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Time gated luminescence imaging of immunolabeled human tissues

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Abstract

Multiplexed immunofluorescence imaging of formalin-fixed, paraffin-embedded tissues is a powerful tool for investigating proteomic profiles and diagnosing disease. However, conventional immunofluorescence with organic dyes is limited in the number of colors that can be simultaneously visualized, is made less sensitive by tissue autofluorescence background and is usually incompatible with commonly used hematoxylin and eosin staining. Herein, we demonstrate the comparative advantages of using time-gated luminescence microscopy in combination with an emissive Tb(III) complex, Lumi4-Tb, for tissue imaging in terms of sensitivity, multiplexing potential and compatibility with common immunohistochemistry protocols. We show that time-gated detection of ms-scale Tb(III) emission increases signal-to-noise ratio relative to conventional steady-state detection of organic dye fluorescence and permits visualization of low-abundance tissue markers such as Bcl-6 or MSH-6. In addition, temporal separation of long- and short-lifetime (~ns) signals adds a second dimension for multiplexing and also permits detection of intermolecular Tb(III)-to-dye Förster resonance energy transfer. Furthermore, we demonstrate that the Lumi4-Tb complex is compatible with tyramide signal amplification and, unlike conventional organic dyes, can be reliably used on tissue stained with hematoxylin and eosin. Our results indicate that time-gated luminescence microscopy using Tb(III) labels can provide a sensitive and robust method to perform multiplexed immunofluorescence on archived or clinical tissue specimens.

Graphical Abstract

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Author Contributions

All authors have given approval to the final version of the manuscript.

CONFLICT OF INTEREST DISCLOSURE

D.M. is a current employee of Lumiphore, Inc., producers of Lumi4-Tb reagent.

SUPPORTING INFORMATION

Supporting figures including micrographs and FRET bleedthrough calibration plots. Supporting table with microscope image acquisition parameters.

Multiplexed immunohistochemistry (mIHC) is needed in the clinic to provide improved diagnoses and therapeutic strategies and at the bench level to characterize the systems biology mechanisms that drive disease progression.^{1–3} Optical microscopy is among the limited number of approaches that can quantitatively determine expression levels, heterogeneity and spatial relationships of multiple markers within dimensions ranging from the sub-cellular to tissue level.^{4–6} Among mIHC methods, immunofluorescence (IF) provides high spatial resolution (sub-micron), offers a large field of view and is compatible with common light microscopes.^{7–9} However, the multiplexing potential of IF suffers from crosstalk between overlapping dye emission spectra and the limited number of secondary antibodies that can be applied to a single sample. Multispectral imaging along with approaches that entail sequential cycles of staining and dye inactivation can overcome these limitations to a certain extent, but incomplete dye quenching can increase background and reduce data quality.^{3,10–12} Mass spectrometry can potentially detect up to 100 elemental mass tag-labeled antibodies, however the field of view with this technique is presently limited to a few hundred μm^2 and implementation is much more difficult and expensive than standard microscopy.^{2,13–16} Another limitation of IF is non-specific background such as autofluorescence that lowers sensitivity and dynamic range. Autofluorescence is particularly pernicious with formalin-fixed, paraffin-embedded (FFPE) tissues.^{17,18} Straightforward approaches to multiplexed IF imaging are needed that can increase the number of markers viewable on a single sample, that maximize sensitivity and that are compatible with commonly used instrumentation and sample preparation techniques.

Time-gated luminescence microscopy (TGLM) can eliminate non-specific background and increase the multiplexing potential of IF.¹⁹ TGLM requires emissive labels that exhibit long excited-state lifetimes such as phosphorescent dyes, platinum porphyrins, inorganic phosphor particles or complexes of Tb(III) or Eu(III). The luminescent lanthanide species are particularly well suited for TGLM because they have lifetimes ranging from 0.1 to 2 ms and, unlike transition metal complexes or organic phosphors, they are insensitive to oxygen quenching.^{20,21} With time-gating, pulsed light is used to excite the specimen, and the detector or camera acquires signal after a μs -scale delay that follows the excitation pulse (Fig. 1A). The delay interval is long enough to eliminate any residual excitation light or short-lived (<100 ns) fluorescence interference. In addition to providing excellent signal-to-background ratio, time-gated detection adds a second dimension for multiplexing in that the gated detection of long-lived luminescence is orthogonal to any fluorescent stains applied to the specimen. Even greater multiplexing is possible when Tb(III) is used as a Förster

resonance energy transfer (FRET) donor in combination with conventional, short-lifetime fluorescent acceptors. The four major emission bands of Tb(III) can sensitize multiple, differently-colored acceptors (Fig. 1B), and six-color Tb-to-dye FRET detection has been demonstrated in solution-phase immunoassays.²²

TGLM with lanthanide emitters requires a pulsed light source emitting in the near-UV and a means of shuttering or switching the camera with μ s-scale temporal resolution. Numerous examples of TGLM have been reported that used high-speed choppers, flashlamps or pulsed lasers for excitation and choppers, liquid-crystal shutters or gated CCD cameras as detectors.^{23–29} The Miller lab has constructed and extensively characterized a TGLM that uses a LED emitting at 365 nm as a pulsed excitation source and an intensified CCD (ICCD) camera for gated, widefield detection with signal amplification.^{30–32} The components of this system are all commercially available, and can be easily added on to any standard, epi-fluorescence microscope.

In this paper, we demonstrate time-gated IF microscopy of FFPE tissue sections using a luminescent Tb(III) complex, Lumi4[®]-Tb that has an excited state lifetime greater than 2 ms. (Fig. 1C).³³ We show that Lumi4-Tb-conjugated, secondary antibodies can be used to visualize various markers including Ki-67, e-cadherin (E-cad), Bcl-6, Bcl-2, MSH-6 and CD-20. TGLM provides higher contrast than conventional, steady state (SS) IF due to the elimination of autofluorescence background, and we demonstrate 2-component imaging of Tb(III) and fluorescent dye signals on the same specimens. In addition, we used tyramide signal amplification (TSA) to co-deposit both Lumi4-Tb alone or with various organic dyes to the same target protein and then visualized intermolecular, Tb(III)-to-dye FRET. Finally, we show that unlike conventional IF using organic dyes, TGLM with Lumi4-Tb is compatible with hematoxylin and eosin (H&E) staining in certain circumstances, a trait potentially useful for further evaluation of archived FFPE specimens.

EXPERIMENTAL SECTION

General chemical reagents and buffer salts were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Primary antibodies used include anti-Ki67 rabbit monoclonal antibody, clone 30-9 (Ventana Medical Systems, Tucson, AZ, USA, 790-4286); anti-E-cadherin rabbit monoclonal antibody, clone EP700Y (Ventana 760-4440); anti-bcl6 (Ventana 760-4241), anti-MSH6 (Ventana 790-4455), anti-CD20 (Ventana 760-2531) and anti-bcl-2 (Ventana 790-4464).

SYNTHESIS OF LUMI4-TB-LABELED ANTIBODIES

Lumi4Tb-NHS ester (Lumiphore, Inc.) was used to label antibodies by mixing 15 eq of Lumi4Tb-NHS (10 mM in DMSO) to antibody in phosphate buffered saline (PBS; pH, 7.4). The reaction mixture was allowed to stand at room temperature for 3 hours and then at 4 °C overnight. The modified antibody was then purified by size exclusion chromatography using a Superdex 200 column. The degree of labeling (DOL) was determined by UV-Vis measurement of the purified antibody using 26,000 M⁻¹ cm⁻¹ as the extinction coefficient for Lumi4Tb at 340 nm. Depending on the specific antibody, DOL ranged from 4 to 7.

SYNTHESIS OF LUMI4-TB-TYRAMIDE (LUMI4TB-TYR)

Lumi4Tb-Tyr was synthesized by following a reported method with minor modifications.³⁴ Briefly, Lumi4Tb-NHS was dissolved in 100 uL DMSO to which was added 0.9 molar equivalents of tyramine solution in DMSO (27.6 mg/mL). The reaction mixture was rotated at room temperature overnight in dark. Three microliters of N,N-Diisopropylethylamine (DIPEA) was added, and the mixture was incubated at RT for another 4 hours. The conjugate was stored at -20 °C until use.

DEPOSITION OF LUMI4-TB-TYR BY HORSE RADISH PEROXIDASE (HRP)

Detection of Ki67 in tonsil tissue was performed to demonstrate HRP-based deposition of Lumi4Tb-Tyr. Three concentrations of Lumi4Tb-Tyr were tested (100 uM, 50 uM and 20 uM) with a negative control of no Lumi4Tb-Tyr. For each slide, 100 uL of Lumi4Tb-Tyr and 100 uL of hydrogen peroxide solution were applied with 16-minute incubation time.

CO-DEPOSITION OF LUMI4TB-TYR AND ORGANIC DYE-TYR FOR FRET

The following combinations of Lumi4Tb-Tyr and various tyramide-derivatized dyes were used: Lumi4Tb/FITC, Lumi4Tb-sCy3 and Lumi4Tb-Cy5. The staining was performed using the same protocol as with Lumi4Tb-Tyr alone.

GENERAL PROCEDURE OF TISSUE TREATMENT AND STAINING

All general IHC reagents were from Ventana unless otherwise specified. IHC staining was performed on a VENTANA BenchMark XT automated slide-processing system (Ventana). Four micron sections of FFPE tissue were mounted onto positive charged glass slides. FFPE sections were first deparaffinized with EZ Prep solution and washed with reaction buffer. Antigen retrieval was carried out in Cell Conditioning and again washed with 1× reaction buffer. Primary antibodies against the targets of interest were applied to the slide and incubated at 37 °C for 16–32 minutes according the specific antibody requirements and washed thoroughly with reaction buffer. For immunofluorescence detection, appropriate species-selective secondary antibody labeled with Lumi4Tb or organic dye was added to slides and incubated at 37 °C for 32 minutes. The slides were then washed thoroughly in reaction buffer to remove any excess reagents. After rinsing in water, the slides were mounted with VECTASHIELD mounting media (VECTOR LABORATORIES, INC., Burlingame, CA). For tyramide deposition, a secondary antibody conjugated horseradish-peroxidase (HRP) (Ventana) was applied and incubated for 16 minutes. After washing, 100 uL each of Lumi4-tyramide and hydrogen peroxide solution was added and incubated for 16 minutes. The slides were thoroughly washed with reaction buffer to remove any excess reagents.

H&E COMPATIBILITY WITH LUMI4-TB STAINING

To examine the compatibility of hematoxylin as counterstain for Lumi4Tb, tissue sections were first processed for deparaffinization and antigen retrieval. Hematoxylin (Ventana 790-2208) was then applied and incubated for 4 minutes followed by bluing (Ventana

760-2037). The target of interest was then stained with primary antibody followed by species-specific secondary antibody labeled with Lumi4Tb. To demonstrate the ability to perform TGLM with H&E stained slides, H&E staining was performed after the deparaffinization with the following reagents: hematoxylin (Gill's II, Leica 3801520), bluing reagent (Scott's tap water, Leica 3802901) and eosin (Leica 3801600). For each step, 100 μ L of the reagent was added onto the slide and incubated for four minutes. After H&E staining, the slides were processed by following the same procedure as described before for Lumi4Tb-Tyr deposition.

TIME-GATED AND STEADY-STATE MICROSCOPIES

Images of Tb(III) labeled markers and Tb-mediated FRET were acquired using a wide-field, TG luminescence microscope that uses a UV light-emitting diode (LED) emitting at 365 nm for pulsed excitation and an ICCD camera for gated detection. The design, operation, and characterization of the microscope system have been described extensively.^{30,32} For each TG image acquisition, the signal from multiple excitation/emission events was accumulated on the ICCD sensor and read out at the end of the camera frame. The camera control software enabled summation of multiple frames to yield a single composite TIFF image with a bit depth equal to 1024 multiplied by the number of frames. All images were summations of four frames (bit depth, 4096), and a feature of the camera control software was enabled that removes large variations in signal resulting from ion feedback noise of the intensifier. The UV LED source and the ICCD timing parameters were the same for all of the time-resolved images and data presented here: excitation pulse width, 1500 μ s, pulse period, 3000 μ s, delay time, 10 μ s, intensifier on-time, 1480 μ s. Sensitivity was modulated by either varying the frame length (and thus, the number of integrations) or the intensifier gain voltage. All TG images were acquired at an intensifier gain of 833 V. The emission filters and all detector timing and gain parameters for each figure in the text are provided in Table S1.

Steady state fluorescence images of markers labeled with various dyes were captured by a conventional CCD camera (Axiocam MrM, Carl Zeiss, Inc.) mounted on the front port of the microscope. A white light LED (UHP-T-LED-White, Stanford Photonics, Inc.) was available for continuous wave fluorescence excitation. Filter cubes containing the appropriate excitation and emission filters and dichroics allowed for wavelength selection.

IMAGE PROCESSING

Raw, 12-bit images were imported into NIH ImageJ (v1.42q or v1.51j8) for all processing operations including cropping, contrast adjustment, and quantitative analysis.³⁵ TG FRET images were corrected for spectral bleedthrough of the Tb donor signal into the FRET channel. Bleedthrough correction was accomplished by measuring signals from many ROIs (>20) of cells containing Lumi4-Tb probe in both the Tb emission channel (494 ± 10 nm) and the FRET emission channels (605 ± 7 nm, 520 ± 10 nm, and 710 ± 20 nm). The FRET channel signal was plotted as a function of the Tb channel signal, a line was fit to the data, and the slope of that line was taken to be the correction constant. The correction constants for the 605 ± 7 emission channels were 0.122. Bleedthrough of Tb signal into the 520 ± 10 nm and 710 ± 10 nm were negligible. The Tb channel image was multiplied by the

appropriate bleedthrough correction constant, and the resulting image was subtracted from the FRET channel image to yield the true FRET image. Heatmap representations of image S/N. were prepared by measuring the mean pixel gray value from multiple ROIs within a given image that lacked cells. Then, the original image was divided by the mean background value, and a color lookup table was applied to generate the S/N map.

Brightfield images of hematoxylin staining were generated with the same monochrome camera used for recording luminescence images by switching to the microscope transmission mode with tungsten lamp illumination. Images were recorded with a red (Edmund Optics, 46545), green (Edmund Optics, 46546), and blue (Edmund Optics, 46547) transmitting filters placed in the light path and flat-field correction in each bright field channel were performed as follows. For each channel, 20 dark frames and 20 bright field images were stacked, converted to 32 bits, and median-filtered (radius 1), and each stack was averaged. The flat-field average was divided by the mean intensity of its central nine pixels to generate a normalized flat-field image. For each sample image, a median filter (radius 1) was applied and the master dark frame was subtracted. The resulting image was then divided by the normalized, master flat-field image, and the mean value of the detector offset was added back to the image. The processed images were then merged to form the brightfield color composite image. For combining with a luminescence image, each pixel of the red-filtered transmission image was divided by the maximum pixel value, the logarithm taken, and multiplied by -1 to approximate absorbance, and the absorbance image was then merged with the luminescence image.

RESULTS AND DISCUSSION

TGLM ON UNAMPLIFIED SPECIMENS

We first sought to qualitatively compare TGLM and SS fluorescence images of indirectly labeled human tonsil tissue. FFPE specimens were stained with primary antibodies targeting various markers including Ki-67, E-cadherin (E-cad), CD 20, Bcl-6, MSH-6, and Bcl-2. Following primary antibody labeling, the tissue specimens were labeled with the appropriate species-selective secondary antibodies that were conjugated to either Lumi4-Tb or Alexa Fluor 488 (AF488) and then imaged. Both SS and TGLM images exhibited the expected phenotype for each marker (Fig. S1).

In order to highlight the orthogonality of time-gated and SS signals, tonsil tissue slides were co-stained with two primary antibodies; one, a rabbit antibody, was selective for Ki-67 or E-cad and the other, a mouse antibody, was selective for Bcl-6, MSH-6 or CD20. The slides were then stained with goat-anti-rabbit (GAR) and goat-anti-mouse (GAM) secondary antibodies conjugated to either Lumi4-Tb or AF488 (GAR-Tb plus GAM-AF488 or GAM-Tb plus GAR-AF488). Visualization of both time-gated Tb(III) photoluminescence (TG Tb PL) and SS AF488 PL made it possible to separately detect Lumi4-Tb-labeled and AF488-labeled markers in the same field of view (Fig. 2A–C), thus adding a second signal dimension for multi-component IF.

The benefits of autofluorescence elimination can be seen by comparing TGLM images of Bcl-6 and MSH-6 to SS images of the same proteins (Fig. 2A–B). Bcl-6 and MSH-6 are

expressed at lower levels than Ki-67, as can be seen in diaminobenzidine (DAB) IHC images (Fig. S2). Whereas the SS AF488 PL signal of these relatively low-abundance markers is difficult to distinguish from background, TG Tb PL is more clearly visible in individual cells. The higher quality of TGLM images is emphasized in heat map representations of signal-to-noise ratio (S/N), calculated by dividing pixel gray value by the standard deviation of gray values measured in background regions of the respective images (Fig. 2D).

TGLM WITH SIGNAL AMPLIFICATION

While unamplified Tb(III) signals are clearly visible above background (Figs. 2 and S1), signal strength, and therefore signal-to-noise ratio, is fundamentally limited by target abundance. Summation or averaging of many frames can be used to increase signal-to-noise ratio, but a strong signal is still needed for imaging rare targets. Therefore, we examined tyramide signal amplification (TSA) as a means of increasing overall Tb(III) signal strength.

TSA is an enzyme-catalyzed reporter deposition method that utilizes peroxidase-antibody conjugates and derivatized tyramine substrates, fluorophores coupled to tyramine in the present study, to covalently label targets with many copies of the tyramine derivatives^{36–38} TSA is particularly useful for multiplexing because individual primary/secondary antibody combinations can be applied and removed and peroxidase inactivated sequentially. We evaluated the feasibility of TSA for TGLM by labeling FFPE tonsil tissue specimens with primary antibodies against Ki-67, Bcl-6 or MSH-6. The specimens were subsequently labeled with complementary secondary antibodies conjugated to horseradish peroxidase (HRP) which catalyzed deposition of Lumi4-tyramide substrate. TSA labeling dramatically increased Tb(III) signal intensity levels and therefore S/N compared to un-amplified specimens. For example, 5-fold shorter exposure times were needed to acquire TGLM images of TSA-labeled Bcl-6 compared to unamplified Bcl-6 specimens, and S/N was substantially higher (Fig. 3, Table S1).

TIME-GATED FRET MICROSCOPY

FRET has an inverse 6th power-dependence on distance, and significant FRET occurs only when the distance between donor and acceptor is <10 nm. Consequently, FRET microscopy can be used to visualize the interaction between two different, fluorescently labeled biomolecules.³⁹ FRET from a Tb(III) donor to an organic dye acceptor results in long-lifetime (~ms), sensitized dye emission that can be sensitively detected with time-gating methods. Moreover, the four major emission bands of Tb can sensitize differently colored fluorescent dyes, and this feature could be leveraged for multiplexing with single-wavelength excitation, as has been demonstrated in solution-phase immunoassays.²²

We investigated the possibility of detecting intermolecular Tb(III)-to-dye FRET by using TSA to co-deposit Lumi4-Tb and one of three dyes (Fluorescein (FITC), sCy3, and Cy5) onto the same marker (Ki-67). Co-labeled tonsil tissue specimens were imaged in SS mode to detect dye fluorescence and in time-gated mode to detect long-lived Tb(III) donor luminescence and Tb(III)-sensitized dye emission. Unambiguous, long-lived photoluminescence signals at the acceptor dye emission wavelengths were observed for all Lumi4-Tb/dye pairs (Lumi4-Tb/FITC and Lumi4-Tb/Cy5, Fig. 4, and Lumi4-Tb/sCy3, Fig.

S3). No long-lifetime signals were observed in specimens labeled only with dye (Fig. S4). We imaged control samples labeled only with Lumi4-Tb, and observed that bleedthrough of Tb(III) luminescence into the FITC or Cy5 channels was negligible ($<3\%$, Fig. S5), while bleedthrough of Tb(III) luminescence into the sCy3 emission channel (605 ± 7 nm) was equivalent to about 12% of the intensity seen in the Tb(III) channel (494 ± 10 nm, Fig. S5). After subtracting the bleedthrough component, we still observed high levels of intensity in the Tb(III)-to-sCy3 channel indicative of FRET (Figs. S3). Tb(III)-to-dye FRET could potentially enhance multiplexing by enabling the use of the same dye to label two different markers on the same specimen. For example, one marker could be stained with FITC for SS imaging and another marker that is not co-localized with the first could be co-labeled with Tb(III) and FITC for visualization by TGLM.

COMPATIBILITY WITH HEMATOXYLIN AND EOSIN (HE) STAINING

Hematoxylin and eosin (H&E) staining is commonly used to distinguish cell components and morphological changes within tissues and is an essential component of contemporary cancer diagnosis. In addition, hematoxylin alone is also commonly used as a nuclear counterstain in IHC. However, due to overwhelmingly strong fluorescent emission for eosin or quenching of specific fluorescence by hematoxylin, these stains are usually incompatible with conventional IF. Here, we sought to determine whether H&E staining might be compatible with TGLM of Lumi4-Tb-labeled tissues. We first examined the impact of hematoxylin counterstain alone on both SS fluorescence microscopy and TGLM.

Counterstained tissue specimens yielded robust, time-gated Tb(III) signals specific to E-cad or Ki-67. Corresponding brightfield images showed good nuclear hematoxylin staining which could be transformed to a darkfield representation and combined with the time gated Tb images to form color composite images (Fig. 5A). However, while membrane-localized E-cad was visible in SS PL images of FITC-labeled specimens, the nuclear marker Ki-67 could not be detected. In addition, no marker-specific fluorescence could be observed on rhodamine-labeled specimens that were counterstained with hematoxylin (not shown).

Since a large number of archived clinical tissue specimens have been stained with H&E (the primary stain for cancer diagnosis), we examined whether these specimens could be further stained for particular biomarkers and visualized using either SS fluorescence or time-gated luminescence detection. To this end, we performed H&E staining prior to a standard IHC staining procedure that included antigen retrieval, primary antibody binding (Ki67, Bcl-6 or MSH-6) and subsequent TSA labeling with either Lumi4-Tb, FITC or AF488. Following this protocol, we observed strong, time-gated Tb(III) luminescence signals with all markers (Fig. 5B). Specific SS AF488 fluorescence could be observed on Ki-67-labeled specimens (Fig. S6), but the less abundant markers Bcl-6 and MSH-6 could not be detected above the autofluorescence background level following labeling with FITC (Fig. 5B). The IHC staining procedure appears to remove detectable H&E stain as evidenced by contrast-free brightfield images (Fig. S6). However, the residual eosin still generated enough background fluorescence to interfere with specific SS signal. These results suggest TGLM using Tb(III) labels can provide a reliable method to perform IF on archived or other tissue specimens that are first subjected to H&E staining.

CONCLUSIONS

In this study, we directly compared TGLM with SS fluorescence microscopy by imaging FFPE tissue sections that had been labeled with either organic dyes or a luminescent Tb(III) complex, Lumi4-Tb. We found that TGLM delivered higher S/N, particularly when visualizing less abundant markers like MSH-6 or Bcl-6. Sensitivity was further enhanced by enzymatic amplified deposition of a Lumi4-tyramide derivative. Moreover, we found that Lumi4-Tb-labeled markers could be imaged in the presence of hematoxylin or on specimens that had been previously stained with H&E prior to IHC. In addition, we demonstrated the multiplexing potential of lanthanide-based TGLM by co-imaging Lumi4-Tb and organic dyes and by detecting intermolecular Tb(III)-to-dye FRET signals. All of these features could be utilized to access valuable information on multi-protein expression patterns and potentially on protein-protein interactions via intermolecular FRET. Moreover, our methods are easy to adopt because they use conventional immunolabeling protocols and standard microscopes with commercially available modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Time-gating detects luminescence signals in the μs – ms range (green-shaded region) and eliminates ns-scale fluorescence background. Depicted are single exponential decay curves with time constants of 2 ns (red line) and 2 ms (green line). X-axis is $\log_{10}$ time in s. (B) Normalized absorption (blue) and emission (green) spectra of Lumi4-Tb. (C) Amine-reactive analog of Lumi4-Tb used in this study.

Figure 2.

Time-gated detection eliminates non-specific fluorescence background, enhances signal-to-noise and separates unamplified Tb(III) luminescence from steady state fluorescence. FFPE tonsil tissue slides were co-labeled with primary rabbit antibodies raised against Ki67 or E-cad and mouse antibodies raised against Bcl-6, MSH-6 or CD-20, then stained with goat, anti-rabbit (GAR) or goat, anti-mouse (GAM) secondary antibodies conjugated to Lumi4-Tb or AF488 as indicated. (A–C) Micrographs depict time-gated Tb(III) photoluminescence (TG Tb PL, cyan; delay, 10 μ s; λ_{ex} , 365 nm; λ_{em} , 494 $\pm$ 10 nm), steady-state AF488 photoluminescence (SS AF PL, magenta; λ_{ex} , 480 $\pm$ 20 nm; λ_{em} , 520 $\pm$ 10 nm) or composite images of slides labeled with indicated antibody combinations. Complete time-gated imaging parameters are given in Table S1. Scale Bars: 20 μ m. (D) Intensity modulated displays indicate signal-to-noise ratios (S/N) calculated from TG Tb PL and SS AF488 PL images of slides labeled with indicated antibody combinations. S/N maps were generated by subtracting the mean pixel gray values measured within image regions lacking specific signals, then dividing the background-subtracted images by the standard deviation of background gray values ($S/N = (S - \bar{B})/\sigma_B$).

Figure 3. Tyramide signal amplification increases Tb(III) signal and S/N relative to indirect labeling with secondary antibodies

Ki-67, MSH-6 or Bcl-6 on FFPE tonsil tissue was labeled with complementary secondary antibody conjugated to horseradish peroxidase (HRP) followed by catalyzed Lumi4-tyramide deposition (TSA-Tb) or else labeled with secondary antibody conjugated to Lumi4-Tb (GAM-Tb) as indicated. Acquisition times of 400 ms and 2 s were needed to obtain images of Bcl-6/TSA-Tb and Bcl-6/GAM-Tb, respectively (see Table S1 for imaging parameters). S/N of Bcl-6/TSA-Tb image was considerably greater than that of Bcl-6/GAM-Tb, as depicted in intensity modulated displays (right middle, right bottom). Scale Bars: 20 μm .

Figure 4. Tyramide Signal amplification (TSA) permitted visualization of intermolecular Tb(III)-to-dye FRET

Ki-67 on human tonsil tissue was detected by co-depositing Lumi4Tb-tyramide as donor and Fluorescein-tyramide (A) or Cy5-tyramide (B) as acceptors. Micrographs depict steady-state (SS) dye, time-gated Tb(III), or time-gated Tb(III)-to-dye (FRET) photoluminescence as indicated: TG Tb PL, delay 10 μ s, λ_{ex} 365 nm; λ_{em} 494 $\pm$ 10 nm; SS FITC PL, λ_{ex} 480 $\pm$ 20 nm, λ_{em} 520 $\pm$ 10 nm; TG FITC PL, delay 10 μ s, λ_{ex} 365 nm, λ_{em} 520 $\pm$ 10 nm; SS Cy5 PL, λ_{ex} 655 $\pm$ 20 nm, λ_{em} 710 $\pm$ 20 nm; TG Cy5 PL, delay 10 μ s, λ_{ex} 365 nm, λ_{em} 710 $\pm$ 20 nm. Scale Bars: 20 μ m. Calibration bars depict ranges of grayscale values shown in corresponding 12-bit images.

Figure 5.

Lumi4-Tb labeling and TGLM are compatible with select hematoxylin and eosin staining protocols. (A) FFPE tonsil tissue were stained with hematoxylin, labeled with primary antibodies against Ki-67 or E-cad and stained with Lumi4-Tb or FITC using tyramide signal amplification. Brightfield images were log-transformed into a pseudo-darkfield presentation and combined with TG Tb PL images. B. Tonsil tissue was stained with H&E prior to antigen retrieval, primary antibody labeling (Bcl-6 or MSH-6) and TSA-mediated deposition of Lumi4-Tb or FITC. Robust, specific Tb(III) signals were observed in time-gated images while SS FITC signals could not be detected above background. Micrographs: time-gated Tb(III) photoluminescence (TG Tb PL; delay, 10 μ s; λ_{ex} , 365 nm; λ_{em} , 494 $\pm$ 10 nm), steady-state FITC photoluminescence (SS FITC PL; λ_{ex} , 480 $\pm$ 20 nm; λ_{em} , 520 $\pm$ 10 nm) Scale Bars: 20 μ m.