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Using FLIM-FRET for characterizing spatial interactions in the spindle

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Abstract

Proper spindle assembly and the attachment of chromosomes to the spindle are key for the accurate segregation of chromosomes to daughter cells. Errors in these processes can lead to aneuploidy, which is a hallmark of cancer. Understanding the mechanisms that drive spindle assembly will provide fundamental insights into how accurate chromosome segregation is achieved. One challenge in elucidating the complexities of spindle assembly is to visualize protein interactions in space and time. The *Xenopus* egg extract system has been a valuable tool to probe protein function during spindle assembly *in vitro*. Tagging proteins with fluorescent proteins and utilizing fluorescence-based approaches, such as Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM), have provided visual clues about the mechanics of spindle assembly and its regulators. However, elucidating how spindle assembly factors are spatially regulated is still challenging. Combining the egg extract system and visual FRET approaches provides a powerful tool to probe the processes involved in spindle assembly. Here we describe how a FLIM-FRET biosensor can be used to study protein-protein interactions in spindles assembled in *Xenopus* egg extracts. This approach should be readily adaptable to a wide variety of proteins to allow for new insights into the regulation of spindle assembly.

Keywords

FLIM; fluorescence; biosensor; protein-protein interactions; spindle assembly; kinesin

1. Introduction

Fluorescent protein FRET is characterized by the energy transfer from a donor fluorophore upon excitation to an acceptor fluorophore that is in close enough proximity to receive the energy and become excited (1). Sufficient proximity can be attained by intermolecular protein-protein interactions of proteins tagged with donor and/or acceptor fluorophores or by intramolecular changes in protein conformation that result in the movement of the donor and acceptor fluorophores closer together. Detection of fluorescent protein FRET can be assessed in multiple ways depending on the chosen donor/acceptor pairs and the equipment used to detect FRET. For a discussion on how to choose a FRET pair and the different

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modes of FRET detection please see Ems-McClung and Walczak (2019) and the references therein.

FRET can be used as a molecular ruler to measure distances between proteins or within proteins *in vitro* because the extent of FRET is proportional to the distance between the donor and acceptor. Alternatively, FRET can be used in physiological contexts through the development of visual biosensors to understand where, when, and to what extent interactions occur. Biosensors are characterized by having two parts: a biological receptor that can bind a specific biological or chemical element, called the analyte, and a transducer that transmits a signal to a detection system (2, 3). The transducer in a FRET biosensor is the FRET pair that responds to the biological receptor upon analyte interaction and results in increases or decreases in energy transfer. The differences in energy transfer can be measured as a change in fluorescence or donor lifetime by spectral or microscopic techniques. Commonly, biosensors are single molecules containing both the donor and acceptor fluorophores separated by a receptor that responds to the analyte in a biological system. For example, the importin β binding domain of importin α is the receptor in the RanGTP gradient FRET biosensor, and responds to the levels of RanGTP, the analyte, in the cytoplasm (4). Alternatively, the Aurora B phosphorylation gradient biosensor uses a short receptor fragment of an Aurora B substrate, which undergoes a phosphorylation-dependent conformational change, resulting in a decrease in FRET emanating outward from the kinetochores along the chromosome arms (5, 6).

For visual detection of FRET, sensitized emission FRET (SE-FRET) and FLIM-FRET are the most commonly utilized approaches (7). FLIM-FRET offers some important advantages over SE-FRET for spatial detection because it measures the change in the decay or lifetime of the donor in the absence or presence of the acceptor, and is predominantly insensitive to acceptor concentration (8). Conversely, for temporal visualization of FRET, SE-FRET is advantageous because acquisition is much faster and requires less signal. It does, however, require additional controls and analysis to account for crosstalk between the donor and acceptor channels (9, 10). For both methods, the signal from the FRET pair is quantitative, which is a critical aspect of a biosensor (11).

Several factors should be considered when designing and performing a FLIM-FRET experiment with a biosensor: decay properties of the donor, competition with endogenous proteins, and localization of the biosensor. Accurately determining the lifetime of the donor requires collecting enough photon counts, which can vary depending on the donor, and can take up to minutes to acquire. Ideally, a donor for FLIM should be mono-exponential such that FRET results in a bi-exponential decay with the second component being the lifetime of the donor undergoing FRET. For a mono-exponential donor, like mTurquoise or EGFP, only 500–1,000 photon counts per pixel may be required; whereas, multi-exponential donors, like CyPet or ECFP, require more photon counts per pixel for accurate determination of lifetimes. For example, CyPet requires at least 2,000 counts per pixel for accurate determination of lifetime with the PicoQuant FLIM system we use. To acquire this number of photons in a reasonable time frame (1–1.5 minutes), CyPet donor concentrations of 1–2 μM are needed. Thus, it is important to know that addition of this much donor protein does not interfere with the biology of the system. For the RanGTP gradient and Aurora

B phosphorylation gradient biosensors, this was not a factor because the receptors were fragments of proteins that did not have a specific activity or function other than interacting with the analyte. In the case of the biosensor highlighted in this chapter, it was important to determine that the added biosensor receptors, which use full-length proteins, do not disrupt spindle assembly by out competing endogenous proteins. Finally, it is important to consider the accessibility of the biosensor to the analyte in your system depending on the questions being asked. In the example presented here, we wanted to probe the effects of the RanGTP gradient on importin α and XCTK2 interaction across the spindle. Thus, it was important to use importin α as the FLIM donor because it is distributed evenly throughout the cytoplasm in contrast to XCTK2, the acceptor, that concentrates toward the poles. In this configuration, we could measure the biosensor response, importin α and XCTK2 interaction, to the RanGTP gradient regardless of the location in the spindle.

In this chapter, we will describe how we used intermolecular FRET biosensors to study the interaction between the Kinesin-14 motor XCTK2 (12, 13) and the RanGTP gradient effector importin α on spindles assembled in *Xenopus* egg extracts using FLIM. We include a description of how to validate the biosensor, how to assemble spindles with the FRET biosensors, and then how to analyze the FLIM data. While the assay may appear specific to this example, the methods described should be easily adapted to monitor other protein-protein interactions in the spindle that respond to different regulatory signals.

2. Materials

2.1. Biosensor Validation by FRET

1. FPLC Buffer: 20 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA. Buffer the pH with KOH and store at 4°C.
2. XB Dialysis Buffer: 10 mM HEPES pH 7.7, 100 mM KCl, 25 mM NaCl, 50 mM sucrose, 0.1 mM EDTA, 0.1 mM EGTA. Store at 4°C.
3. 10 mg/ml casein: made in water and stored in aliquots at −20°C.
4. 10 mg/ml LPC: 10 mg/ml each leupeptin, pepstatin, and chymostatin in DMSO; stored at −20°C in 100 μl aliquots.
5. ≥ 5 μM YPet-XCTK2: Baculoviral expressed and purified by traditional chromatography with a final buffer composition of 300 mM KCl, FPLC Buffer, 1 mM DTT, 10 μM MgATP, 0.1 μg/ml LPC, 10% sucrose (13). Flash frozen in liquid nitrogen as small single use aliquots (5–10 μl) and stored at −80°C.
6. ≥ 20 μM 6His-importin α-CyPet: bacterially expressed, NiNTA purified, and dialyzed into XB Dialysis Buffer (12). Flash frozen in liquid nitrogen as small single use aliquots (10–20 μl) and stored at −80°C.
7. ≥ 20 μM 6His-S-importin β: bacterially expressed, NiNTA purified, and dialyzed into XB Dialysis Buffer (12). Flash frozen in liquid nitrogen as small single use aliquots (10–20 μl) and stored at −80°C.

8. ≥ 350 μM GST-RanQ69L: bacterially expressed, glutathione agarose purified, loaded with GTP, and dialyzed into XB Dialysis Buffer (14). Flash frozen in liquid nitrogen as small single use aliquots (5–10 μl) and stored at −80°C.
9. Plate Dilution Buffer: FPLC Buffer, 1 mM DTT, 0.2 μg/μl casein.
10. Costar #3964 half area 96-well non-treated black plate.
11. Monochromator-based plate reader: Synergy H1.
12. Plate centrifuge: Eppendorf 5810R centrifuge with A-4–81 MTP rotor and 96-well plate adapters.

2.2. CSF and Cycled Extract

1. 100 unit/ml pregnant mare serum gonadotropin (PMSG): diluted with sterile MilliQ water and stored in small aliquots at −20°C. This hormone can be thawed and refrozen several times.
2. 1,000 unit/ml human chorionic gonadotropin (hCG): diluted with sterile MilliQ water and stored at 4°C. This hormone can be stored in small aliquots at −20°C but avoid repeated freezing and thawing.
3. 20X MMR: 100 mM HEPES, pH 7.8, 2 mM EDTA, 2M NaCl, 40 mM KCl, 20 mM $MgCl_2$, 40 mM $CaCl_2$; pH with NaOH and sterilize by autoclaving (*see* Note 1).
4. 20X XB Salts: 2M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$; sterile filter and store at 4°C (*see* Note 1).
5. 1M HEPES pH 7.7: pH with KOH; sterile filter and store at 4°C.
6. 2M sucrose: sterile filter and store at 4°C.
7. 1M $MgCl_2$: autoclaved.
8. 0.5M EGTA: pH with KOH and sterile filter.
9. 10 mg/ml LPC: 10 mg/ml each leupeptin, pepstatin, and chymostatin in DMSO; stored at −20°C in 100 μl aliquots.
10. 1 L 1X MMR (5 mM HEPES, pH 7.8, 0.1 mM EDTA, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$): 50 ml 20X MMR diluted to 1 liter with MilliQ water (*see* Note 1).
11. 500 ml XB: 10 mM HEPES, pH 7.7, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, 50 mM sucrose. Make with 5 ml 1M HEPES pH 7.7, 25 ml 20X XB Salts, 12.5 ml 2M sucrose, and pH with 55 μl 10 N KOH (*see* Note 1).
12. 250 ml CSF-XB: 10 mM HEPES, 7.7, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, 5 mM EGTA, 50 mM sucrose. Make with 250 ml XB from step 11 and add 250 ul 1M $MgCl_2$, 2.5 ml 0.5M EGTA (*see* Note 1).
13. 100 ml CSF-XB, 0.01 mg/ml LPC: 100 ml CSF-XB from step 12 and add 100 μl 10 mg/ml LPC (added fresh) (*see* Note 1).

14. 200 ml Dejellying Solution: 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2% L-cysteine, pH 7.8; pH by the addition of 0.9 ml 10 N NaOH. Make with 10 ml XB Salts, 4 g L-cysteine (free base) (*see* Note 1). L-cysteine should be added right before collecting eggs.
15. Spindle Fix: MMR, 60% glycerol, 10 μg Hoechst; stored in 100 μl aliquots at −20°C; add 37.5 μl 37% formaldehyde before first use. This solution can be thawed and refrozen numerous times.
16. Cytochalasin B: 10 mg/ml in DMSO and stored at −20°C.
17. 5% gelatin: made in water and stored at −20°C.
18. 50X Energy Mix: 150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$. Stored at −20°C in 50 μl aliquots (15).
19. ≥ 100 μM X-rhodamine labeled tubulin: stored in 3–6 μl aliquots at −80°C (see Note 2).
20. ~6×10^3 sperm/μl; de-membranated (16).
21. 4-liter laying bucket: Rubbermaid FG630400CLR.
22. 600 ml glass beaker.
23. 150 × 75 mm glass petri dish: VWR #89000–294.
24. 4 ml disposable polystyrene transfer pipets.
25. Nyosil M25: Nye Lubricants.
26. 13 × 51 mm ultraclear centrifuge tubes.
27. Clinical centrifuge.
28. Ultracentrifuge with swinging bucket rotor: Beckman SW55i rotor.
29. 26-gauge needle with 1 cc syringe.
30. 18-gauge needle with 3 cc syringe.
31. Coverslips: 18 × 18 mm #1.5.
32. Microscope slides.

2.3. FLIM-FRET

1. 25X calcium chloride solution: 10 mM HEPES pH7.7, 1 mM $MgCl_2$, 100 mM KCl, 150 mM sucrose, 10 μg/ml cytochalasin D, 10 mM $CaCl_2$.
2. ≥ 5 μM YPet-XCTK2: Baculoviral expressed and purified by traditional chromatography with a final buffer composition of 300 mM KCl, FPLC Buffer, 1 mM DTT, 10 μM MgATP, 0.1 μg/ml leupeptin, pepstatin, chymostatin (LPC), 10% sucrose (13). Flash frozen in liquid nitrogen as small single use aliquots (5–10 μl) and stored at −80°C.

3. ≥ 20 μM 6His-importin α-CyPet: bacterially expressed, NiNTA purified, and dialyzed into XB Dialysis Buffer (12). Flash frozen in liquid nitrogen as small single use aliquots (10–20 μl) and stored at −80°C.
4. Coverslips: 22 × 22 mm #1.5.
5. Microscope slides.
6. FLIM system: Leica SP8 with PicoQuant FLIM module controlled by LASX v3.1.1 software and SymPhoTime 64 v1.6 software, respectively.

3. Methods

When developing a biosensor, it is critical to characterize the different aspects of the biosensor in terms of the affinity and sensitivity to the analyte. In our example, the biosensor transducer is the CyPet/YPet FRET pair, and the biosensor receptor is the Kinesin-14 XCTK2 complex with importin α/β that responds to the RanGTP analyte. We previously described how to measure the XCTK2 interaction with importin α by solution-based FRET (17). Similarly, the RanGTP gradient biosensor Rango, which uses the importin β binding domain of importin α, was shown to respond quantitatively to different levels of RanGTP in *Xenopus* egg extract and in cells (4, 18). Thus, to validate our bipartite biosensor prior to using it in egg extracts, we first describe a solution-based FRET assay based on our previous work to demonstrate that the complex of YPet-XCTK2 and importin α-CyPet/importin β is sensitive to the RanGTP-bound mutant, RanQ69L (12, 14). We then provide the method we use to make *Xenopus* egg extracts, which closely follows other published methods (19, 20). Finally, we describe how we collect FLIM data on spindles containing our biosensors using a hybrid Leica SP8-PicoQuant system followed by step-by-step instructions for the analysis of lifetimes in SymPhoTime. When designing your biosensor and FLIM-FRET experiments, consider additional controls to support your results, such as mutations that disrupt binding or affect the analyte concentration or location. For example, mutation of the importin α binding site in XCTK2 could be made to inhibit importin α binding, which should result in FRET and lifetimes like the importin α-CyPet donor only controls.

3.1. Biosensor Validation by FRET

1. Assemble the following enzyme mixes on ice.
   a. 1.2 μM YPet-XCTK2 (acceptor only) in FPLC buffer, 0.2 μg/μl casein, 110 mM KCl (see Notes 3 and 4): 15 μl final volume.
   b. 1.2 μM 6His-importin α-CyPet (donor only) + 4.8 μM 6His-S-importin β in FPLC buffer, 0.2 μg/μl casein, 110 mM KCl (*see* Notes 3 and 4): 15 μl final volume.
   c. 1.2 μM 6His-importin α-CyPet + 1.2 μM YPet-XCTK2 in FPLC buffer, 0.2 μg/μl casein, 110 mM KCl (*see* Notes 3 and 4): 15 μl final volume.
   d. 1.2 μM 6His-importin α-CyPet + 4.8 μM 6His-S-importin β + 1.2 μM YPet-XCTK2 in FPLC buffer, 0.2 μg/μl casein, 110 mM KCl (*see* Notes 3 and 4): 30 μl final volume.

e. 60 μM GST-RanQ69L in FPLC buffer, 0.2 μg/μl casein (*see* Note 3): 20 μl final volume.

f. Ran Control Buffer in FPLC buffer, 0.2 μg/μl casein, 8.71 mM KCl: 50 μl final volume (*see* Note 5).

g. Plate Dilution Buffer: 200 μl final volume.

2. Add 40 μl of Plate Dilution Buffer to five wells of a half area 96-well plate.
3. Add 10 μl of 1.2 μM YPet-XCTK2 to the first well (*see* Note 5).
4. Add 10 μl of 1.2 μM 6His-importin α-CyPet + 4.8 μM 6His-S-importin β to the second well (*see* Note 5).
5. Add 10 μl of 1.2 μM 6His-importin α-CyPet + 1.2 μM YPet-XCTK2 to the third well (*see* Note 5).
6. Add 10 μl of 1.2 μM 6His-importin α-CyPet + 4.8 μM 6His-S-importin β + 1.2 μM YPet-XCTK2 to the fourth and fifth wells (*see* Note 5).
7. Perform a spectral FRET scan by exciting the donor at 433 nm and collecting the emission from 460–600 nm with 5 nm steps, which is specific to CyPet and YPet. Alternately, measure FRET using an end point read with excitation at 433 or 405 nm and emission at 535 nm (17).
8. Add 10 μl of control buffer to the first through fourth wells and 10 μl 60 μM GST-RanQ69L to the fifth well (*see* Note 5).
9. Measure FRET as in step 6 and export the data to Excel.
10. Analyze in the data in Excel by background subtracting the YPet fluorescence and plotting the corrected normalized FRET emission intensity by the wavelength as described (17) (Fig. 1).

3.2. CSF Extract

1. Day 1: Inject frogs with 0.5 ml PMSG (50 units) using 26-gauge needle and 1 cc syringe noting day, date, and time (*see* Note 6).
2. Day 3: Boost the same frogs with 0.25 ml PMSG (25 units) using 26-gauge needle and 1 cc syringe noting day, date, and time.
3. Day 5–13: Inject 2–3 frogs with fresh 0.5 ml hCG (500 units) using 26-gauge needle with 1 cc syringe and place in 2 L 1X MMR in clear laying bucket noting day, date and time (*see* Note 6).
4. Warm 5% gelatin to 37°C in water bath.
5. Get all solutions ready, making cysteine last, and thaw cytochalasin B and energy mix.
6. Coat Petri dish with 100 μl 5% gelatin in MMR, swirl and replace with XB.
7. Add 1 ml CSF-XB + LPC to centrifuge tubes and then add 10 μl cytochalasin B to each centrifuge tube.

8. Collect laid eggs by removing frogs from laying buckets and placing them in a holding tank for 24–48 hours for observation and until they are done laying eggs. If eggs are distinguishably different in quality, select only the highest quality eggs (*see* Note 7).

9. Wash eggs in MMR until all debris and dirt is removed and garden away bad eggs (*see* Note 7) with plastic Pasteur pipet.

10. Gently pour washed eggs into a 600 ml beaker and remove as much MMR as possible.

11. Dejelly in 2% cysteine. Pour dejellying solution into the beaker, using approximately half of the solution, incubate ~5 min or until the eggs start to settle. Continue gardening away the bad eggs if present. Remove cysteine by carefully pouring off cysteine, and then add remaining cysteine until the eggs are packed (7–10 min) (*see* Note 8). Pour the cysteine solution with eggs into the coated Petri dish, and then remove all cysteine by pouring off and removing solution with a transfer pipet (*see* Note 9).

12. Wash dejellied eggs 3–4 times with XB, pouring off XB in between washes. Garden away any eggs that activate (*see* Note 7).

13. Wash dejellied eggs with CSF-XB 2–3 times, pouring off in between, and then removing all CSF-XB with a transfer pipet (*see* Note 9).

14. Wash 2 times with CSF-XB+PI, removing some of solution with the last wash.

15. Carefully transfer eggs to 13 × 51 mm ultraclear centrifuge tubes containing CSF-XB+PI+100 μg/ml cytochalasin B with polished cut-off Pasteur pipet (*see* Note 10). Let eggs drop in slowly below surface!

16. Remove all buffer from top of each tube using an aspirator until the egg surface is nearly dry.

17. Using forceps, gently lower the 13 × 51 ultraclear tube into a 15 ml plastic round bottom tube and spin 10 sec at ~400 x *g* in a clinical centrifuge. Start the time at start of the run, not when the rotor is at top speed.

18. Remove all buffer from the top of the eggs with an aspirator and pipet in ~1 ml Nyosil M25.

19. Spin in the clinical centrifuge at ~600 x *g* for 30 sec followed by full speed for 15 sec.

20. Remove all buffer and Nyosil M25 getting the surface as dry as possible without sucking up eggs.

21. Crush eggs at 18°C in SW55i at 10,000 rpm (~12,000 x *g*) for 15 min.

22. Wipe sides of tubes with EtOH. Slowly collect extract with 18-gauge needle right above pigment with open side of needle facing up; tilt needle up to extract being careful not to collect pigment. Remove needle to dispense extract into fresh tube on ice (*see* Note 11).

23. Add the following per volume of extract: 1/1,000 LPC, 1/1,000 cytochalasin B, 1/50 Energy Mix, and 1/40 2 M sucrose. Gently mix by rocking tube back and forth and place on ice.

24. Add X-rhodamine labeled tubulin to ~0.3 μM final concentration (*see* Notes 2 and 12).

25. Test extract quality by adding sperm to a final concentration of 150 sperm/μl to 20 μl of CSF extract containing X-rhodamine labeled tubulin. Squash 1 μl extract with 3 μl spindle fix on slide with 18 × 18 mm coverslip at 20, 40, and 60 minutes (*see* Note 13).

3.3. FLIM-FRET Imaging

1. Cycle extract into interphase by adding 25X calcium chloride solution to 1X and sperm to 600 sperm/μl (*see* Note 14) and moving extract to room temperature or to a 20°C water bath.

2. Monitor cycling by squashing 1 μl extract with 3 μl spindle fix at 20, 40, and 60 minutes (*see* Note 13).

3. Once most nuclei are in interphase, place the extract on ice for 10 min, combine multiple tubes of extract to one tube, and add an equal volume of CSF extract + X-rhodamine tubulin.

4. While the extract is cycling, make 20X master mixes of donor only and donor + acceptor proteins on ice. For our example, we made the following mixes in CSF-XB with a final volume of 7.5 μl.

    a. Donor only: 20 μM importin α-CyPet + 3 μM XCTK2 (unlabeled).

    b. Donor + acceptor: 20 μM importin α-CyPet + 3 μM YPet-XCTK2.

5. Aliquot 25 μl of cycled extract to the desired number of tubes on ice and add 1.25 μl of protein mix to each tube (*see* Note 12). Mix gently by finger-flicking the tube.

6. Set up the acquisition settings for the Leica SP8 confocal microscope and PicoQuant PicoHarp 300 time-correlated single photon counting FLIM system (*see* Note 15).

    a. Image bit depth changed from the default 8-bit to 12-bit in the Configuration/Hardware menu.

    b. White light laser set to 70% output for YPet and X-rhodamine imaging.

    c. Objective: HC PL APO CS2 63× 1.2 NA water immersion objective.

    d. HyD SMD1 detector for CyPet (importin α-CyPet) confocal imaging: 440 nm excitation at 90%, 460–490 nm emission, 500% gain, 40 MHz.

    e. HyD SMD2 detector for YPet (YPet-XCTK2) confocal imaging: 514 nm excitation at 50%, 520–580 nm emission, 20% gain, 40 MHz.

f. HyD5 detector for X-rhodamine (microtubule) confocal imaging: 594 nm excitation at 25%; 610–700 nm emission, 100% gain, 40 MHz.

g. Confocal imaging: 256 × 256-pixel images, 400 MHz scanning, 2X zoom, 1 AU pinhole, and 2 frame averaging.

h. FLIM imaging: 128 × 128-pixel images (*see* Note 16), 200 MHz scanning, 2X zoom. The 440 nm laser was set to 90% and 20 MHz with 50 ns pulses. For acquisition of 2,000 photons in 1–1.5 minutes, average photon count rate should be 200–300 thousand counts per sec (kcps).

i. The maximum number of photons/px to reach per image: 2,000 photons.

7. Name the LASX and SymPhoTime workspaces with the experiment date and a brief description and save to a known location and folder.

8. Incubate reactions at room temperature for 45–60 minutes, staggering reaction start times by 45–60 minutes so that all structures will be imaged at approximately the same time of spindle assembly.

9. Squash 4 μl of extract between a slide and a 22 × 22 mm #1.5 coverslip.

10. Focus on a spindle in the microtubule channel, and acquire confocal images of the microtubule, YPet, and CyPet channels according to conditions set in step 6.

11. Run FLIM test in LASX program, which engages the PicoQuant FLIM system and samples the image to determine whether the image matches the desired average count rate of 200–300 kcps and does not saturate the system count rate of 10^7 counts/sec. If the image is not satisfactory, choose another spindle structure to image, or if no counts are detected, double check the settings.

12. Run FLIM in LASX program, which acquires a FLIM image according to the settings set in steps 6 and 11.

13. Periodically save your results in the LASX software as you acquire images. The SymPhoTime software automatically saves the data after acquisition.

3.4. FLIM-FRET Lifetime Analysis with SymPhoTime Software

1. Open your SympPhoTime saved Workspace “File\Open Workspace” and open the FLIM file labeled as “filename_OFLIM.pqres”. In the display window, the Events[Cnts] (counts) and Fast Lifetime[ns] (FLIM) images open as an overlaid image (Fig. 2A) along with the model parameter menus (Fig. 2B), and the decay curve and residual graph (Fig. 2C).

2. Expand the FLIM menu under the Region of Interest and Frame menus to the left of the overlay image if it was not automatically expanded (Fig. 2A). Binning, Time Gate, and Threshold options become visible.

3. Adjust the bin size to improve the accuracy of the lifetime calculation (*see* Note 16). In this example, the FLIM image was acquired until 2,000 counts were

reached and then the counts were binned 2 by 2 by setting “Binning” to a value of 2 Pts for a total of ~8000 counts per binned pixel.

4. Creating a threshold for the photon count image to isolate the photon counts that are due to the donor will reduce noise in the analysis and reduce processing time for images containing isolated structures or cells on a blank background. This can be done empirically or using the “Set Visually” feature (*see* Note 17). In our example, the donor is homogenous throughout the cytoplasm, thus setting a threshold was not needed.

5. Select “Calculate FastFLIM” to the left of the image under the “Threshold” option to update the counts and FLIM overlay image based on the binning and thresholding to prepare the data for fitting (*see* Note 18).

6. Select the fitting model in the “Fitting Model” dropdown menu of the “Decay Fitting” window tap to the left of the decay curve based on the predicted lifetime of the donor (Fig. 2B) (*see* Note 19). In our example with CyPet as the donor, the n-Exponential Reconvolution fit was chosen (*see* Note 20).

7. Choose which decay data to use by selecting an option from the “Decay” dropdown menu under the “Fitting Model” (Fig. 2B): overall image (“Overall Decay”), region of interest (“ROI”), or a single pixel (“Pixel 0, 0”) (*see* Note 21). For our example, we did not define an ROI, thus the overall decay was used for fitting.

8. Choose the type of instrument response function (IRF) from the “IRF” dropdown menu (Fig. 2B). SymPhoTime estimates an IRF for every image called the “Calculated IRF” (red curve in decay histogram). Alternatively, an IRF can be calculated for the system by using a quenched dye (21) or urea crystal (22) and imported into SymPhoTime using the “Import” option. In our example, we used the calculated IRF.

9. Adjust boundaries of the decay data by manually adjusting the blue lines to the left and right of the decay histogram to best approximate the decay (Fig. 2C). In this example image, the boundaries were set to 0.5 ns to the left where the trace is level immediately before the rise and to 46 ns on the right of the trace to eliminate the pulse at the end of the trace, which were kept constant for all subsequent manipulations.

10. Choose the number of “Model Parameters n” to fit to the decay data empirically (Fig. 2B), typically 1–3, and repeats steps 11–13 until the X^2 approaches 1 (*see* Note 22).

11. Determine an initial fit to the decay data by clicking the orange “Initial Fit” button (Fig. 2B, bottom), which returns the best fit curve overlaid on the decay data as a black line, the corresponding residuals (Fig. 2C), as well as the calculated model parameters in the “Decay Fitting” window (Fig. 2B).

12. Assess the goodness of fit based on the overlaid best fit curve, the residuals plotted below the decay curve (Fig. 2C), and the X^2 value (Fig. 2B, bottom) based on the following characteristics (*see* Note 22).

    a. Does the best fit curve (black) drawn over the decay data (green, Intensity [Cnts]) follow and not deviate from the data?

    b. Do the residuals (Resids. [StdDev]) resonate around 0 without large fluctuations?

    c. Does the X^2 approach 1?

13. Incrementally increase the number of model parameters to get a X^2 value close to 1 (Fig. 3). Be sure to press the "Clear" button between changes in the model parameter settings (Fig. 2B). In our example, single exponential (n=1) (Fig. 3A) and double exponential (n=2) (Fig. 3B) models do not fit the decay curve well because the best-fit curve fits the data poorly, the residuals deviate significantly from 0, and the X^2 value is much greater than 1. A three exponential (n=3) model fits much better with better residuals and a X^2 value approximately equal to 1 (Fig. 3C). Increasing the number of model parameters to 4- or 5- (Fig. 3, D and E) improves the X^2 value slightly, but not enough to prefer either of these over a 3-exponential model.

14. Calculate the lifetimes for each pixel in the image once the optimal fitting parameters are determined: manually fix the decay background to zero by typing 0.00 into the $Bkgr_{Dec}$[Cnts] field and unchecking the corresponding checkbox next to the value (Fig. 4A, blue boxes); manually fix the lifetime values (τ_1[ns], τ_2[ns] and τ_3[ns]) to the values determined from the initial fit by unchecking the corresponding boxes next to the values (Fig. 4A, red boxes); and click the "FLIM Fit" button above the "Decay Fitting" window (Fig. 4B). The FLIM and photon count overlay image (Fig. 4C) will be updated with the calculated lifetimes.

15. Set the scale of the photon count image for saving and exporting by manually setting the "Events[Cnts]" scale in the "Min" and "Max" boxes (Fig. 4D). In this example, 0 and 8,000 counts were chosen because these are the estimated minimum and maximum possible photon counts from 2,000 acquired counts binned 2×2.

16. Set the scale of the FLIM image for saving and exporting by changing the FLIM image to display the $\tau_{Av/Amp}$[ns] values by selecting "tau_Av_Amp[ns]" from the dropdown menu (*see* Note 23) and setting the scale to best reflect lifetimes by manually adjusting the "Min" and "Max" values (Fig. 4D). In this example, a scale of 1.1–1.8 ns was used.

17. Apply a scale bar by right clicking on the image and selecting "Show ScaleBar". The scale bar will be included in the Bitmap and Bitmap with Colorscale formats (Fig. 4C).

18. Save the lifetimes and the X^2 values by clicking the "Export as ASCII" or "Export to Clipboard" buttons above the table of results (Fig. 4A). This is a little

cumbersome, so it may be simpler to write down the lifetimes (τ_1[ns], τ_2[ns] and τ_3[ns]), average lifetime ($\tau_{Av/Amp}$[ns]), and X^2 values for reference. The decay histogram results can also be exported in multiple formats by right clicking on the histogram and selecting from the pop-up menu.

19. Save your results to the SymPhoTime workspace file by clicking “Save Result” under the “File” drop down menu (Fig. 4B). It will be designated as FLIM.pqres. Subsequent saved modifications will be saved incrementally as FLIM_X.pqres where X is +1 increments.

20. Save photon count, FLIM, and/or overlay images. These images can be saved or exported in a variety of formats by right clicking on the image and selecting “Export” from the pop-up menu. To save images individually deselect one of the images by unchecking the desired “Active” box (Fig. 4D). To save an overlay keep both images “Active” by having them checked in the dialog box.

a. Bitmap: saves an RGB .bmp image that will include the image scale bar if selected from the “Show ScaleBar” dropdown menu on both the photon counts and FLIM image (Fig. 5A). The rainbow color of the FLIM image is preserved. This format is preferred for publication to preserve the crisp pixel edges that are lost in the 1 to 1 Bitmap image in step 20c (*see* Note 24).

b. Bitmap with Colorscale: saves an RGB bitmap image of the photon count and/or FLIM image with the photon count or lifetime color scale to the right of the image (Fig. 5B). An image scale bar will be included on both the photon and FLIM images if selected, and the rainbow color of the FLIM image is preserved. This is a nice format for record keeping because it will open in standard image programs on computers and provides a record of the scales used in the images.

c. 1 to 1 Bitmap: saves a one to one representation of the photon count and FLIM images (Fig. 5C). A scale bar will not be included. The rainbow color of the FLIM image is preserved, but the pixels are smoothed.

d. TIFF Export: Saves a .tif image that reports the photon counts or lifetime in seconds per pixel in grayscale (Fig. 5D). A scale bar will not be included, and the rainbow color is not preserved. This format or the ASCII format is preferred for analysis in other imaging programs like NIH ImageJ.

e. ASCII Export: Saves a .dat file in a text file image format in which the first three lines are descriptive followed by the counts and/or lifetimes in nanoseconds in rows and columns of the dimensions of the images (*see* Note 25). If an overlay image is exported, both the counts and the lifetime values will be exported. For our example the file descriptor lines would look like this when opened:

tau_Av_Amp[ns]

(x0|y0) = (0.00[um]|0.00[um])

(x1|y1) = (92.288[um]|92.288[um]

21. The count and lifetime images can be analyzed quantitatively in third party software like ImageJ by drawing ROIs or lines in areas of interest. We recommend making stacks of the fluorescence, photon, and FLIM images so that quantification can be done simultaneously on all channels, which will prevent repetitive quantification. Care must be taken if the fluorescence and FLIM images are taken with different settings, i.e. 256×256 pixels versus 128×128 pixels, or if the FLIM images are binned during analysis. In our example, the photon count and FLIM images are downsized from 256×256 pixels 16bit images to 64×64 pixels 32bit images relative to the fluorescence images. The fluorescence images can readily be scaled down in ImageJ using the “Image/Scale” feature. Images can then be opened in Photoshop, scaled, and copied into other programs like Adobe Illustrator for generation of publication quality figures (*see* Note 24).

4. Notes

1. It is important to make the MMR, XB, CSF-XB, and dejellying solutions the day of the extract so that they can be at room temperature. Cool solutions will adversely affect the extract. To simplify making the solutions the day of the extract, we typically make 20X MMR and 20X XB Salts stocks (2M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$) and dilute them accordingly. They need not be sterilized after diluting them.

2. We typically label tubulin in house using X-rhodamine succinimidyl ester and determine the labeling stoichiometry so that we can add a defined amount of labeled tubulin to the extract (23).

3. Adjust KCl concentration with appropriate volumes of XB Dialysis Buffer.

4. Some proteins are sensitive to dilution, and because FRET is a sensitive assay and requires very little protein, it is sometimes necessary to do an intermediate dilution prior to the final dilution.

5. The high concentrations of Ran needed in the experiment results in a high KCl concentration; therefore, add an equivalent volume of XB Dialysis Buffer in the Ran Control Buffer to account for salt concentration. When adding the enzyme mixes or RanQ69L to the wells, mix gently by pipetting up and down several times being careful to not introduce bubbles.

6. Frogs must be kept at 18°C under conditions of a regulated day/night light cycle. For standard spindle assembly reactions, inject frogs with hCG 17–18 hrs before harvesting the eggs. Freshly diluted or freshly thawed hCG can be used for several weeks.

7. Cycling extract and imaging require very good quality extracts; thus, high-quality eggs are required. Eggs are laid arrested in meiosis II, and good eggs

have a distinct dark animal pole and light/white vegetal pole with no visible loose jelly coat. Bad eggs can be removed with a plastic Pasteur pipet if in low amounts (<5%), but the batch of eggs should be discarded if there are high amounts of poor-quality eggs. Bad eggs can consist of activated eggs, which are mostly white with a small dark remnant of the animal pole, apoptotic eggs, which are puffed white eggs, or strings of immature eggs. If the MMR is cloudy with debris, these batches should be discarded as well. If the eggs activate during the processing, remove as many activated eggs as possible, and make a Note as the extract quality may suffer.

8. During the removal of the zona pellucida (dejellying) the eggs become very fragile; thus, gentle handling is essential. Once the dejellying solution is added, carefully and slowly swirl the beaker occasionally to mix, alternating directions. As the zona pellucida is removed, the eggs begin to settle toward the bottom of the beaker and will become packed against one another.

9. To remove solution from the Petri dish effectively, rock the dish back and forth slightly to move the buffer away from the eggs so that the buffer can be removed easily with a transfer pipet without pulling up eggs.

10. A smooth wide-bore pipet is needed to transfer the eggs from the Petri dish to the tubes because the eggs are very fragile. To create a reusable polished cut-off Pasteur pipet, scratch the side of Pasteur pipet with a diamond tipped pen or similar object and carefully break it off leaving a 3–4 mm opening. Then flame polish the end of the pipet with a Bunsen burner until the edges are smooth and not sharp. It is a good idea to make several of these in case one breaks during the prep.

11. Do not pipet the extract to measure!! Measure volume of extract by placing it in a graduated tube or by comparing the volume of extract to another tube containing an equivalent volume of water.

12. Extract is very sensitive to dilution. Thus, it is desirable to have labeled tubulin at high concentrations with ≥ 0.5 labeling stoichiometry. Similarly, we found it ideal to add proteins back at $1/20^{th}$ of the extract volume for live imaging rather than $1/10^{th}$ the volume of the extract for typical reactions that are spun down onto coverslips.

13. A good CSF extract will have asters and some half spindles at 20 minutes, half spindles and spindles at 40 minutes, and mostly bipolar spindles at 60 minutes. The sperm nuclei will maintain their S-shape until assembled into half spindles and spindles when they will become more oblong. An activated extract will either have microtubules throughout the cytoplasm without any defined organization or will be devoid of microtubules with sperm nuclei that become rounded or sausage shaped. When cycling an extract into interphase, the S-shaped sperm nuclei will round up into round nuclei, and the cytoplasm will be mostly devoid of microtubules. In general, spindles prepared from cycled

extracts are more robust and uniform in shape and are therefore preferred for imaging studies.

14. When making cycled extracts, it is important to remember to add twice as many nuclei per μl to the initial extract such that the concentration will be at 1X after the second CSF extract addition. Each tube should contain no more than 50–75 μl of extract to ensure sufficient oxygenation of the extract. We also find that it is helpful to add extra sperm for live imaging experiments to aid in finding structures.

15. For our system, the Leica SP8 was controlled by the Leica LASX v3.1.1 software, and the PicoQuant FLIM system was controlled by SymPhoTime 64 v1.6 software. The FLIM wizard in the LASX program was used to coordinate the microscope and the PicoQuant imaging. Settings for the imaging and FLIM were optimized empirically and kept constant from experiment to experiment. The settings represented here can serve as a starting point for optimization on your own system.

16. Accurate calculations of lifetimes from the decay of multi-exponential donors, like CyPet, require high photon counts. To achieve this, the image size for FLIM can be down-sized relative to the confocal images to aggregate the photons. Additionally, the image can be binned during the analysis to further concentrate the photon counts per pixel. Both downsizing the image and binning sacrifices the resolution of the FLIM image, which needs to be taken into consideration if the question being asked in the experiment involves localization to small structures in the spindle/cell.

17. Thresholding makes the background blue in the SymPhoTime software, which is the color of your fastest/shortest lifetime on the rainbow color scale. This may not be desirable. Alternatively, designating ROIs makes the background grey, which is distinct from the rainbow color scale of lifetimes. SymphoTime has several ROI tools (Paint, Free, Rectangle, Ellipse, and Magic Wand) that are available through a dropdown menu, which appears by right clicking on the image.

18. Once you have recalculated the lifetimes based on the binning and thresholding or ROI selection, deselect the photon counts image in the dialog box between the overlay image and Lifetime Histogram by unchecking the “Active” box next to the “Events[Cnts]” overlay option (Fig. 2A). This will display only the FLIM image. If the object or area of interest is selected properly, the thresholded area will appear blue in the Fast Lifetime image whereas the omitted area from the ROI will appear grey. If needed, adjust threshold or ROI and re-calculate the FastFLIM before fitting.

19. To calculate the lifetimes in a FLIM image, the appropriate model and parameters need to be determined. This will require multiple rounds of initial fitting to determine the best parameters based on the X^2 of the fit.

20. The n-Exponential Reconvolution fit is preferable because it considers the complete decay curve and should be chosen when the fitted lifetimes are significantly longer than the instrument response function (IRF). Note that the left-hand blue boundary is positioned before the rise of the decay trace (Fig. 2C), which includes the IRF. The n-Exponential Tail fit may give a better fit if the lifetimes are within the IRF window. For this fit, the left-hand blue boundary is shifted to the start of the photon decay trace.

21. To improve the fitting accuracy, use as many pixels as possible that contain a good number of photon counts. Thus, the whole image or regions of interest are preferred rather than individual pixels or small areas.

22. While a donor may be predicted to have a 1- or 2-exponential decay, in practice the decay of the donor may best fit an exponential decay with additional parameters. For example, EGFP is predicted to be mono-exponential, and CyPet is predicted to be bi-exponential, but with the PicoQuant Pico Harp 300 TSCPC system and SymPhoTime software, they best fit 2- and 3-exponential decay models respectively. Also Note that the best fit model is the model with the least number of parameters that no longer significantly changes the goodness of fit.

23. The $\tau_{Av/Int}$[ns] is the intensity weighted average lifetime of the individual model lifetime parameters and equals the average amount of time the fluorophore remains in its excited state after the onset of excitation. The $\tau_{Av/Amp}$[ns] is the amplitude weighted average lifetime of the individual model lifetime parameters and is proportional to the steady state intensity. The $\tau_{Av/Amp}$[ns] is the preferred lifetime to report when comparing lifetimes between conditions.

24. To generate quality confocal and FLIM images of the same size for publication, scale the intensities of your confocal images appropriately in software like Fiji and then save as 8-bit images. Next open the images in Photoshop, change image resolution to 300 pixel/in, and Note the image dimensions. Open the “Bitmap” (.bmp) FLIM image in Photoshop, change resolution to 300 pixel/in, change image dimensions to the same dimensions as the confocal image size, and resample image as “Nearest Neighbor (hard edges)” to preserve the FLIM color scale look. To modify the confocal and FLIM images identically, e.g. cropping and/or rotating, add a layer to the FLIM image in Photoshop and then copy and paste the desired confocal image into the new layer. Save the combined image with a new name and then modify as desired.

25. To be opened in ImageJ, the ASCII file needs to be modified by deleting the descriptive lines using a text editor and then imported using the File/Import/Text Image command. If the counts or lifetime image is exported as a TIFF image, it will open directly in ImageJ, but the values will be in seconds and not nanoseconds.

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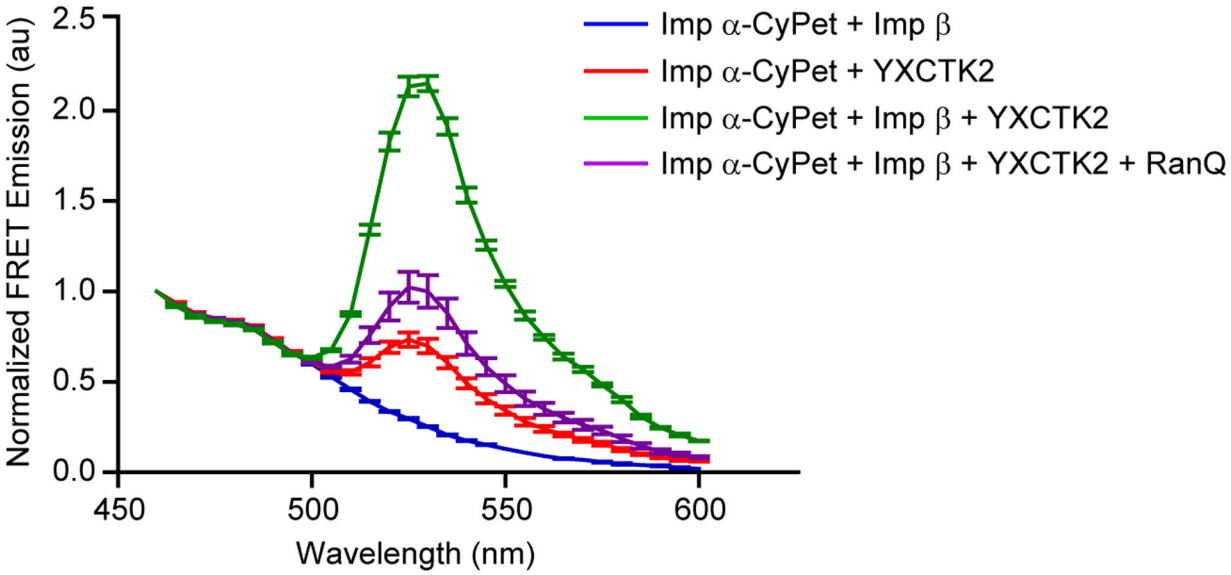

Fig. 1.

Example spectral data from three independent experiments graphed as the average normalized emission fluorescence ± SD for importin α-CyPet (imp α-CyPet) + imp β (donor only), imp α-CyPet + YPet-XCTK2 (YXCTK2) (no imp β control), and imp α-CyPet + imp β + YXCTK2 ± RanQ69L (RanQ). This example shows that the FRET biosensor, imp α-CyPet/imp β + YXCTK2, has reduced FRET in the presence of the analyte RanQ69L.

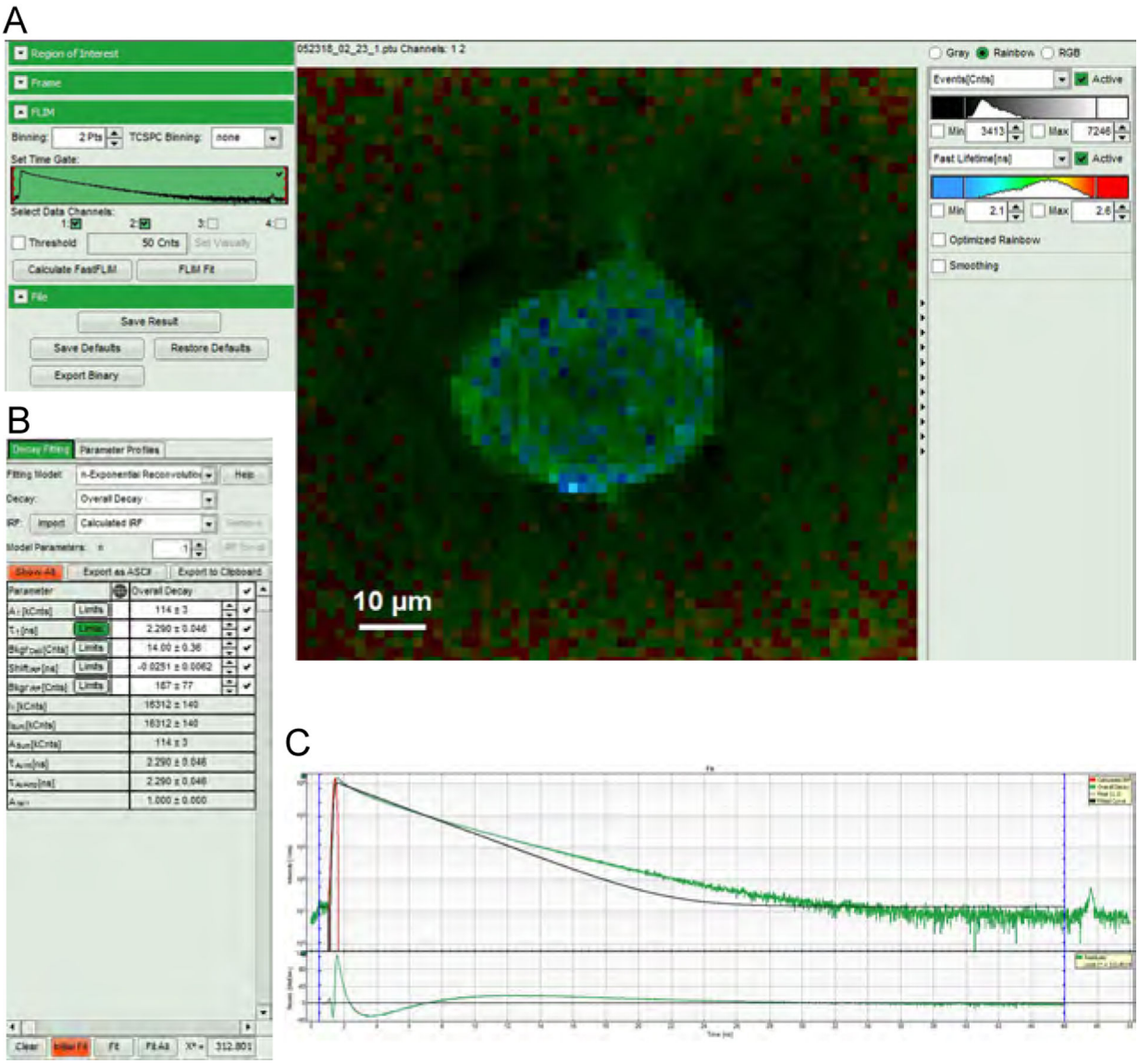

Fig. 2.

A, Screenshots of the FLIM (Fast Lifetime[ns]) and photon count (Events[Cnts]) overlay image opened in the image display window; **B**, Decay Fitting window with model parameter menus and results table; and **C**, decay data (Intensity [Cnts]) and residual (Resids. [StdDev.]) graphs.

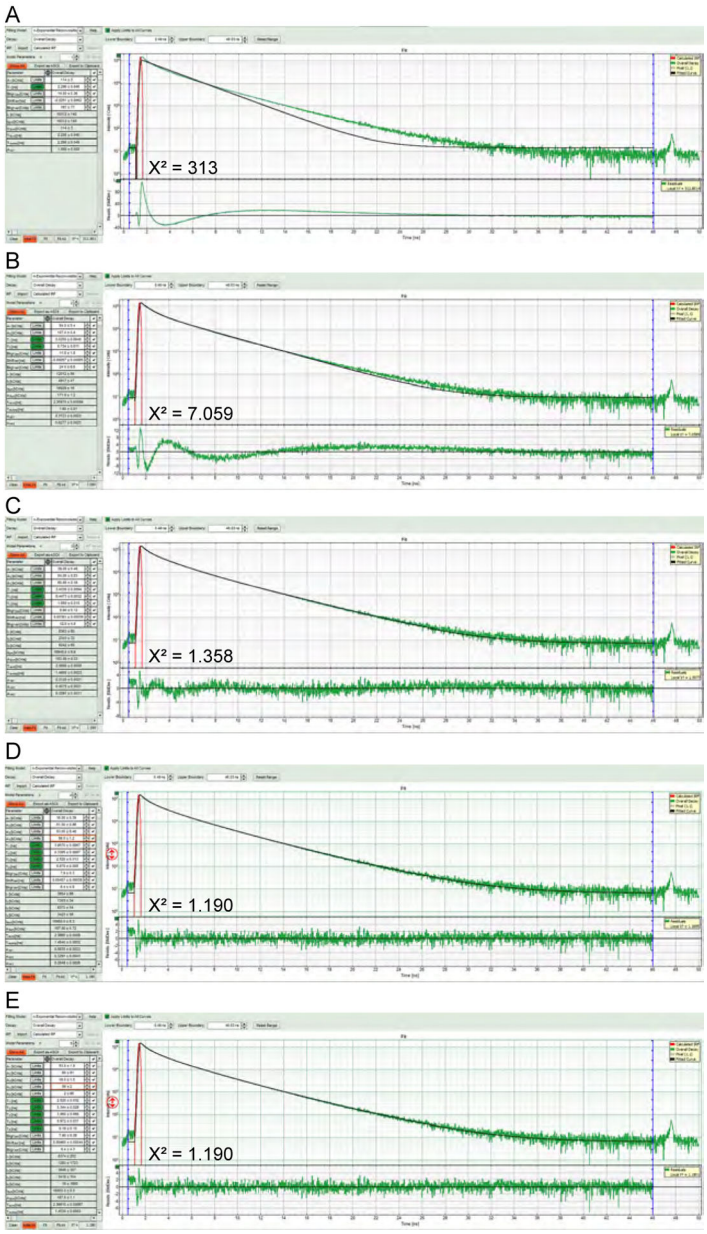

Fig. 3.

Screenshots of the decay data with different n-exponential reconvolution model fits and their corresponding X^2 values. The single (n=1) exponential (**A**) and double (n=2) exponential (**B**) reconvolution models do not fit the data well as their X^2 values are much greater than 1. A 3-exponential model (**C**) fits the data much better with a X^2 value close to 1. Additional model parameters (n=4 or 5) do not improve the X^2 value sufficiently to justify using these in the decay model (**D** and **E**).

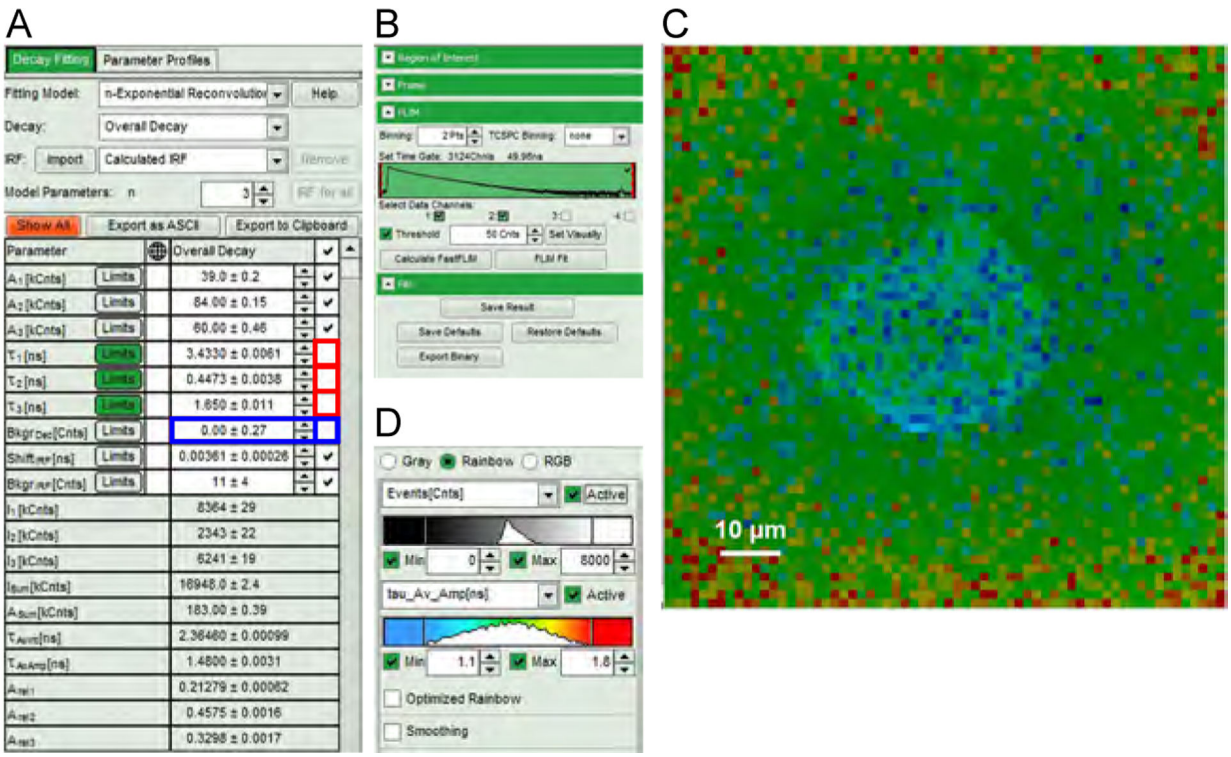

Fig. 4.

Screenshots of the display window for the calculated lifetimes for the 3-exponential reconvolution fit. **A**, Decay Fitting window for selecting the model parameters and fixing the best fit lifetimes and background for the lifetime calculation by selecting the “FLIM Fit” button in **B**. The calculated lifetimes are displayed in the overlay image window (**C**) and can be scaled by typing in the desired minimum and maximum counts or lifetimes in the appropriate boxes in **D**. Once scaled, save the results using the “Save Results” button in **B**.

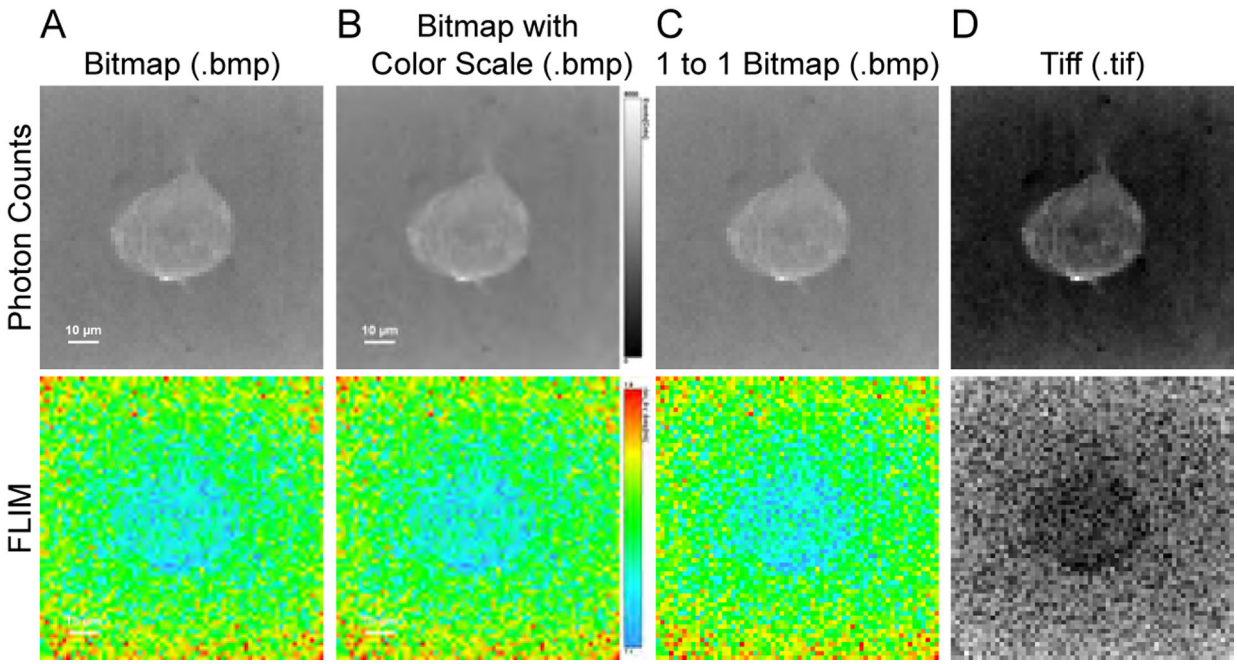

Fig. 5.

The photon count and FLIM images can be saved individually or as an overlay in a variety of formats. The Bitmap (**A**), Bitmap with Colorscale (**B**), and 1 to 1 Bitmap (**C**) preserve the rainbow color scale of the FLIM image, whereas the Tiff format (**D**) does not. However, the tiff format is useful for quantification in other programs.