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Long wavelength TCF-based fluorescence probe for the detection of Alkaline Phosphatase in live cells

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12 **Keywords: Reaction-based fluorescent probe₁, Alkaline Phosphatase₂, Cell Imaging₃**

13 **Abstract**

14 A long wavelength TCF-based fluorescent probe (**TCF-ALP**) was developed for the detection of
15 alkaline phosphatase (ALP). ALP-mediated hydrolysis of the phosphate group of **TCF-ALP** resulted
16 in a significant fluorescence ‘turn on’ (58-fold), which was accompanied by a colorimetric response
17 from yellow to purple. **TCF-ALP** was cell-permeable, which allowed it to be used to image ALP in
18 HeLa cells. Upon addition of bone morphogenic protein 2, **TCF-ALP** proved capable of imaging
19 endogenously stimulated ALP in myogenic murine C2C12 cells. Overall, TCF-ALP offers promise as
20 an effective fluorescent/colorimetric probe for evaluating phosphatase activity in clinical assays or live
21 cell systems.

22 **1 Introduction**

23 Alkaline phosphatase (ALP) is an ubiquitous enzyme found in the majority of human tissues, where it
24 catalyses the dephosphorylation of various substrates such as nucleic acids, proteins and other small
25 molecules (Millán, 2006, Coleman, 1992). ALP also plays an important role in signal transduction and
26 regulation of intracellular processes (cell growth, apoptosis and signal transduction pathways) (Julien
27 et al., 2011). Abnormal levels of ALP in serum are an indicator of several diseases including bone
28 disease (Garnero and Delmas, 1993), liver dysfunction (Rosen et al., 2016), breast and prostatic cancer
29 (Ritzke et al., 1998, Wymenga et al., 2001) and diabetes (Tibi et al., 1988). As a result, ALP is regarded
30 as a key biomarker in medical diagnosis (Coleman, 1992, Ooi et al., 2007). Therefore, it is important
31 to develop a fast, reliable and selective detection system for monitoring ALP activity that is amenable
32 to clinical diagnostics.

33 There have been numerous approaches to determine ALP levels, including colorimetric (Yang et al.,
34 2016, Hu et al., 2017), chemiluminescent (Jiang and Wang, 2012), electrochemical (Zhang et al.,
35 2015b), surface-enhanced Raman methods (Ruan et al., 2006) and fluorescence (Cao et al., 2016, Fan

36 et al., 2016). This work focused on the development of fluorescent probes for the detection of
37 biologically relevant analytes (Sedgwick et al., 2017a, Sedgwick et al., 2018b, Wu et al., 2017,
38 Sedgwick et al., 2017b, Sedgwick et al., 2018a, Zhang et al., 2019). Fluorescence has many advantages
39 over other methods owing to its simplicity and high sensitivity/selectivity, providing rapid, non-
40 invasive, real-time detection (Wu et al., 2017). Whilst there have been many fluorophores developed
41 for assaying ALP activity such as organic dyes (Zhang et al., 2015a, Zhao et al., 2017), conjugated
42 polymers (Li et al., 2014), inorganic semiconductor dots (Qian et al., 2015), and noble metal clusters
43 (Sun et al., 2014), most require high probe concentrations and crucially rely on short wavelength
44 emission, thus limiting their applicability in biological systems. Therefore, ALP probes that operate at
45 long wavelengths are urgently required. Such probes should allow for deeper tissue penetration and be
46 subjected to less cell-based autofluorescence (Liu et al., 2017, Zhang et al., 2017, Tan et al., 2017).

47 2 Results and Discussion

48 2.1 Chemistry

49 Here we report a TCF-based fluorescent probe that allows for the detection of ALP and/or ACP. As
50 shown in Scheme 1, this probe (TCF-ALP) is based on the conjugation of 2-dicyanomethylene-3-
51 cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) to an electron-donating moiety, a phosphorylated
52 phenol; this affords an internal charge transfer (ICT) donor- π -acceptor (D- π -A) system whose
53 fluorescence properties vary dramatically following ALP-mediated phosphate group cleavage
54 (Gopalan et al., 2004, Liao et al., 2006, Lord et al., 2008, Bouffard et al., 2008, Jin et al., 2010, Teng
55 et al., 2018, Sedgwick et al., 2017b). TCF-ALP was synthesised in four steps with an overall yield of
56 27% (Scheme 2). In brief, 3-hydroxy-3-methyl-2-butanone, malononitrile and NaOEt were heated at
57 reflux in EtOH for 1 h. The resultant precipitate TCF (1) was then treated with a mixture of piperidine
58 (cat.) and 4-hydroxybenzaldehyde in EtOH to afford intermediate 2 (TCF-OH). Intermediate 2 was
59 then treated with diethylchlorophosphate, DMAP (cat.) and NEt₃ in THF to give the phosphonate ester
60 3. Hydrolysis using trimethylsilyl iodide in dichloromethane (DCM) afforded TCF-ALP as a
61 crystalline solid (Et₂O).

62 2.2 Spectroscopic studies of TCF-ALP

63 UV-Vis and fluorescence spectroscopic titrations of TCF-ALP were performed in 50 mM Tris-HCl
64 buffer in the absence and presence of ALP from porcine kidney. In the absence of ALP, TCF-ALP
65 was found to have no UV absorption features above ~550 nm; however, upon addition of ALP a
66 bathochromic shift in the UV absorption maximum was observed (from 440 to 580 nm), which was
67 accompanied by a change in colour from yellow to purple (Figure S1). ALP-mediated hydrolysis of
68 TCF-ALP to form highly fluorescent phenol (2), was confirmed by ³¹P NMR studies and HRMS (See
69 Figure S1 – S4). The effect of pH on the rate of ALP mediated hydrolysis of TCF-ALP was evaluated.
70 It was found that incubation with 0.8 U/mL of ALP at pH 9.2 resulted in the largest fluorescence
71 response (Figure S5). Consequently, all *in vitro* experiments to determine ALP activity were carried
72 out in 50 mM Tris-HCl buffer at pH 9.2.

73 The kinetics of ALP towards TCF-ALP were determined *via* fluorescence spectroscopy (Figure S6
74 and S7), with the resultant fluorescence data analysed using the Michaelis-Menten equation (Figure
75 S8). This revealed a K_m of 35.81 ± 2.63 μM and a V_{max} of 3029 ± 157.3 min⁻¹ for hydrolysis of TCF-
76 ALP by ALP at pH 9.2 (see SI for details). TCF-ALP was then incubated with various concentrations
77 of ALP (0.0 – 0.2 U/mL) for 15 minutes to evaluate its ability to monitor ALP activity. As shown in
78 Figure 1, a significant fluorescence response was observed in the presence of ALP (58-fold) with a
79 limit of detection (LOD) calculated as 0.12 mU/mL (Figure S9). This sensitivity is comparable to other

80 fluorescent probes found in literature (**Table S3**). Although serum alkaline phosphatase levels vary
81 with age in normal individuals (Lowe et al., 2018), it is widely accepted that serum ALP levels in
82 healthy adults lies between 39 – 117 U/mL (Sahran et al., 2018, Saif et al., 2005). This suggests that
83 **TCF-ALP** is capable of detecting ALP in human serum, and therefore could be used in clinical assays.

84 Inhibition studies were carried out in the presence of sodium orthovanadate (Na_3VO_4), which is known
85 to be a strong inhibitor of ALP activity. Addition of Na_3VO_4 resulted in a decrease in the fluorescence
86 response in the **TCF-ALP** hydrolysis assay (see **Figure S10**) (Swarup et al., 1982). These inhibition
87 studies enabled an IC_{50} of 6.23 μM to be calculated (**Figure S11**), which is similar in value to other
88 ALP substrates that have been reported in the literature (Tan et al., 2017, Zhang et al., 2015a).

89 The selectivity of **TCF-ALP** towards other biologically relevant enzymes (at their optimal pH values)
90 was then determined (**Figure 2** and **S12**), with **TCF-ALP** displaying high substrate selectivity for ALP
91 over other common hydrolytic enzymes (e.g. trypsin, porcine liver esterase) or non-specific binding
92 proteins (e.g. bovine serum albumin (BSA)). Interestingly, **TCF-ALP** produced a fluorescence
93 response when treated with acid phosphatase (ACP). The detection of this enzyme is of significance
94 since it is a tumour biomarker for metastatic prostate cancer (Makarov et al., 2009). Normal levels of
95 ACP in serum range from 3.0 – 4.7 U/mL, and elevated ACP levels can be indicative of a variety of
96 other diseases (Bull et al., 2002). Furthermore, **TCF-ALP** proved capable of detecting ACP (25-fold
97 fluorescence enhancement) and ALP (38-fold enhancement) at a physiological pH of 7.1 (**Figure S13**
98 and **S14**). Kinetic determination of ALP and ACP towards **TCF-ALP** at pH 7.1 was conducted, and
99 the resultant K_m and V_{\max} were compared (see **SI 2.1** and **Figures S15-S18**). It was found that ALP has
100 a smaller K_m value in comparison to ACP ($0.38 \pm 0.042 \mu\text{M}$ and $99.22 \pm 13.16 \mu\text{M}$ respectively) and
101 a lower V_{\max} ($208 \pm 3.81 \text{ min}^{-1}$ and $1962 \pm 223.6 \text{ min}^{-1}$ respectively). Hence, ALP has higher affinity
102 towards **TCF-ALP** compared to ACP, thus is more selective towards ALP at physiological pH.

103 According to current standards, determination of ALP and ACP is undertaken at the phosphatase's
104 optimum pH. For example, the Centers for Disease Control and Prevention (CDC) procedure for ALP
105 determination is carried out in 2-amino-2-methyl-1-propanol (AMP) buffer at pH 10.3 ((CDC), 2012).
106 This is in accordance with other literature sources (Guo et al., 2018, Di Lorenzo et al., 1991, Radio et
107 al., 2006, Pandurangan and Kim, 2015). Likewise, ACP determination is carried out at pH 4-6 (Myers
108 and Widlanski, 1993, Boivin and Galand, 1986, LI et al., 1984). Following these observations, further
109 studies were conducted to determine selectivity at pH 5.0 and 9.2 (**Figures S19 – S22**). Results showed
110 that **TCF-ALP** acts selectivity towards ACP at acidic pH, and ALP at alkaline pH. Therefore, **TCF-**
111 **ALP** can be used to selectively detect ALP/ACP in clinical assays, or live cell systems (provided the
112 buffer solution is optimal for the phosphatase under study).

113 2.3 Imaging of ALP in living cells

114 Prior to exploring whether **TCF-ALP** could be used to image ALP activity levels in live cells, the
115 cytotoxicity of **TCF-ALP** was assessed using a MTT assay (**Figure S23**). Negligible cell toxicity was
116 observed for **TCF-ALP** concentrations between 0 – 5 μM , and cell viability was only slightly reduced
117 (91%) when incubated with 10 μM **TCF-ALP**, indicating good biocompatibility.

118 **TCF-ALP** proved cell permeable to HeLa cells that express ALP and provided a clear 'turn on'
119 response (**Figure 3**). In contrast, pre-treatment of HeLa cells with 5 mM Na_3VO_4 prior to incubation
120 with **TCF-ALP** resulted in minimal 'turn on'. This was taken as evidence that the increase in **TCF-**
121 **ALP** fluorescence levels seen for HeLa cells in the absence of Na_3VO_4 is due to ALP activity. We thus
122 conclude **TCF-ALP** is a probe that allows for the selective cellular imaging of ALP activity.

123 Bone morphogenetic protein 2 (BMP-2) is capable of inducing osteoblast differentiation into a variety
124 of cell types (Guo et al., 2014, Wang et al., 2015) *via* pathways that result in increased ALP mRNA
125 expression, leading to increased ALP activity (Kim et al., 2004). Treatment of myogenic murine C2C12
126 cells with **TCF-ALP** resulted in a low fluorescence intensity (low ALP levels) being observed (**Figure**
127 **4**); however, pre-treatment of these cells with BMP-2 (300 ng/mL, 3 days) resulted in a significant
128 increase in **TCF-ALP**-derived fluorescence intensity (high ALP levels). Once again, pre-incubation
129 with 5 mM Na₃VO₄ led to no fluorescence response being observed in the cells treated with **TCF-ALP**
130 (with or without BMP-2). This provided support for the notion that **TCF-ALP** is capable of imaging
131 endogenous ALP activity induced by BMP-2.

132 **3 Conclusions**

133 In summary, a long wavelength TCF-based fluorescent probe (**TCF-ALP**) has been prepared with the
134 goal of detecting ALP activity. ALP hydrolyses the phosphate group of **TCF-ALP** resulted in a
135 significant ‘turn on’ fluorescence response (58-fold) within 15 minutes. These spectroscopic changes
136 were accompanied by a colorimetric change from yellow to purple. This enables **TCF-ALP** to be used
137 as a simple assay for the evaluation of ALP activity. Further analysis revealed that **TCF-ALP** could
138 also be used as a probe for detecting ACP activity. **TCF-ALP** was shown to be cell permeable, enabling
139 its use as a fluorescent probe for monitoring ALP levels in HeLa cells. **TCF-ALP** also proved capable
140 of imaging endogenously stimulated ALP produced in myogenic murine C2C12 cells through the
141 addition of bone morphogenetic protein 2. We thus suggest that **TCF-ALP** offers promise as a tool for
142 measuring ALP and ACP activity levels in clinical assays or in live cell systems.

143 **4 Conflict of Interest**

144 The authors declare that the research was conducted in the absence of any commercial or financial
145 relationships that could be construed as a potential conflict of interest.

146 **5 Author Contributions**

147 LG and ACS carried out synthetic and spectroscopic experiments and co-wrote the manuscript with
148 TDJ and JLS. JEG and GTW carried out background experiments. GK carried out cellular imaging
149 experiments. JPL carried out the ³¹P NMR titrations. J-YM and ATAJ are supervisors of LG and GTW.
150 SDB, JY, JLS and TDJ both conceived the idea and helped with the manuscript.

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159

160

161 **8 Supplementary Material**

162 Data supporting this study are provided as supplementary information accompanying this paper, which
163 is available free of charge.

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297 10 Captions for Figures and Schemes

298 **Scheme 1** – A TCF-based fluorescence probe (**TCF-ALP**) for the detection of alkaline phosphatase.

299 **Scheme 2** – Synthesis of **TCF-ALP**.

300 **Figure 1** - Fluorescence spectra of **TCF-ALP** (10 μM) produced via the addition of alkaline
 301 phosphatase (ALP; 0 – 0.2 U/ mL) in 50 mM Tris-HCl buffer, pH = 9.2 at 25 $^{\circ}\text{C}$. λ_{ex} = 542-15 nm. All
 302 measurements were made 15 min after the addition of ALP.

303 **Figure 2** - Fluorescence spectra of **TCF-ALP** (10 μM) recorded in the presence of trypsin (0.8 BAEE
 304 U/ mL), porcine liver esterase, protease from *Streptomyces griseus*, proteinase K, bovine serum
 305 albumin (0.1 mg/ mL), acid phosphatase (50 mM Tris-HCl, pH = 5.0) and alkaline phosphatase (50
 306 mM Tris-HCl, pH = 9.2). All enzymes were standardised to 0.8 U/ mL in Tris-HCl buffer pH 7.1 unless
 307 otherwise stated. λ_{ex} = 542-15 nm/ λ_{em} = 606 nm. Fluorescence measurements were made 30 min after
 308 adding the enzyme in question.

309 **Figure 3** - HeLa cells incubated under the following conditions: (a) No treatment. (b) **TCF-ALP** (10
 310 μM , 30 min). (c) Pre-treated with Na_3VO_4 (5 mM, 30 min), followed by the addition of **TCF-ALP** (10
 311 μM , 30 min). (d) Pretreated with Na_3VO_4 (0.5 mM, 30 min) and **TCF-ALP** (10 μM , 30 min). Cells
 312 were washed with DPBS before their fluorescence images were acquired using a confocal microscope.
 313 Top half: fluorescence images, bottom half: fluorescence images merged with its corresponding DIC
 314 image. Ex. 559 nm/ em. 575-675 nm. Scale bar : 20 μm . DIC - differential interference contrast.

315 **Figure 4**- **TCF-ALP** in C2C12 cell. C2C12 cells were treated with 300 ng/ mL BMP-2 for 3 days and
 316 then pretreated with 5 mM levamisole for 30 min and stained with 10 μM probe for 30 min. After
 317 washing with DPBS, fluorescence images were acquired by confocal microscopy. (a) only probe, (b)
 318 levamisole + probe, (c) BMP-2 + probe (d) BMP-2 + levamisole + probe. Top : fluorescence images,
 319 bottom : merged with DIC image. Ex. 559 nm/ em. 575-675 nm. Scale bar : 20 μm . DIC - differential
 320 interference contrast.

Figure 1.TIF

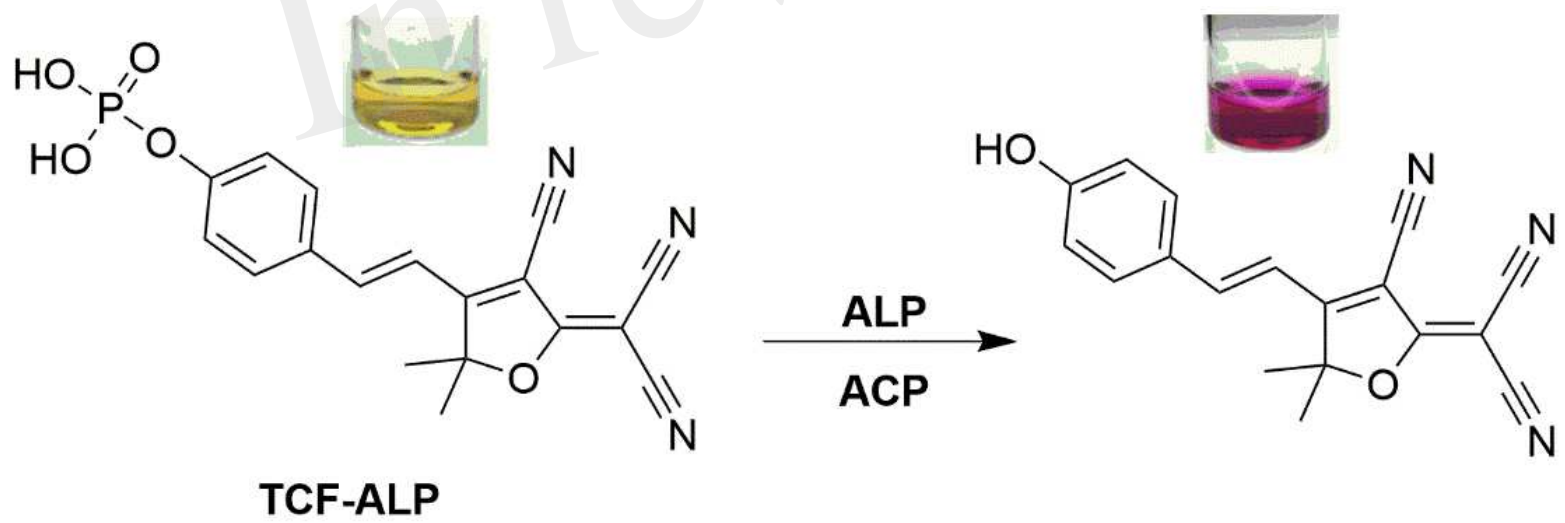


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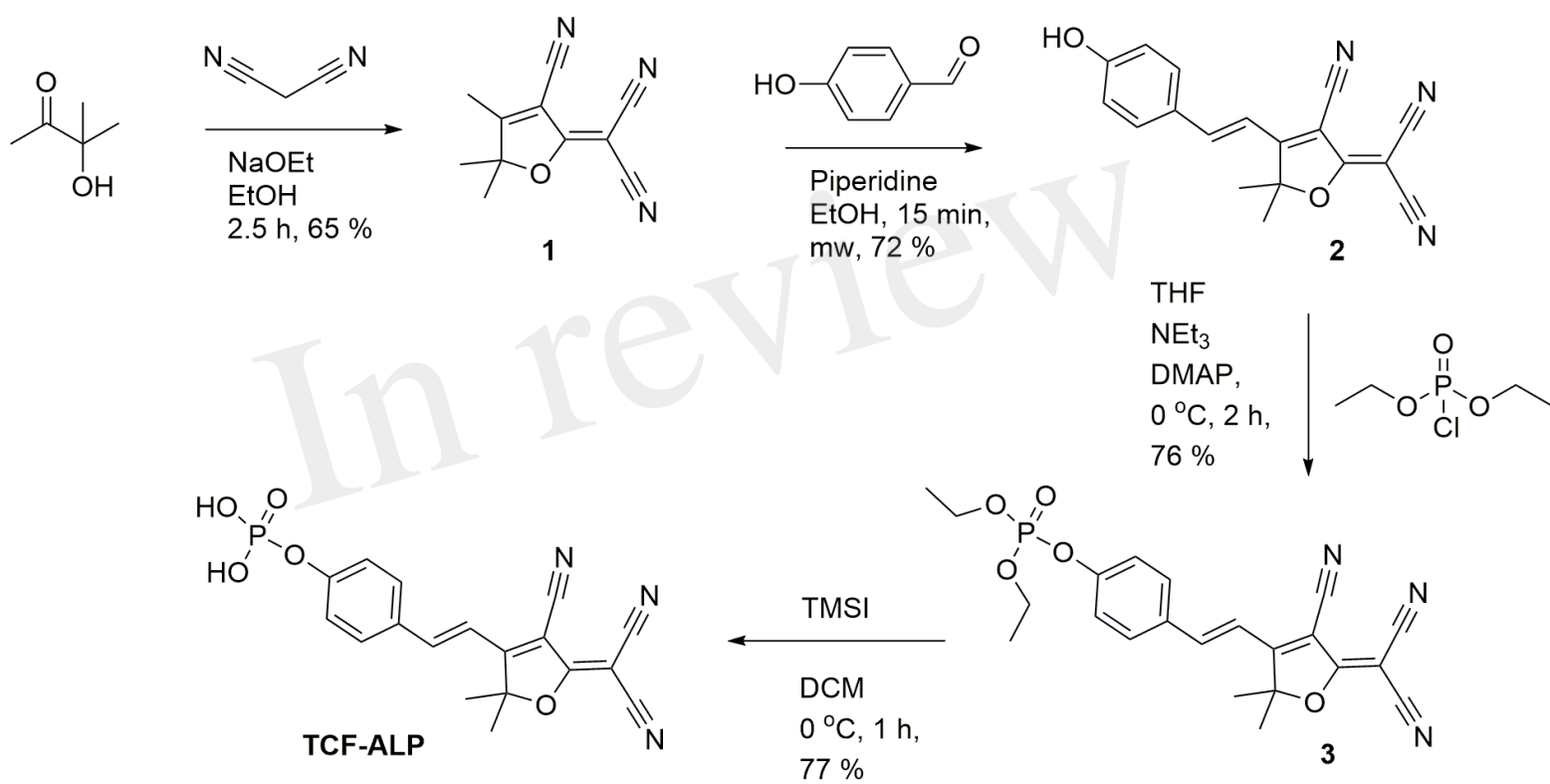


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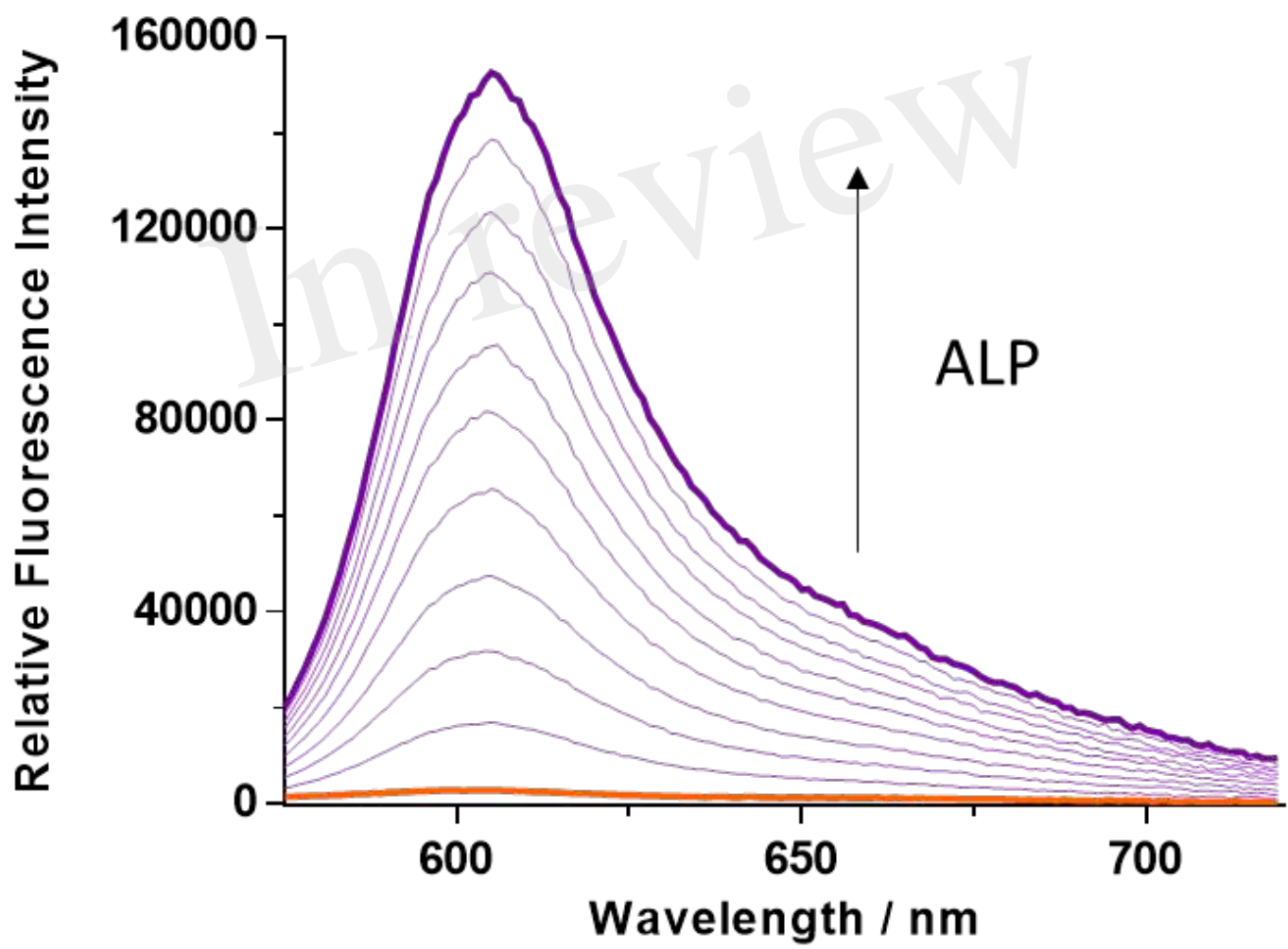


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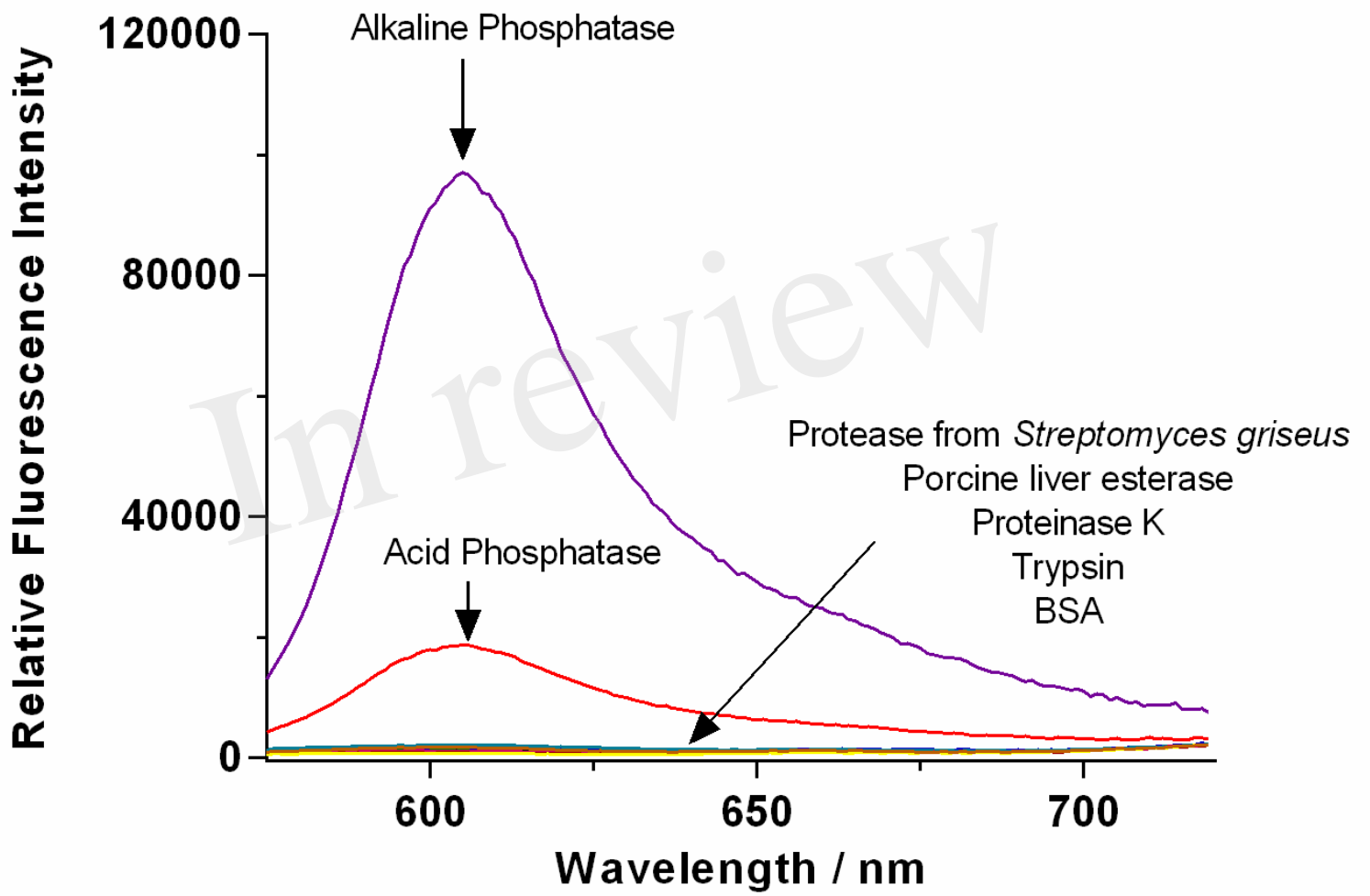


Figure 5.TIF

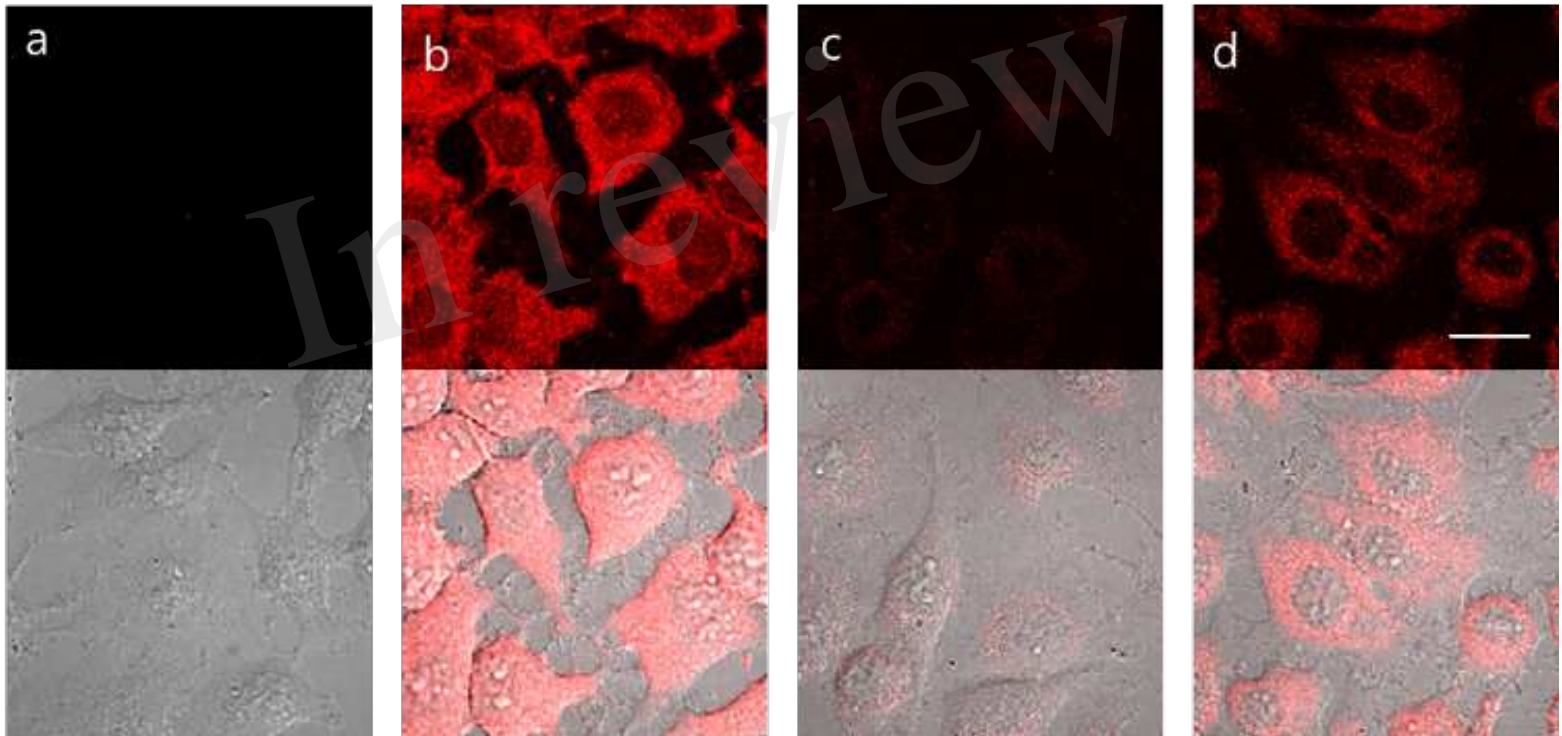


Figure 6.TIF

