Tagging Live Cells that Express Specific Peptidase Activity with Solid-State Fluorescence

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A three-component probe harnesses the extraordinary properties of a solid-state fluorophore for the detection of living cells exhibiting a particular peptidase activity. The off–on mode by which the probe operates, the bright fluorescence of the resulting precipitate, and the rapid response allow an exceptional signal-to-background ratio during microscopic imaging. A tertiary carbamate link between the spacer and phenolic fluorophore is at the heart of the probe’s long-term stability. The degree of chlorination of the probe determines its response time and thus its suitability for live-cell analysis. Our probe also allows highly resolved localization of peptidase activity during gel analysis or on agar. In comparison, probes releasing soluble fluorophores demonstrate complete diffusion of the fluorescent signal. These results demonstrate the probe’s potential for diverse biomedical applications, including high-fidelity flow cytometry and sensitive colony assays.

Introduction

Microscopic imaging and metabolic mapping[1,2] of enzyme activity in live cells is severely hampered by diffusion/dilution of the fluorescent signal. Indeed, the majority of fluorogenic probes release a water-soluble fluorophore, thus delocalizing (to a greater or lesser extent) the fluorescent signal—even staining the entire culture plate including the supernatant.[3] Today’s histochemical techniques get around this problem by fixation of the cells and subsequent incubation[4] with an antibody–fluorophore conjugate to highlight the mere presence of the enzyme. However, the imaging of enzyme activity in live cells requires new innovation in probe design.

Retention of the fluorescent signal within the living cell can be achieved either by tagging the protein of interest with activity-based probes[5,6] or by using probes that release a precipitating fluorophore, thus tagging the cell in which the enzyme is active. Although the former technique theoretically allows precise localization of the protein, it irreversibly inhibits the enzyme, thus accounts for low detection sensitivity, and catalytic turnover cannot be demonstrated. The latter solution, by contrast, can achieve high sensitivity by acting as a true substrate for the target enzyme, thus leading to significant catalytic signal amplification. The release of a solid-state fluorophore into the medium from an enzyme-responsive probe was recognized as highly attractive in the early nineties.[7–9] More recent reports based on aggregation-induced emission attest to its renewed interest.[10,11] The only commercial “precipitation-based probe” (ELF97-phosphate, Life Technologies)[12,13] relies on highly insoluble hydroxyphenylquinazolinone (3a, Scheme 1),[14] and targets generic phosphatase activity.[15,16] Six other probes releasing 3a were marketed during the 2000s for diverse enzyme activities (esters,[17,18] lipases, glycosidases,[19–22] β-lactamas,[23]), but all have been discontinued. We believe that the very electron-deficient nature of phenol 3a makes it such a good leaving group that it leads to swift degradation of the probe under physiological conditions. Users have thus lamented the impossibility to design robust probes that take advantage of the attractive properties[24] of 3a for the targeting of enzymes other than phosphatase.[22,23] This is true in particular for the large class of peptidases that play a pivotal role in biomedical research.

Herein, we report a precipitation-based probe that allows persistent tagging of live cells expressing leucine aminopeptidase (LAP). LAPs are metallopeptidases involved in the processing of bioactive peptides.[24] In mammals, they process peptides for MHC I antigen presentation, contribute to vesicle trafficking to the plasma membrane, and are overexpressed in human embryo-derived extravillous trophoblasts that invade the uterus during placentation.[25] High LAP activity was also reported in endometrial adenocarcinomas.[26] As a solution to the stability problem, we proposed a three-component probe (1d), in which phenol 3d is separated from the substrate portion by a cyclizing spacer.[29,30] However, although this construct gave satisfactory results in terms of stability and in vitro detection, no fluorescence could be observed in cellulo. We first considered slow spacer cyclization to account for our inability to detect a fluorescent signal. Indeed, most of the reported cyclizing spacers are the cause of extended response times by their respective probes.[31,32] Slow spacer cyclization promotes excessive signal dilution because the fluorophore–spacer conjugate released by the enzyme diffuses before fragmenting.

Results and Discussion

We synthesized the three-component probe 4, which releases water-soluble 4-methylumbelliferone 5 and its two-component
counterpart 6 (which does not comprise the trigger group for LAP; see the Supporting Information for experimental details). Comparison of the conversion and fragmentation kinetics with those of the commercial probe 7 should allow dissociation of the kinetics stemming from spacer cyclization from those of the peptidase action.

Dissolution of 6 in buffer over the tested pH range triggered cyclization–fragmentation and thus signal generation (Figure 1A). This furnished the half-lives of spacer cyclization at physiological pH (pH 7.4, $t_{1/2} = 7$ s), at neutral pH ($t_{1/2} = 1$ min), and at pH 6 ($t_{1/2} = 12$ min; all determined at 37 °C). This was as expected: slowing of spacer cyclization with increasing acidity is consistent with a reduction in the proportion of the free nucleophilic amino group. Not surprisingly, the spacer immolation at physiological pH (almost instant at 37 °C) was somewhat slower at room temperature (22 °C, $t_{1/2} = 1$ min). The improvement achieved by our approach can be appreciated by comparing these half-lives with the 47 min for cyclization of an un-preorganized ($N,N'$-dimethylethylene)diamine spacer contained in an enzyme-responsive prodrug. [33]

Incubation of LAP with 4 resulted in $t_{1/2} = 7$ min, whereas commercial AMC-Leu (7) showed $t_{1/2} = 3$ min (Figure 1B). The faster conversion of 7 can be explained in terms of the energetically less demanding task of extruding an aniline rather than an aliphatic amine (as in our spacer). We previously showed that aniline versions of 3 are not fluorescent in the solid state;[34] and thus a two-component solution (as found in 7) must be ruled out. In conclusion, it is evident that spacer hydrolysis is very fast compared to enzyme turnover, which represents the rate-determining step. In this context, it should be noted that our spacer (an aliphatic amide) better mimics the true substrate of the enzyme, and the observed kinetics might better reflect biological peptide turnover than those obtained with the anilide substrate, as would be preferred for a probe assessing enzymatic activity.[1]

As the performance of our spacer was high, the failure to observe an intracellular signal might be attributable to delayed precipitation of fluorophore 3d. We thus prepared a series of chlorinated derivatives of 3 (3a–c) that were predicted to have decreased solubility, and their corresponding probes (1a–c, Scheme 1). The kinetics of signal appearance for 1a–c caused by LAP action (Figure 2A) were significantly shorter than for those of nonchlorinated 1d. Inspection of the curves argues in favor of the monochloro derivative 1b, but closer inspection of the initial response (Figure S1) reveals that the dichloro derivative 1a reacts faster. A comparison of the performances of 1a and 1b at lower concentrations demonstrates the clear superiority of 1a (Figure 2B). A lower solubility threshold for...
3a and a slightly faster precipitation rate for 3b are possible explanations. When 100 µM 1a was incubated with LAP for 2 h, the initial signal increased by a factor of 40 (Figure S2), thus attesting to the remarkable detection sensitivity of 1a.

Next, we determined the robustness of our best candidate, 1a. We incubated it at four different pH values exhibited by various cell organelles (pH 4.5 to 9.0). No signal stemming from probe degradation was detected, even after long periods of incubation (>15 h, Figure 3A), in contrast to a slight rise in signal for 7 (Figure 3B). In this respect, it is particularly important to note that inclusion of two electron-withdrawing chlorine substituents into the fluorophore does not impair robustness thanks to the tertiary carbamate link [35–37] at the heart of our design. We also assessed the specificity of 1a for LAP by incubations with three broad-spectrum peptidases. To our satisfaction, probe 1a remained silent in the presence of chymotrypsin, trypsin, and cathepsin B (Figure 4A). Finally, we synthesized the non-natural enantiomer (R) of probe 1a to confirm that LAP catalytic activity is responsible for the rise in fluorescence. In contrast to 1a, probe 1a-(R) remained silent when incubated with LAP (Figure 4B).

We now turned our attention to the performance of 1a in cellulo. We first verified that LAP is active in mammalian cells (HeLa). For this, we performed a colorimetric dosage assay with leucyl para-nitroanilide and a western blot of cell extract (Figures S4 and S7); this proved the presence of active LAP. To illustrate the usefulness of a precipitation-based probe, we first prepared a zymogram of purified LAP by employing native polyacrylamide gel electrophoresis (PAGE; Figure 5A); a highly resolved fluorescent band indicated catalytic activity inside the gel. A band for the zymogram of cell extract was not observed, but the same cell extract, when spotted on agar plates containing probe 1a, led to the formation of a strong and precisely located fluorescent spot (Figure 5B). The conditions for native PAGE do not apparently prevent slight denaturation, which pushes enzyme activity of the cell extract below the detection limit. In both cases, the use of 7 caused signal diffusion over the whole gel/plate; these experiments demonstrate the need to avoid fluorochrome diffusion.

Incubation of HeLa cells with 1a (concentrations ranging from 5 to 100 µM) over two hours resulted in an increasing number of finely resolved intense green fluorescent spots within the cells (Figures 6A, B and S9). This demonstrates passive cell penetration of our probe (the ELF97 phosphatase sub-

![Figure 2](image2.png)

**Figure 2.** Solid-state fluorescence. Rate dependence of LAP response on A) chlorination level (a–d) of probe 1 (100 µM) and B) on concentration of probes 1a and 1b.

![Figure 3](image3.png)

**Figure 3.** In vitro pH stability over 15 h of A) 100 µM probe 1a and B) 100 µM commercial AMC-Leu (7). F = fluorescence.

![Figure 4](image4.png)

**Figure 4.** Solid-state fluorescence. A) Specificity of 1a with four peptidases (10 µM); B) specificity of 1a and its enantiomer (10 µM) with LAP.

![Figure 5](image5.png)

**Figure 5.** A) Zymogram of LAP by native PAGE (lane i, purified enzyme; lane ii, cell extract); B) demonstration of the presence of active LAP in cell extract spotted onto agar containing 1a (1) or 7 (2).
whereas 20 μm number of fluorescent spots (Figure 6 C, Figures S12 and S13). Of fluorescence, just 5 μm competitive inhibitors (Bestatin and 26 min. ing Movies) demonstrates that the signal appears after only moved (Figure S4). A 1 h time-lapse experiment (see the Support-
strate is known not to penetrate). The technical advance achieved with the present technology is highlighted when comparing these microscopy images with those obtained with probe 4, which releases water-soluble fluorophore 5 (Figure 6D and E); its total diffusion over the incubation time renders this approach useless. Although soluble 5 can be completely removed by rinsing the cells with PBS, stains of 3a are unaffected (Figure S4). A 1 h time-lapse experiment (see the Supporting Movies) demonstrates that the signal appears after only 26 min.

Inhibition of LAP with increasing concentrations of two competitive inhibitors (Bestatin and L-Leucinethiol) decreased the number of fluorescent spots (Figure 6C, Figures S12 and S13). Whereas 20 μm Bestatin was required for total disappearance of fluorescence, just 5 μm L-Leucinethiol was sufficient for this, thus clearly indicating the superiority of the latter as an inhibitor. These experiments highlight our ability to specifically detect cells in which LAP is still active and thus the potential cleavage reactions of fluorochrome 3a in the design of a stable, responsive fluorogenic probe for a peptidase, in spite of its highly electron-deficient phenolic nature. We demonstrated that the probe penetrates mammalian cells effectively and that wild-type levels of leucine aminopeptidase are sufficient to convert it into intracellular and highly resolved fluorescent stains. We believe that the ease of synthetic access to our three-component probe and its demonstrated ability to tag living cells bode well for its widespread use. Future attention will be given to its adaptation to a variety of peptidase activities and their use in more complex media.

Conclusions

We have succeeded in harnessing the unique physical properties of fluorochrome 3a in the design of a stable, responsive fluorogenic probe for a peptidase, in spite of its highly electron-deficient phenolic nature. We demonstrated that the probe penetrates mammalian cells effectively and that wild-type levels of leucine aminopeptidase are sufficient to convert it into intracellular and highly resolved fluorescent stains. We believe that the ease of synthetic access to our three-component probe and its demonstrated ability to tag living cells bode well for its widespread use. Future attention will be given to its adaptation to a variety of peptidase activities and their use in more complex media.

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Figure 6. Cell tagging: intracellular conversion (2 h) of A–C 1a (10 μm) and D and E 4 (100 μm) by LAP activity in live HeLa cells: fluorescence channel (A and D), merged with bright-field (B and E); scale bars: 20 μm. C) Fluorescence image in the presence of LAP-selective inhibitor leucine-thiol (5 μm). F) MTS cell viability test over 24 h for the analogous probes, releasing a solid-state and a soluble fluorophore.
