Supplementary Note 1: Comparison of SF with Conventional Sequential Imaging Approaches

Multicolor 4Pi-SMS imaging has been demonstrated by sequential imaging in a single section close to one of the coverslips and with fiducial markers in the past. Our salvaged fluorescence approach (SF), in contrast, can image structures far away from the coverslips or in whole cells, which is difficult by sequential imaging. Below, we discuss several advantages of our approach.

(1) Better dyes for the second color channel.
Many of the best-performing dyes for SMS, including AF647, are clustered in the far-red range\(^1\). As sequential imaging requires the dyes to be well-separated in spectrum, one cannot select a second dye in the far-red range in that imaging modality. As has been reported recently\(^2\), while Cy3B has been a preferred red dye in the past, CF568 shows better image quality in AF647-compatible imaging buffers. Based on this information as well as its use in other studies\(^3\), we therefore selected CF568 as a reference for sequential imaging in combination with AF647. For ratiometric imaging, as discussed in the main text, we used two dyes, CF660C and CF680. CF680 has been used in classical ratiometric imaging\(^4\) in combination with AF647. CF660C, while showing the best performance among the three dyes (CF568, CF660C and CF680), has been used much less widely in classical ratiometric imaging because it is spectrally very close to AF647. Our salvaged fluorescence approach, in contrast, allows us to image CF660C together with AF647 to achieve similar resolutions in both channels (Supplementary Fig. 4). Below we show additional data to prove that CF660C performs better than CF568. All samples were imaged at 100 fps with 7.5 kW/cm\(^2\) excitation intensity (642 nm or 560 nm) on the 4Pi-SMS microscope.

In comparison to CF568, CF660C shows more easily separable blinking events and does not bleach as quickly, has higher photon numbers per localization (median: 1825 vs 837) and consequently higher localization precisions (median: 8.7 nm vs 12.3 nm) (Supplementary Note Fig. 1).
Supplementary Note Figure 1: Comparing the performance of CF660C and CF568 in single-color imaging. (a) Example images of CF660C and CF568 at different time points. t = 0 s denotes the moment the excitation laser was turned on. (b) Distributions of photons per localization per frame from the data shown in (a). (c) Distributions of lateral localization precision. Representative images of three (a, CF660C) or two (a, CF568) independent experiments are shown.

Additionally, CF660C achieves a much higher localization density when imaging microtubules (localizations per μm of microtubule, mean ± s.d.: CF660C: 30904 ± 5664; CF568: 4213 ± 636) (Supplementary Note Fig. 2).

Supplementary Note Figure 2: Comparing the localization density of CF660C and CF568 in microtubules. (a,b) Histogram images of localization density. Colormap indicates the number of localizations per pixel (pixel size = 10 nm). The samples were labeled with the same concentration of primary and secondary antibodies and imaged at the same conditions. We used the single-label-for-STORM versions of both dyes, which have 1 dye per secondary antibody according to manufacturer specifications. (c) Example time traces of normalized localization numbers from the images shown in (a,b). Steps in these time traces were caused by manual adjustment of the 405-nm activation laser power. The black arrow denotes the time that the 405-nm laser reached the maximum power. CF568 was mostly photobleached at the end of the imaging session while CF660C still showed a high number of blinking molecules. (d) Localizations per μm of microtubule. 180,000 frames were recorded for CF568 and 360,000-432,000 frames were recorded for CF660C. Error bars show mean ± s.d. (n = 60 segments from 5 cells for CF660C, n = 48 segments from 4 cells for CF568). Representative images of two (a) or one (b) independent experiments are shown.
Furthermore, when sequentially imaging microtubules dual-labeled with AF647 and CF568, we were able to resolve the tubules as “hollow” with AF647 well but barely with CF568 (Supplementary Note Fig. 3a-c and cut outs on the top right). In contrast, the CF660C, when imaged simultaneously with AF647, provided a comparable resolution as AF647 (Supplementary Note Fig. 3d, and cut outs on the bottom right; same data shown in Supplementary Fig. 4i-k).

Supplementary Note Figure 3: Comparing the performance of CF660C and CF568 in two-color 4Pi-SMS imaging. (a,b) Two-color 4Pi-SMS image of dual-labeled microtubules by sequential imaging of AF647 and CF568, where AF647 was imaged before CF568. (c) Merged image of the two labels shown in (a,b). 20-nm thick x-y slices of the boxed regions are shown in the right panel. (d) Two-color 4Pi-SMS image of dual-labeled microtubules by simultaneously imaging AF647 and CF660C (the same data is shown in Supplementary Fig. 4i-k). 20-nm thick x-y slices of the boxed regions are shown in the right panel. Single-label-for-STORM secondary antibodies which have 1 dye per antibody were used for CF568 and CF660C. Representative images of one (a-c) or seven (d) independent experiments are shown.

(2) Negligible registration errors between color channels.

Channel registration is necessary for sequential multi-color imaging mainly due to chromatic aberrations. The most common way to register channels is to use the transformation information provided by imaging fiducial markers in each channel. This approach also works in 3D when imaging close to the coverslips. However, the registration becomes more difficult when imaging deep inside the sample and even more challenging when imaging whole cells due to the depth-dependent distortions in both lateral and axial directions and refractive index heterogeneities within the specimen. Therefore, it is challenging to precisely register multiple channels deep in the specimen even for single-objective microscopes. Channel registration is even more complicated in 4Pi-SMS systems when imaging deep in the specimen. Chromatic aberrations vary between the two objectives despite carefully picking a well-matched pair (based on magnification and chromatic shifts). In addition, the depth-dependent distortions vary from sample to sample as the distance between the two coverslips changes.
To show the depth-dependent effects, we imaged dual-labeled microtubules with our 4Pi-SMS system by sequentially imaging AF647 and CF568 using the same detection beam path with different detection bandpass filters in front of the camera (Supplementary Note Fig. 4). First, we imaged microtubules in both color channels at the surface of the coverslip and generated a 3D affine transformation. Then we applied the transformation to a different region of microtubules at the surface of the coverslip in the same sample. We found that the two labels were well aligned in 3D, which indicates that the transformation is almost the same when imaging at the same depth (Supplementary Note Fig. 4a). However, when we applied the same transformation to a region of microtubules about 2 μm above the coverslip, we found a ~30 nm shift in xy and ~40 nm shift in z (Supplementary Note Fig. 4b). This showed that the transformation varies when imaging at different depths.

To show the effects of sample-induced aberrations, we imaged dual-labeled mitochondria in the same way. Similarly, we generated a transformation from a region close to the coverslip. When it was applied to another region close to the coverslip, the two labels were well aligned in 3D (Supplementary Note Fig. 4c). However, when it was applied to a region above the nucleus (> 3 μm), we found much larger shifts along the z direction (Supplementary Note Fig. 4d, Correction 1). This is caused by the bottom objective needing to image through the nucleus which induced strong aberrations. When we applied a transformation from another region also above the nucleus, the axial shift was mostly corrected as the aberrations were mostly canceled out (Supplementary Note Fig. 4d, Correction 2). However, the residual shifts suggested that the local aberrations cannot be corrected in the same way.

In contrast, the two labels were well aligned without any channel registration when imaged using our salvaged fluorescence approach (Supplementary Note Fig. 4e).
Supplementary Note Figure 4: Channel registration in sequential and SF 4Pi-SMS imaging. (a) Example two-color image of microtubules close to the coverslip. Channel registration was done by applying a transformation from another image of microtubules close to the coverslip in the same sample. (b) Example two-color image of microtubules ~2 μm above the coverslip in the same sample. The same channel registration was applied as in (a). (c) Example two-color image of mitochondria close to the coverslip. Channel registration was done by applying a transformation from another image of mitochondria close to the coverslip in the same sample. Boxed regions 1 and 2 are shown in the right panels. (d) Example two-color image of mitochondria above the nucleus (> 3 μm). Correction 1: The same channel registration was applied as in (c). Correction 2: Channel registration was performed by applying a transformation generated from another image of mitochondria above the nucleus (> 3 μm) in the same sample. (e) Example two-color image of mitochondria above the nucleus (> 4 μm) labeled with both AF647 and CF660C and imaged with our salvaged fluorescence approach. No channel registration was applied. Representative images of one experiment are shown (a-e).

Overall, our data confirms that standard channel registration in sequential imaging on 4Pi-SMS microscopes is not sufficient for imaging deep in the samples. In contrast, 3D chromatic shifts in the salvaged fluorescence approach are negligible (see Fig. 1f,j).
(3) Potential for multi-color live-cell imaging.
Sequential imaging is not ideal for multi-color live-cell imaging as one needs to observe the dynamic behavior of multiple targets at the same time. Typically, multi-color SMS or single-particle-tracking (SPT) imaging in live cells is performed by using several fluorophores with well-separated spectra excited simultaneously by multiple illumination lasers. This approach has two drawbacks. First, the use of multiple illumination lasers increases the phototoxicity. In particular, it has been reported that light-induced photodamage is dramatically increased at shorter irradiation wavelength. Second, the shorter wavelength laser will cause significant photobleaching of the longer wavelength fluorophore, which reduces the total number of localizations. Please also see Supplementary Note 2 for further discussion on this topic.
Supplementary Note 2: Potential for Live-cell Imaging

With the ability to image multiple fluorophores excited with a single laser, our salvaged fluorescence approach has great potential for live-cell imaging. Fluorescent proteins are commonly used in live-cell imaging as they can be genetically encoded. To investigate the possible performance of our SF approach in live-cell imaging, we performed a simulation using three photoactivatable fluorescent proteins: Dendra2, PAmCherry1 and PAmKate (Supplementary Note Fig. 5). We selected these proteins because the separation between their peak emission wavelengths is about 20-30 nm and they have been used in classical ratiometric imaging before\(^9\). Assuming a realistic photon number distribution\(^10\) and a reasonable level of background and readout noise (see below), we found that our SF approach should allow for three-color imaging with cross-talk of less than 5%.

Supplementary Note Figure 5: Simulation of the performance of salvaged fluorescence imaging using fluorescent proteins. (a) The emission spectra of the fluorescent proteins Dendra2, PAmCherry1 and PAmKate and assumed collection windows for the salvaged and conventional fluorescence. The collection efficiencies in the conventional fluorescence channel are 47%, 73% and 93% for Dendra2, PAmCherry1 and PAmKate, respectively. The collection efficiencies in the salvaged fluorescence channel are 44%, 19% and 2% for Dendra2, PAmCherry1 and PAmKate, respectively. (b) Assumed photon number distribution used for all proteins in the simulation. (c) Scatter plot of simulated salvaged fluorescence versus conventional fluorescence signal levels of localized molecules. (d) Cross-talk and rejection rates for the three proteins on a logarithmic scale.
The drawback of fluorescent proteins is that they are not as photostable as organic dyes. We have synthesized a new photoactivatable version of JF669 that can be used together with PA-JF646. PA-JF646-SNAP-tag was synthesized as described previously\(^\text{11}\). PA-JF669-HaloTag ligand was synthesized in an analogous fashion from JF669 (ref. \(^\text{12}\)). Supplementary Note Fig. 6 shows that our approach is able to separate these two live-cell compatible far-red dyes (PA-JF646 and PA-JF669) with low cross-talk. The imaging was done in fixed cells (dye-labeling was done in live cells before fixation) as we do not yet have a perfusion chamber for live-cell imaging on our 4Pi-SMS microscope. Nevertheless, this shows the potential of our approach for two-color live-cell imaging with a single excitation laser (642 nm).

Supplementary Note Figure 6: Two-color 4Pi-SMS imaging of ER using two far-red PA-JF dyes. (a) Two-color image of KDEL (PA-JF669, top) and Rtn4 (PA-JF646, bottom) of a U2OS cell, dye-labeled in living cells using Halo-Tag and SNAP-Tag fusion proteins and fixed before imaging. Rainbow color denotes depth. (b) The binned 2D intensity histogram of PA-JF646 and PA-JF669 from more than 1 million localizations of each dye based on the same data shown in (a). The plot shows salvaged fluorescence intensity (y-axis) versus conventional fluorescence intensity (x-axis). The solid lines show the threshold
where the intensity value is 10% of each peak intensity. (c) Merged image of the two labels in (a). Inset shows the cross-section image along the yellow line. (d) Magnified image of the blue boxed-region in (c). Representative images of two independent experiments are shown (a,c-d).

**Simulation of the SF approach performance using fluorescent proteins**

To explore the potential of our SF approach for live-cell imaging, we performed a simulation using three photoactivatable fluorescent proteins: Dendra2, PAmCherry1 and PAmKate (Supplementary Note Fig. 5). For the simulation, we assumed an excitation laser at 552 nm and commercially available optical filters: a dichroic beamsplitter (Chroma T585lpvr), an emission filter for the conventional fluorescence (Semrock FF632/148) and an emission filter for the salvaged fluorescence (Semrock FF572/28). The simulation was based on the optical configuration shown in Supplementary Fig. 13c, where only one objective was used for fluorescence detection. The collection efficiencies in the conventional fluorescence channel were 47%, 73% and 93% for Dendra2, PAmCherry1 and PAmKate, respectively. The collection efficiencies in the salvaged fluorescence channel were 44%, 19% and 2% for Dendra2, PAmCherry1 and PAmKate, respectively. We based our simulation on published values of detected photon numbers for Dendra2 and PAmCherry1 (686 photons for Dendra2 and 706 photons for PAmCherry1)\(^1\). For PAmKate, we assumed the same value as for PAmCherry1 since they had been reported to be similar\(^2\) and simulated lognormal distributions with a mean of these photon numbers. These distributions were corrected for the difference in transmission of the used filters to simulate signal expectation values in the conventional and salvaged fluorescence channels. We then generated synthetic single-molecule images based on these photon number expectation values in each channel. In the conventional fluorescence channel, we added a background of 40 photons per pixel, applied shot noise to the sum of signal and background and added readout noise of 10 photons per pixel. In the salvaged fluorescence channel, the same process applied assuming 20 photons per pixel background and a readout noise of 5 photons per pixel. Under these conditions, we found that the three proteins exhibited well-separated populations in the photon distribution (Supplementary Note Fig. 5c). Cross-talk of less than 5% was achieved when about 15% molecules were rejected (Supplementary Note Fig. 5d). We expect the performance of two-color imaging to be slightly better than three-color imaging.

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