Time-resolved luminescence resonance energy transfer imaging of protein–protein interactions in living cells

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Förster resonance energy transfer (FRET) with fluorescent proteins permits high spatial resolution imaging of protein–protein interactions in living cells. However, substantial non-FRET fluorescence background can obscure small FRET signals, making many potential interactions unobservable by conventional FRET techniques. Here we demonstrate time-resolved microscopy of luminescence resonance energy transfer (LRET) for live-cell imaging of protein–protein interactions. A luminescent terbium complex, TMP-Lumi4, was introduced into cultured cells using two methods: (*i*) osmotic lysis of pinocytic vesicles; and (*ii*) reversible membrane permeabilization with streptolysin O. Upon intracellular delivery, the complex was observed to bind specifically and stably to transgenically expressed *Escherichia coli* dihydrofolate reductase (eDHFR) fusion proteins. LRET between the eDHFR-bound terbium complex and green fluorescent protein (GFP) was detected as long-lifetime, sensitized GFP emission. Background signals from cellular autofluorescence and directly excited GFP fluorescence were effectively eliminated by imposing a time delay (10 μs) between excitation and detection. Background elimination made it possible to detect interactions between the first PDZ domain of ZO-1 (fused to eDHFR) and the C-terminal YV motif of claudin-1 (fused to GFP) in single microscope images at subsecond time scales. We observed a highly significant ($P < 10^{-6}$), six-fold difference between the mean, donor-normalized LRET signal from cells expressing interacting fusion proteins and from control cells expressing noninteracting mutants. The results show that time-resolved LRET microscopy with a selectively targeted, luminescent terbium protein label affords improved speed and sensitivity over conventional FRET methods for a variety of live-cell imaging and screening applications.

cellular imaging | dihydrofolate reductase | Forster resonance energy transfer | lanthanide luminescence | protein labeling

Protein–protein interactions, often mediated by modular interaction domains, play a fundamental role in the dynamic organization of cells (1). Various experimental techniques such as immunoprecipitation, affinity chromatography, and yeast two-hybrid analysis have been used to identify putatively interacting proteins and deduce the biomolecular mechanisms of cell function (2, 3). However, cell-free studies and screening assays do not provide information about the spatio-temporal organization of protein networks in the natural environment of the living cell or organism. A variety of optical methods are available for monitoring protein interactions in cells, including fluorescence cross correlation spectroscopy (FCCS) (4, 5), bimolecular fluorescence complementation (6), translocation-based assays (7–9), and methods that detect intermolecular Förster resonance energy transfer (FRET). Among these methods, only FRET allows dynamic and reversible imaging of protein–protein interactions while simultaneously preserving information about their subcellular distribution (10–12).

FRET occurs when a fluorescent donor molecule is brought within close proximity (<10 nm) to an acceptor whose absorption spectrum overlaps with the donor emission spectrum. FRET can be detected in appropriately configured microscopes as a decrease in the fluorescence intensity, lifetime, or anisotropy of the donor or as an increase in the fluorescence intensity of the acceptor upon donor excitation (if the acceptor is fluorescent). Fluorescent proteins (FPs), especially cyan (CFP) and yellow (YFP) variants, are the most common donors and acceptors used in live-cell FRET imaging (13). Intermolecular FRET between two FP-labeled proteins has been used to visualize receptor oligomerization and interactions between transcription factors and cell signaling components (11, 13–15).

Steady-state monitoring of sensitized acceptor emission upon donor excitation is the most popular FRET microscopy method. This technique involves acquiring three sets of images using different filter combinations: donor excitation/donor emission, acceptor excitation/acceptor emission, and donor excitation/acceptor emission (11, 12, 16, 17). The images are then processed postacquisition to correct for cross-detection of donor fluorescence in the acceptor emission channel (and vice versa), and to normalize for the dependence of FRET on the relative concentrations of donor and acceptor. However, image processing propagates noise and uncertainty from each individual image, lowering the sensitivity and precision of steady-state FRET imaging. Changes in donor emission intensity upon acceptor photobleaching enable direct determination of FRET efficiency (the fraction of the photon energy absorbed by the donor that is transferred to the acceptor), but this method destroys the sample and precludes time-lapse imaging. Alternatively, fluorescence lifetime imaging microscopy (FLIM) allows direct detection of FRET effects on donor excited-state lifetime and, therefore, overcomes artifacts associated with donor/acceptor spectral overlap. However, FLIM image acquisition can take several minutes and requires complicated, expensive instrumentation to accurately resolve changes in nanosecond-scale lifetimes of FPs or organic fluorophores (11). Finally, the maximum FRET dynamic range for nondimerizing fluorescent proteins is <5-fold (11, 18), and the signal observed by microscopy in living cells may only change ~10% (14, 15). The small signal variation and considerable noise factors make many interactions difficult or impossible to measure by existing FRET approaches. Therefore, despite numerous reported successes, FRET imaging has significant limitations.

Lanthanide coordination complexes are well established probes for detecting biomolecular interactions in cell-free systems at high signal-to-background ratio (19–22). As lanthanide emission is not technically fluorescence (singlet-to-singlet transi-

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tion), the term luminescence (or lanthanide-based) resonance energy transfer (LRET) is commonly used to describe transfer from a lanthanide donor to an organic fluorophore or fluorescent protein acceptor. Lanthanide complexes have two distinct properties that enable sensitive LRET measurements. Firstly, large Stokes shifts (separation between excitation and emission wavelengths) and narrow emission lines facilitate efficient spectral separation of donor and acceptor emission signals. Secondly, long-lived (μs-ms) luminescence enables time-resolved detection, where pulsed light excites the sample, and the detector is switched on after decay of short-lived (<100 ns) autofluorescence and directly excited acceptor fluorescence background signals (21). Direct conjugation of terbium or europium complexes to oligonucleotides, antibodies and proteins has permitted the development of sensitive time-resolved LRET assays of biomolecular interactions in purified biochemical preparations, cellular extracts, and on cell surfaces (22–24). Recently, lanthanide complexes have been developed as probes for live cell imaging applications using time-resolved microscopy with pulsed, near-UV single photon or two photon excitation (25–28). Time-resolved microscopy in the microsecond domain has also been demonstrated using platinum or palladium complexes (29–31). However, to date, lanthanide complexes have not been used as LRET donors for intracellular experiments because: (*i*) suitably bright and photostable complexes were not available; (*ii*) methods to deliver membrane-impermeant lanthanide complexes from culture medium to the cytoplasm were lacking; and (*iii*) novel approaches were needed to specifically attach probes to proteins or subcellular structures.

Herein we report LRET imaging of protein–protein interactions in living cells. We show that a luminescent terbium complex, TMP-Lumi4, can be delivered to the cytoplasm and specifically targeted to *Escherichia coli* dihydrofolate reductase (eDHFR) fusion proteins. Time-resolved microscopy was used to image LRET between the terbium complex and green fluorescent protein (GFP) by detecting long-lifetime (>10 μs) terbium-sensitized GFP emission (Fig. 1). Interactions between the first PDZ domain of ZO-1 and the C-terminal YV motif of claudin-1 were detected unambiguously in a single microscope image at time scales (seconds) comparable to steady-state FRET imaging, but without the need for multiple control measurements and associated postimage processing. Whereas steady-state microscopy of intermolecular FRET may yield signal changes of only 10% (14, 15), we observed >6-fold change in mean, donor-normalized LRET signal between cells expressing interacting proteins and control cells expressing noninteracting mutants. This article shows that time-resolved LRET microscopy enables faster, more sensitive and simpler analyses of molecular interactions in living cells than currently available FRET imaging methods.

Results and Discussion

Properties of the Selective Terbium Protein Label. We recently reported a method to impart terbium luminescence to recombinant fusion proteins (32). Heterodimeric conjugates of the common antibiotic trimethoprim (TMP) covalently linked to various terbium chelates were developed that bind noncovalently to eDHFR. TMP and eDHFR interact exclusively with one another in mammalian systems, and this interaction has been previously exploited to develop TMP-fluorophore conjugates that specifically label eDHFR fusion proteins in a variety of wild-type mammalian cell lines (LigandLink™ Universal Labeling Technology, Active Motif, Inc.) (33–35). In our previous study, we found that one particular conjugate, TMP-Lumi4 (Fig. 2*A*), bound tightly (K_D = ~2 nM) to a GFP-eDHFR fusion protein and that intramolecular terbium-to-GFP LRET could be detected with >100-fold dynamic range as long-lifetime, sensitized GFP emission in a cell-free assay (32).

The effectiveness of TMP-Lumi4 as a LRET donor to GFP results from its overall brightness, long lifetime, and the ability to spectrally discriminate GFP emission from terbium emission. Lumi4®-Tb is an exceptionally bright ($\varepsilon \approx 26{,}000\ M^{-1}\,cm^{-1}$ at

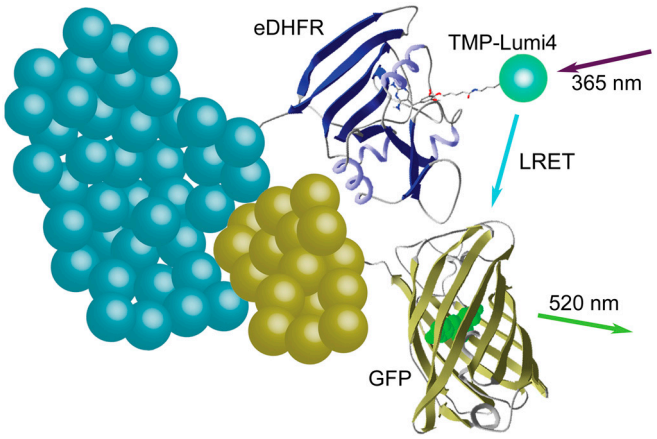

Fig. 1. Schematic representation of intermolecular terbium-to-GFP LRET. TMP-Lumi4 binds specifically and tightly to eDHFR (K_D = ~2 nM). Interaction between eDHFR and GFP fusion proteins and excitation of TMP-Lumi4 (the donor) results in LRET-sensitized emission of GFP (the acceptor). Time-resolved microscopy detects long-lifetime, sensitized GFP emission while eliminating cellular autofluorescence and directly excited GFP fluorescence background signals.

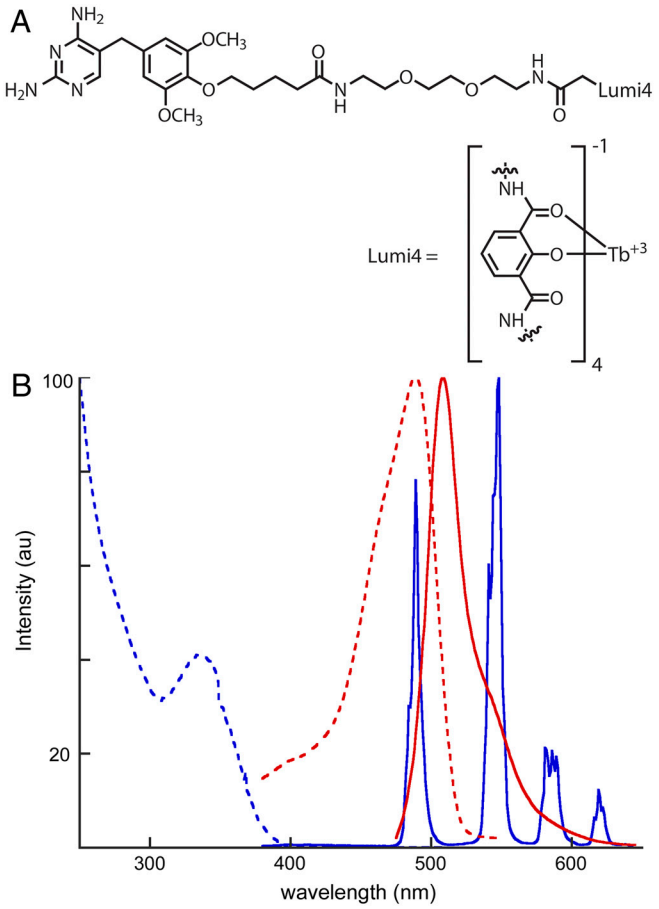

Fig. 2. (*A*) Structure of TMP-Lumi4, a conjugate of trimethoprim covalently linked to a proprietary analog of a multidentate 2-hydroxyisophthalamide terbium chelate (Lumi4®-Tb) (36). The molecule has a molecular weight of 1879 and a net charge of −1 when complexed with terbium. (*B*) Normalized absorption (dotted) and emission (solid) spectra of TMP-Lumi4 (blue) and EGFP (red). The characteristically narrow terbium emission bands enable efficient spectral separation of signals.

354 nm, quantum yield = 0.59) and long-lived ($\tau \approx 2.7$ ms) proprietary analog of a multidentate 2-hydroxyisophthalamide terbium chelate previously reported by Raymond and coworkers (36). TMP-Lumi4 exhibits characteristic terbium luminescence upon excitation with near-UV light and absorbs strongly at 365 nm, enabling efficient excitation with commercially available light-emitting diode (LED) sources (Fig. 2*B*). Both TMP-Lumi4 and unconjugated Lumi4®-Tb were found to exhibit single-exponential decay kinetics with the same apparent lifetime (~2.3 ms) under identical experimental conditions (Fig. S1). Terbium-sensitized GFP emission from a complex of TMP-Lumi4 bound to GFP-eDHFR occurred with single-exponential kinetics and an apparent lifetime of ~0.8 ms (Fig. S1). From lifetime data, we calculated an efficiency of ~67% for energy transfer from terbium to GFP in the TMP-Lumi4/GFP-eDHFR complex (*SI Materials and Methods*).

Intracellular Probe Delivery and Specific Protein Labeling. TMP-Lumi4 will not diffuse passively into cells from culture medium (32). Therefore, to perform intracellular LRET imaging, it was first necessary to establish methods of cytoplasmic probe delivery. Microinjection is one possible approach for loading adherent cells, and it has been successfully used for lanthanide complex delivery (25). However, microinjection requires specialized apparatus and can only be used to load relatively few cells at a time. Two techniques were therefore adapted to simultaneously deliver TMP-Lumi4 to the cytoplasm of many cells: (*i*) reversible plasma membrane permeabilization with streptolysin O (SLO) (37); and (*ii*) osmotic lysis of pinocytic vesicles (38, 39). Both methods consistently yielded >50% loading efficiency while maintaining ~95% cell viability 2 h posttreatment (Table S1).

Whereas both loading techniques have negligible effects on cell viability, the pinocytosis method should be less perturbative to cellular physiology, as the cell membrane is not compromised at any point during the process. With the pinocytic loading method, cells are allowed to undergo pinocytosis in a hypertonic medium containing sucrose, polyethylene glycol, and TMP-Lumi4. Upon transfer to a hypotonic medium, the pinocytic vesicles (containing TMP-Lumi4) burst due to the lowering of osmotic pressure, releasing their contents into the cytoplasm. The overall amount of TMP-Lumi4 delivered into cells can be controlled by varying the concentration in the hypertonic loading medium, or alternatively, by repeated applications of the pinocytosis/lysis process. Given a natural rate of pinocytosis equaling $\sim 10^{-16}$ L/min for most cell types (39), and assuming typical cell volumes of $\sim 10^{-12}$ L, the estimated cellular concentration of TMP-Lumi4 was $< 10^{-7}$ M for the experimental conditions used in this study (single loading application, 50 μM TMP-Lumi4, 10 min. pinocytosis) (*SI Materials and Methods*).

Intracellular delivery of TMP-Lumi4 and specific labeling of eDHFR fusion proteins was visualized using time-resolved microscopy. A conventional epifluorescence microscope was adapted for time-resolved imaging by incorporating a fast-modulated, UV LED ($\lambda_{em} = 365$ nm) as the excitation source and an intensified CCD camera for image acquisition. The LED circuitry allowed for either continuous wave emission or external on/off modulation with ~1 μs time resolution. The excitation intensity could be varied; however, we held the intensity constant at a measured value of 1.6 mW at the objective back aperture, yielding an estimated illumination intensity of ~0.6 W/cm^2 at the image plane (see *SI Materials and Methods*). The image intensifier component of the camera served as both a fast shutter and emission signal amplifier. By synchronizing the LED and intensifier with a digital delay generator, the excitation pulse width, the gate delay (time between pulse and detection), the gate width (intensifier on-time), and the pulse/detection repetition rate could be varied independently. The output from multiple excitation/detection cycles could be integrated on the CCD during a single camera frame, and the camera control software allowed summation of multiple frames (see *SI Materials and Methods* and Table S2 for microscope operation parameters used in this study).

Madin Darby Canine kidney (MDCKII) epithelial cells were transiently cotransfected with two plasmid DNA vectors; one that expressed nucleus-localized eDHFR and another that expressed nucleus-localized CFP as a positive control for transfection. After SLO-mediated delivery of TMP-Lumi4, a time-resolved image (gate delay = 10 μs) of broadband emission (>400 nm) revealed specific localization of terbium luminescence in the nucleus of a transfected cell loaded with probe (Fig. 3*A*). When unconjugated TMP (final conc. ~ 10 μM) was added to the imaging medium, it diffused into cells, competed with TMP-Lumi4 for eDHFR binding, and markedly diminished nuclear luminescence (Fig. 3*A*). Specific labeling of nucleus-localized eDHFR was also observed in NIH3T3 fibroblast cells that were loaded with TMP-Lumi4 by SLO-mediated membrane permeabilization (Fig. S2), and results seen with pinocytic delivery were similar.

Cytoplasmic delivery of TMP-Lumi4 and specific labeling of eDHFR was further assessed by imaging terbium-to-GFP LRET in time-resolved mode. MDCKII cells were transfected with DNA encoding a GFP-eDHFR fusion protein. The cells were loaded with TMP-Lumi4 by pinocytic delivery, and intramolecu-

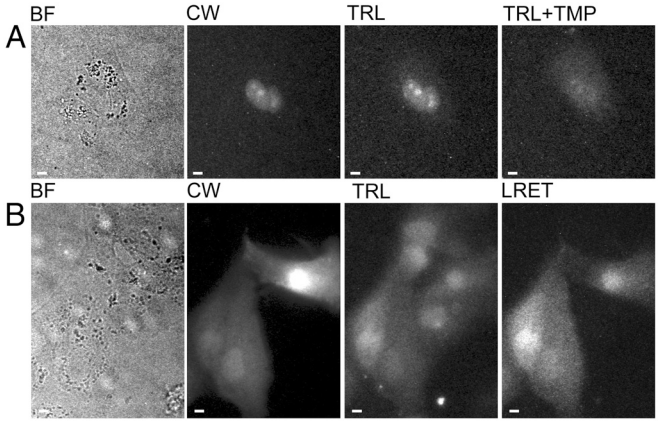

Fig. 3. Both streptolysin O (SLO)-mediated membrane permeabilization and osmotic lysis of pinocytic vesicles deliver TMP-Lumi4 to the cytoplasm of MDCKII cells, and specific labeling of eDHFR fusion proteins can be visualized by time-resolved luminescence microscopy. (*A*), (*B*) Micrographs: BF, bright field; CW, continuous wave fluorescence ($\lambda_{ex} = 480 \pm 20$ nm, $\lambda_{em} = 535 \pm 25$ nm); TRL, time-resolved luminescence ($\lambda_{ex} = 365$ nm, $\lambda_{em} > 400$ nm, gate delay = 10 μs); TRL + TMP, 20 min after addition of TMP (final conc. = 10 μM) to culture medium; LRET, time-resolved luminescence ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 520 \pm 10$ nm, gate delay = 10 μs). TRL and TRL + TMP images were adjusted to identical contrast levels. Complete details of time-resolved microscopy parameters are provided in *SI Materials and Methods* and Table S2. Scale bars, 10 μm. (*A*) MDCKII cells transiently cotransfected with DNA encoding nucleus-localized CFP and nucleus-localized eDHFR were incubated with TMP-Lumi4 (15 μM) and streptolysin O (SLO, 50 ng/mL) for 10 min. Time-resolved detection of broadband (>400 nm) terbium emission reveals localization of TMP-Lumi4 in nucleus of transfected cell (corresponding to continuous wave fluorescence image of CFP emission). A time-resolved image taken 20 min. after addition of TMP (final conc. = 10 μM) to the culture medium shows diminished nuclear luminescence because TMP out-competes TMP-Lumi4 for binding to eDHFR. (*B*) MDCKII cells transiently transfected with DNA encoding GFP-eDHFR. Cells were incubated in hypertonic medium containing TMP-Lumi4 (50 μM) for 10 min to allow pinocytosis and subsequently exposed to hypotonic medium to effect lysis of pinocytic vesicles and release of probe into the cytoplasm. Time-resolved detection of broadband emission (>400 nm) reveals terbium luminescence in TMP-Lumi4-loaded cells. Luminescence resonance energy transfer (LRET) is seen only in transfected cells (indicated in continuous wave fluorescence image) coincidentally loaded with TMP-Lumi4 when visualized in time-resolved mode through a narrow-pass filter (520 ± 10 nm), indicating specific labeling of the GFP-eDHFR fusion protein.

lar, terbium-sensitized GFP emission was seen only in GFP-eDHFR-expressing cells when visualized in time-resolved mode through a narrow-pass filter ($\lambda_{em} = 520 \pm 10$ nm) that blocked terbium emission (Fig. 3*B*). Intramolecular, terbium-to-GFP LRET was also observed in NIH3T3 fibroblasts that were pinocytically loaded with TMP-Lumi4 (Fig. S2). These results and those above show that both SLO-mediated membrane permeabilization and osmotic lysis of pinocytic vesicles can effectively deliver TMP-Lumi4 into the cytoplasm of at least two cell types (MDCKII and NIH3T3). Upon cellular delivery, TMP-Lumi4 diffuses freely throughout the cytoplasm and nucleus without detectable nonspecific binding and binds selectively to eDHFR, suggesting that other methods of cytoplasmic delivery should prove equally useful. Moreover, time-resolved microscopy can visualize intramolecular terbium-to-GFP LRET as long-lifetime (>10 μs), sensitized GFP emission.

Quantification of LRET signals. The concentration of GFP-eDHFR or other fusion proteins expressed from a strong promoter (1–10 μM) (40) likely exceeds the estimated concentration of TMP-Lumi4 (<100 nM) in pinocytically loaded cells. Given its high affinity for eDHFR ($K_D = \sim 2$ nM), TMP-Lumi4 was assumed to be mostly bound to fusion protein, and therefore the LRET signal should vary with the amount of TMP-Lumi4 in the cell. To quantify the LRET signal, two time-resolved images were obtained using identical acquisition settings but for different emission wavelengths: (*i*) the pure sensitized GFP emission signal ($\lambda_{em} = 520 \pm 10$ nm); and (*ii*) a signal comprising terbium emission plus sensitized GFP emission ($\lambda_{em} = 540 \pm 10$ nm). For each cell in an image, the background-subtracted mean gray value was used as an indicator of emission intensity (*SI Materials and Methods* and Fig. S3). From these pairs of images, a donor-normalized LRET signal (LRETN) could then be calculated as the 520/540 emission ratio for each measured cell.

TMP-Lumi4 Stability and Photoresistance in cells. To be useful as cellular imaging probes, lanthanide complexes must remain stably luminescent in cells over time and must also be resistant to photobleaching. Long-term cellular stability may be compromised by endogenous chelators that could remove metal from the complex, rendering it nonluminescent. The stability of TMP-Lumi4 in MDCKII cells expressing GFP-eDHFR was determined by imaging terbium-to-GFP LRET. LRETN was observed to be constant over ~23 h (Fig. S4). However, the unnormalized LRET signal decreased approximately 50% over the same time period, from an initial value of 230 ± 100 to a value of 110 ± 65 at 23 h (mean ± s.d., >10-cell samples). The results suggest that TMP-Lumi4 remains stably luminescent within cells but may slowly leak out of the cells over time, and therefore the LRET signal remains quantitatively useful over the time course of the experiment.

The photoresistance of TMP-Lumi4 in MDCKII cells expressing GFP-eDHFR was determined by monitoring the LRET signal as a function of accumulated irradiation time. A field of view was exposed to continuous wave LED excitation at the standard illumination intensity (~0.6 W/cm^2). The LRET signal ($\lambda_{em} = 520 \pm 10$ nm) was measured on a cell-by-cell basis at successive timepoints, normalized to each cell's initial intensity, and fit to a single exponential decay, yielding an estimated photobleaching lifetime of ~2.0 min (Fig. S4). The measured photobleaching lifetime is considerably longer than typical image exposure times (seconds), and therefore time-lapse LRET imaging should be possible without concern for loss of signal due to photobleaching of the donor.

LRET Imaging of Protein–Protein Interactions. The ability of this unique LRET system to image protein–protein interactions was next used to measure the association of a PDZ domain with a carboxyl-terminal binding motif in living cells (41, 42). Such PDZ-mediated interactions are fundamental to biological function in species from Drosophila to mammals. In epithelia, a direct, high-affinity ($K_D = \sim 10$–20 nM) interaction between the C-terminal cytoplasmic tail of claudin-1 and the most N-terminal PDZ domains (PDZ1) of ZO-1 and the related proteins ZO-2 and ZO-3 has been demonstrated using isolated recombinant proteins (43). Moreover, PDZ1 domains of ZO-1, ZO-2, and ZO-3 are recruited to sites of claudin-1 polymerization in transfected fibroblasts (43) but not to sites enriched in mutant claudin-1 lacking the C-terminal YV motif (43). The relevance of this interaction to disease is emphasized by the observations that mutations within the ZO-2 PDZ1 domain are linked to familial hypercholanemia (44), a disease of hepatic bile transport, whereas loss of claudin-1 expression is associated with neonatal sclerosing cholangitis (45), another hereditary cholestatic disease. However, a direct interaction between the C-terminal claudin-1 YV motif and the first PDZ domain of ZO-1 has not been previously demonstrated in live cells.

To examine the interaction between claudin-1 and ZO-1 in living cells, MDCKII cells were transfected with expression vectors encoding a C-terminal fusion of eDHFR to the PDZ1 domain (residues 19–113) of ZO-1 (ZO-1/PDZ1-eDHFR) and an N-terminal fusion of EGFP to the C-terminal cytoplasmic domain (residues 187–211) of claudin-1 (GFP-cldn1/tail). Transfected cells were loaded with TMP-Lumi4 using both SLO-mediated and pinocytic delivery methods. In both cases, LRET imaging revealed terbium-sensitized GFP emission only in transfected cells loaded with TMP-Lumi4, providing an unambiguous image of protein–protein interaction (Fig. 4*A* and Fig. S2). The LRETN for pinocytically loaded cells equaled 0.38 ± 0.10 (mean ± s.d., Table S3). Addition of excess TMP to growth med-

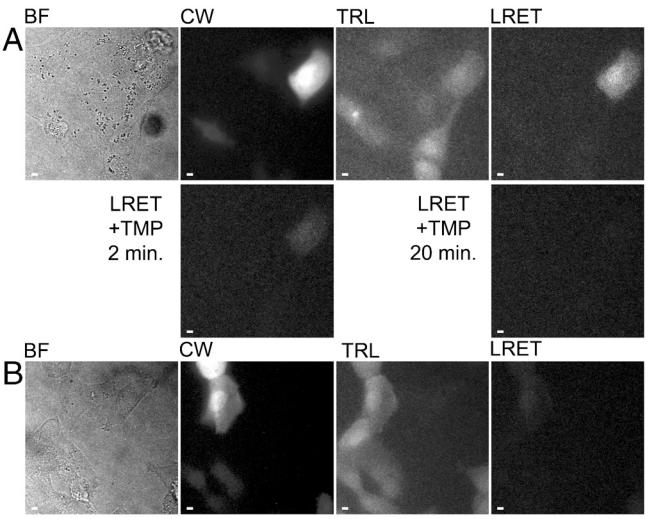

Fig. 4. Time-resolved LRET imaging reveals interaction between ZO-1/PDZ1-eDHFR and GFP-cldn1/tail in MDCKII cells. (*A*), (*B*) Micrographs: BF, bright field; CW, continuous wave fluorescence ($\lambda_{ex} = 480 \pm 20$ nm, $\lambda_{em} = 535 \pm 25$ nm); TRL, time-resolved luminescence ($\lambda_{ex} = 365$ nm, $\lambda_{em} > 400$ nm, gate delay = 10 μs); LRET, time-resolved luminescence ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 520 \pm 10$ nm, gate delay = 10 μs); LRET + TMP, after addition of TMP (final conc. = 10 μM) to growth medium at indicated time-points. LRET and LRET + TMP images were adjusted to identical contrast levels. Complete details of time-resolved microscopy parameters are provided in *SI Materials and Methods* and Table S2. Scale bars, 10 μm. (*A*) MDCKII cells coexpressing ZO-1/PDZ1-eDHFR and GFP-cldn1/tail loaded with TMP-Lumi4 by osmotic lysis of pinosomes. Terbium-to-GFP LRET is seen only in transfected cells loaded with TMP-Lumi4. When TMP is added to culture medium, it displaces TMP-Lumi4 from the ZO-1/PDZ1-eDHFR/GFP-cldn1/tail complex, eliminating the LRET signal. (*B*) MDCKII cells coexpressing ZO-1/PDZ1-eDHFR and GFP-cldn1/tail$^{\Delta YV}$, loaded with TMP-Lumi4 as in (*A*). No LRET signal is seen in expressing cells loaded with TMP-Lumi4.

ium eliminated the LRET signal (Fig. 4*A*), further confirming that long-lifetime, sensitized GFP emission results from specific binding of TMP-Lumi4 to ZO-1/PDZ1-eDHFR. The predominantly nuclear localization of interactions between ZO-1/PDZ1-eDHFR and the GFP-cldn1/tail is consistent with previous reports showing that GFP-tagged ZO-1 PDZ1 domain accumulates within the nucleus (43) and that ZO-1 directs subcellular claudin trafficking (46).

To assess specificity of the observed interaction, a GFP-cldn1/tail construct lacking the C-terminal YV motif (GFP-cldn1/tail$^{\Delta YV}$) was developed and expressed in MDCKII cells with ZO-1/PDZ1-eDHFR. Whereas continuous widefield GFP fluorescence and broadband, time-resolved terbium luminescence signals were easily detected in cells loaded with TMP-Lumi4, only extremely faint LRET was seen (Fig. 4*B*), verifying that the LRET signal observed between ZO-1/PDZ1-eDHFR and GFP-cldn1/tail reflects a specific PDZ-mediated interaction. There was a highly significant ($P < 10^{-6}$), >6-fold difference between the mean LRETN signal from cells expressing ZO-1/PDZ1-eDHFR and GFP-cldn1/tail (0.38 ± 0.10) and that seen from cells expressing ZO-1/PDZ1-eDHFR and GFP-cldn1/tail$^{\Delta YV}$ (0.06 ± 0.04, mean $\pm$ s.d., Table S3).

The data above show that LRET imaging is faster, more sensitive, and in many respects, less complex than widefield, steady-state FRET microscopy. With steady-state FRET, the sensitized emission signal is contaminated by directly excited donor and acceptor fluorescence. Therefore, multiple control images must be collected, requiring acquisition times of at least ~3 s on optimized systems (12, 16, 17). Even with careful image acquisition and proper analysis, signal changes of only ~10% are typically observed between FRET-positive samples and negative controls (14, 15). By contrast, LRET imaging only detects signals emanating from interacting molecules. We observed a ~500% increase in mean LRETN between cells expressing interacting and noninteracting ZO-1/PDZ1-eDHFR and GFP-cldn1/tail pairs (Table S3), and this result represents a ~50-fold increase in signal-to-background ratio over that typically observed with steady-state FRET imaging of protein–protein interactions.

The dramatic difference in measured signal between LRET-positive cells and negative controls is partly due to the high affinity between ZO-1/PDZ1 and cldn1/tail (in vitro $K_D = 13$ nm) (45). The high affinity makes it likely that most of the TMP-Lumi4 delivered into cells was bound to ZO-1/PDZ1-eDHFR and donated energy to proximal GFP-cldn1/tail. Measurements of lower-affinity interactions should be possible using the LRET method, however, longer image acquisition times may be necessary to generate sufficient signal. Alternatively, increased loading of TMP-Lumi4 (up to saturation of eDHFR binding) would yield a higher measured signal level. For LRETN calculations, we used images that were acquired at relatively long acquisition times (8 s, Table S2), although intermolecular LRET could be detected with acquisition times as short as 0.67 s (Fig. S5). Thus, in addition to enhanced sensitivity, LRET imaging displays increased time resolution that may allow analysis of interaction dynamics that cannot be resolved by traditional steady-state FRET or FRET-FLIM. Whereas other optical methodologies such as induced translocation of fluorescent protein fusions (7–9) and FCCS (4, 5) can monitor interactions in cells with much better time resolution (0.1–1 s for FCCS), these methods do not provide a spatial map of interactions.

Conclusion

Our results show that eDHFR fusion proteins can be specifically and stably labeled with a luminescent terbium complex, TMP-Lumi4, in living, wild-type mammalian cells. The ability to selectively impart terbium luminescence enables dynamic, nondestructive LRET imaging of intracellular interactions between eDHFR and GFP fusion proteins without additional control measurements and mathematical processing. By detecting terbium-sensitized GFP emission at long lifetimes, we effectively eliminate detection of cellular autofluorescence, unbound terbium probe luminescence, and directly excited GFP fluorescence, thereby imaging LRET in living cells at time scales (<1 s) that are substantially faster than steady-state FRET imaging (~3 s) and orders of magnitude faster than FLIM imaging of FPs.

Whereas the techniques described here should improve live-cell studies of protein function, there is broad scope for further enhancements and modifications. For instance, intramolecular LRET could be exploited to develop biosensors that incorporate eDHFR and GFP in a single fusion protein, analogous to CFP/YFP sensors designed to detect cellular analytes or enzyme activity (13). Time-resolved microscopy can also be adapted to measure donor or sensitized acceptor lifetimes by capturing and analyzing a series of images at different detector delay intervals (47, 48). The multiple terbium emission peaks of TMP-Lumi4 can serve as a LRET donor to both GFP and red fluorescent proteins, potentially enabling the simultaneous detection of more than one molecular interaction in a single living cell (Fig. 2*B*). Finally, both SLO and pinocytosis probe delivery methods can be easily adapted for use in multiwell plate format, enabling high-throughput screening assays of intracellular protein–protein interactions using commercially available time-resolved fluorescence plate readers.

Materials and Methods

Chemical Synthesis. Details describing the synthesis and characterization of TMP-Lumi4 can be found in *SI Materials and Methods* and in Rajapakse et al. (32).

Cell Culture, Transfection, and Probe Delivery Methods. NIH3T3 and MDCKII cells were cultured and and transfected with plasmid DNA according to standard protocols. Complete details of these as well as methods for pinocytic and SLO-mediated probe delivery and cell viability assay protocols are provided in *SI Materials and Methods*.

Live Cell Imaging and Data Analysis. Comprehensive descriptions of the time-resolved microscope system, image acquisition parameters and methods of image processing and data analysis are offered in *SI Materials and Methods*.

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