigma-Aldrich)

Luna C18, 3 µm, 150 × 2 mm (Phenomenex, Torrance, CA)

Discovery C18, 5 µm, 250 x 4.6 mm (Supelco Analytical; Sigma-Aldrich)

Spherisorb S5W, 5 µm 150 × 4.6 mm column (Waters, Herts, UK)

Formatted: Indent: Left: 0 cm, First line: 0 cm

Formatted: Indent: Left: 0 cm

Formatted: Indent: Left: 0 cm, First line: 0 cm

Reagent setup.

Tyrode's buffer (Ca^{2+} free) 1 L

NaCl	7.83 g	(134 mM)
$NaHCO_3$	1.01 g	(12 mM)
KCl	0.22 g	(2.9 mM)
Na_2HPO_4	0.09 g	(0.34 mM)
$MgCl_2$	0.20 g	(1.0 mM)
HEPES	2.38 g	(10 mM)
Glucose	0.90 g	(5 mM)

Formatted: German (Germany)

Prepared by adding chemicals to sterile water to make 1 L in a volumetric flask. pH values are checked and adjusted to pH 7.4. Aliquots (50 ml) are then dated before being frozen and stored at -20 ^{o}C, retain for no longer than 12 months. **Do not repeat freeze and thaw process**.

Phosphate buffered saline (PBS)

Dulbecco's phosphate-buffered saline (no calcium, no magnesium), Gibco, Catalog Number 14190-094

Comment [DB9]: Storage conditions / shelf-life? Prepared freshly?

ACD (Acid citrate dextrose) 1 L

Trisodium Citrate	25.0 g	(85 mM)
Citric acid	13.7 g	(65 mM)
Glucose	20.0 g	(111 mM)

Prepared by adding chemicals to sterile water to make 1 L in a volumetric flask. pH values are checked and adjusted to pH 5.0. Aliquots (20 ml) are then dated before being frozen and stored at -20 ^{o}C, retain for no longer than 12 months. **Do not repeat freeze and thaw process**.

$CaCl_2$ (100mM): 10 ml

1.10 g Calcium Chloride (anhydrous)

Prepared by adding chemical to 10 ml of sterile water before aliquoting in to 1 ml dated eppendorfs for storage at -20 ^{o}C, retain for no longer than 12 months.

2.8 % Citrate 1 L

Sodium Citrate Tribasic 28 g

in PBS, pH 7.3, filter sterilised

Comment [DB10]: Storage conditions / shelf-life? Prepared freshly?

0.4 % Citrate 1 L

Sodium Citrate Tribasic 4 g

in PBS, pH 7.3, filter sterilised

Comment [DB11]: Storage conditions / shelf-life? Prepared freshly?

Hypotonic Saline (0.2 %) 1 L

Sodium Chloride 2 g

in water, filter sterilised

Comment [DB12]: Storage conditions / shelf-life? Prepared freshly?

Hypertonic Saline (1.6 %) 1 L

Sodium Chloride 16 g

in water, filter sterilised

Comment [DB13]: Storage conditions / shelf-life? Prepared freshly?

Krebs buffer (Ca^{2+} free) 1 L

HEPES	11.38 g	(500 mM)
NaCl	5.8 g	(100 mM)
KCl	0.38 g	(5 mM)
$NaH_2PO_4.2H_2O$	0.16 g	(1 mM)
Glucose	0.36 g	(2 mM)

pH 7.4

Comment [DB14]: Storage conditions / shelf-life? Prepared freshly?

EZ-link Sulfo-NHS-Biotin in 5 ml of Tyrode's buffer

EZ-link Sulfo-NHS-Biotin 25mg (11 mM)

Note: this needs to be made up immediately before use.

Lysine in 5ml of Tyrode's buffer

Lysine	183 mg	(250 mM)

Solvent mix 1

Chloroform	100 ml
Methanol	200 ml

Comment [DB15]: Storage conditions / shelf-life? Prepared freshly?

Solvent mix 2

Hexane	300 ml
Isopropanol	200 ml
1M acetic acid	20 ml

Comment [DB16]: Storage conditions / shelf-life? Prepared freshly?

PROCEDURE

Synthesis of Biotinylated Standards **<TIMING>** ~~Generation of standards takes~~ 2-3 days including synthesis and purification.

1. Open fresh ampoule of lipid standard in chloroform as in reagent list (supplied as 25 mg/ml in chloroform), and remove 1 mg into a 1.5 ml glass vial. Aliquot remaining lipid into vials and store in chloroform at -80°C under argon (if not available, nitrogen can be used instead).

2. Evaporate 1 mg lipid standard under nitrogen stream and reconstitute in 220 µl chloroform and 110 µl methanol in a small glass vial. Weigh approximately 6 mg NHS-Biotin (powder) on a weigh boat and transfer to the vial (to give 52 mM), vortex and add 3.3 µl triethylamine. Incubate at room temperature for 30 minutes.

Comment [DB17]: Please define a range at first use of this term.

Formatted: Highlight

3. Sediment the excess NHS-Biotin reagent using a 500 x g, 5 minute centrifugation. Carefully transfer the solvent to a clean glass vial. "Wash" the remaining NHS-Biotin with a chloroform/methanol (2:1) solution, vortex and repeat centrifugation step. Combine solvent fractions and dry, re-suspending biotinylated lipids in 500 µl methanol for HPLC purification.

4. Purify using reverse phase HPLC on a Discovery C18 column (25 cm x 4.6 mm, 5µm) with the following conditions; temperature 22 °, flow rate - 1 ml min^{-1}, gradient elution profile - 50 % mobile phase B to 100 % mobile phase B (A: 5 mM ammonium acetate in water, B: 5 mM ammonium acetate in methanol) over 15 min. Hold at 100 % B for 20 min before re-equilibration to 50% B, UV absorbance - 205 nm.

5. Inject 100 µl of sample. Collect fraction and dry using RapidVap or dryblock with nitrogen stream with temperature at 30 °C and re-suspend sample in 200 µl methanol.

~~5.~~6. Accurately weigh the glass vial that the sample is to be stored in ensuring the vial is clean, dry and gloves are worn at all times when handling. Transfer 200 µl sample into pre-weighed glass vial and dry solvent. Re-weigh glass vial and record difference in weight. Use a high accuracy analytical or micro balance for this, for example the Mettler Toledo XP2U.

~~6.~~7. Re-suspend in methanol at 100 ng.µl^{-1} which allows simple dilution for standard curve preparation, and store under argon gas (or nitrogen if argon is not available) at -80 ºC. MS/MS spectra and fragmentation patterns of a biotinylated PE and PS are shown for reference in Figure 5.

Note: Before purifying biotinylated standards it is recommended that a small fraction of lipid substrate be analyzed using the HPLC method to determine accurate retention times. Typically the substrate lipid DMPE/DMPS eluted around 27 minutes with the longer fatty acyl chain lipids eluting around 30 minutes.

Formatted: List Paragraph, No bullets or numbering

Comment [DB18]: I think that this should be a step in its own right. Please consider where this could be inserted. The steps might go something like:
4. Setup the HPLC.
5. Run the lipid substrate sample to get starting material retention time(s).
6. Run a small amount of the crude product to check that all separates nicely.
7. Purify the product.
If you don't think that it should be its own step, then please consider carefully where this advice should be placed; probably as a CRITICAL STEP callout before the purification step. Alternatively, the whole HPLC setup plus this bit of advice, could be moved to the EQUIPMENT SETUP.

~~TIMING. Generation of standards takes 2-3 days including synthesis and purification.~~

PAUSE POINT. Standards can be kept for up to a year in storage at -80 ℃.

Isolation of cell samples

~~7.~~8. Perform option A below for isolation and activation of human platelets; perform option B for isolation and activation of human neutrophils. Platelets can be activated to externalize APL using several stimuli, including thrombin, Ca^{2+} ionophore (e.g. A23187), collagen, as well as during apoptosis and in vitro ageing (*4*). In option A, ~~T~~thrombin activation is described ~~below~~.

Comment [DB19]: This has been moved here as we cannot have any hanging text in the procedure that is not part of a step or callout.

A. Isolation and Activation of Human Platelets TIMING: 1-1.5 hrs.

CRITICAL STEP: Platelets are sensitive to shear stress, physical deformation and negatively charged surfaces such as glass or polystyrene. To avoid activation, do not pipette vigorously with small-bore pipette tips or generate bubbles during handling. Small aggregates resembling grains of sand will be evident if platelets have become activated during isolation.

Comment [DB20]: I think that it might be helpful to repeat this sentence (or re-word) in the Troubleshooting section to make it clear how the user would know that there was a problem that needed to be solved.

(i) Draw venous blood through a 21 gauge needle from healthy volunteers ~~free from~~(that have not taken any nonsteroidal anti-inflammatory drugs for at least 14 days), through a butterfly needle into a syringe. Immediately mix whole blood with ACD at a ratio blood:ACD of 8.1:1.9 eg. 40.5 ml blood into 9.5 ml ACD in a plastic tube.

(ii) Centrifuge at 250 x g for 10 min at room temperature.

CRITICAL STEP: Ensure that a brake mechanism is NOT employed on the centrifuge as this will disturb the separation of the cells.

(iii) Gently aspirate the upper layer of platelet-rich plasma with a 3 ml plastic Pasteur pipette into a 50 ml plastic tube, and centrifuge this at 900 x g for 10 min to pellet the platelets. Discard the red cell lower phase. Aspirate the platelet poor plasma and discard. Re-suspend the cell pellet in 10 ml of ACD in Tyrode's buffer (ACD:Tyrode's 1:9, v/v).

(iv) Centrifuge at 800 x g for 10 min. Aspirate the supernatant and discard. Carefully re-suspend in 2 ml Tyrode's buffer. At this point count the platelets, using either a haemocytometer or coulter counter, and dilute to a concentration of 2 x 10^8 platelets.ml^{-1} in Tyrode's buffer.

(v) Allow platelets to rest for 20 minutes at room temperature before further manipulation, but no longer than 2 hrs.

~~Platelets can be activated to externalize APL using several stimuli, including thrombin, Ca^{2+} ionophore (e.g. A23187), collagen, as well as during apoptosis and in vitro ageing (*4*). Thrombin activation is described below.~~

(vi) **Activation using thrombin** Incubate 200 µl of 2 × 10^8 platelets.ml^{-1} with 1 mM $CaCl_2$ at 37 ºC in a water bath for 10 minutes. Add 0.2 U.ml^{-1} thrombin and incubate for 30 min. Proceed to biotinylation of externalized aminophospholipids (Section 17).

Formatted: Font: Bold

~~TIMING: Platelet isolation takes 1-1.5 hrs.~~

B. Isolation of human neutrophils and induction of apoptosis. TIMING: 2-2.5 hrs

CRTITICAL STEP: Neutrophils are sensitive to shear stress, physical deformation, negatively charged surfaces such as glass or polystyrene and contaminating microorganisms. To avoid activation, do not pipette vigorously with small-bore pipette tips or generate bubbles during handling. Reagents and equipment should be sterile and procedures should occur under aseptic technique for all procedures described. Exposure to bacteria or bacterially-derived contaminants can increase neutrophil lifespan and confound studies of cell death. However, handling of samples over short intervals (such as for lipid profiling) can be undertaken on the bench because contamination will be minor.

(i) Draw venous blood through a 21-gauge needle from healthy volunteers (~~free from~~that have not taken nonsteroidal anti-inflammatory drugs for at least 14 days), through a butterfly needle into a syringe. Immediately mix whole blood with 2.8 % citrate and hetasep (Stemcell Technologies) at a ratio blood:citrate:Hetasep of 10:2:2, *eg* 20 ml blood into 4 ml 2.8 % Citrate and 4 ml Hetasep.

(ii) Allow this mixture to settle under gravity for 35-50 minutes at room temperatur~~e~~ure (until a distinct upper layer of at least 50 % of the volume is seen). This will be easily visible since the lower layer contains the majority of red cells. Recover the upper, red cell-deficient layer. Underlay with lymphoprep (Axis-Shield) at ratio of erythrocyte poor blood:lymphprep of 2:1, *eg* 10 ml with 5 ml Lymphoprep.

(iii) Centrifuge at 800 x g for 20 min at 4 ºC.

~~Note:~~<CRITICAL STEP> Ensure that a brake mechanism is NOT employed on the centrifuge as this will disturb the separation of the cells.

(iv) Carefully remove and discard the monocytic cells at the interface (a white layer between two more transparent phases) without allowing them to mix with the pelleted cells with a 3 ml plastic Pasteur pipette, then remove all remaining plasma and Lymphoprep. Recover the pelleted cells with 2 ml of 0.4 % citrate, transfer to a clean tube and dilute to 10 ml volume with 0.4 % citrate.

~~Note:~~<CRITICAL STEP> Ensure that the neutrophils and erythrocytes in the pellet do not mix with the monocytic cells that were at the interface and that will be stuck to the side of the tube.

(v) Centrifuge at 400 x g for 10 min at 4 ℃. Aspirate the supernatant and discard. Lyse red cells by hypotonic shock: Resuspend in 3 ml of ice cold hypotonic saline with a 3 ml plastic Pasteur pipette adding slowly down the side of the tube, swirling gently. After 35-45 seconds, restore osmolality by addition of 3 ml ice cold hypertonic saline and make up the volume to 10 ml with PBS.

(vi) Centrifuge at 400 x g for 10 min at 4 ℃ and repeat the hypotonic shock if red cells are visible in the pellet. Finally, resuspend in 1 ml Krebs (Recipe 9) and dilute to a concentration of 2×10^{7} neutrophils.ml^{-1}.

CRITICAL ~~POINT~~STEP: In the absence of stromal cells or an inflammatory environment, neutrophils will begin to spontaneously apoptose in culture after only a few hours. Many cells will be apoptotic by 24 hours. Serum must not be included as the biotinylation reagents will be consumed by reaction with primary amines in these proteins.

(vii) Add 2.5 x 10^6 freshly isolated neutrophils in 0.5 ml volume in the presence of 2.5 mM $CaCl_2$ and 1.25 mM $MgCl_2$ to wells of a 48 well plate, in triplicate for each time point or condition. Incubate at 37 ºC 5 % CO_2 for up to 24 hours. At each time point, harvest cells by gently pipetting with a 3 ml plastic Pasteur pipette for 10 seconds before removal of 200 µl into an eppendorf tube. Proceed to biotinylation of externalised aminophospholipids (Section 8).

~~TIMING: Neutrophil isolation takes 2-2.5 hrs~~.

Extraction of native (un-biotinylated) lipids from cells ~~by~~ using acidified hexane:isopropanol. TIMING. Extraction of lipids takes approximately 1 hr with 1-2 hrs drying time.

CRITICAL STEP: In order to determine which PE and PS species to monitor for externalization, the primary molecular species need to be first characterized in the cells of interest. Two samples are isolated: a PE sample and a PS sample. The PE sample requires an additional purification//fractionation step using normal phase chromatography. This is because there are a number of isobaric lipids in the PE sample and fractionation makes it easier to tell them apart. ~~For PE, the presence of isobaric lipids means that the PE fraction must first be purified using normal phase chromatography.~~ Fractionation of PE is described in ~~the method below~~steps…..

Comment [DB21]: I thought that it was a little confusing as written so tried to re-write it. Now I am not sure that I understood it correctly! Please edit as appropriate and try to clarify what is meant here.

~~8.~~9. Prepare labeled glass extraction tube with 2.5 ml of solvent mix 2. Add 1 ml un-activated, freshly prepared cells to the glass extraction tubes. For platelets, typically this contains 2 x 10^8 cell.ml^{-1}. For neutrophils, use 1 x 10^6.ml^{-1}.

~~9.~~10. Vortex thoroughly for 1 minute. Add 2.5 ml hexane and vortex thoroughly for 1 minute. Centrifuge samples at 500 x g for 5 minutes. Carefully remove the ***upper*** layer with a glass Pasteur pipette and transfer to a clean, labeled glass vial. Re-extract the lower layer by addition of a further 2.5 ml hexane and vortex thoroughly for 1 minute.

~~10.~~11. Repeat centrifuge step and again remove the ***upper*** hexane layer and combine with the previous one. Dry samples using nitrogen gas or by use of a vacuum extraction system and re-suspend in 100 µl methanol, storing under argon gas (or nitrogen if argon is not available) at -80 ºC until further analysis.

~~TIMING. Extraction of lipids takes approximately 1 hr with 1-2 hrs drying time.~~

PAUSE POINT. Samples can be kept in storage for up to a week before analysis.

Fractionation of lipid species to purify PE. TIMING. Approximately 1 day.

CRITICAL: Undertake purification of PE from cell samples of interest to generate a fraction that can be used for characterizing the major PE species, as follows. Ensure HPLC grade solvents are used, as lower grades can absorb significantly in the low UV. Note that retention times can vary a little on normal phase chromatography, but not significantly between runs.

~~11.~~12. Set up normal phase HPLC with a Spherisorb S5W 5 µm column (150 × 4.6 mm) with guard column, at the following conditions at 22 ° C; flow rate 1.5 ml.min^{-1}, gradient elution - 50 % mobile phase B to 100 % mobile phase B (A: hexane:isopropranol 3:2, B: Mobile phase A:water, 94.5:5.5) over 25 min before re-equilibration to 50% B, UV absorbance - 205 nm.

~~12.~~13. Inject brain or egg PE (approx. 1-10 □g) and determine retention time (PE - typically 5.5 min). Ensure no carry over from standard is evident by injecting a blank sample consisting of mobile phase. Dry down lipid sample under nitrogen stream and resuspend in mobile phase at starting composition.

~~13.~~14. Inject sample and collect fractions corresponding to elution time for PE. A typical trace of elution is shown in Figure 6 A. Dry using RapidVap or dryblock with nitrogen stream with temperature at 30 °C, and resuspend in 100 µl methanol and store under argon gas (or nitrogen if argon is not available) at -80 °C until LC-MS analysis.

~~TIMING. Purification of PE takes approximately 1 day.~~

PAUSE POINT. Samples can be kept in storage for up to a week before analysis.

Characterization of cellular PE and PS molecular species using direct infusion MS.

TIMING. Characterization of PE and PS species takes approximately 1 hr.

15 | Determination of PS is achieved by neutral loss MS/MS of crude lipid extracts for the PS headgroup (87 amu) (Figure 6 B), while PE is determined by MS scanning of the purified PE fraction in positive or negative ion mode (Figure 6 C), as described in Experimental design (p6).

A heavily diluted extract should initially be used for direct infusion to prevent source contamination. Once putative structural assignments are generated, they need to be verified using LC/MS/MS, where PE and PS are monitored in negative mode using the transition corresponding to parent → fatty acid carboxylate anion. The assignment of the ions in platelet PE is described in more detail in *(4)*. Ions identified as PEs are circled (Figure 6 C). Follow option A and B for PS and PE characterization respectively.

> **Comment [DB22]:** Is it clear what you mean by this? Seems that it is mentioned for the first time in the subheading above…

> **Comment [DB23]:** Consider whether it might be better to move this to the step where the data is analysed.

A PS characterization

> **Formatted:** Font: Bold

i. ~~For PS, d~~Dilute the unfractionated lipid extract ~~into~~ with methanol. To ensure the MS is not contaminated by concentrated lipid extracts, start with 1:1,000 and gradually work up 10 x until a signal is detected.

> **Formatted:** Numbered + Level: 1 + Numbering Style: i, ii, iii, … + Start at: 1 + Alignment: Right + Aligned at: 1.9

ii. Using a syringe pump, inject into the mass spectrometer at 10µl.min^{-1}. Scan for negative neutral loss of 87 amu from *m/z* 600 - 1,000.

iii. On a 4000 Q-Trap, acquire spectra using a 2 second scan time and the following settings: Q1 unit resolution, Q3 unit resolution, MR Pause 5 msec, MCA – Yes, Step size 0.10 amu, CUR 10, IS -4500, TEM 0, GS1 12, GS2 0, ihe On, CAD Medium, DP -40, EP -10, CE -30, CXP -6.

PE characterisation

i. For PE, dilute fractionated lipid into methanol and using a syringe pump, inject into the mass spectrometer at 10µl.min^{-1}.

> **Formatted:** Numbered + Level: 1 + Numbering Style: i, ii, iii, … + Start at: 1 + Alignment: Right + Aligned at: 0.63

> **Comment [DB24]:** Is the dilution similar to that for PS? Please clarify in the text.
> Please make sure that it is clear how many fractions are obtained on PE fractionation and which of these are analysed in this step.

~~14.~~ii. Scan in positive MS mode from 600 to 900 amu using settings as follows on a 4000 Q-Trap: unit resolution, MR pause 5 msec, step size 0.1 amu, CUR 10, IS 4500, TEM, GS1 12, GS2 0, ihe On, DP 120, EP 10.

> **Formatted:** Font: Arial, (Intl) Arial Unicode MS, English (U.K.)

~~15.~~iii. Generate putative structural assignments using either Lipid Calculator (http://pharmacology.ucdenver.edu/lipidcalc/) or LIPID MAPS (http://www.lipidmaps.org/data/standards/index.html).

Formatted: Numbered + Level: 1 + Numbering Style: i, ii, iii, … + Start at: 1 + Alignment: Right + Aligned at: 0.63

~~16.~~iv. Set up a reverse phase HPLC system with a Luna C18, 3 µm (150 × 2 mm) column and conditions at 22 ° C as follows; flow rate 0.2 ml.min^{-1}, gradient elution profile - 50 % mobile phase B to 100 % mobile phase B (A: methanol:acetonitrile:water, 60:20:20, 1 mM ammonium acetate, B: methanol, 1 mM ammonium acetate) over 10 min followed by 30 min hold at 100 % B before re-equilibration to 50 % B.

Formatted: Numbered + Level: 1 + Numbering Style: i, ii, iii, … + Start at: 1 + Alignment: Right + Aligned at: 0.63

~~17.~~v. Inject 10-20 □l lipid extract and monitor multiple reaction monitoring (MRM) transition (parent → daughter ion) in negative mode for all species of interest. Daughter ions represent the carboxylate anion for PE, and the neutral loss of 87 amu for PS, which is the serine headgroup. The MRM transitions to monitor for the platelet lipids identified in our study are shown in Table 1 (native MRM transitions). This table also shows MRM transitions subsequently used to monitor their biotinylated analogs. In that case, PE is still monitored by formation of the *sn2* carboxylate anion, with PS instead monitored by neutral loss of 313 amu (biotinylated serine). Full details of instrument settings used on our platform are given in Table 1 but we point out that these are specific for the AB Sciex 4000 A-Trap platform, and so users need to conduct a full tune with standards on their own instruments before measuring cell samples.

Formatted: Numbered + Level: 1 + Numbering Style: i, ii, iii, … + Start at: 1 + Alignment: Right + Aligned at: 0.63

~~TIMING. Characterization of PE and PS species takes approximately 1 hr.~~

Biotinylation of externalized APL. TIMING. Biotinylation of PE and PS takes approximately 30 min.

CRITICAL STEP: ~~This is the protocol for~~Steps 16-25 describes the procedure for labeling external facing APL on activated or apoptotic human cells. *Perform this reaction with 200 □l samples of platelets at 2 x 10^8.ml^{-1} and nucleated cells at 1 x 10^6.ml^{-1} after stimulation to undergo activation or apoptosis.* *(Steps xy-yy describe the procedure for determining the total concentration of APL in the cell membranes by labeling with a cell permeable reagent.)*

~~18.~~16. Remove sample from water bath/incubator and add 86 µl of EZ-link Sulfo-NHS-Biotin.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~19.~~17. Incubate at room temperature for 10 min with occasional gentle inversion.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~20.~~18. Add 72 µl of lysine.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~21.~~19. Incubate at room temperature for a further 10 min with occasional gentle inversion.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~22.~~20. Add 10 µl of DMPE-B (1 ng.μl^{-1}) and DMPS-B (1 ng.μl^{-1}) internal standards, thus giving a final concentration of 10 ng.μl^{-1} of each standard.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~23.~~21. Extract lipids as below (do not pause, continue straight onto next section).

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~TIMING. Biotinylation of PE and PS takes approximately 30 min.~~

Extraction of biotinylated lipids according to modified Bligh and Dyer (*16*) TIMING. Extraction of lipids takes approximately 1 hr with 1-2 hrs for drying.

~~This is the protocol for labeling external facing APL on activated or apoptotic human cells.~~

~~24.~~22. In advance, prepare a labeled glass extraction tube for each sample containing 1.5 ml of Solvent mix 1. Add sample to extraction tube, followed by 42 µl of Tyrode's Buffer to yield a total volume of 400 µl aqueous, and a 1:3.75 ratio of aqueous sample:solvent mix 1. If using a different volume, then maintain the ratio of aqueous:solvent to ensure efficient extraction.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~25.~~23. Vortex thoroughly for 1 minute. Add 0.5 ml chloroform and vortex thoroughly for a further minute. Add 0.5 ml HPLC-grade water and again vortex thoroughly for 1 minute. Centrifuge the samples at 500 x g for 5 minutes.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~26.~~24. Carefully remove the ***lower*** layer with a glass Pasteur pipettes and transfer to a clean, labeled glass vial. Dry samples using RapidVap or dryblock with nitrogen stream with temperature at 30 °C.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

CRITICAL STEP: To limit degradation of sample, do not over heat the samples in the drying process.

~~27.~~25. Re-suspend in 100 µl methanol and store under argon gas (or nitrogen if argon is not available) at -80 ºC until LC-MS analysis.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

TIMING. Extraction of lipids takes approximately 1 hr with 1-2 hrs for drying.

PAUSE POINT. Samples can be kept in storage for up to a week before analysis.

Biotinylation of total cellular lipids. TIMING. Biotinylation takes approximately 30 min.

<CRITICAL> This protocol is used to calculate the % of a particular phospholipid pool externalized. In this,In steps 26-yy, total all of the APLs in the samples are biotinylated using the cell permeable analog NHS-biotin (NB). Externalized APL is then calculated as a fraction of the total, to give %.

28.26. Activate cells or stimulate to undergo apoptosis as described above, but with samples of 400 µl total volume (keep cell concentration as previously specified). After activation, remove from water bath and split into two eppendorf tubes with equal volumes.

29.27. To one half, biotinylate extracellular aminophospholipids, – as described in steps 45-53 above. To the other half add 20 µl of 20 mM NB (6.82 $mg.ml^{-1}$) in DMSO and incubate at 22 °C for 10 min.

30.28. Add 10 µl of 1 $ng.\mu l^{-1}$ DMPE-B and DMPS-B internal standards. Extract both samples as per modified Bligh and Dyer, and store under argon gas (or nitrogen if argon is not available) -80 °C until analysis.

TIMING. Biotinylation takes approximately 30 min.

Comment [DB25]: I am not actually clear on why 16-25 and 26-28 need to be formatted as two sections. Please consider whether they should be made into one section with two options introduced by the text currently in Step 27.
If they should be two different experiments, then please make this more clear in the text? (apologies if I have misunderstood, and edited incorrectly).

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

PAUSE POINT. Samples can be kept in storage for up to a week before analysis.

Reverse phase HPLC-ESI-MS of biotinylated APL. TIMING. Analysis of samples takes 25 min per sample.

31.29. Set up reverse phase HPLC with an Ascentis C18 column (5 µ□, 150 × 2.1 mm, Sigma Aldrich) with the following conditions at 22 ° C; flow rate 400 µl.min^{-1}, isocratic mobile phase – methanol with 0.2 % ammonium acetate, for 25 min.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

32.30. Detect using MRM transitions (biotinylated MRM transitions) and corresponding declustering potential (DP), collision energy (CE) and cell exit potential (CXP) as Table 1. Further instrument details are as follows; Q1 and Q3 unit resolution, CUR 20, CAD High, V-4500 Tem 500°C, GS1 40, GS2 30. Examples of chromatograms for the major platelet species are shown in Figure 6 D,E.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

TIMING. Analysis of samples takes 25 min per sample.

Generation of Standard Curves for Quantification TIMING. Generation of standard curves takes 1 hr preparation, 5 hrs analysis.

Comment [DB26]: Please comment on replicates?

33.31. Prepare internal standard mix as follows: Dilute DMPE-B 100 ng.µl^{-1} stock to 1 ng.µl^{-1} in methanol by adding 1 µl of DMPE-B into 99 µl of methanol in a clean glass vial. To a separate clean glass vial add 1 µl of DMPS-B stock (100 ng.µl^{-1}) to 99 µl of methanol, using a 0.5 µl pipette. To a fresh vial with 960 µl methanol add 20 µl of DMPE-B (1ng.µl^{-1}) and 20 µl of DMPS-B (1ng.µl^{-1}) solution as prepared above. This will

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

generate 1 ml of final internal standard solution containing 20 pg.µl^{-1} of each standard. Vortex the solution thoroughly.

~~34.~~32. Prepare analyte mix as follows. To a fresh vial with 270 µl methanol add 6 µl of each biotinylated analyte standard (100 ng.ul^{-1}), SOPS-B, DOPS-B, SAPS-B, SpAPE-B and SAPE-B (do not add DMPE-B or DMPS-B internal standards). Vortex thoroughly to yield standard #1 with a final analyte solution containing 2 ng.µl^{-1} of each analyte.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~35.~~33. Prepare the rest of the analyte standards by serial dilution as follows: To prepare standard #2 add 150 µl of standard #1 to 150 µl methanol. To prepare standard #3 – add 60 µl standard #2 to 240 µl methanol. To prepare standard #4 – add 150 µl standard #3 to 150 µl methanol. To prepare standard #5 – add 60 µl standard #4 to 240 µl methanol. To prepare standard #6 – add 150 µl standard #5 to 150 µl methanol. To prepare standard #7 – add 60 µl standard #6 to 240 µl methanol. To prepare standard #8 – add 150 µl standard #7 to 150 µl methanol. To prepare standard #9 – add 60 µl standard #8 to 240 µl methanol

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~36.~~34. To prepare the standard curve, mix 100 µl analyte solution no. 1 with 100 µl internal standard mix (leaving 10 pg.µl^{-1} of internal standards per sample). Repeat with analyte standards 2 – 9 to yield the analyte concentrations as Table 2.

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

~~37.~~35. Analyze using LC-MS as section 32 with an injection volume of 10 µl. Integrate peak area for all lipids and calculate the ratio of Analyte (A):Internal Standard (IS) for ng

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

amounts of lipid in each standard vial and for A:IS peak area in counts per second (c.p.s.) for each standard analyzed.

~~38.~~36. Plot A:IS (c.p.s.) versus A:IS (ng) as shown by examples in Figures 6 F,I. Calculate slope for each analyte. For all samples use the following equation to determine ng per sample.

$$A_{ng} = (A_{cps}/IS_{cps})(I_{ng} / \text{slope})$$

~~39.~~37. Determine ng lipid per cell number to give amounts of externalized APL. An example of quantitative data generated on neutrophils undergoing apoptosis is shown in Figure 2.

~~TIMING. Generation of standard curves takes 1 hr preparation, 5 hrs analysis.~~

TROUBLESHOOTING

Platelets and neutrophils are easy to activate during isolation. To prevent this, ensure that you are gentle (no shaking or vortexting of cells), and maintain platelets at 20 - 22 ºC and neutrophils on ice at all times before stimulation. Resuspend very gently after centrifugation. At the end of the isolation procedure, they may resuspend into small clumps, but these should disperse after 10 - 15 min resting on the bench.

ANTICIPATED RESULTS

Yield of biotinylated (?) standards from synthesis is approximately quantitative, but during purification by…, up to 30 % maybe lost. Thus, we typically generate up to 0.6 - 0.7 mg per 1 mg starting material. Typical chromatography traces for biotinylated species are shown in

Comment [DB27]: In panels F to I, I would have expected the data points to have error bars with an explanation of the error bars plus the number replicates stated in the legend?

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

Formatted: Numbered + Level: 1 + Numbering Style: 1, 2, 3, … + Start at: 16 + Alignment: Left + Aligned at: 0.63 cm +

Comment [DB28]: Please consider whether more troubleshooting information could be included. This usually takes the form of a table with columns: Step (where problem becomes evident), Problem, Cause, Solution. The steps where the problems originated can be mentioned in the other columns.

If this really is all the troubleshooting advice, then I would like you to consider incorporating this information into the main part of the procedure, and not having a troubleshooting section.

Figure 6 D,E. These should appear as single, well-resolved peaks around 5 - 10 min using the chromatography set up described. When setting up the method, several columns were tested and the Ascentis gave the best results, particularly for PS species.

Typical amounts of APL externalized in thrombin-activated human platelets were around 70 ng and 100 ng/2 x 10^8 cells, for PS and PE, respectively. These increased 5-fold on activation using calcium ionophore (*4*). Neutrophils typically externalized 20 - 30 ng PS and up to 500 ng PE/1 x 10^6 cells during 24 hrs incubation (Figure 2). The molecular species did not differ significantly between the cell types. In our laboratory, the method has also been used to detect APL externalization on platelets undergoing activation, apoptosis and during energy deficiency ageing, as well as on apop~~o~~totic nucleated cells. Thus, the procedure is equally applicable to cells undergoing all three cellular processes, and could easily be adapted to additional cell types and to other relevant cellular events that involve APL externalization, including cell division and vesiculation.

Comment [DB29]: As discussed before, please consider whether you might be able to include a figure for this: either in the main part of the manuscript, or as supplementary information.
At least: please state that these findings are unpublished, and give the initials/names of the people involved in this work.

Comment [DB30]: Please add the figure legends (and any supplementary information legends) here.

Author contributions: CPT, SRC, VJH, MA conducted experiments, VBO, CPT wrote the paper, PWC edited the paper. VBO, SRC, CPT, PWC designed the protocols.

Acknowledgments: The studies were funded by the Wellcome Trust (VBO, VJH, PWC). CPT is a Marie Curie International Outgoing Fellow.

Competing interests: The authors declare no competing interests.

REFERENCES

1. D. L. Daleke, Phospholipid flippases. *J Biol Chem* **282**, 821 (Jan 12, 2007).
2. K. S. Ravichandran, Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med* **207**, 1807 (Aug 30, 2010).
3. J. W. Heemskerk, E. M. Bevers, T. Lindhout, Platelet activation and blood coagulation. *Thrombosis and haemostasis* **88**, 186 (Aug, 2002).
4. S. R. Clark *et al.*, Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proc Natl Acad Sci U S A* **110**, 5875 (Apr 9, 2013).
5. J. Shi, G. E. Gilbert, Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid-binding sites. *Blood* **101**, 2628 (Apr 1, 2003).
6. D. E. Otzen, K. Blans, H. Wang, G. E. Gilbert, J. T. Rasmussen, Lactadherin binds to phosphatidylserine-containing vesicles in a two-step mechanism sensitive to vesicle size and composition. *Biochimica et biophysica acta* **1818**, 1019 (Apr, 2012).
7. P. Meers, T. Mealy, Phospholipid determinants for annexin V binding sites and the role of tryptophan 187. *Biochemistry* **33**, 5829 (May 17, 1994).
8. G. Koopman *et al.*, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415 (Sep 1, 1994).
9. C. Gao *et al.*, Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticles release in patients with nephrotic syndrome. *Thrombosis and haemostasis* **107**, 681 (Apr, 2012).

10. D. L. Daleke, Regulation of transbilayer plasma membrane phospholipid asymmetry. *Journal of lipid research* **44**, 233 (Feb, 2003).

11. H. A. Andree *et al.*, Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *The Journal of biological chemistry* **265**, 4923 (Mar 25, 1990).

12. F. Hullin, M. J. Bossant, N. Salem, Jr., Aminophospholipid molecular species asymmetry in the human erythrocyte plasma membrane. *Biochimica et biophysica acta* **1061**, 15 (Jan 9, 1991).

13. P. K. Schick, K. B. Kurica, G. K. Chacko, Location of phosphatidylethanolamine and phosphatidylserine in the human platelet plasma membrane. *The Journal of clinical investigation* **57**, 1221 (May, 1976).

14. J. E. Rothman, E. P. Kennedy, Rapid transmembrane movement of newly synthesized phospholipids during membrane assembly. *Proc Natl Acad Sci U S A* **74**, 1821 (May, 1977).

15. W. L. Hurley, E. Finkelstein, Identification of leukocyte surface proteins. *Methods Enzymol* **184**, 429 (1990).

16. E. G. Bligh, W. J. Dyer, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911 (Aug, 1959).

Author Contributions. CPT, SRC, VJH, MA conducted experiments, PWC edited the paper, VOD wrote the paper. CPT, SRC and VOD designed experiments. The authors indicate no competing financial interests.

Analyte	Mass	m/z [M-H]$^-$	Native MRM transition	Biotinylated Mass	m/z [M-H]$^-$	Biotinylated MRM transition	DP (V)	CE (V)	CXP (V)
DMPE (14:0/14:0-PE)	635	634	634 → 227	861	860	860 → 227	-135	-60	-13
SpAPE (18:0p/20:4-PE)	751	750	750 → 303	977	976	976 → 303	-160	-60	-5
SAPE (18:0a/20:4-PE)	767	766	766 → 303	993	992	992 → 303	-170	-58	-5
PpAPE (16:0p/20:4-PE)	723	722	722 → 303	949	948	948 → 303	-160	-60	-5
SOPE (18:0a/18:1-PE)	745	744	744 → 281	971	970	970 → 281	-170	-58	-5
OpAPE (18:1p/20:4-PE)	749	748	748 → 303	975	974	974 → 303	-160	-60	-5
DMPS (14:0/14:0-PS)	679	678	678 → 591	905	904	904 → 591	-150	-42	-17
SOPS (18:0a/18:1-PS)	789	788	788 → 701	1015	1014	1014 → 701	-140	-44	-23
DOPS (18:1a/18:1-PS)	787	786	786 → 699	1013	1012	1012 → 699	-150	-46	-23

SAPS (18:0a/20:4-PS)	811	810	810 →723	1037	1036	1036 →723	-145	-42	-23

Table 1. Analyte mass and transition data

#	Concentration	Final analyte concentration	Amount on column
1	2 ng/µl	1 ng/µl	10 ng
2	1 pg/µl	0.5 pg/µl	5 ng
3	0.2 ng/µl	0.1 ng/µl	1 ng
4	0.1 ng/µl	50 pg/µl	500 pg
5	20 pg/µl	10 pg/µl	100 pg
6	10 pg/µl	5 pg/µl	50 pg
7	2 pg/µl	1 pg/µl	10 pg
8	0.5 pg/µl	0.5 pg/µl	5 pg
9	0.2 pg/µl	0.1 pg/µl	1 pg

Table 2. Standard curve analyte dilution protocol.