Supporting Information

Live-Cell Fluorescence Lifetime Multiplexing Using Synthetic Fluorescent Probes
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Table of Contents
Supporting Methods S3-5
Supporting Figures
Figure S1: Phasor plots of the 18 synthetic probes tested. S6
Figure S2: Spectral characterization of synthetic probes used for fluorescence lifetime multiplexing by confocal microscopy. S7
Figure S3: Exemplary crosstalk characterization of SPY555-DNA and SPY555-Tubulin in U-2 OS cells. S8
Figure S4: Live-cell fluorescence lifetime multiplexing using synthetic probes in different spectral regions. S9-10
Figure S5: Live-cell fluorescence lifetime multiplexing using synthetic probes in the orange spectral region. S11-12
Figure S6: Live-cell fluorescence lifetime multiplexing in different cell lines. S13
Figure S7: Multiplexing synthetic probes and self-labeling protein tags. S14-15
Figure S8: Multiplexing synthetic probes and HaloTag in the red channel. S16
Figure S9: Four species images combining fluorescence lifetime multiplexing in the green and orange spectral channel. S17
Figure S10: Four species images combining fluorescence lifetime multiplexing in the green and red spectral channel. S18
Figure S11: Six species images combining fluorescence lifetime multiplexing in the green, orange, and NIR spectral channel. S19
Figure S12: Dynamic six species images combining fluorescence lifetime multiplexing in the green, orange, and NIR spectral channel. S20
Figure S13: Eight species image combining fluorescence lifetime multiplexing in the green, orange, red, and NIR spectral channel. S21
Supporting Figure S14: Flow cytometry plots and statistical analysis of cell viability. S22

Supporting Tables
Table S1: Structures of all 18 synthetic probes tested. S23-26
Table S2: Comparison of intensity weighted fluorescence lifetimes (τ) of different synthetic probes in three different cell lines.

Table S3: Crosstalk analysis for all eight probe pairs.

Table S4: Comparison of intensity weighted fluorescence lifetimes (τ) of MaP555-BG on SNAPf-tag fused to different proteins of interest.

Table S5: Plasmids used and generated as well as the stable cell lines derived thereof.

Table S6: Fluorescence microscopy data acquisition parameters.

References
Supporting Methods

Plasmids: A pcDNA5/FRT/TO vector (ThermoFisher Scientific) was used for transient expression and stable cell line establishment in mammalian cells. HaloTag7-Pro30-SNAP-tag was fused to CalR/KDEL for ER localization. SNAPf-tag was fused to CEP41, H2B, TOMM20, NES, COX8, CalR/KDEL, β4Gal-T1, LAMP1, SKL, Lyn11, Ig-κ/-PDGFR, or Lifeact for expression in mammalian cells. Cloning was performed by Gibson assembly\(^5\). DNA was subsequently electroporated in *E. coli*10G (Lucigene) and plated on agar Lysogenic Broth plates with 100 μg/mL ampicillin and incubated at 37 °C overnight. CEP41 (Addgene plasmid # 135446)\(^2\), TOMM20 (Addgene plasmid # 135443)\(^2\), H2B (Addgene plasmid # 135444)\(^2\), COX8 (Addgene plasmid # 113916)\(^3\), Ig-κ/-PDGFR\(^6\), and SNAPf (Addgene plasmid # 167271)\(^5\) were available in house and used as template plasmids. mCherry-LaminB1-10 was a gift from Michael Davidson (Addgene plasmid # 55069), pAAV_hsyn_NES-his-CAMPARI2-F391W-WPRE-SV40 was a gift from Eric Schreiter (Addgene plasmid # 101061)\(^6\), pmTurquoise2-Golgi was a gift from Dorus Gadella (Addgene plasmid # 36205)\(^7\), and LAMP1-mGFP was a gift from Esteban Dell’Angelica (Addgene plasmid # 34831)\(^8\). For more information see Supporting Table S5. Plasmids generated in this work have been deposited on Addgene and the accession codes are listed in Supporting Table S5.

Stable cell line establishment: The Flp-In™ T-REx™ System (ThermoFisher Scientific) was used to generate stable cell lines exhibiting inducible expression of CalR-HaloTag7-Pro30-SNAP-tag-KDEL or CalR-HaloTag7-KDEL-T2A-Lyn11-SNAPf. Briefly, pCDNA5-FRT-TO-GOI and pOG44 were co-transfected into the host cell line U-2 OS FlpIn TReX\(^9\). Homologous recombination between the FRT sites in pCDNA5-FRT-TO-GOI and the host cell chromosome, catalyzed by the Flp recombinase expressed from pOG44, produced the U-2 OS FlpIn TReX cells expressing stable GOI. Selection was performed using 100 μg mL\(^{-1}\) hygromycin B (ThermoFisher Scientific) and 15 μg mL\(^{-1}\) blasticidin (ThermoFisher Scientific).

Cell culture and transfection: U-2 OS (ATCC), HeLa (ATCC), HEK 293 (ATCC), U-2 OS Flp-In TReX CalR-HaloTag7-Pro30-SNAP-tag-KDEL, and U-2 OS Flp-In TReX CalR-HaloTag7-KDEL-T2A-Lyn11-SNAPf cells were cultured in high-glucose phenol red free DMEM (Life Technologies) medium supplemented with GlutaMAX (Life Technologies), sodium pyruvate (Life Technologies) and 10% FBS (Life Technologies) in a humidified 5% CO\(_2\) incubator at 37 °C. Cells were split every 3–4 days or at confluency. Cell lines were regularly tested for mycoplasma contamination. Cells were seeded on 8 well glass bottom dishes (Ibidi) or 96 well plates (Eppendorf) three to one day before imaging. U-2 OS Flp-In TReX cells were induced using 100 μg ml\(^{-1}\) doxycycline (SigmaAldrich) for 24–48 h before imaging. Transient transfections were performed using Lipofectamine™ 2000 reagent (Life Technologies) according to the manufacturer’s recommendations: the DNA (0.3 μg) was mixed with OptiMEM I (10 μL, Life Technologies) and Lipofectamine™ 2000 (0.75 μL) was mixed with OptiMEM I (10 μL, 8 well). The solutions were incubated for 5 min at room temperature, then mixed and incubated for an additional 20 min at room temperature. The prepared DNA-Lipofectamine complex was added to one of the wells in an 8 well glass bottom dish with cells at 50–70% confluence. After 12 h incubation in a humidified 5% CO\(_2\) incubator at 37 °C the medium was changed to fresh medium. The cells were incubated under the same conditions for 24–48 h before imaging.
**Labeling and sample preparation:** Cells were labeled with the respective fluorophores:

- **MitoTracker-Green:** 200 nM, 30 min, 37 °C, one wash
- **LysoTracker-Green:** 75 nM, 30 min, 37 °C, one wash
- **SPY555-Actin:** 500 nM, 1 h, 37 °C, no wash
- **SPY555-DNA:** 1 μM, 1 h, 37 °C, no wash
- **SPY555-Tubulin:** 500 nM, 1 h, 37 °C, no wash
- **MaP555-CA:** 500 nM, 1 h, 37 °C, one wash
- **MaP555-BG:** 2 μM, 1 h, 37 °C, two washes
- **MitoTracker-Orange:** 500 nM, 30 min, 37 °C, one wash
- **BioTracker 560 Orange Lysosomes:** 10 μg of the fluorophore were diluted in 200 μL DMSO and a 1:1,000 dilution was used for labeling, 30 min, one wash
- **Rodamine B:** 1 μM, 30 min, 37 °C, one wash
- **MitoTracker-Red:** 500 nM, 30 min, 37 °C, one wash
- **LysoTracker-Red:** 75 nM, 30 min, 37 °C, one wash
- **SPY620-Actin:** 1 μM, 1 h, 37 °C, no wash
- **SPY620-DNA:** 500 nM, 1 h, 37 °C, no wash
- **MaP618-CA:** 500 nM, 1 h, 37 °C, one wash
- **SiR-DNA:** 500 nM, 1 h, 37 °C, no wash
- **SiR-Tubulin:** 500 nM, 1 h, 37 °C, no wash
- **SiR-Actin:** 500 nM, 1 h, 37 °C, no wash
- **SPY700-DNA:** 1 μM, 1 h, 37 °C, no wash
- **SiR700-Tubulin:** 500 nM, 1 h, 37 °C, no wash
- **SiR700-Actin:** 500 nM, 1 h, 37 °C, no wash

in phenol-red free DMEM medium supplemented with GlutaMAX, sodium pyruvate, and 10% FBS (all Life Technologies), washed with the same medium as indicated above. If no-wash and wash probes were used simultaneously, labeling with wash probes was performed first followed by labeling with no-wash probes. Imaging was performed in the same medium.

**Fluorescence excitation and emission spectra:** Fluorescence excitation and emission spectra of synthetic probes were measured in living U-2 OS cells by confocal microscopy. Acquisition settings were as follows:

- **Green excitation:** excitation between 470-534 nm in 2 nm steps; collection at 555 – 600 nm.
- **Green emission:** excitation at 470 nm and collection between 480–567 nm in 3 nm steps with a bandwidth of 10 nm.
- **Orange excitation:** excitation between 475–575 nm in 2 nm steps; collection at 595–700 nm.
- **Orange emission:** excitation at 520 nm and collection between 530–617 nm in 3 nm steps with a bandwidth of 10 nm.
- **Red excitation:** excitation between 550–650 nm in 2 nm steps; collection at 670–780 nm.
- **Red emission:** excitation at 600 nm and collection between 610–697 nm in 3 nm steps with a bandwidth of 10 nm.
- **Except LysoTracker-Red excitation:** excitation between 500–600 nm in 2 nm steps; collection at 620–700 nm.
- **LysoTracker-Red emission:** excitation at 540 nm and collection between 550–647 nm in 3 nm steps with a bandwidth of 10 nm.
- **NIR excitation:** excitation between 630–670 nm in 2 nm steps; collection at 720–780 nm.
NIR emission: excitation at 670 nm and collection between 690–768 nm in 3 nm steps with a bandwidth of 10 nm.

**Cell viability test**
U-2 OS cells stably expressing CalR-HaloTag7-KDEL-T2A-Lyn11-SNAPf (induced with 100 μg ml⁻¹ doxycycline) were transiently transfected with a plasmid expressing Golgi-HaloTag11 one day prior labeling. The cells were labeled with MaP618-CA (0.5 μM), MaP555-BG (2.0 μM) for 1 h whereby after 30 min LysoTracker-Green (75 nM) and MitoTracker-Green (200 nM) were added. Subsequently the cells were washed with growth medium and then labeled with SPY555-Actin (500 nM), SiR700-Tubulin (500 nM), and SPY700-DNA (1 μM) for ~20 h. The medium was collected from unlabeled and labeled cells. Additionally, the adherent cells were trypsinised and also added into the same tube to collect all dead and live cells. After spinning the cells at 300 g for 3 min, the cell pellet was resuspended in 2% FBS/PBS containing 1 μM SYTOX Blue dead cell stain (ThermoFisher). Technical triplicates were measured at the BD LSRFortessa™ X-20 Flow Cytometer (Becton, Dickinson and Company) using the software BD FACSDiva™. The following settings were used to detect the various channels:

| Channel                        | Laser | LP Filter | BP Filter |
|--------------------------------|-------|-----------|-----------|
| SYTOX Blue                     | 405nm |           | 450/50    |
| LysoTracker-Green/MitoTracker-Green | 488nm | 505LP     | 530/30    |
| MaP555-BG/ SPY555-Actin        | 561nm | 570LP     | 586/15    |
| MaP618-CA/ SiR700-Tubulin/ SPY700-DNA | 640nm | 665LP     | 670/30    |

For flow cytometry analysis, 10,000 events were recorded and analysed using FlowJo 10.7.1.

**Software and image processing:** All images were processed in LASX and ImageJ/Fiji¹⁰,¹¹ unless otherwise stated. Excitation and emission spectra were visualized using OriginLab¹².
Supporting Figure S1: Phasor plots of the 18 synthetic probes tested. A Green spectral region. B Red spectral region. C Orange spectral region. D Far-red spectral region. E NIR spectral region.
Supporting Figure S2: Spectral characterization of synthetic probes used for fluorescence lifetime multiplexing by confocal microscopy. A-E Normalized emission and excitation spectra of green probes (A), red probes (B), orange probes (C), orange probes (D), and NIR probes (E). For comparison of the orange probes the spectra of SPY555-Tubulin are given both in (C) and (D). The excitation maximum of the NIR probes (~680 nm) could not be measured as the laser only reached 670 nm. The spectra were normalized to 0.5 instead of 1.0.
Supporting Figure S3: Exemplary crosstalk characterization of SPY555-DNA and SPY555-Tubulin in U-2 OS cells. Singly stained samples were used for the characterization and their phasor plots overlaid to define the position of the cluster circles. The two individual images were then separated resulting in the images of the pure species (SPY555-DNA – SPY555-DNA and SPY555-Tubulin-SPY555-Tubulin) as well as the images showing the crosstalk into the other lifetime component (SPY555-DNA-SPY555-Tubulin and SPY555-Tubulin-SPY555-DNA). These images were used to quantify the crosstalk (Supporting Table S3). To visualize the crosstalk and its relation to the pure species, the separated images were given in two different brightness scaling. Scale bars, 10 μm.
Supporting Figure S4: Live-cell fluorescence lifetime multiplexing using synthetic probes in different spectral regions. A Green spectral region labeling U-2 OS cells with MitoTracker-Green and LysoTracker-Green. B Red spectral region labeling U-2 OS cells with SPY620-DNA and SPY620-Actin. C-D NIR spectral region labeling U-2 OS cells with SPY700-DNA and SiR700-Actin (C) or SPY700-DNA and SiR700-Tubulin (D). The composite, the FastFLIM image with the respective color scale, the total fluorescence intensity, the two individual separated species as well as the corresponding wavelet-filtered phasor plot used for separation are given. Species separation was achieved
using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S5: Live-cell fluorescence lifetime multiplexing using synthetic probes in the orange spectral region. A-D U-2 OS cells labeled with SPY555-DNA and BioTracker 560 Orange Lysosome (A), SPY555-DNA and SPY555-Actin (B), SPY555-DNA and RhodamineB (C), or SPY555-DNA and SPY555-Tubulin (D). The composite, the FastFLIM image with the respective color scale, the total fluorescence intensity, the two individual separated species as well as the corresponding wavelet-filtered phasor plot used for separation are given. Species separation
was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 µm.
Supporting Figure S6: Live-cell fluorescence lifetime multiplexing in different cell lines. A-D HEK 293 (A-B) or HeLa cells (C-D) labeled with SPY555-DNA and BioTracker 560 Orange Lysosome (A, C) or SPY555-DNA and SPY555-Tubulin (B, D). The composite, the FastFLIM image with the respective color scale, the total fluorescence intensity, the two individual separated species as well as the corresponding wavelet-filtered phasor plot used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S7: Multiplexing synthetic probes and self-labeling protein tags. **A-D** U-2 OS cells stably expressing the endoplasmic reticulum marker calreticulin (CalR) as a HaloTag7-SNAP-tag fusion additionally fused to a KDEL targeting peptide were labeled with MaP555-CA (**A-B**) or MaP555-BG (**C-D**) and SPY555-DNA (**A, C**) or SPY555-Actin (**B, D**). The composite, the FastFLIM image with the respective color scale, the total fluorescence intensity, the two individual separated species as well as the corresponding wavelet-filtered phasor plot used for
Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
**Supporting Figure S8**: Multiplexing synthetic probes and HaloTag in the red channel. A U-2 OS cells stably expressing CalR-HaloTag7-SNAP-tag-KDEL were labeled with MaP618-CA and SPY620-DNA. The composite, the FastFLIM image with the respective color scale, the total fluorescence intensity, the two individual separated species as well as the corresponding wavelet-filtered phasor plot used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S9: Four species images combining fluorescence lifetime multiplexing in the green and orange spectral channel. A-B U-2 OS cells were labeled with MitoTracker-Green, LysoTracker-Green, SPY555-DNA, and either SPY555-Tubulin (A) or SPY555-Actin (B) and imaged in the two spectral channels giving access to four species images. The composite, the total fluorescence intensity, the four individual separated species as well as the corresponding wavelet-filtered phasor plots used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S10: Four species images combining fluorescence lifetime multiplexing in the green and red spectral channel. U-2 OS cells were labeled with MitoTracker-Green, LysoTracker-Green, SPY620-DNA, and SPY620-Actin and imaged in the two spectral channels giving access to a four species image. The composite, the total fluorescence intensity, the four individual separated species as well as the corresponding wavelet-filtered phasor plots used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S11: Six species images combining fluorescence lifetime multiplexing in the green, orange, and NIR spectral channel. A-C U-2 OS cells stably expressing CalR-HaloTag7-SNAP-tag-KDEL were labeled with MitoTracker-Green, LysoTracker-Green, MaP555-CA, SPY555-Actin, SPY700-DNA and SiR700-Tubulin and imaged in the three spectral channels giving access to six species images. The composite, the total fluorescence intensity, the six individual separated species as well as the corresponding wavelet-filtered phasor plots used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S12: Dynamic six species images combining fluorescence lifetime multiplexing in the green, orange and NIR spectral channel. U-2 OS cells stably expressing CalR-HaloTag7-SNAP-tag-KDEL were labeled with MitoTracker-Green, LysoTracker-Green, MaP555-CA, SPY555-Actin, SPY700-DNA, and SiR700-Tubulin and repeatedly imaged in the three spectral channels giving access to six species images. Composites at the four different time points, a zoom as well as the corresponding summed wavelet-filtered phasor plots used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S13: Eight species image combining fluorescence lifetime multiplexing in the green, orange, red, and NIR spectral channel. U-2 OS cells stably expressing CalR-HaloTag7-KDEL and Lyn11-SNAP-tag were transiently transfected with β-4-Gal-T1-HaloTag11 and labeled with MitoTracker-Green, LysoTracker-Green, MaP555-BG, SPY555-Actin, MaP618-CA, SPY700-DNA, and SiR700-Tubulin and imaged in the four spectral channels giving access to eight species images. The composite, the total fluorescence intensity, the eight individual separated species as well as the corresponding wavelet-filtered phasor plots used for separation are given. Species separation was achieved using the phasor approach by positioning the cluster circles on the phasor plot at the position of the pure species. Scale bars, 10 μm.
Supporting Figure S14: Flow cytometry plots and statistical analysis of cell viability. U-2 OS cells stably expressing CalR-HaloTag7-KDEL and Lyn11-SNAP-tag were transiently transfected with β-4-Gal-T1-HaloTag11 and labeled with MitoTracker-Green, LysoTracker-Green, MaP555-BG, SPY555-Actin, MaP618-CA, SPY700-DNA, and SiR700-Tubulin. After 20 h, all cells both dead (supernatant) and living (trypsination) were collected and labeled with SYTOX Blue. Flow cytometry analysis of four channels clearly showed labeled and unlabeled (Green, Orange, and the combined Red and NIR) as well as living and dead cells (SYTOX Blue). The difference in cell viability of labeled and unlabeled cells was not statistically significant (two sided t-test, p = 0.27, degrees of freedom = 4). The mean ± 95%CI and the three individual data points from technical replicates are given.
Supporting Tables

Supporting Table S1: Structures of all 18 synthetic probes tested. In addition, the vendor/reference and their excitation and emission maximum (as specified by the vendor) are given.

| Name               | Vendor/Reference | Ex/Em [nm] | Structure |
|--------------------|------------------|------------|-----------|
| MitoTracker-Green  | ThermoFisher Scientific | 490/516    | ![Structure](image1) |
| LysoTracker-Green  | ThermoFisher Scientific | 504/511    | ![Structure](image2) |
| SPY555-Actin       | Spirochrome      | 555/580    | Unpublished |
| SPY555-DNA         | Spirochrome      | 555/580    | Unpublished |
| SPY555-Tubulin     | Spirochrome      | 555/580    | Unpublished |
| MaP555-CA          |                  | 558/578    | ![Structure](image3) |
| MaP555-BG          |                  | 556/576    | ![Structure](image4) |
| Stain                  | Supplier          | Excitation/Emission | Structure |
|------------------------|-------------------|---------------------|-----------|
| MitoTracker-Orange     | ThermoFisher      | 554/576             | ![MitoTracker-Orange](image) |
| Rhodamine B            | TCI               | -                   | ![Rhodamine B](image) |
| BioTracker 560 Orange Lysosomes | Merck           | 532/560             | ![BioTracker 560 Orange Lysosome](image) |
| MitoTracker-Red        | ThermoFisher      | 579/599             | ![MitoTracker-Red](image) |
| LysoTracker-Red        | ThermoFisher      | 577/590             | ![LysoTracker-Red](image) |
| SPY620-Actin           | Spirochrome       | Unpublished         | ![SPY620-Actin](image) |
| SPY620-DNA             | Spirochrome       | Unpublished         | ![SPY620-DNA](image) |
| MaP618-CA              | ¹³                 | 618/635             | ![MaP618-CA](image) |
**Supporting Table S2:** Comparison of intensity weighted fluorescence lifetimes (τ) of different synthetic probes in three different cell lines. Fluorescence lifetimes were measured by FLIM (mean ± SEM., N = 4 field of views (FOVs) from 2 biological replicates, average N = 12 FOVs from 6 biological replicates).² bi-exponential fit, ³ tri-exponential fit, * tail fit (all others n-exponential reconvolution fit).

| Fluorophore                        | U-2 OS [ns] | HeLa [ns] | HEK 293 [ns] | Average [ns] |
|-----------------------------------|-------------|-----------|--------------|--------------|
|                                   | τ           | SEM       | τ            | SEM          | τ            | SEM          |
| **Green**                         |             |           |              |              |              |              |
| MitoTracker-Green                  | 0.96³       | 0.03      | 0.88³        | 0.03         | 0.96³        | 0.02         | 0.93          | 0.02         |
| LysoTracker-Green                  | 3.92²       | 0.06      | 3.78²        | 0.05         | 4.24²        | 0.01         | 3.98          | 0.06         |
| Rhodamine B                        | 2.26³       | 0.03      | 2.12³        | 0.01         | 2.30³        | 0.02         | 2.23          | 0.03         |
| **Orange**                        |             |           |              |              |              |              |              |              |
| BioTracker Orange Lysosome         | 2.30²       | 0.07      | 1.80³        | 0.13         | 2.04²        | 0.05         | 2.05          | 0.08         |
| SPY555-Actin                       | 1.79²       | 0.09      | 1.91²        | 0.02         | 1.88²        | 0.03         | 1.86          | 0.03         |
| SPY555-DNA                         | 3.00²       | 0.02      | 3.01²        | 0.04         | 3.01²        | 0.01         | 3.01          | 0.01         |
| SPY555-Tubulin                     | 2.00²       | 0.01      | 1.99²        | 0.01         | 2.02²        | 0.01         | 2.00          | 0.01         |
| **Red**                           |             |           |              |              |              |              |              |              |
| LysoTracker-Red                    | 3.88²       | 0.07      | 3.57²        | 0.09         | 3.63²        | 0.06         | 3.70          | 0.06         |
| SPY620-Actin                       | 2.93²       | 0.01      | 2.94²        | 0.01         | 2.88²        | 0.03         | 2.91          | 0.01         |
| SPY620-DNA                         | 3.62²       | 0.01      | 3.59²        | 0.01         | 3.61²        | 0.01         | 3.60          | 0.01         |
| **NIR**                           |             |           |              |              |              |              |              |              |
| SiR700-Actin                       | 1.90³       | 0.01      | 1.88²        | 0.01         | 1.93²        | 0.01         | 1.90          | 0.01         |
| SiR700-DNA                         | 2.28²       | 0.01      | 2.26²        | 0.01         | 2.30²        | 0.01         | 2.28          | 0.01         |
| SiR700-Tubulin                     | 1.90³       | 0.01      | 1.90³        | 0.01         | 2.02³        | 0.01         | 1.94          | 0.02         |
**Supporting Table S3:** Crosstalk analysis for all eight probe pairs. Images of singly stained U-2 OS cells were separated for the two species under question and the resulting images used to calculate crosstalk (mean ± SEM, \( N = 4 \) field of views (FOVs) from 2 biological replicates).

| Fluorophore                         | Pure | Crosstalk |
|-------------------------------------|------|-----------|
|                                     | %    | SEM       | %    | SEM       |
| MitoTracker-Green                   | 96.6 | 0.5       | 3.4  | 0.5       |
| LysoTracker-Green                   | 97.0 | 0.6       | 3.0  | 0.6       |
| Rhodamine B                         | 95.5 | 0.3       | 4.5  | 0.3       |
| SPY555-DNA                          | 90.7 | 1.0       | 9.3  | 1.0       |
| BioTracker Orange Lysosome          | 88.3 | 4.7       | 11.7 | 4.7       |
| SPY555-DNA                          | 85.8 | 1.6       | 14.2 | 1.6       |
| SPY555-Actin                        | 94.0 | 0.5       | 6.0  | 0.5       |
| SPY555-DNA                          | 94.5 | 0.7       | 5.5  | 0.7       |
| SPY555-Tubulin                      | 96.0 | 0.7       | 4.0  | 0.7       |
| SPY555-DNA                          | 93.8 | 0.7       | 6.2  | 0.7       |
| SPY620-Actin                        | 96.8 | 0.5       | 3.2  | 0.5       |
| SPY620-DNA                          | 91.6 | 0.7       | 8.4  | 0.7       |
| SiR700-Actin                        | 91.7 | 1.3       | 8.3  | 1.3       |
| SPY700-DNA                          | 95.2 | 1.3       | 4.8  | 1.3       |
| SiR700-Tubulin                      | 90.8 | 1.1       | 9.2  | 1.1       |
| SPY700-DNA                          | 86.6 | 1.9       | 13.4 | 1.9       |
Supporting Table S4: Comparison of intensity weighted fluorescence lifetimes (τ) of MaP555-BG on SNAPf-tag fused to different proteins of interest. H2B: nucleus, labeling histone 2B, LMNB1: nuclear lamina, labeling Lamin B1, Cytosol: untargeted (no fusion), NES: cytosol, fusion with a nuclear export signal, Tomm20: outer mitochondrial membrane, labeling the membrane receptor Tomm20, COX8: inner mitochondrial membrane, labeling the cytochrome c oxidase subunit 8, CalR-KDEL: endoplasmic reticulum, labeling calreticulin and additionally fused to a KDEL targeting peptide, β4-Gal-T1: Golgi Apparatus, labeling beta-1,4-galactosidase, LAMP1: lysosomes, labeling lysosome-associated membrane glycoprotein 1, SKL: peroxisomes, targeted via a peroxisomal targeting signal, Lyn11: inner leaflet of the plasma membrane, labeling tyrosine protein kinase Lyn11, Ig-κ-PDGF: outer plasma membrane, labeling platelet-derived growth factor receptor and additionally fused to an immunoglobulin kappa light chain leader sequence, CEP41: microtubules, labeling the microtubule-binding protein CEP41, lifeact: filamentous actin (F-actin), labeling the actin-binding peptide lifeact. Fluorescence lifetime was measured by FLIM (mean ± SEM, \( N = 4 \) FOVs from 2 biological replicates). Unless otherwise stated all mono-exponential fits. \(^2\) bi-exponential. Data for HaloTag7 from reference\(^{17}\).

| Fluorophore       | SNAP-tag [ns] | HaloTag7 [ns] |
|-------------------|---------------|---------------|
|                   | τ             | SEM           | τ              | SEM           |
| H2B               | 2.55\(^2\)    | 0.03          | 2.41           | 0.01          |
| LMNB1             | 2.57\(^2\)    | 0.04          | 2.38           | 0.00          |
| Cytosol           | 2.49\(^2\)    | 0.01          | 2.33           | 0.01          |
| NES               | 2.57\(^2\)    | 0.02          | 2.41\(^2\)    | 0.01          |
| Tomm20            | 2.51\(^2\)    | 0.02          | 2.38           | 0.00          |
| COX8              | 2.46\(^2\)    | 0.04          | 2.40\(^2\)    | 0.02          |
| CalR-KDEL         | 2.52\(^2\)    | 0.03          | 2.36           | 0.00          |
| β4Gal-T1          | 2.58\(^2\)    | 0.14          | 2.35           | 0.03          |
| LAMP1             | 2.62\(^2\)    | 0.02          | 2.38           | 0.00          |
| SKL               | 2.40\(^2\)    | 0.05          | 2.37           | 0.01          |
| Lyn11             | 2.37\(^2\)    | 0.05          | 2.37\(^2\)    | 0.01          |
| Ig-κ-PDGF         | 2.34\(^2\)    | 0.03          | 2.31\(^2\)    | 0.01          |
| CEP41             | 2.40\(^2\)    | 0.06          | 2.51\(^2\)    | 0.02          |
| Lifeact           | 2.48\(^2\)    | 0.03          | 2.36\(^2\)    | 0.01          |
| Average           | 2.49          | 0.06          | 2.38           | 0.01          |
**Supporting Table S5:** Plasmids used and generated as well as the stable cell line derived thereof.

| Name                        | Addgene# | Plasmid                  | Gene              | Localization tag | Addgene# | Stable cell lines |
|-----------------------------|----------|--------------------------|-------------------|------------------|----------|-------------------|
| pCDNA5/FRT/TO_H2B-SNAPf-tag | 181998   | pCDNA5/FRT/TO H2B-SNAPf-tag | 135444^2          | -                |          |                   |
| pCDNA5/FRT/TO_SNAPf-tag-LMNB1| 181999   | pCDNA5/FRT/TO SNAPf-tag-LMNB1| 55069             | -                |          |                   |
| pCDNA5/FRT/TO_SNAPf-tag     | 182000   | pCDNA5/FRT/TO SNAPf-tag   | 167271^5          | -                |          |                   |
| pCDNA5/FRT/TO_NES-SNAPf-tag | 182001   | pCDNA5/FRT/TO NES-SNAPf-tag| 101061^6          | -                |          |                   |
| pCDNA5/FRT/TO_TOMM20-SNAPf-tag| 182002  | pCDNA5/FRT/TO TOMM20-SNAPf-tag| 135443^2          | -                |          |                   |
| pCDNA5/FRT/TO_COX8-SNAPf-tag| 182003   | pCDNA5/FRT/TO COX8-SNAPf-tag| 113916^3          | -                |          |                   |
| pCDNA5/FRT/TO_CalR-SNAPf-tag-KDEL | 182004 | pCDNA5/FRT/TO CalR-SNAPf-tag-KDEL | synthetic         | -                |          |                   |
| pCDNA5/FRT/TO_β4Gal-T1-SNAPf-tag | 182005 | pCDNA5/FRT/TO β4Gal-T1-SNAPf-tag | 36205^7          | -                |          |                   |
| pCDNA5/FRT/TO_LAMP1-SNAPf-tag | 182006  | pCDNA5/FRT/TO LAMP1-SNAPf-tag| 34831^8          | -                |          |                   |
| pCDNA5/FRT/TO_SNAPf-tag-SKL | 182007   | pCDNA5/FRT/TO SNAPf-tag-SKL| synthetic         | -                |          |                   |
| pCDNA5/FRT/TO_Lyn11-SNAPf-tag | 182008 | pCDNA5/FRT/TO Lyn11-SNAPf-tag| synthetic         | -                |          |                   |
| pCDNA5/FRT/TO_Ig-k-κ-SNAPf-tag-PDGFR | 182009 | pCDNA5/FRT/TO Ig-k-κ-SNAPf-tag-PDGFR | 135446^2          | -                |          |                   |
| pCDNA5/FRT/TO_CEP41-SNAPf   | 182010   | pCDNA5/FRT/TO CEP41-SNAPf| 135446^2          | -                |          |                   |
| pCDNA5/FRT/TO_Lifeact-SNAPf-tag | 182011 | pCDNA5/FRT/TO Lifeact-SNAPf-tag| synthetic         | -                |          |                   |
| pCDNA5/FRT/TO_CalR-HaloTag7-P30-SNAPf-tag-KDEL | 182012 | pCDNA5/FRT/TO CalR-HaloTag7-P30-SNAPf-tag-KDEL | synthetic | U-2 OS Flp-In TREx |          |                   |
| pCDNA5/FRT/TO_CalR-HaloTag7-KDEL-T2A-Lyn11-SNAPf | -       | pCDNA5/FRT/TO CalR-HaloTag7-KDEL and Lyn11-SNAPf | synthetic | U-2 OS Flp-In TREx |          |                   |
| pCDNA5/FRT/TO_β4Gal-T1-HaloTag11 | 182013 | pCDNA5/FRT/TO β4Gal-T1-HaloTag11 | 36205^7          | -                |          |                   |
**Supporting Table S6:** Fluorescence microscopy data acquisition parameters. *See methods for details.

| Image  | Probe                    | Microscope | Excitation [nm] | Pixel dwell time [μs] | Pinhole [Airy Units] | Objective | Pixel size [pixels] | Emission [nm] | Comment          |
|--------|--------------------------|------------|-----------------|-----------------------|----------------------|-----------|--------------------|---------------|------------------|
| Fig. 1C| MitoTracker-Green        | SP8-FALCON | 489             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| LysoTracker-Green        | SP8-FALCON | 489             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| RhodB                    | SP8-FALCON | 550             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| BioTracker Orange Lysosome| SP8-FALCON | 550             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY555-Actin             | SP8-FALCON | 550             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY555-DNA               | SP8-FALCON | 550             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY555-Tubulin           | SP8-FALCON | 550             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY620-Actin             | SP8-FALCON | 615             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY620-DNA               | SP8-FALCON | 615             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SiR700-Actin             | SP8-FALCON | 670             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SPY700-DNA               | SP8-FALCON | 670             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig. 1C| SiR700-Tubulin           | SP8-FALCON | 670             | 3.16                  | 1                    | 40x1.10   | water              | 142           | 512x512          |
| Fig2A  | MitoTracker-Green LysoTracker-Green | SP8-FALCON | 489             | 2.30                  | 1                    | 40x1.10   | water              | 90            | 704x704          |
| Fig2B  | RhodB SPY555-DNA         | SP8-FALCON | 550             | 2.70                  | 1                    | 40x1.10   | water              | 102           | 600x600          |
| Fig2C  | SPY620-DNA               | SP8-FALCON | 615             | 4.06                  | 1                    | 40x1.10   | water              | 112           | 400x400          |
| Figure | Reporter | Fura Schneider | Object | Magnification | NA | Gain | Multiplication | Accumulation | Imaging Parameters |
|--------|----------|----------------|--------|---------------|-----|------|---------------|---------------|-------------------|
| Fig2D  | SPY620-Actin | SPY700-DNA, Sir700-Tubulin | SP8-FALCON | 670 | 8.49 | 1 | 40x1.10 water | 122 | 464x464 | 710-760 | 80 MHz | 10 line accumu. |
| Fig3B  | MitoTracker-Green, LysoTracker-Green | SP8-FALCON | 489 | 2.54 | 1 | 40x1.10 water | 114 | 840x840 | 510-540 | 80 MHz | 10 line accumu. |
| Fig3B  | Lyn11-SNAPf, Map555-BG, SPY555-Actin | SP8-FALCON | 550 | 2.54 | 1 | 40x1.10 water | 114 | 840x840 | 570-600 | 80 MHz | 10 line accumu. |
| Fig3B  | CalR-HaloTag7-KDEL, β4Gal-T1-HaloTag11, Map618-CA | SP8-FALCON | 615 | 2.54 | 1 | 40x1.10 water | 114 | 840x840 | 635-660 | 80 MHz | 10 line accumu. |
| S1A    | MitoTracker-Green | SP8-FALCON | 489 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 510-540 | 80 MHz | 500 photons |
| S1A    | LysoTracker-Green | SP8-FALCON | 489 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 510-540 | 80 MHz | 500 photons |
| S1B    | MitoTracker-Red | SP8-FALCON | 575 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 595-625 | 80 MHz | 500 photons |
| S1B    | LysoTracker-Red | SP8-FALCON | 575 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 595-625 | 80 MHz | 500 photons |
| S1B    | SPY620-Actin | SP8-FALCON | 615 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 635-700 | 80 MHz | 500 photons |
| S1B    | SPY620-DNA | SP8-FALCON | 615 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 635-700 | 80 MHz | 500 photons |
| S1C    | RhodB | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
| S1C    | BioTracker Orange Lysosome | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
| S1C    | SPY555-Actin | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
| S1C    | SPY555-DNA | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
| S1C    | SPY555-Tubulin | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
| S1C    | MitoTracker-Orange | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz | 500 photons |
|   |     |     |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|-----|-----|
| S1D | SiR-DNA | SP8-FALCON | 631 | 3.16 | 1 | 40x1.10 water | 569 | 512x512 | 650-700 | 80 MHz 1,000 4 line accumu. |
| S1D | SiR-Actin | SP8-FALCON | 631 | 3.16 | 1 | 40x1.10 water | 569 | 512x512 | 650-700 | 80 MHz 1,000 4 line accumu. |
| S1D | SiR-Tubulin | SP8-FALCON | 631 | 3.16 | 1 | 40x1.10 water | 569 | 512x512 | 650-700 | 80 MHz 1,000 4 line accumu. |
| S1E | SiR700-Actin | SP8-FALCON | 670 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 80 MHz 500 photons |
| S1E | SPY700-DNA | SP8-FALCON | 670 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 80 MHz 500 photons |
| S1E | SiR700-Tubulin | SP8-FALCON | 670 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 80 MHz 500 photons |
| S2A | MitoTracker-Green or LysoTracker-Green | SP8 | * | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | * | Spectra |
| S2B | MaP618-CA or SPY620-DNA or SPY620-Actin | SP8 | * | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | * | Spectra |
| S2B | LysoTracker-Red | SP8 | * | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | * | Spectra |
| S2C-D | MaP555-CA or MaP555-BG or SPY555-DNA or SPY555-Actin or SPY555-Tubulin or Rhodamine B or Bio Tracker Orange Lysosome | SP8 | * | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | * | Spectra |
| S2E | SPY700-DNA or SiR700-Actin or SiR700-Tubulin | SP8 | * | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | * | Spectra |
| S3 | SPY555-DNA | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| S3 | SPY555-Tubulin | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| S4A | MitoTracker-Green | SP8-FALCON | 489 | 2.30 | 1 | 40x1.10 water | 90 | 704x704 | 510-540 | 80 MHz |
|     | LysoTracker-Green          |     |     | 6 line accumu.         |
|-----|---------------------------|-----|-----|-----------------------|
| S4B | SPY620-DNA                | SPY620-Actin | SP8-FALCON | 615 | 4.06 | 1 | 40x1.10 water | 112 | 400x400 | 635-700 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S4C | SPY700-DNA                | Sir700-Actin | SP8-FALCON | 670 | 6.65 | 1 | 40x1.10 water | 123 | 592x592 | 710-760 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S4D | SPY700-DNA                | Sir700-Tubulin | SP8-FALCON | 670 | 8.49 | 1 | 40x1.10 water | 122 | 464x464 | 710-760 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S5A | Bio Tracker Orange        | Lysosome SPY555-DNA | SP8-FALCON | 550 | 2.85 | 1 | 40x1.10 water | 101 | 568x568 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S5B | SPY555-Actin              | SPY555-DNA | SP8-FALCON | 550 | 2.50 | 1 | 40x1.10 water | 101 | 648x648 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S5C | RhodB                     | SPY555-DNA | SP8-FALCON | 550 | 2.70 | 1 | 40x1.10 water | 102 | 600x600 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S5D | SPY555-Tubulin            | SPY555-DNA | SP8-FALCON | 550 | 1.56 | 1 | 40x1.10 water | 101 | 1040x1040 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S6A | Bio Tracker Orange        | Lysosome SPY555-DNA | SP8-FALCON | 550 | 3.69 | 1 | 40x1.10 water | 101 | 440x440 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S6B | SPY555-Tubulin            | SPY555-DNA | SP8-FALCON | 550 | 9.85 | 1 | 40x1.10 water | 100 | 400x400 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S6C | Bio Tracker Orange        | Lysosome SPY555-DNA | SP8-FALCON | 550 | 4.06 | 1 | 40x1.10 water | 101 | 400x400 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S6D | SPY555-Tubulin            | SPY555-DNA | SP8-FALCON | 550 | 7.24 | 1 | 40x1.10 water | 100 | 544x544 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S7A | SPY555-DNA                | CalR-Halo-KDEL MaP555-CA | SP8-FALCON | 550 | 2.25 | 1 | 40x1.10 water | 101 | 728x728 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S7B | SPY555-Actin              | CalR-Halo-KDEL MaP555-CA | SP8-FALCON | 550 | 1.70 | 1 | 40x1.10 water | 101 | 952x952 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S7C | SPY555-DNA                | CalR-SNAP-KDEL MaP555-BG | SP8-FALCON | 550 | 2.25 | 1 | 40x1.10 water | 101 | 720x720 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| S7D | SPY555-Actin              | CalR-SNAP-KDEL MaP555-BG | SP8-FALCON | 550 | 1.93 | 1 | 40x1.10 water | 101 | 840x840 | 570-600 | 80 MHz |
|     |                           |     |     |                       |     |     |               |     |         |         | 10 line accumu. |
| Sample | Description | Filter | Gain | Zoom | Exposure | Magnification | MSS | Accumulation |
|--------|-------------|--------|------|------|----------|---------------|-----|--------------|
| S8     | SPY620-DNA  | SP8-FALCON | 615  | 2.50 | 1        | 40x1.10 water | 114 | 648x648 635-700 | 80 MHz 10 line accumu. |
| S9A    | MitoTracker-Green | SP8-FALCON | 489  | 1.96 | 1        | 40x1.10 water | 90  | 824x824 510-540 | 80 MHz 10 line accumu. |
| S9A    | SPY555-DNA  | SP8-FALCON | 550  | 1.96 | 1        | 40x1.10 water | 90  | 824x824 570-600 | 80 MHz 10 line accumu. |
| S9B    | MitoTracker-Green | SP8-FALCON | 489  | 1.83 | 1        | 40x1.10 water | 90  | 888x888 510-540 | 80 MHz 10 line accumu. |
| S9B    | SPY555-DNA  | SP8-FALCON | 550  | 1.83 | 1        | 40x1.10 water | 90  | 888x888 570-600 | 80 MHz 10 line accumu. |
| S10    | MitoTracker-Green | SP8-FALCON | 489  | 1.89 | 1        | 40x1.10 water | 90  | 856x856 510-540 | 80 MHz 12 line accumu. |
| S10    | SPY620-DNA  | SP8-FALCON | 615  | 1.89 | 1        | 40x1.10 water | 90  | 856x856 635-700 | 80 MHz 12 line accumu. |
| S11A   | MitoTracker-Green | SP8-FALCON | 489  | 2.01 | 1        | 40x1.10 water | 90  | 776x776 510-540 | 80 MHz 10 line accumu. |
| S11A   | CalR-Halo-KDEL MaP555-CA SPY555-Actin | SP8-FALCON | 550  | 2.01 | 1        | 40x1.10 water | 90  | 776x776 570-600 | 80 MHz 10 line accumu. |
| S11A   | SPY700-DNA SiR700-Tubulin | SP8-FALCON | 670  | 2.01 | 1        | 40x1.10 water | 90  | 776x776 710-760 | 80 MHz 10 line accumu. |
| S11B   | MitoTracker-Green | SP8-FALCON | 489  | 1.46 | 1        | 40x1.10 water | 90  | 712x712 510-540 | 80 MHz 10 line accumu. |
| S11B   | CalR-Halo-KDEL MaP555-CA SPY555-Actin | SP8-FALCON | 550  | 1.46 | 1        | 40x1.10 water | 90  | 712x712 570-600 | 80 MHz 10 line accumu. |
| S11B   | SPY700-DNA SiR700-Tubulin | SP8-FALCON | 670  | 1.46 | 1        | 40x1.10 water | 90  | 712x712 710-760 | 80 MHz 10 line accumu. |
| S11C   | MitoTracker-Green | SP8-FALCON | 489  | 2.00 | 1        | 40x1.10 water | 90  | 1160x1160 510-540 | 80 MHz 10 line accumu. |
| S11C   | CalR-Halo-KDEL MaP555-CA SPY555-Actin | SP8-FALCON | 550  | 2.00 | 1        | 40x1.10 water | 90  | 1160x1160 570-600 | 80 MHz 10 line accumu. |
| S11C   | SPY700-DNA SiR700-Tubulin | SP8-FALCON | 670  | 2.00 | 1        | 40x1.10 water | 90  | 1160x1160 710-760 | 80 MHz 10 line accumu. |
| S12    | MitoTracker-Green | SP8-FALCON | 489  | 3.14 | 1        | 40x1.10 water | 122 | 680x680 510-540 | 80 MHz 10 line accumu. |
|   | Fluorochrome                          | # Channels | Filter Set | Scale Factor | Objective | ExC | 1st det | 2nd det | Speed | Gain | Photomultiplier |
|---|--------------------------------------|------------|------------|--------------|-----------|-----|---------|---------|-------|------|-----------------|
| S12 | CalR-Halo-KDEL MaP555-CA SPY555-Actin | 3.14       | 1          | 40x1.10 water | 122       | 680x680 | 570-600 | every 2 min |
| S12 | SPY700-DNA Sir700-Tubulin             | 3.14       | 1          | 40x1.10 water | 122       | 680x680 | 710-760 | every 2 min |
| S13 | MitoTracker-Green LysoTracker-Green   | 2.54       | 1          | 40x1.10 water | 114       | 840x840 | 510-540 | every 2 min |
| S13 | Lyn11-SNAPf MaP555-BG SPY555-Actin   | 2.54       | 1          | 40x1.10 water | 114       | 840x840 | 570-600 | every 2 min |
| S13 | CalR-HaloTag7-KDEL β4Gal-T1-HaloTag11 MaP618-CA | 2.54 | 1 | 40x1.10 water | 114 | 840x840 | 635-660 | every 2 min |
| Tab S2-3 | MitoTracker-Green | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 510-540 | 80 MHz 500 photons |
| Tab S2-3 | LysoTracker-Green | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 510-540 | 80 MHz 500 photons |
| Tab S2-3 | RhodB | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S2-3 | BioTracker Orange Lysosome | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S2-3 | SPY555-Actin | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S2-3 | SPY555-DNA | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S2-3 | SPY555-Tubulin | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S2 | LysoTracker-Red | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 595-625 | 80 MHz 500 photons |
| Tab S2-3 | SPY620-Actin | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 635-700 | 80 MHz 500 photons |
| Tab S2-3 | SPY620-DNA | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 635-700 | 80 MHz 500 photons |
| Tab S2-3 | Sir700-DNA | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 80 MHz |

36
| Tab S2-3 | SPY700-DNA | SP8-FALCON | 670 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 500 photons |
| Tab S2-3 | SiR700-Tubulin | SP8-FALCON | 670 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 710-760 | 80 MHz 500 photons |
| Tab S4 | H2B-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | SNAP-LMN1 MaP555-BG | SP8-FALCON | 550 | 5.64 | 1 | 40x1.10 water | 101 | 288x288 | 570-600 | 80 MHz 500 photons |
| Tab S4 | SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 500 photons |
| Tab S4 | NES-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 500 photons |
| Tab S4 | Tomm20-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | COX8-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | CalR-SNAP-KDEL MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | β4Gal-T1-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | LAMP1-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | SNAP-SKL MaP555-BG | SP8-FALCON | 550 | 7.69 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | Lyn11-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | Ig-k-SNAP-PDGFR MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | CEP41-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
| Tab S4 | Lifeact-SNAP MaP555-BG | SP8-FALCON | 550 | 3.16 | 1 | 40x1.10 water | 142 | 512x512 | 570-600 | 80 MHz 500 photons |
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