

High-Throughput Spectral and Lifetime-Based FRET Screening in Living Cells to Identify Small-Molecule Effectors of SERCA

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Tory M. Schaaf¹, Kurt C. Peterson², Benjamin D. Grant²,
Prachi Bawaskar¹, Samantha Yuen¹, Ji Li¹, Joseph M. Muretta¹,
Gregory D. Gillispie², and David D. Thomas^{1,3}

Abstract

A robust high-throughput screening (HTS) strategy has been developed to discover small-molecule effectors targeting the sarco/endoplasmic reticulum calcium ATPase (SERCA), based on a fluorescence microplate reader that records both the nanosecond decay waveform (lifetime mode) and the complete emission spectrum (spectral mode), with high precision and speed. This spectral unmixing plate reader (SUPR) was used to screen libraries of small molecules with a fluorescence resonance energy transfer (FRET) biosensor expressed in living cells. Ligand binding was detected by FRET associated with structural rearrangements of green fluorescent protein (GFP, donor) and red fluorescent protein (RFP, acceptor) fused to the cardiac-specific SERCA2a isoform. The results demonstrate accurate quantitation of FRET along with high precision of hit identification. Fluorescence lifetime analysis resolved SERCA's distinct structural states, providing a method to classify small-molecule chemotypes on the basis of their structural effect on the target. The spectral analysis was also applied to flag interference by fluorescent compounds. FRET hits were further evaluated for functional effects on SERCA's ATPase activity via both a coupled-enzyme assay and a FRET-based calcium sensor. Concentration-response curves indicated excellent correlation between FRET and function. These complementary spectral and lifetime FRET detection methods offer an attractive combination of precision, speed, and resolution for HTS.

Keywords

fluorescence lifetime, time-resolved FRET, biosensor, drug screening, spectral unmixing

Introduction

The preceding article¹ reports the performance of a novel microplate reader that records fluorescence emission spectra with an unprecedented combination of speed and precision. That study indicated that this spectral unmixing plate reader (SUPR), when combined with a previously described fluorescence lifetime plate reader (FLTPR) that achieves a similarly high level of performance using nanosecond time resolution, is ready for high-throughput screening (HTS). This study demonstrates an initial application.

The specific target in this work is sarco/endoplasmic reticulum calcium ATPase (SERCA),^{2,3} which has therapeutic relevance for a wide range of diseases, including heart failure,³ multidrug-resistant leukemia,⁴ and type II diabetes.⁵ SERCA is a critical enzyme, as it maintains calcium homeostasis by actively pumping calcium from the cytosol into the endoplasmic or sarcoplasmic reticulum. Over a dozen human SERCA isoforms have been described, each with tissue-specific expression and distinct structural and functional characteristics. Specialized SERCA isoforms are predominantly found in

electrically excitable cells, such as myocytes and cardiomyocytes, where calcium cycling is necessary for the contractile apparatus to function properly.⁶

Recently, SERCA-based therapy based on calcium upregulation by percutaneous administration of gene therapy was tested in cardiac disease clinical trials (CUPID study). SERCA overexpression by administration of adeno-associated virus (AAV), delivered directly to the hearts of

¹Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA

²Fluorescence Innovations, Inc., Minneapolis, MN, USA

³Photonic Pharma LLC, Minneapolis, MN, USA

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Corresponding Author:

David D. Thomas, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA.
Email: ddt@ddt.umn.edu

patients experiencing end-stage heart failure, was shown to correct deficits in SERCA2a (cardiac-isoform) expression and activity, known to be correlated to impairment in cardiac (diastolic) function.⁷ Despite encouraging early results, SERCA AAV gene therapy failed to meet primary end goals in phase IIb clinical trials. This failure was attributed to the limitations of AAV gene therapy, including the development of neutralizing antibodies⁸ and difficulties of maintaining constant, long-term expression of the large 110-kD enzyme. We continue to explore alternative SERCA-based gene therapy strategies^{9–11} but are also actively pursuing the search for small-molecule SERCA effectors capable of ameliorating the SERCA malfunction found in numerous degenerative diseases.⁶

Initial screening campaigns evaluated structural perturbations using a reconstituted membrane system and fluorescence resonance energy transfer (FRET) detection between SERCA and its regulatory partner phospholamban (PLB). In these studies, conventional fluorescence emission spectral recording was used for large-scale screening and resulted in the discovery of small-molecule activators of SERCA.² We continued our development of SERCA biosensors by engineering a genetically encoded intramolecular FRET sensor; donor and acceptor fluorescent proteins were fused to specific locations on SERCA's cytoplasmic headpiece, known to undergo large-scale, physiologically relevant, structural changes (5–10 nm), as depicted by the known crystal structures.^{3,12} This type of assay lends itself naturally to a structure-based screening campaign, in which the results are related not only to variation of compound structure but also to variation of specific structural changes in the labeled target.

This two-color SERCA (2CS) biosensor uses green fluorescent protein (GFP) and red fluorescent protein (RFP) as a FRET pair. These red-shifted fluorescent proteins are less sensitive to cell autofluorescence and fluorescent compound interference, compared with their blue-shifted cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) counterparts. The existence of multiple 2CS FRET populations was previously identified using single-molecule fluorescence lifetime microscopy.¹³ These studies elucidated SERCA's sensitivity to PLB and calcium, revealing insights into residues involved in this structure-activity relationship.¹⁴ This set the stage for a proof-of-principle structure-based small-molecule screen, using a prototype fluorescence lifetime (FLT) microplate reader.^{3,15} This seminal study proved that a genetically encoded FRET sensor could be stably expressed in human embryonic kidney (HEK293) cells and used for HTS in a microplate format.

The present work uses a novel top-read fluorescence lifetime plate reader, which involves an epi-illumination geometry (**Fig. 1A**), thereby allowing for temperature control and the use of inexpensive black-bottom 384- or 1536-well plates. The preceding article¹ established the technical

feasibility of decomposing the fluorescence emission spectra into a linear combination of component spectra, from which the FRET efficiency (FRET) can be calculated. That study demonstrated high screening quality (high Z' value) and the ability to resolve minute FRET changes (0.5%), even when the cellular autofluorescence was artificially increased. This study involves screening a small-molecule library (National Clinical Collections 1 and 2), which contains a collection of compounds that have already been evaluated in preclinical and clinical trials. The ability to accurately determine changes in FRET from the 2CS biosensor from two independent fluorescence measurements (spectral and lifetime) increases the confidence of hit selection.

Materials and Methods

Cell Culture

HEK293 (originally derived from human embryonic kidney) cells were maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) from Gibco (Waltham, MA) supplemented with 2 mM GlutaMAX (Gibco) and 10% fetal bovine serum (FBS) from Atlanta Biologicals (Lawrenceville, GA) and 1 IU/mL penicillin/streptomycin (Gibco) and grown at 37 °C with 5% CO₂. HEK293 cell lines were used to generate stable clones overexpressing the FRET-based biosensors and corresponding donor- and acceptor-labeled control cell lines.³ Three days prior to screening, the stable cell lines were expanded in five T225 flasks from Corning (Corning, NY). On each day of screening and FRET hit retesting, approximately 300 million cells were harvested by treatment of Tryple from Invitrogen (Carlsbad, CA), washed three times in phosphate buffer solution (PBS) with no magnesium or calcium from Thermo Scientific (Waltham, MA) by centrifugation at 300 × *g*, filtered using 70-μm cell strainers (Corning), and diluted to 10⁶ cells/mL using an automated countess cell counter (Invitrogen). On each day of screening, cell viability was assessed using the trypan blue assay.

After resuspension and dilution in PBS, the cells were constantly and gently stirred using a magnetic stir bar at room temperature, keeping the cells in suspension and evenly distributed to avoid clumping. During screening, cells were then dispensed into five 384-well assay plates, one containing no compound, one containing eight-point concentration curves of three known SERCA effectors, and three containing the NCC libraries 1 and 2. The same methods were applied for subsequent FRET testing of the reproducible hits identified in the pilot screen. Concentration-response curves (CRCs) of the FRET hits were assessed at multiple time points by repeatedly scanning the 384-well plates. HEK293 stable clones expressing either the DIER calcium FRET sensor (CFP/YFP) or the 2CS biosensor were used to evaluate the hits. The DIER calcium

sensor monitored changes in endoplasmic reticulum $[Ca^{2+}]$.^{16,17} The methods and protocols for the DIER cells were identical to those for 2CS, with the exception that they were evaluated only in spectral mode and the 384-well plates containing compound CRC were scanned every 3 min for 2 h.

Liquid Dispensing

Cells were dispensed using a Multidrop Combi liquid dispenser from Thermo (Pittsburg, PA), at a density of 10^6 cells/mL. Compounds were diluted in DMSO and dispensed using an automated Echo acoustic liquid dispenser from Labcyte (Sunnyvale, CA) or a Mosquito liquid dispenser from TTP Labtech (Melbourn, UK).

Cells and compound mixtures were dispensed into 384-well flat, black-bottom polypropylene plates from Greiner (Kremsmünste, Austria). The cells were dispensed at room temperature into plates containing test compounds. They were incubated with compound for 20, 60, 90, and 120 min and then scanned in both lifetime and spectral modes. In total, 727 compounds from the NCC 1 and 2 compound libraries were purchased from Evotec (Hamburg, Germany), formatted into 96-well mother plates using a Biomek FX liquid dispenser from Beckman Coulter (Brea, CA), and subsequently formatted across three 384-well plates at 50 nL (10 μ M final concentration per well) using an Echo liquid dispenser from Labcyte. Control wells containing matching %v/v DMSO were formatted into unused wells and columns 1, 2, 23, and 24 of the assay plates. The 11 reproducible FRET hits were purchased from three different vendors—Sequoia Sciences (St. Louis, MO), Tocris (Minneapolis, MN), or Santa Cruz (Santa Cruz, CA)—depending on their availability.

Instrumentation and Data Analysis

An in-depth description of the fluorescence instrumentation is described in the previous article¹ and in the supplemental material. For lifetime mode, the observed fluorescence waveform was convolved with the instrument response function, and the average energy transfer efficiency ($E = 1 - \tau_{DA}/\tau_D$) was calculated from the average lifetimes of donor τ_D and donor-acceptor, τ_{DA} , FRET cell lines. The structural correlates for FRET were modeled as previously described^{18–20} assessing the nanosecond time dependence of the TR-FRET waveforms according to equations (1) to (6):

$$F_D(t) = \sum_{i=1}^2 A_i \exp\left(-t/\tau_i\right), \quad (1)$$

$$F_{DA}(t) = \sum_{j=1}^2 X_j \cdot T_j(t), \quad (2)$$

$$F(t) = x_D F_D(t) + x_{DA} F_{DA}(t), \quad (3)$$

$$T_j(t) = \int_{-\infty}^{\infty} \rho_j(R) \cdot \sum_{i=1}^3 A_i \exp\left(\frac{-t}{\tau_i} \cdot \left[1 + \left(\frac{R_{0i}}{R}\right)^6\right]\right) dR, \quad (4)$$

$$\rho_j(R) = \frac{1}{\sigma_j \sqrt{2\pi}} \exp\left(\frac{-[R - R_j]^2}{2\sigma_j^2}\right), \quad (5)$$

$$\sigma_j = \text{FWHM}_j / (2\sqrt{2\ln 2}), \quad (6)$$

where F_D is the time-resolved fluorescence decay function of the GFP donor (equation (1)), best fit by a two-exponential decay (Suppl. Fig. S6). F_{DA} (equation (2)) is the time-resolved fluorescence decay function of the GFP donor-acceptor FRET sample. F_D and F_{DA} were fit to a linear combination of mole fractions of x_D and x_{DA} and x_D equals zero for the intramolecular FRET sensor (equation (3)). F_{DA} is a linear combination with molar fraction X_j of two FRET-affected fluorescence decays $T_j(t)$ (equation (4)). ρ_j is the probability of each distance distribution, determined by least squares minimization of the distance (nm) R associated with each donor-acceptor lifetime species τ_i (equation (5)). (σ_j) Gaussian interprobe distance distributions centered at $R_j = 5.5$ nm and 10.2 nm with distribution widths defined by the standard deviation and full-width half-maximum (equation (6)). The Förster distance (R_0) for the eGFP and tagRFP FRET pair is 5.8 nm. The parameters in this system of equations were optimized using simultaneous least squares minimization to waveforms from donor-only and donor-acceptor cell lines. The best-fit model was indicated by minimized χ^2 and by evaluation of the parameter error surface as described in our previous publications.^{18–20}

For spectral detection, the observed fluorescence emission spectrum was fitted by least squares minimization to a linear combination of component spectra:

$$F_{Fit}(\lambda) = aF_D(\lambda) + bF_A(\lambda) + cF_C(\lambda) + dF_W(\lambda), \quad (7)$$

where D is donor, A is acceptor, C is cell autofluorescence, and W is water Raman, and a , b , c , d are the coefficients determined from the fit.

$$\text{FRET} = \frac{FR \times QR - AR}{1 + FR \times QR}. \quad (8)$$

For an intramolecular FRET sensor, having both donor D and acceptor A , FRET was determined from equation (8), where QR is the ratio of quantum yields (Q_D/Q_A) in the absence of FRET, and AR is the ratio of molar absorptivities (ϵ_A/ϵ_D), both obtained from reported values.²¹ QR is corrected for spectrograph sensitivity at the appropriate wavelength (Suppl. Fig. S5). The only experimental observable in equation (8) was FR .

$$FR = \frac{\text{Acceptor emission}}{\text{Donor emission}} = \frac{bF_A}{aF_D} \quad (9)$$

Full derivation of equations (7) to (9) can be found in the supplementary material.

HTS Data Analysis

Fluorescent compounds were identified and flagged as potential false positives by evaluating the similarity index (SI) between an observed compound spectrum $I^{(a)}$ and DMSO control spectra $I^{(b)}$.

$$SI = 1 - \frac{\sum I_i^{(a)} \cdot I_i^{(b)}}{\sqrt{\sum I_i^{(a)} \cdot I_i^{(a)}} \sqrt{\sum I_i^{(b)} \cdot I_i^{(b)}}} \quad (10)$$

The spectra of 192 DMSO control wells (%v/v DMSO) were averaged for each screen to generate a single control spectrum ($I^{(b)}$). The fluorescence spectra of the two-color SERCA biosensor, screened against 727 separate compounds ($I^{(a_{1-727})}$), were compared with the single DMSO control spectrum. The similarity index between the spectra was computed over the GFP emission spectrum ($i = 500\text{--}540$ nm). The fluorescent compound threshold was set to flag potential false positives with an SI greater than 2×10^{-4} throughout the screens.

Enzymatic SERCA Activity Assays of FRET Hits

Functional assays were performed using rabbit light skeletal sarcoplasmic (SR) vesicles.³ An enzyme-coupled, NADH-linked ATPase assay was used to measure SERCA ATPase activity in 96-well microplates. Each well contained 50 mM MOPS (pH 7.0), 100 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2 mM NADH, 1 mM phosphoenol pyruvate, 10 IU/mL pyruvate kinase, 10 IU/mL lactate dehydrogenase, 1 μ M of the calcium ionophore A23187 from Sigma (St. Louis, MO), and CaCl₂ added to set free [Ca²⁺] to 10 μ M.²² Then, 4 μ g/mL of SR vesicle, calcium, compound, and assay mix was incubated for 20 min. The assay was started upon the addition of adenosine triphosphate (ATP), at a final concentration of 5 mM (total volume to 200 μ L), and absorbance read in a SpectraMax Plus microplate spectrophotometer from Molecular Devices (Sunnyvale, CA).

Results

High-Precision FRET Efficiency Determinations from Two Independent Fluorescence Measurements

The SERCA cycles through multiple conformations as it pumps calcium into the sarcoplasmic reticulum. Briefly, a GFP was fused to the N-terminus of SERCA and an RFP

was fused to a flexible intrasequence loop located on the nucleotide-binding domain of SERCA. The distance between these two fluorescent proteins can be measured by determining the rate of FRET. This two-color SERCA (2CS) biosensor was stably expressed in a human embryonic kidney (HEK293) cell line and grown in sufficient quantities for HTS.³ The binding of potential small-molecule effectors is directly evaluated as changes in FRET (Fig. 1B).

Conceptual fluorescence lifetime waveforms are depicted in Figure 1C. In the actual lifetime measurements, typically 1000 laser pulses are averaged over a 200-ms interval to generate an entire decay waveform for each well of a 384-well plate. The fluorescence lifetimes τ_{DA} (FRET biosensor with donor and acceptor) and τ_D (donor-only control) are used to determine the energy transfer efficiency $FRET = 1 - \tau_{DA}/\tau_D$. A representative complete fluorescence emission spectrum acquired with a 100-ms integration time at a 0.5-nm spectral resolution in spectral mode is shown in Figure 1D. The high-quality emission spectrum, acquired from a single well, was decomposed into a linear combination of its spectral components, GFP and RFP. These components were then used to solve for the contribution of the fluorescence emission from GFP (donor fit) and RFP (acceptor fit), allowing for a high-precision determination of an ensemble-averaged FRET from the 2CS biosensor.

Global Lifetime Analysis Resolves Structural Status of 2CS Biosensor

Global analysis of the fluorescence intensity decay rate (lifetime mode) was used to resolve two distinct structural states of the 2CS biosensor (equations (1)–(6)). These distinct structural states of 2CS were previously resolved using single-molecule fluorescence microscopy,¹³ which is not a high-throughput detection method. Here, we demonstrate analogous structural resolution of the 2CS biosensor, except with an ensemble-averaged FRET measurement, acquired in 200 ms/well from live-cell suspensions.

FRET is a sensitive spectroscopic molecular ruler, due to the R^{-6} distance dependence of the rate of energy transfer from an excited donor fluorophore (GFP) to an acceptor (RFP) in the ground state.²³ The exceptionally good precision of direct waveform recording (DWR) and global analysis of the fluorescence decay waveforms produced in this fashion allow FRET measurements to be evaluated in terms of distance distributions and mole fractions of physiologically relevant structural states. Fluorescence lifetime waveforms were analyzed using a global two-component model (equations (1)–(6)), yielding a two-state structural model for SERCA's cytosolic headpiece, describing an equilibrium between the open (10.2 nm) and closed (5.5 nm) structural states with full-width at half-maximum (FWHM) of 6.9 and 2.2 nm, respectively. The Gaussian distance distribution was determined for each structural state and plotted

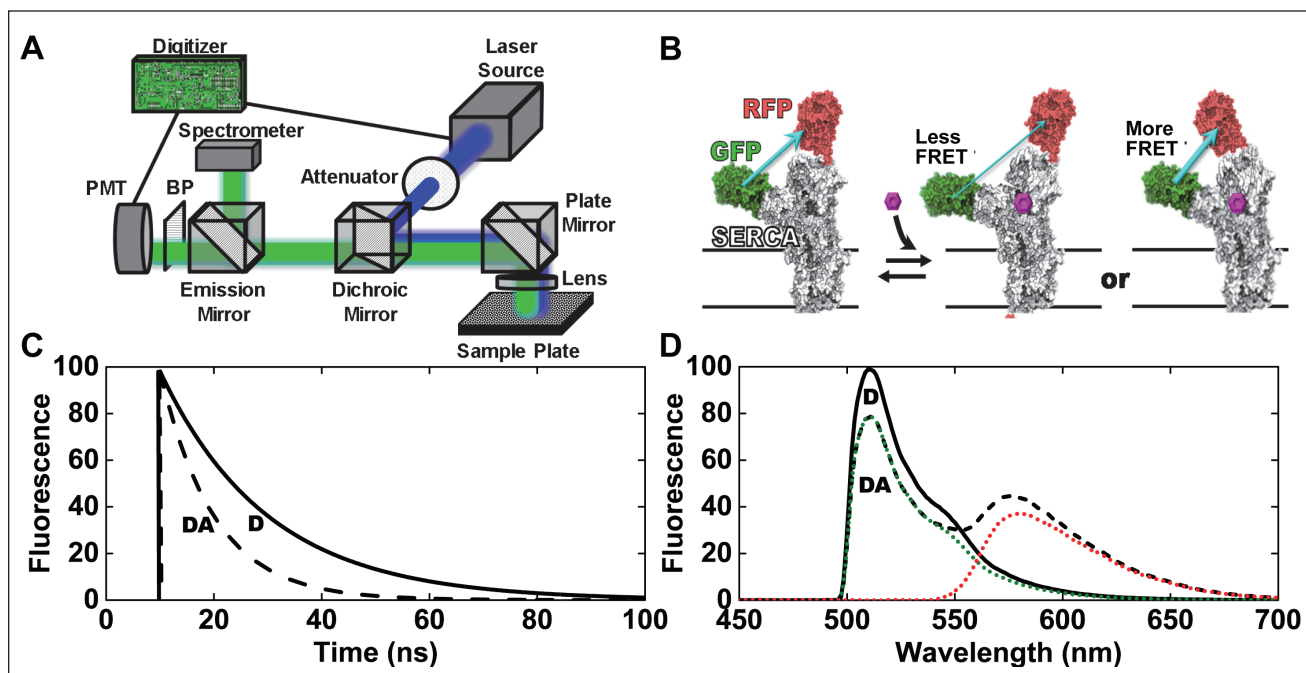


Figure 1. (A) Diagram of the instrument. (B) The two-color sarco/endoplasmic reticulum calcium ATPase (SERCA) (2CS) intramolecular fluorescence resonance energy transfer (FRET) biosensor.^{2,12,22} As depicted, the FRET efficiency is dependent on the structural status of SERCA's domains, which may be affected by the binding of small molecules. Conceptual data (C, lifetime mode) and (D, spectral mode) illustrate the dependence of fluorescence signals on FRET. Solid black curve (D): donor only (no FRET). Dashed black curve (DA): donor plus acceptor (FRET). (D) Dotted curves show the resolution of the spectrum into components corresponding to donor (green fluorescent protein [GFP]) and acceptor (red fluorescent protein [RFP]) emission.

as a histogram in **Figure 2A**. The distance R and FWHM fitting parameters were allowed to vary globally, and the mole fraction of each state was determined according to the two-component global fit (equations (1)–(6)). Thapsigargin inhibits SERCA at nanomolar concentration and perturbs SERCA2a's cytosolic headpiece, greatly increasing the population of the more open and disordered structural state at 50 nM (**Fig. 2B**). The full concentration dependence is illustrated in **Figure 2C**, and the concentration-response curve of the closed state mole fraction (**Fig. 2D**) yields an EC_{50} value of 2.2 nM thapsigargin, in agreement with the known EC_{50} for SERCA inhibition.²⁴

Pilot Screening of NCC Libraries to Evaluate Both Spectral and Lifetime FRET Detection

A small-molecule library (National Clinical Collections 1 and 2), consisting of 727 compounds previously evaluated in preclinical and clinical trials, was used to evaluate both spectral and lifetime modes of the SUPR. After an initial quality control check of the 2CS cell line on each day of screening (response to known effectors and signal level), a HEK stable clone overexpressing the 2CS biosensor was dispensed, using a Multidrop liquid dispenser into 384-well plates, and then scanned in both spectral and lifetime modes

after 20, 60, 90, and 120 min of incubation with the compounds or control wells.

A single-exponential fit was used to determine the lifetime τ_{DA} from 2CS and τ_D from the one-color SERCA2a donor-only control cell line. These lifetimes were used to determine $FRET = 1 - \tau_{DA}/\tau_D$. In spectral mode, the observed spectrum acquired from each well was decomposed into a linear combination of components (GFP, RFP, cellular autofluorescence, and water Raman). The reference spectrum of each was used to solve for the total contribution of fluorescence emission from each component (equation (7)). These values were used to calculate a fitted ratio of the total fluorescence emission of RFP/GFP (equation (9)), and then FRET from the 2CS biosensor was determined using the simplified FRET equation for intramolecular FRET sensors as described in the preceding article¹ (equation (8)).

Both lifetime and spectral fluorescence measurements are prone to interference from fluorescent compounds. We took advantage of the information contained in the full-emission spectrum to develop a streamlined process to flag these potential false positives. A spectral similarity index (equation (10)), which monitors differences from the spectra of control wells with no compound added, was computed in the donor-only region. A stringent similarity index threshold (2×10^{-4}) was used to flag 44 compounds as

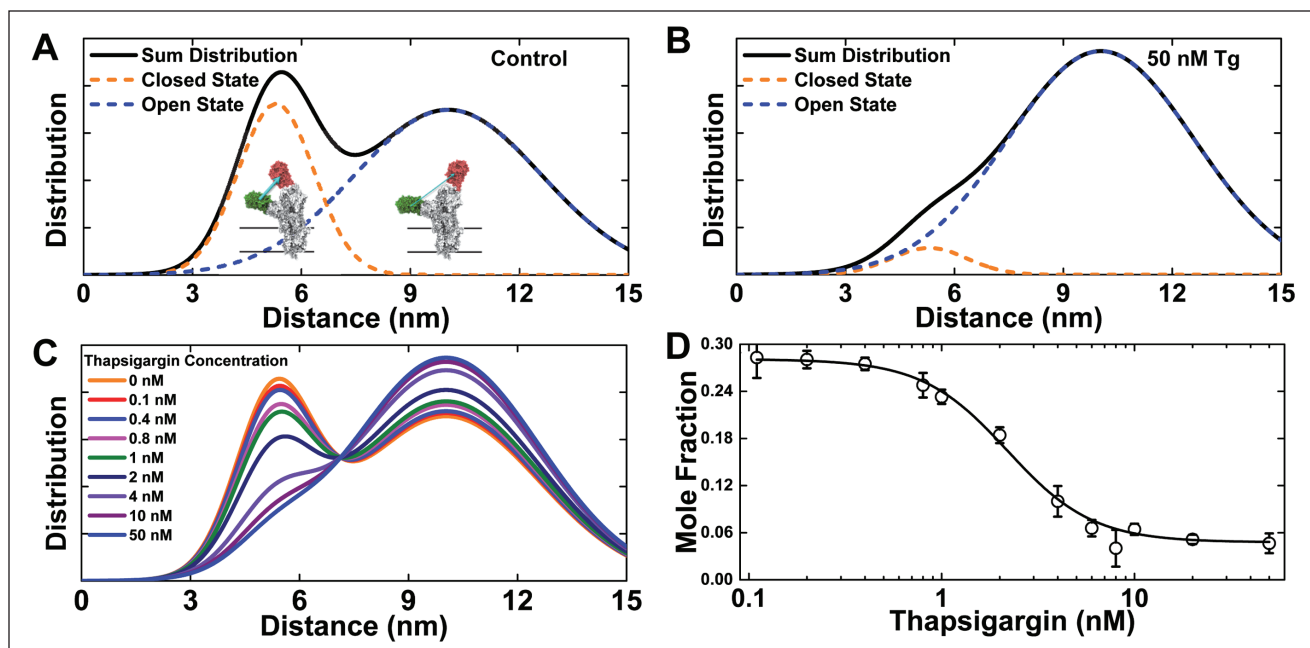


Figure 2. Lifetime analysis of time-resolved fluorescence decay waveforms resolves structural states of the 2CS biosensor. **(A)** Two structural states of sarco/endoplasmic reticulum calcium ATPase (SERCA) are resolved, corresponding to two Gaussian interprobe distance distributions, consistent with a two-state structural model with an equilibrium between closed (5.5-nm fluorescence resonance energy transfer [FRET] distance, orange) and open (10.2-nm FRET distance, blue) structural states. **(B)** The addition of a saturating dose (50 nM) of the known inhibitor thapsigargin (Tg) shifts this equilibrium substantially toward the open state. **(C)** Concentration dependence of Tg effect on the structural distribution ($n = 8$ wells for each concentration). **(D)** Plot, based on **(C)**, of closed-state mole fraction versus Tg.

potential false positives due to interference from compound fluorescence (Fig. 3A).

Histogram plots from all wells that passed the fluorescence compound filter, from a single NCC screen, demonstrated a three-fold increase in precision from the spectral mode in comparison to lifetime mode, as exemplified by a narrower distribution (Fig. 3B). These two complementary determinations of FRET were used to identify hits, quickly rule out false positives, and increase reproducibility across screens.

From three independent screens of the NCC libraries at a time point of 20 min after compound incubation, 11 reproducible FRET hits were found. These hits were identified using the spectral unmixing method and a hit threshold set at a 0.02 change in FRET. The results from one 2CS NCC screen are depicted in Figure 3C,D. Based on triplicate screens, 11 of 16 compounds were identified as reproducible FRET hits, shown as blue circles in Figure 3C. In lifetime mode, 9 of the 11 reproducible FRET hits found using spectral mode were also reproducible lifetime hits (Fig. 3D).

Reproducibility of Hit Identification across Independent Screens and Time Course Studies

The reproducibility of 2CS FRET hits identified across three independent screens in spectral mode, after a 20-min

compound incubation at a 10- μ M concentration, is depicted in Figure 4A. The change in 2CS FRET (Δ FRET) of each hit from three independent NCC screens remained nearly constant from one screen to the next. For purposes of directly evaluating the use of the spectral recording method, a hit threshold of a 0.02 negative change in FRET (red bar) was applied and will be used from here on. When these same hits were evaluated in lifetime mode, 9 of the 11 spectral FRET hits were found to be reproducible hits (Fig. 4B). Compounds 106 and 190 were not identified as lifetime FRET hits in one of the three independent screens.

The ability to acquire lifetime and spectral measurements with scan times under 3 min for an entire 384-well microplate allowed for the examination of 2CS FRET changes in response to the full NCC library of compounds at multiple compound incubation time points. Time course screening may not be directly amenable to large-scale screening but is potentially highly applicable for assessing the reproducibility of a large number of FRET hits identified during a large-scale HTS campaign at multiple concentrations. Time-dependent compound effects may also elucidate compounds with low binding affinities or delayed effects from low membrane permeability. These types of studies may also be useful for other fluorescence bioassays solely based on monitoring time-dependent effects, as we

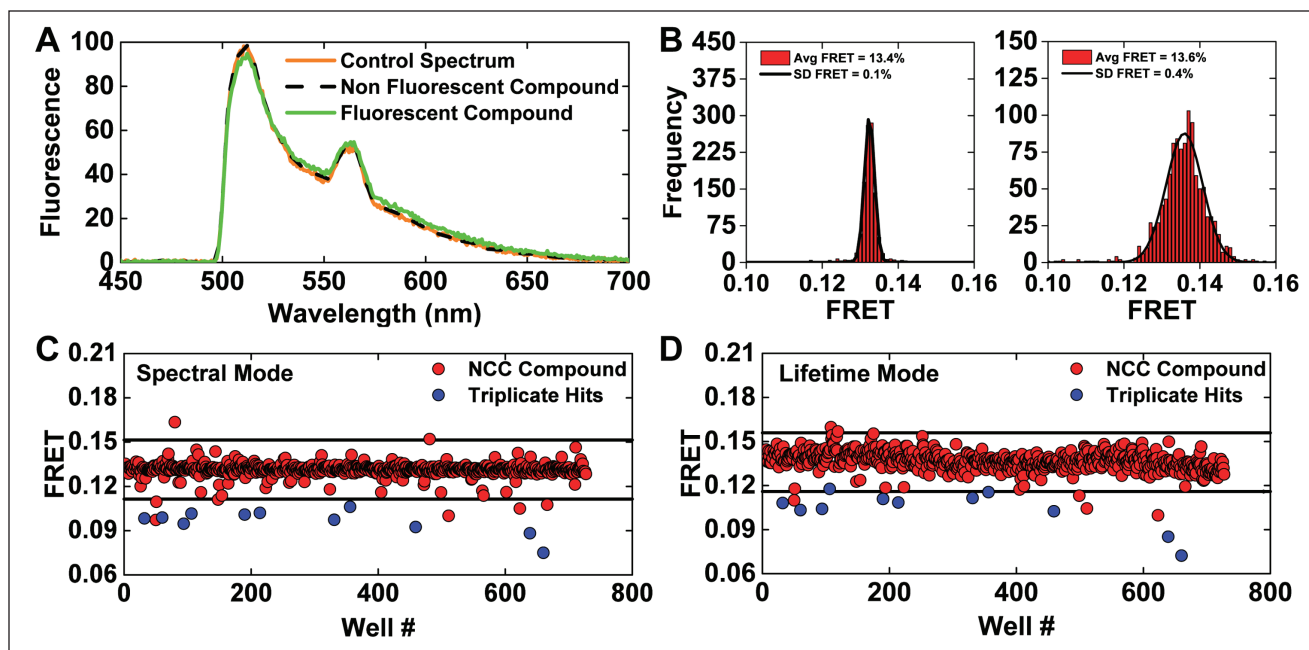


Figure 3. (A) Fluorescence emission spectra were used to identify and flag potential interference from fluorescent compounds by assessing the similarity index (equation (10)) of each well from a pilot screen of the NCC 1 and 2 small-molecule libraries. A control spectrum (%v/v DMSO well) and nonfluorescent compound (not identified as a fluorescence resonance energy transfer [FRET] hit during screening with 2CS) have a high degree of similarity, as shown as direct overlap of the orange and black spectrum. A slightly fluorescent compound is depicted by the green spectrum and was flagged as a potential false-positive hit. The fluorescent profile of all 1152 wells from one NCC screen was assessed using a stringent similarity index threshold; 44 compounds were flagged as potential false positives due to interference from compound fluorescence. (B) Histogram plots of the wells from one NCC screen after removing potential fluorescent compounds. Gaussian fits depict an increase in precision from spectral mode (left) in comparison to lifetime mode (right), shown as the frequency of FRET efficiency determined by either method and a narrower distribution from spectral mode (average FRET calculated by spectral unmixing or lifetime and the standard deviation determined from the Gaussian fit). (C) One 2CS pilot NCC screen (spectral mode) is shown with a hit threshold set at a 0.02 change in 2CS FRET (4 SD). Sixteen FRET hits were identified in this screen. Eleven of these 2CS FRET hits were found to be reproducible across three independent screens (blue). (D) The same 384-well plates were scanned in lifetime mode. Sixteen hits were identified using the same threshold set at a 0.02 change in 2CS FRET efficiency (3 SD). In this screen, nine of the reproducible 2CS FRET hits identified in spectral mode were also FRET hits as assessed by lifetime mode (blue).

will depict later using theameleon calcium FRET sensor. For these 2CS pilot screening studies, time-dependent screening was used to determine the interscreen reproducibility of FRET hit identification as assessed in both spectral and lifetime modes.

Time-dependent scans of reproducible 2CS FRET hits were consistent over multiple time points (20, 60, 90, and 120 min after compound incubation). The 2CS FRET hits analyzed here were from screen 2 (turquoise bars in Fig. 4A,B) and show excellent reproducibility across time points. Five compounds (60, 359, 459, 639, and 660) exhibited an increased FRET change over time (Fig. 4C). Reproducible hits were evaluated in lifetime mode (Fig. 4D) Very subtle differences in the 2CS FRET changes were found across spectral and lifetime methods. Ten compounds from screen 2 were identified as hits after 20 min of compound incubation. Compounds 32 and 356 displayed a modest reduction in 2CS Δ FRET at the later time points.

Compounds 60, 94, 459, 639, and 660 again exhibited an increased FRET change over time. Overall, both methods show excellent agreement in terms of the direction and magnitude of FRET change.

Multiparameter Concentration-Dependent Effects of FRET Hits

The reproducible 2CS FRET hits were further evaluated as a function of concentration. Compounds were dispensed into 384-well plates, across an 8-point concentration gradient ($n = 4$ for each concentration). Three independent dose-dependent FRET tests were performed on the 2CS FRET hits. Six compounds that produced the largest reproducible FRET change after a 120-min compound incubation are depicted. These compounds dose-dependently decreased 2CS FRET as determined by spectral mode (Fig. 5A). Each FRET curve was fit using the Hill equation. Decreased 2CS

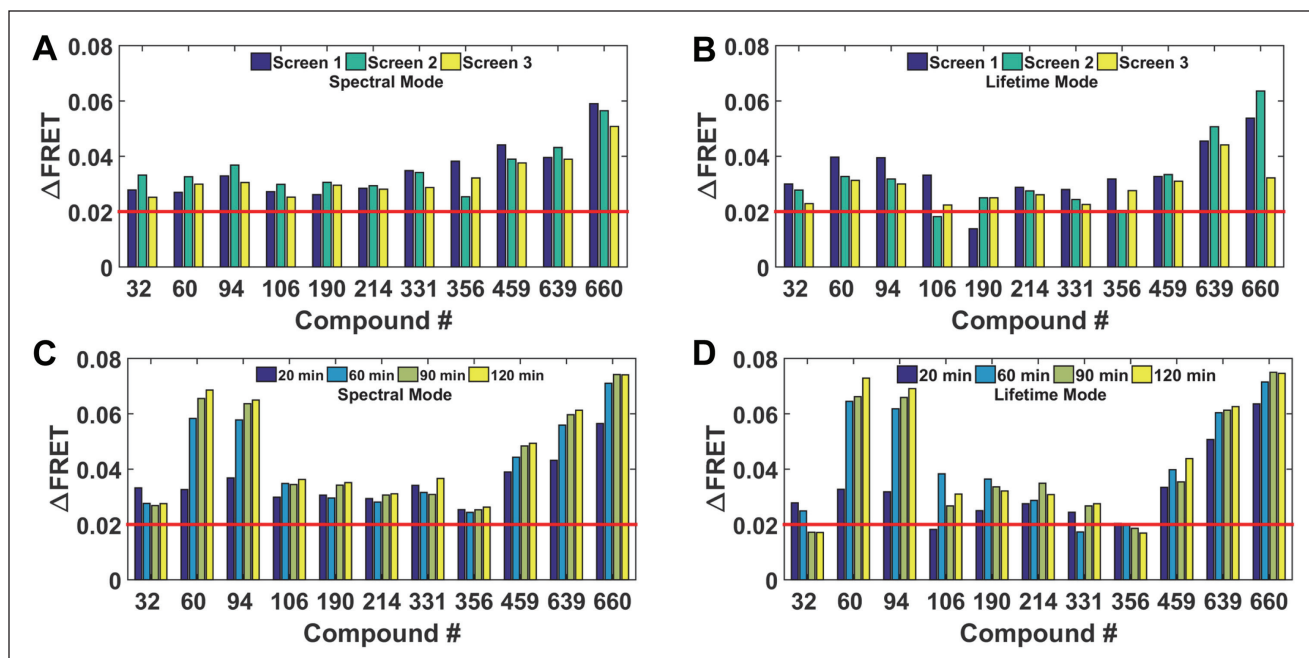


Figure 4. Reproducible fluorescence resonance energy transfer (FRET) hits assessed across independent screens and time course studies. **(A)** Spectral mode identified 11 reproducible 2CS FRET hits using a threshold of 0.02 change in FRET (red line). The reproducibility of each 2CS FRET hit, after a 20-min incubation, across three independent screens is shown. The $\Delta FRET$ from each compound remains consistent from screen to screen. $\Delta FRET$ was calculated by assessing the change in FRET of 2CS from the average FRET, determined by the Gaussian fit of all wells not flagged as fluorescence compounds. **(B)** Lifetime mode assessment of the 11 reproducible spectral FRET hits. Nine of the 11 FRET hits were reproducible (triplicate) using a 0.02 FRET threshold. Compounds 106 and 190 were not identified as lifetime FRET hits in one of the three independent screens. **(C)** Spectral FRET hits evaluated by time course studies. Each independent pilot screen was scanned in spectral mode after 20, 60, 90, and 120 min of compound incubation. The change in 2CS FRET efficiency ($\Delta FRET$) of each compound is plotted, and each reproducible FRET hit, identified as a hit using the spectral unmixing method, remained a hit over multiple time points. The 2CS FRET hits depicted here were from screen 2 (turquoise bars in **A** and **B**). Compounds 60, 94, 459, 639, and 660 exhibited an increased FRET change over time. **(D)** Lifetime FRET change evaluated by time course studies. Ten compounds from screen 2 were identified as hits, after 20 min of compound incubation, using a threshold set at 0.02 FRET change (red bar). Compounds 32 and 356 displayed a modest reduction in 2CS $\Delta FRET$ at the later time points. Compounds 60, 94, 459, 639, and 660 again exhibited an increased FRET change over time.

FRET was observed at micromolar concentrations with notable differences in the apparent EC_{50} (half-maximal effective concentration) of the FRET curve. The same 384-well plate, containing the reproducible hits, was evaluated in lifetime mode with a simple, single exponential fit (equations (1) and (2)) and demonstrated great agreement between the FRET change across the two modes of independent FRET measurements (**Fig. 5B**).

The compounds found to be reproducible hits and that exhibited dose-dependent FRET changes all decreased 2CS FRET. The imidazole antifungal clotrimazole was a hit and has been previously shown to inhibit SERCA function.²⁵ The related compounds, oxiconazole, bifonazole, and miconazole, were also hits. The antibacterial triclosan has been shown to increase cytosolic calcium²⁶ but to our knowledge not through interaction with SERCA.

Global analysis of the lifetime data (equations (1)–(6)) resolved a dose-dependent change in the mole fractions of the open and closed structural states. Using this distance

distribution model, the reproducible hits perturbed the 2CS structural equilibrium between open and closed states in **Figure 5C** (5.5-nm distance distribution shown). The high sensitivity of the spectral mode is shown by the fluorescence signal of the water Raman spectrum. Raman scattering was acquired from compound-only wells of the known aggregator miconazole.²⁷ Compound aggregation dose-dependently causes more light to be absorbed and decreases inelastic light scattering (Raman band) (**Fig. 5D**). This information may become useful for flagging potential false positives due to compound aggregation.

Functional Characterization of FRET Hits on SERCA ATPase Activity and ER Calcium Content

Functional assays of the confirmed reproducible NCC hits were used to assess the relationship of hits that perturb 2CS structure and their effects on SERCA function. The ATPase activity of purified SERCA was measured after a 20-min

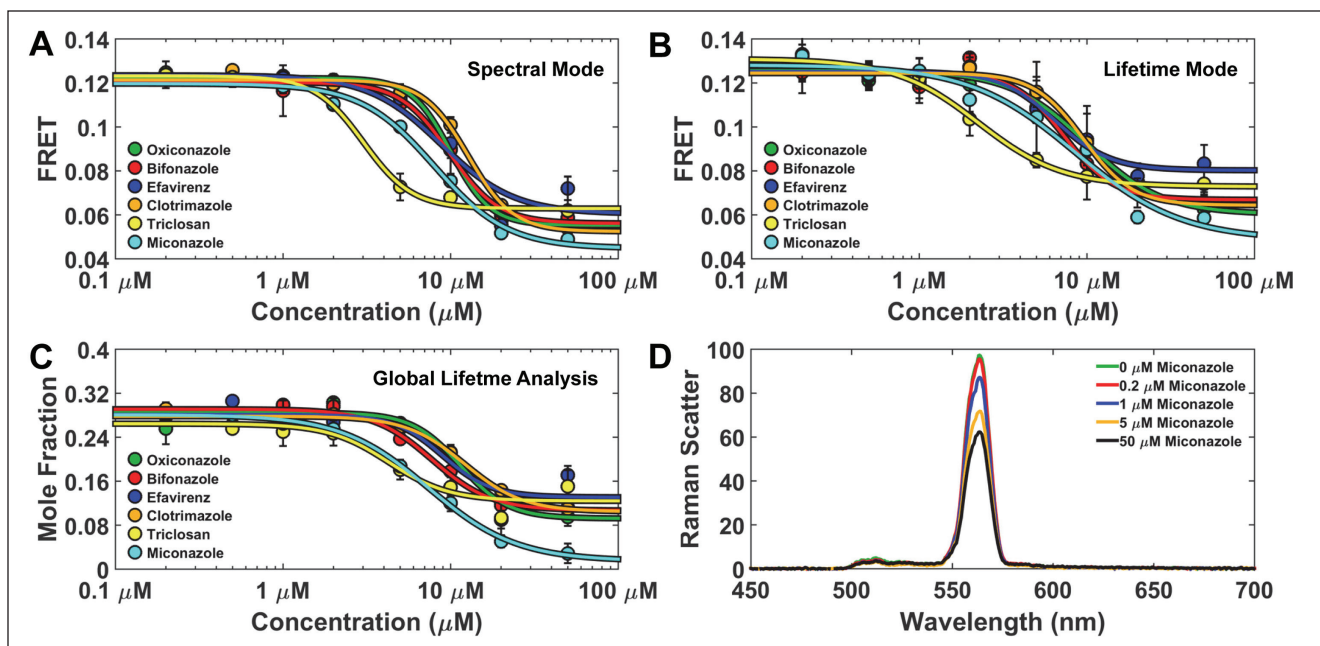


Figure 5. Multiparameter concentration-dependent effect of fluorescence resonance energy transfer (FRET) hits. **(A)** Spectral mode analysis of the reproducible 2CS FRET hits. Compounds were dispensed into 384-well plates, across an 8-point concentration gradient ($n = 4$ for each concentration). Six representative compounds produced a dose-dependent FRET change as evaluated in spectral mode. These compounds altered FRET with micromolar affinities with subtle differences across the compounds. **(B)** The same 384-well plate was evaluated using lifetime mode and demonstrated excellent agreement in the dose-dependent FRET change across two independent FRET measurements. **(C)** Global analysis of the lifetime data depicts a dose-dependent change in the mole fraction of the closed 2CS headpiece (5.5-nm distance distribution). Using this distance distribution model, the confirmed reproducible hits perturbed the 2CS structural equilibrium between open and closed states. All of the hits decreased 2CS FRET, indicating increased distance between green fluorescent protein (GFP) and red fluorescent protein (RFP). **(D)** Water Raman spectrum acquired from compound-only wells of the known compound aggregator miconazole demonstrates ultra-high sensitivity of spectral recording. Compound aggregation dose-dependently causes more light to be absorbed and decreases inelastic light scattering (Raman band).

incubation with a saturating dose of buffered free calcium (10 μM) and titration of each compound. Experiments were performed in triplicate with 8-point concentration curves. The top six hits were found to dose-dependently inhibit SERCA's ATPase function (**Fig. 6A**). The antifungal miconazole showed almost complete inhibition (92.4%), with a K_i of 2.8 μM . Clotrimazole's ability to inhibit SERCA's ATPase was slightly reduced in comparison, with a K_i of 17.3 μM , which is in agreement with previous steady-state measurements (7–35 μM).²⁵

SERCA malfunction can result in decreased ATPase activity and/or calcium pumping efficiency. ER calcium content was monitored over time using the endoplasmic-localized cameleon calcium FRET sensor (D1ER).¹⁶ As demonstrated in the preceding article,¹ known SERCA inhibitors deplete ER calcium in a time- and dose-dependent manner and can be monitored using live-cells expressing D1ER. Briefly, D1ER FRET changes were monitored over time by repeatedly (every 3 min) scanning a 384-well plate containing varying concentrations of the 2CS FRET hits. D1ER cells were assessed immediately after compound incubation. These plates were scanned only in spectral mode using

434-nm excitation with a laser-driven light source to acquire a full-emission spectrum from each well. The appropriate CFP/YFP reference spectra were used to determine FRET using equations (8) and (9).

D1ER FRET curves were determined for each time point scan, after compound incubation, over a period of 120 min (40 scans total). The 2CS FRET hits displayed time-dependent and compound-specific ER calcium depletion. Miconazole (turquoise) exhibited both maximal SERCA ATPase V_{max} inhibition and the largest amount of ER calcium depletion, as depicted at the final 120-min time point in **Figure 6B**. Maximal ER calcium depletion in the presence of a saturating dose (50 μM) of each compound (decreased D1ER FRET) was assessed over a 120-min period (**Fig. 6C**). The 2CS FRET hits displayed time-dependent and compound-specific ER calcium depletion. The K_i and EC_{50} s from the ATPase activity and D1ER FRET curves showed good agreement at the 20-min time points. The structure-activity relationship of the 2CS FRET hits was further analyzed by comparing 2CS FRET (both spectral and lifetime mode), ATPase activity, and ER calcium depletion (**Fig. 6D**). The maximal change (shown as percent

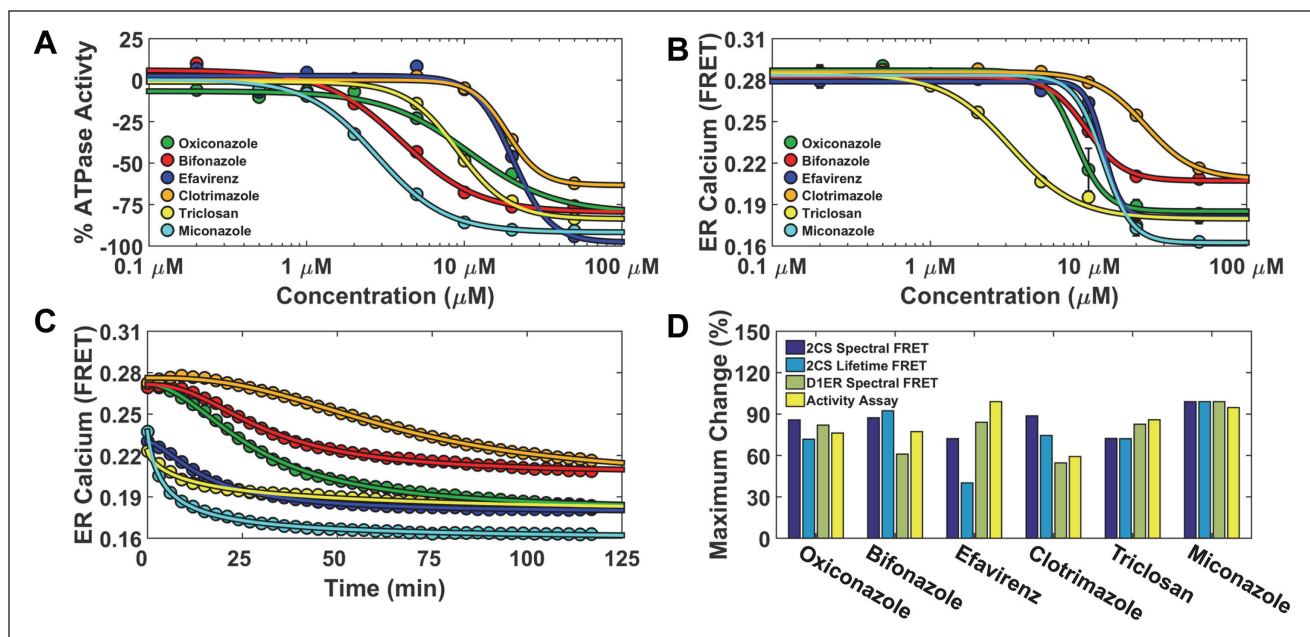


Figure 6. Functional characterization of fluorescence resonance energy transfer (FRET) hits on sarco/endoplasmic reticulum calcium ATPase (SERCA) ATPase activity and endoplasmic reticulum (ER) calcium content. **(A)** The 2CS FRET hits inhibit SERCA ATPase activity. NADH enzyme-coupled activity assay of purified SERCA was measured at eight different concentrations of the reproducible FRET hits. The maximal rate of SERCA activity was measured at saturating calcium (10 μM) after a 20-min incubation with compounds, and dose-dependent inhibition was observed. **(B)** ER calcium was depleted by the 2CS FRET hits. ER calcium was monitored in live cells overexpressing the ER-localized calcium FRET sensor (DIER). DIER FRET is dependent on calcium concentration, where less calcium causes a reduction in FRET. ER calcium levels were monitored over time in response to an 8-point concentration gradient of each hit compound. A 384-well plate was repeatedly scanned (every 3 min) with DIER cells. The dose-dependent FRET change (ER calcium depletion) after a 120-min compound incubation is shown and depicts differential depletion at each compound concentration. **(C)** Maximal ER calcium depletion in the presence of a saturating dose (50 μM) of each compound (decreased DIER FRET) was assessed over a 120-min period. The 2CS FRET hits displayed time-dependent and compound-specific ER calcium depletion. Miconazole (turquoise) exhibited both maximal SERCA ATPase V_{max} inhibition and the largest amount of ER depletion. **(D)** Structure and activity assay correlation of 2CS FRET hits. The maximal change (percent change) of the structural FRET change from the 2CS FRET biosensor and the maximal change from two different functional assay (ATPase activity assay and DIER calcium depletion) are shown.

change) of the structural FRET change from the 2CS FRET biosensor had excellent agreement with the maximal change from two different functional assays (ATPase activity assay and ER calcium depletion). Structural perturbation of the 2CS FRET biosensor directly relates to a compound's effect on SERCA function.

Discussion

This study illustrates the complementary combination of spectral and lifetime fluorescence detection for the purposes of HTS. The spectral unmixing method increases the precision of hit identification and reproducibility of the hits in concentration-response curves (Fig. 4). The fluorescence lifetime detection mode offers excellent precision and offers the additional advantage of structural resolution, revealed by multiexponential global lifetime fitting. This approach resolves multiple FRET populations and assesses them in terms of distance distributions and mole fractions, assigned

to structurally relevant perturbations of SERCA effectors (Fig. 2). This resolution of multiple FRET-detected structural states from a live-cell biosensor is highly advantageous for screening, offering the potential to elucidate chemotypes or classes of compounds, identified in large-scale screens, which differentially alter the structural status of a biosensor. This high-content information can be used to generate structure-activity relationships based on binding affinities, structural dynamics, and disorder.

The capability to couple two independent measurements of FRET, thereby substantially decreasing the false-positive rate, would be of significant value to the HTS community. Spectral recording does not offer the resolution of structural information, in terms of resolving multiple structural states, but can be used to identify fluorescent compounds, eliminate artifacts due to dispenser error or contaminated samples, and increase assay precision across screens (Fig. 3).

This is the first microplate reader capable of direct waveform recording in both lifetime and spectral domains. A

recent review of fluorescence lifetime imaging (FLIM) plate readers demonstrates the medium-throughput capabilities currently offered by other technologies (20-min scan times per 96-well plate).²⁸ The approach described here is considerably faster yet offers very high precision.

Beyond developing new fluorescence technology, the overarching goal of this research is to identify novel small-molecule SERCA effectors with therapeutic potential for multiple disease states. These studies employed a 2CS biosensor based on the SERCA2a isoform,³ which is the primary isoform expressed in the heart. We have engineered constructs based on the other human isoforms, with the intent of performing drug discovery campaigns to identify isoform-specific SERCA effectors. Furthermore, we are currently developing new synthetic analogues based on our previously identified SERCA activators and inhibitors, where our HTS approach allows us to quickly assess and triage the most prominent candidates from a large pool of synthetically derived analogues. We concluded this assay-based demonstration by investigating hits identified through structural-based screening, using an NADH-enzyme coupled ATPase activity assay and the DIER endoplasmic reticulum-targeted calcium sensor to evaluate the correlation between the structure and function of hits identified in this pilot screening campaign (Fig. 6).

The compounds identified during these pilot screens all decreased FRET from the 2CS biosensor, corresponding to opening of SERCA's cytoplasmic headpiece. This may be a consequence of their similarity in the mode of binding or mechanism of inhibition. However, the 2CS biosensor is not limited to detection of decreases in FRET. In broken cells, we have previously shown that ligands such as calcium increase FRET, due to closure of SERCA's cytoplasmic headpiece.²⁹ It is plausible that the maximal FRET effect is essentially reached for the HEK293 live cells, in which calcium and ATP maintain SERCA in its closed structural state.

The novel paradigm used in the present study enables the measurement of multiple FRET parameters. These high-content assays are ideally suited for HTS campaigns, with potential to discover novel allosteric effectors, which may differentially perturb FRET. This strategy is now being evaluated for use on homogenate and microsomal cellular preparations using FRET-based biosensors. These applications allow for fine control of environment (calcium, nucleotide, pH, etc.). Preliminary results have demonstrated that these purified preparations of FRET-based biosensors are suitable for counterscreens and also in-depth structural evaluations of novel SERCA effectors.

The high-resolution FRET approach, coupled to functional assays, is applicable to a wide range of protein targets, including the ryanodine receptor,³⁰ myosin,^{19,31} phospholamban,¹¹ multiple-drug resistance receptor,³² and the tumor necrosis receptor.³³ The ability to quickly and reliably assess structural

perturbations from biosensors in relation to physiologically relevant functional changes holds high promise for the development of allosteric effectors and potentially valuable lead compounds.

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Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Thomas holds equity in and serves as an executive officer for Photonic Pharma LLC. This relationship has been reviewed and managed by the University of Minnesota.

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