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Quantitative Measurement of Protease-Activity with Correction of Probe Delivery and Tissue Absorption Effects

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Abstract

Proteases play important roles in a variety of pathologies from heart disease to cancer. Quantitative measurement of protease activity is possible using a novel spectrally matched dual fluorophore probe and a small animal lifetime imager. The recorded fluorescence from an activatable fluorophore, one that changes its fluorescent amplitude after biological target interaction, is also influenced by other factors including imaging probe delivery and optical tissue absorption of excitation and emission light.

Fluorescence from a second spectrally matched constant (non-activatable) fluorophore on each nanoparticle platform can be used to correct for both probe delivery and tissue absorption. The fluorescence from each fluorophore is separated using fluorescence lifetime methods.

Keywords

Fluorescence Imaging; Fluorescence Lifetime; Activatable Probe; Ratiometric

1. Introduction

Proteases, enzymes that break down proteins, play an important role in a variety of pathologies including cancer and cardiovascular disease [1], the two leading causes of death the United States [2]. In cancer, high levels of protease activity are associated with invasive and metastatic cancers [3]. In cardiovascular disease, proteases are active in apoptosis, tissue remodeling, and modification of cardiac proteins [4].

Protease activity has been used to visualize these diseases with protease activatable probes. Because these probes are injected in a quenched state and activated *in vivo*, the background signal from nonspecific probe uptake is minimized for accurate measurements of low levels of activity [5]. This makes it possible to accurately delineate tumor margins *in vivo* and quantify that the protease activity in tumors is more than ten times the level in healthy tissue using *ex vivo* samples [6]. Imaging of protease activity in cancer can be used as a screening tool or for

measuring treatment efficacy [7]. Atherosclerosis treatment decisions may be made based on in vivo evaluation of plaques by fluorescence angiography [8].

While these probes are useful for locating lesions, the measured fluorescence cannot be used to quantify protease activity because the measured fluorescence is a combination of probe delivery, probe activation, and optical tissue absorption. Probe activation can be measured independently by using a dual fluorophore probe. In these probes, a constitutively active fluorophore is used to measure probe distribution and optical absorption. An activatable fluorophore measures the combined effect of distribution, optical absorption, and activation. The percentage of activation is the ratio of the two signals [8], since the other terms cancel out due to a common carrier platform.

The accuracy of the dual fluorophore technique is limited by the matching of optical absorption of tissue at the excitation and emission wavelengths of the two fluorophores. The quantitative protease activity measurement system presented in this paper guarantees that optical absorption cancels out exactly in ratiometric imaging. The system, shown in Fig. 1, consists of the two parts: a nanoparticle imaging probe and an imager. The probe is small enough to travel throughout the animal and interact with biomolecules. Spectrally matched fluorophores are used to report distribution and activation. The imager measures fluorescence from the two fluorophores using the property of fluorescence lifetime by taking nanosecond images of a large imaging area.

2. Small Animal Lifetime Imager (SALI)

Fluorescence lifetime imaging is an instrumentation challenge because the fluorescence lifetimes of fluorophores suitable to in vivo imaging vary from hundreds of picoseconds to a few nanoseconds. The Small Animal Lifetime Imager (SALI) captures images for measuring these lifetimes and the fluorescence from multiple fluorophores by combining nanosecond excitation pulses with nanosecond exposures. Two of these nanosecond images are sufficient to calculate the lifetime of a single exponential decay or the fluorescence from two fluorophores with known lifetimes [9].

The small animal lifetime imager (SALI) captures fluorescence lifetime images of in vitro and in vivo samples. This system comprises three parts: a nanosecond illuminator, imaging optics, and a commercial nanosecond imager as shown in Fig. 2.

The nanosecond illuminator is based on an electronically pulsed laser diode [9]. A custom designed printed circuit board houses the timing circuitry. Inverters A, B, and C form an oscillator with the 47 k Ω resistor and 10 pF capacitor setting the frequency to 2 MHz. The 5.6 k Ω resistor and inverters D and E introduce the delay required between triggering the nanosecond imager and exciting the fluorophores. The 10 pF capacitor and the 560 Ω resistor transform the square wave into a pulse train. Inverter F and the power mosfet switch the 658 nm laser diode. The laser beam is diffused to illuminate the entire sample by a square-pattern engineered light diffuser (Thorlabs ED1-S20.) The size of the illumination area is then adjusted by the placement of the sample with respect to the illuminator.

A commercial gated-optical-intensifier camera, the Picostar HR (LaVision), captures the nanosecond exposures when triggered by an electrical signal from the illuminator. A programmable delay in the Picostar HR varies the delay between excitation and exposure across a 20 ns range. The fluorescence light first passes through a chromatically corrected lens assembly for focusing and aperture control (Schneider 17 mm/f1.4 Xenoplan.) The light is then infinity focused by a 40 mm achromatic lens (Edmund Optics.) A six-position motorized filter wheel (Thorlabs FW102B) selects the wavelengths with a 680 nm-710 nm bandpass filter

(Omega Filters 3RD680-710) used for far-red imaging. The image is then focused using another 40 mm achromatic lens (Edmund Optics) on the Picostar HR.

Operation of the SALI was first verified by imaging a reflective white surface with the filter wheel turned to an empty slot. In this configuration, the excitation beam is imaged. Images were taken at delays at one-nanosecond intervals with respect to the excitation pulse. The mean signal intensity was then measured within a region of interest in the illumination area using Matlab (Mathworks.) This measured pulse waveform is plotted in a solid line in Fig. 3. The pulse is 10ns wide with both on and off transients faster than one order of magnitude per nanosecond, sufficient for measuring single nanosecond fluorescent lifetimes.

Two fluorophores with published lifetimes were then imaged with the bandpass filter replaced. ATTO 647 and ATTO 647N are far-red fluorophores from ATTO Tec. The fluorophores were placed in black 384-well plates. As with the excitation pulse, images were taken at one-nanosecond delay intervals and the mean intensity calculated. Plotting the intensity on a log scale versus time on a linear scale as in Fig. 3 creates a straight line from the single exponential decay as seen in both fluorophores. The slope of the line is the fluorescent lifetime. The lifetimes of 2.5ns and 3.8ns agree well with the manufacturer's reported values.

3. Fluorescent Lifetime of Activatable Probe

The SALI was then used to measure unknown fluorescent lifetimes, the fluorescent lifetimes of an activatable probe before and after activation. This activatable probe is a member of a class of enzyme-activatable fluorescence probes using an iron-oxide nanoparticle, cleavable peptide linker, and fluorophore [10]. The iron-oxide nanoparticle is used as a quencher and nanoparticle platform. The peptide linker is chosen for enzyme specificity. The fluorophore is chosen for fluorescence properties, generally excitation and emission wavelengths. In this work, a long fluorescence lifetime fluorophore was used to investigate changes in fluorescence lifetime during activation. A poly-arginine linker was used because of its susceptibility to trypsin cleavage [11].

This single fluorophore activatable probe in Fig. 4(A) was synthesized by first making CLIO nanoparticles [10]. A mixture of ferrous and ferric iron chlorides was dissolved with T-10 dextran. The pH was then modified with ammonia to form the particles. The resulting material was further reacted with epichlorohydrin to crosslink the protective dextran coating, followed with ammonia to provide amine attachment points.

The cleavage peptide (GRRRRGC) (Tufts University Peptide Core Facility) was labeled with ATTO 647N by reacting 1 mg of the lyophilized peptide with 100 μ L of ATTO-647N-NHS ester (1 mg/200 μ L DMSO) overnight, using 2 μ L of diisopropylethyl amine as a catalyst. After the reaction, 1 mL of PBS and 10 μ L of 0.5M TCEP (tris(t-carboxyethyl) phosphine) solution were added to form the labeled peptide stock solution.

The labeled cleavage peptide was attached to CLIO by first activating 10 mg of CLIO with 9 mg of SIA (succinimidyl iodoacetate) in 100 μ L of DMSO. After 15 minutes at room temperature, the CLIO was purified by size exclusion chromatography (sephadex G-25 resin) in PBS. Activated CLIO was then mixed with 450 μ L of the labeled cleavage peptide. This was allowed to react overnight at room temperature, and again purified by size exclusion chromatography (sephadex G-50 resin) in PBS.

As with the free dye, the lifetime of this probe was measured by imaging 50 μ L of probe in a black 384-well plate. Trypsin was added to the wells to create concentrations ranging from 0 to 6 μ M. The SALI was then used to measure fluorescence intensity in each 1 ns window from 0 to 20 ns with laser excitation between 2 ns and 9 ns. The linear plot in Fig. 4(B) demonstrates

four-fold activation with increasing concentrations of trypsin. Plotting the same data with a log scale for fluorescence intensity demonstrates that the fluorescence lifetime, the slope of the straight-line decay in this plot, is unchanged during activation. A pure exponential decay with a 3.9 ns time constant is plotted in Fig. 4(C) for comparison to probes.

4. Fluorescent Lifetime Multiplexing

Because the lifetime is unchanged with activation, it is possible to apply the recently developed technique of fluorescent lifetime multiplexing to activatable probes. Fluorescence lifetime imaging was first applied to in vivo imaging to generate contrast between normal and diseased tissue [12–14]. It was then applied to exogenous fluorophores to increase the signal-to-background ratio [15]. Recently, a new application has been developed, fluorescent lifetime multiplexing, the independent measurement of fluorescence from multiple fluorophores in a single spectral window [16–17].

A variety of methods have been used for fluorescent lifetime multiplexing. In this work the two-window method is used [9]. The fluorescence is measured in two time windows of equal length, F_{win1} and F_{win2} . The time between the start of the first window and the start of the second window is t_{win} . Equations for the fluorescence in each window are written as the sum of fluorescence with fluorescence lifetimes of τ_1 and τ_2 and relative amplitudes A_1 and A_2 respectively.

$$F_{win1} = A_1 + A_2 \quad (1)$$

$$F_{win2} = A_1 e^{-\frac{t_{win}}{\tau_1}} + A_2 e^{-\frac{t_{win}}{\tau_2}} \quad (2)$$

The two equations are then solved for A_1 and A_2 .

$$A_1 = \frac{F_{win2} - F_{win1} e^{-\frac{t_{win}}{\tau_2}}}{\tau_1 \left(1 - e^{-\frac{t_{win}}{\tau_1}} \right) \left(e^{-\frac{t_{win}}{\tau_1}} - e^{-\frac{t_{win}}{\tau_2}} \right)} \quad (3)$$

$$A_2 = \frac{F_{win1} e^{-\frac{t_{win}}{\tau_1}} - F_{win2}}{\tau_2 \left(1 - e^{-\frac{t_{win}}{\tau_2}} \right) \left(e^{-\frac{t_{win}}{\tau_1}} - e^{-\frac{t_{win}}{\tau_2}} \right)} \quad (4)$$

This multiplexing method was verified using free dyes in the SALI. The spectrally matched fluorophores measured independently for Fig. 3, ATTO 647 and ATTO 647N, were used for the multiplexing experiment. First, it was determined that fluorescence intensity varied linearly with fluorophore concentration in the range from 0.5 μM to 4.5 μM for both fluorophores when 50 μL of fluorophore solution is imaged in a standard a black 384-well plate with the SALI. Below this range, the fluorescence signal was indistinguishable from noise. Above this range, fluorescence increases more slowly, likely due to significant absorption of excitation and emission light.

Then, a phantom for testing multiplexing was developed using a four-by-four grid of wells in a 384-well plate. Each well contained a mixture of the two fluorophores, as shown in Fig. 5. Each row had a constant concentration of ATTO 647 increasing from no fluorophore in the bottom row to 3 micromolar in the top row. Each column had a constant concentration of ATTO 647N increasing from no fluorophore in the right column to 3 micromolar in the left column. Because these fluorophore intensities are within the linear range, the fluorescence from each fluorophore should vary with fluorophore concentration.

The well plate was imaged in the SALI at 2 ns and 11 ns following excitation. The images in Fig. 5 demonstrate that the 2 ns image shows signal from both fluorophores, while the contribution from the short lifetime fluorophore, ATTO 647, is decreased in the 11 ns image. Applying Eqns. (3) and (4) to each pixel independently in the measured images produced the calculated images of fluorescence from the two fluorophores. The agreement between the calculated fluorescence and fluorophore concentration demonstrates the success of this technique.

4. Dual Fluorophore Ratio Imaging

The two technologies of activation and fluorescent lifetime multiplexing offer even greater possibilities when used with a dual fluorophore probe. Dual fluorophore probes make it possible to separate the activation signal from probe delivery and optical tissue absorption. The operation of dual fluorophore probes can be seen by considering the variables effecting in vivo fluorescence intensity. The fluorescence that reaches an imager from an in vivo fluorophore is given by Eq. (5) with geometric terms independent of wavelength and quantum yield included in the constant k . The illumination photons, $I(\lambda_{ex})$, pass through tissue where a fraction of the photons, $ab(\lambda_{ex})$, are absorbed before they interact with the fluorophore. The light that reaches the fluorophore excites a number of fluorophores proportionate to the number of fluorophores, n , and the extinction coefficient, $\varepsilon(\lambda_{ex})$. The quantum yield, $\varphi(x)$, which may be a function of activation, determines the percentage of excited fluorophores that emit photons. A fraction, $ab(\lambda_{em})$, of these emitted photons are then absorbed by the tissue.

$$F(\lambda_{ex}, \lambda_{em}) = k \cdot I(\lambda_{ex}) \cdot ab(\lambda_{ex}) \cdot n \cdot \varepsilon(\lambda_{ex}) \cdot \varphi(x) \cdot ab(\lambda_{em}) \quad (5)$$

Activation of activatable probes changes the quantum yield, $\varphi(x)$. Unfortunately, variations in the other variables also affect the signal. Uneven tissue delivery of the nanoparticles changes n . Different tissue absorption due to either depth of probe or tissue type can change $ab(\lambda)$. Dual fluorophore probes attempt to solve this problem by taking the ratio of two fluorescence intensities, F_{act} and F_{const} .

$$\frac{F_{act}(\lambda_{ex}, \lambda_{em})}{F_{const}(\lambda_{ex}, \lambda_{em})} = \frac{k_1 \cdot I(\lambda_{ex,1}) \cdot ab(\lambda_{ex,1}) \cdot n_1 \cdot \varepsilon(\lambda_{ex,1}) \cdot \varphi(x) \cdot ab(\lambda_{em,1})}{k_2 \cdot I(\lambda_{ex,2}) \cdot ab(\lambda_{ex,2}) \cdot n_2 \cdot \varepsilon(\lambda_{ex,2}) \cdot \varphi(1) \cdot ab(\lambda_{em,2})} \approx R \frac{\varphi(x)}{\varphi(1)} \quad (6)$$

$$R = \frac{k_1 \cdot I(\lambda_{ex,1}) \cdot ab(\lambda_{ex,1}) \cdot n_1 \cdot \varepsilon(\lambda_{ex,1}) \cdot ab(\lambda_{em,1})}{k_2 \cdot I(\lambda_{ex,2}) \cdot ab(\lambda_{ex,2}) \cdot n_2 \cdot \varepsilon(\lambda_{ex,2}) \cdot ab(\lambda_{em,2})} \quad (7)$$

This technique works well when R is relatively constant. The values of k , n , and $\varepsilon(\lambda_{ex})$ are kept at a fairly constant ratio, but the absorbance terms, $ab(\lambda)$, can lead to errors. The small amount of tissue between the illumination source and shallow fluorophores only creates a small

variation between different wavelengths of light. The larger section of tissue between the illumination source and deeper fluorophores creates a larger wavelength dependent variation in excitation light. The cause of this variation can be seen by considering a homogenous sample. Here $ab(\lambda)$ is given by Beer's law in Eqn. (8), where $\alpha(\lambda)$ is the absorption coefficient. While the ratio of absorption coefficients is constant for the homogeneous sample, changes in position vary l changing the ratio of absorbance.

$$ab(\lambda) = 10^{-\alpha(\lambda)l} \quad (8)$$

Fluorescent lifetime multiplexing solves this problem by forcing the excitation and emission wavelengths to be equal. In this case the absorbance terms cancel out exactly.

A comparison of spectrally separated ratiometric fluorescence imaging to lifetime separated ratiometric fluorescence imaging was performed using a tissue phantom and mixtures of free fluorophores. For spectral separation, Cy3.5 and ATTO 647 were used. For lifetime separation, ATTO 647 and ATTO 647N were used.

The tissue phantom was fabricated using murine blood mixed with agarose to mimic optical absorption of tissue. 600 μ L of murine blood was combined with 16 mL of heated 2.5 % agarose (Sigma-Aldrich) in PBS along with an additional 3 mL of PBS, and molded in 100 mm Petri dishes (Fisher Scientific.)

The spectrally separated fluorophores were prepared by placing 25 μ L of 2 μ M Cy3.5 into each of 9 wells in a black 384 well plate. Then 25 μ L of 0, 2 μ M, and 4 μ M ATTO 647 were added to 3 wells each with the Cy3.5 forming fluorophore ratios of 0, 1, and 2. Two pieces of the tissue phantom were then placed atop the wells to create thicknesses of 2mm and 4mm as shown in Fig. 6(A). The fluorescence lifetime separated fluorophores were prepared in the same manner with each well containing 1 μ M ATTO 647N and varying concentrations of ATTO 647.

The fluorescence in each well was then measured in the SALI. The fluorescence-lifetime-separated fluorescence was measured using the fluorescence demultiplexing method in section 3. The spectrally separated fluorescence was measured using SALI as a steady-state fluorescence imager. A halogen illuminator (Edmund Optics) was used with a 550 nm to 580nm interference filter to excite the Cy 3.5. A 600nm to 650nm bandpass filter was used in the imaging path. The Picostar HR took 1ns exposure images at approximately 1 MHz rate independent of the illumination. The ATTO 647 fluorescence was measured with the same illumination and filtering as used in the fluorescence-lifetime-separated fluorescence measurement. The delay between excitation and imaging was set to maximize fluorescence intensity.

The fluorescence intensities for spectrally separated and fluorescence lifetime experiments are plotted in Fig. 6(B) and Fig. 6(C) respectively. In both cases, measured fluorescence from both fluorophores decreases with increased phantom thickness for the same fluorophore ratio, and the ATTO 647 fluorescence increases with increasing fluorophore ratio (i.e. increasing ATTO 647 concentration) for a given tissue phantom thickness. The difference between the spectrally separated and fluorescence lifetime experiments is in the ratios of the fluorescence. The measured fluorescence from Cy3.5 decreases more quickly with increasing phantom thickness than the ATTO 647 fluorescence. This difference in tissue absorbance at the different wavelengths causes the difference in measured fluorescence for the same fluorophore ratio as shown in Fig. 6(D). In contradistinction, the measured fluorescence ratios match the

fluorophore ratios for the fluorescence lifetime separated fluorophores because the tissue absorption is matched as shown in Fig. 6(E).

5. Dual Fluorophore Activatable Probe

The spectrally matched fluorophores were then used to synthesize a dual fluorophore fluorescent lifetime activatable probe (FLAP). The CLIO particles were synthesized as described in section 3. After creation of amine attachment points in that protocol, 10 mg of CLIO (Fe basis) dissolved in 2.1 mL PBS was reacted with 60 μ L of ATTO 647-NHS ester (1 mg/300 μ L in DMSO) and allowed to sit overnight at room temperature. An aliquot was purified by size exclusion chromatography (sephadex G-50 resin) to characterize the amount of dye directly bound to the nanoparticle. The cleavage peptide was then labeled with ATTO 647N and attached to the CLIO as before. The iron concentration and total dye concentration of both this conjugate and the aliquot of the ATTO-647-CLIO conjugate were measured by UV-Vis spectroscopy. The final product contains 1.97 mg Fe/mL, 17 μ M ATTO 647, and 12 μ M ATTO 647N.

Probe activation was measured using prepared concentrations of trypsin of 1, 2, 3, 4, and 5 μ g/mL solutions. 10 μ L of probe solution was placed in each of six wells in a black 384-well plate. 40 μ L of PBS/trypsin solution was added to each well varying from pure PBS to 5 μ g/mL trypsin. After allowing ten minutes for the trypsin to cleave the poly-arginine linker, the wells were imaged in the SALI. Fluorescent multiplexing analysis was performed to separate out the fluorescence signals from ATTO 647 and ATTO 647N. As shown in Fig. 7, the demultiplexing clearly separated a constant signal from a signal with only a 0.16 correlation to trypsin concentration from an activatable signal with a 0.92 correlation to trypsin concentration.

6. Conclusion

Protease activity can be quantified using a new spectrally matched dual fluorophore nanoparticle probe with a small-animal lifetime imager. Because the fluorescent lifetime is unchanged during activation of the CLIO based activatable probes used in this work, it is possible to separate fluorescence from spectrally matched fluorophores on a dual fluorophore probe. The use of spectrally matched fluorophores reduces the effect of tissue absorption on fluorescence ratios by 75 percent. The challenge of separating fluorescence from these fluorophores based on nanosecond time scale emission was solved by developing a small animal lifetime imager.

This system is now ready for in vivo imaging. Far-red fluorophores were chosen for optical transparency of tissue. Single fluorophore protease activatable probes based on similar technology have been previously used for in vivo imaging [7–8]. The SALI may now be used to improve the accuracy of these in vivo experiments characterizing tumors and atherosclerotic plaques.

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Biographies

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Quantitative Protease Activity Measurement System

Probe

- Submicron Size
- Linear Activation with Enzyme Activity
- Distribution Correction
- Optical Tissue Absorption Correction

Imager

- Large Field of View
- Nanosecond Time Resolution

Figure 1.

Protease Activity is measured using a two-part system. A nanoparticle probe circulates through the organism interacting with biomolecules. Proteases activate this probe. The imager then takes nanosecond images of the entire imaging area simultaneously to measure the fluorescence from the activatable and constant fluorophores. The protease activity is given by the ratio of the fluorescence intensities.

Figure 2.

Small Animal Lifetime Imager (SALI). Custom circuitry on a printed circuit board drives a laser diode for 10 ns excitation and generates trigger pulses for the nanosecond imager. The laser light is diffused through a square pattern diffuser. Fluorescence is gathered by a lens assembly, infinity focused by a single lens, reflected off of a gold mirror, filtered by interference filters mounted in a motorized filter wheel, focused by an additional lens, and imaged by a Picostar nanosecond imager.

Figure 3. Fluorescence Lifetime Measurement. The excitation laser was pulsed from 0 ns to 10 ns, plotted in a solid line. The straight-line decay in this plot of fluorescence intensity on a log scale represents a single exponential decay. The slopes of the two fluorescence lines are the fluorescent lifetimes of the fluorophores: 2.5 ns for ATTO 647 and 3.8 ns for ATTO 647N.

Figure 4.

Trypsin Activation of Single Fluorophore Probe. (A) Trypsin cleaves the linker that attaches the fluorophore to the iron oxide quencher. (B) The linear plot of fluorescence intensity versus time for various trypsin concentrations demonstrates greater than 4-fold activation. (C) A plot with the fluorescence intensity on a log scale shows that the fluorescent lifetime (shown in this graph as the slope of the straight-line decay) is a constant 3.9ns regardless of activation.

Figure 5.

Fluorescent Lifetime Multiplexing. Spectrally similar ATTO 647 and ATTO 647N are mixed in varying concentrations in 16 wells of a 384-well plate. Images captured 2ns and 11ns after excitation are used to calculate fluorescence from the two fluorophores. Comparison of calculated images at the bottom to actual concentration at the top demonstrates good agreement.

Figure 6.

Ratiometric Imaging Comparison. (A) A ratiometric imaging phantom was prepared in a black 384-well plate. Each well contains 50 μ l of fluorophore solution. Every well is a 1 micromolar solution of the constant fluorophore. The fluorescence ratio is adjusted by varying the concentration of the second fluorophore from 0 to 2 micromolar. Tissue was simulated with 2mm and 4mm sections of blood agarose phantom. (B) Spectral separation was performed using Cy3.5 as the constant fluorophore and ATTO 647 as the variable fluorophore. (C) Fluorescent multiplexing was used to separate the spectrally matched fluorophores ATTO 647 and ATTO 647N. (D) The fluorescence ratio increases with tissue thickness in the spectral separation case as the fluorescence from the Cy3.5 decreases more quickly with tissue thickness than ATTO 647 fluorescence. A 2mm increase in phantom thickness is equivalent to doubling fluorophore ratio. (E) The fluorescence ratio is much more constant when using spectrally matched fluorophores with a 2mm increase in phantom thickness causing less than 25% error in fluorophore ratio.

Figure 7.

Protease activity was varied by adjusting trypsin concentration across microwells. 10 μL of spectrally matched dual fluorophore probe was added to 40 μL of PBS/trypsin solution. Fluorescent demultiplexing of the fluorescence from each well yields an activatable fluorescence signal with a 0.92 correlation coefficient to trypsin concentration and a constant fluorescence signal with only a 0.16 correlation coefficient to trypsin concentration.