
Selective-plane-activation structured illumination microscopy

In the format provided by the
authors and unedited

Supplementary Note.1 : Light distribution for SPA-SIM

The electric field distribution $E(\mathbf{r})$ of light after passing through a lens is represented as an integral of plane waves as follows:

$$E(\mathbf{r}) = \int_{-\infty}^{\infty} P(\mathbf{r}') e^{i(\mathbf{k}(\mathbf{r}') \cdot \mathbf{r} - \omega t)} d\mathbf{r}', \quad (1)$$

where $\mathbf{r}$ and $\mathbf{r}'$ are the position vectors in the object space and pupil plane, respectively; $P(\mathbf{r})$ is the complex-valued pupil transmission function of the lens; t is the time; and $\mathbf{k}(\mathbf{r})$ and ω are the wave number vector and angular frequency, respectively. When illumination for activation is provided by a lens and objects including photoswitchable fluorophores are placed near the focus of the lens, the distribution of the activated fluorophores is represented as

$$c_{\text{act}}(\mathbf{r}) = |E_{\text{act}}(\mathbf{r})|^2 o(\mathbf{r}), \quad (2)$$

where $E_{\text{act}}(\mathbf{r})$ and $o(\mathbf{r})$ are the illuminating electric field distribution for the activation and distribution of the object, respectively. The activated fluorophores were then excited by a second illumination. The resultant distribution of the excited fluorophores is described as

$$c_{\text{ex}}^{nm}(\mathbf{r}) = |E_{\text{ex}}^{nm}(\mathbf{r})|^2 c_{\text{act}}(\mathbf{r}), \quad (3)$$

where $E_{\text{ex}}^{nm}(\mathbf{r})$ is the electric field distribution of the structured excitation illumination, and $n, m = 1 \cdots 3$ are the indices of the angle and phase of the illumination pattern for the SIM reconstruction. For two-dimensional (2D) SIM, the intensity distribution of the structured illumination is represented as

$$|E_{\text{ex}}^{nm}(\mathbf{r})|^2 = 1 + \frac{1}{2} e^{i(\mathbf{k}_n \cdot \mathbf{r} + \phi_{nm})} + \frac{1}{2} e^{-i(\mathbf{k}_n \cdot \mathbf{r} + \phi_{nm})}, \quad (4)$$

where $\mathbf{k}_n$ is the wave vector, and ϕ_{nm} is the phase of a sinusoidal illumination pattern with an angle-dependent global phase. Fluorescence emitted from the excited

fluorophores was measured using detection optics. The distribution of the detected fluorescence $I(\mathbf{r})$ was

$$I^{nm}(\mathbf{r}) = h_{\text{det}}(\mathbf{r}) \otimes \left[\left| E_{\text{ex}}^{nm}(\mathbf{r}) \right|^2 c_{\text{act}}(\mathbf{r}) \right], \quad (5)$$

where $h_{\text{det}}(\mathbf{r})$ is the point spread function (PSF) of the detection optics. By substituting (2) into (5), the relationship between the illumination patterns and the resultant image can be described as

$$I^{nm}(\mathbf{r}) = h_{\text{det}}(\mathbf{r}) \otimes \left[\left| E_{\text{ex}}^{nm}(\mathbf{r}) \right|^2 \left| E_{\text{act}}(\mathbf{r}) \right|^2 o(\mathbf{r}) \right]. \quad (6)$$

(6) indicates that the integration of sheet illumination for activation and structured illumination for excitation determines the distribution of excited fluorophores.

Image reconstruction of SIM for super-resolution

Conventional SIM reconstruction methods¹ are directly applicable to SPA-SIM, because the mechanism of image formation is the same as that of SIM. We now briefly explain the reconstruction procedure in the case of the 2DSIM.

SIM Reconstruction was performed based on the Fourier transform and matrix-based unmixing. The Fourier transform of (5) is represented as

$$\begin{aligned} \tilde{I}^{nm}(\mathbf{k}) &= \tilde{h}_{\text{det}}(\mathbf{k}) \left[\left| \tilde{E}_{\text{ex}}^{nm}(\mathbf{k}) \right|^2 \otimes \tilde{c}_{\text{act}}(\mathbf{k}) \right] \\ &= \tilde{h}_{\text{det}}(\mathbf{k}) \left[\tilde{c}_{\text{act}}(\mathbf{k}) + \frac{e^{-i\phi_{nm}}}{2} \tilde{c}_{\text{act}}(\mathbf{k} + \mathbf{k}_n) + \frac{e^{i\phi_{nm}}}{2} \tilde{c}_{\text{act}}(\mathbf{k} - \mathbf{k}_n) \right] \end{aligned} \quad (7)$$

where $\tilde{X}$ represents Fourier transform of X . There are 3 unknowns: $\tilde{c}'_{\text{act}}(\mathbf{k})$, $\tilde{c}'_{\text{act}}(\mathbf{k} + \mathbf{k}_n)$ and $\tilde{c}'_{\text{act}}(\mathbf{k} - \mathbf{k}_n)$ to be estimated for the reconstruction. We used 3 different phases of structured illumination in the imaging to estimate these unknowns. Then, we got,

$$\begin{bmatrix} \tilde{I}^{n1}(\mathbf{k}) \\ \tilde{I}^{n2}(\mathbf{k}) \\ \tilde{I}^{n3}(\mathbf{k}) \end{bmatrix} = \begin{bmatrix} 1 & \frac{1}{2}e^{-i\phi_{n1}} & \frac{1}{2}e^{i\phi_{n1}} \\ 1 & \frac{1}{2}e^{-i\phi_{n2}} & \frac{1}{2}e^{i\phi_{n2}} \\ 1 & \frac{1}{2}e^{-i\phi_{n3}} & \frac{1}{2}e^{i\phi_{n3}} \end{bmatrix} \begin{bmatrix} \tilde{h}_{\text{det}}(\mathbf{k}) & 0 & 0 \\ 0 & \tilde{h}_{\text{det}}(\mathbf{k}) & 0 \\ 0 & 0 & \tilde{h}_{\text{det}}(\mathbf{k}) \end{bmatrix} \begin{bmatrix} \tilde{c}_{\text{act}}(\mathbf{k}) \\ \tilde{c}_{\text{act}}(\mathbf{k} + \mathbf{k}_n) \\ \tilde{c}_{\text{act}}(\mathbf{k} - \mathbf{k}_n) \end{bmatrix} \quad (8)$$

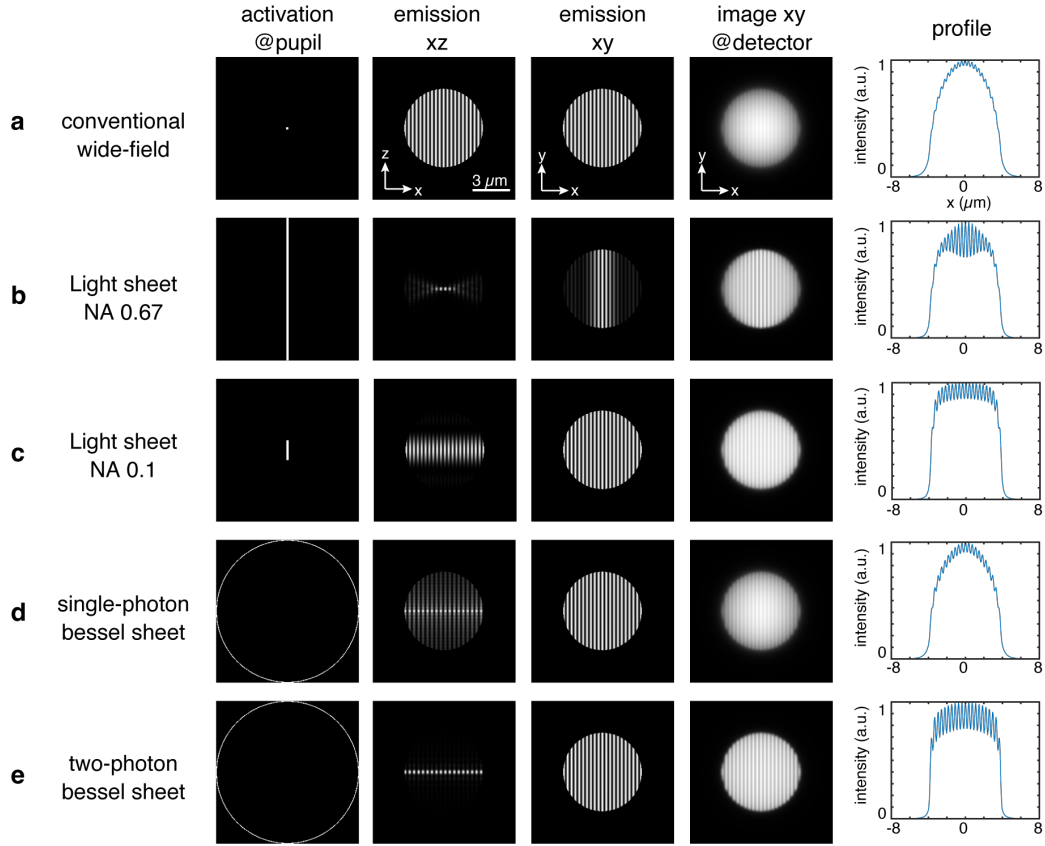
We estimated three components for reconstruction, $\tilde{c}'_{\text{act}}(\mathbf{k})$, $\tilde{c}'_{\text{act}}(\mathbf{k} + \mathbf{k}_n)$ and $\tilde{c}'_{\text{act}}(\mathbf{k} - \mathbf{k}_n)$ by solving this equation with the estimated or measured detection PSF: $\tilde{h}'_{\text{det}}(\mathbf{k})$. $\tilde{c}'_{\text{act}}(\mathbf{k} + \mathbf{k}_n)$ and $\tilde{c}'_{\text{act}}(\mathbf{k} - \mathbf{k}_n)$ are shifted back to superpose the DC components in the frequency domain and are represented as $\tilde{c}'_{\text{act-shifted}}(\mathbf{k} + \mathbf{k}_n)$ and $\tilde{c}'_{\text{act-shifted}}(\mathbf{k} - \mathbf{k}_n)$, respectively. The estimation is performed in three different $\mathbf{k}_n$ for a homogenous optical passband, and the summation of these nine components is the Fourier transform of the reconstructed super-resolution image:

$$\tilde{I}_{\text{SPA-SIM}}(\mathbf{k}) = \sum_{n=1}^3 \tilde{c}'_{\text{act}}(\mathbf{k}) + \tilde{c}'_{\text{act-shifted}}(\mathbf{k} + \mathbf{k}_n) + \tilde{c}'_{\text{act-shifted}}(\mathbf{k} - \mathbf{k}_n). \quad (9)$$

A similar reconstruction was performed using 3DSIM². In particular, in 3DSIM, selective plane activation can modify the effective illumination pattern depending on the sheet thickness. This effect changes the weights of the frequency components derived from the structured illumination, which should be considered when choosing the coefficients in the unmixing process. In our reconstruction, the coefficients were optimized according to the weights of the frequency components in the raw dataset.

	Activation		Excitation & Detection
objective	NA 0.1 water (n = 1.33)	NA 0.67 water (n = 1.33)	NA 1.1 water (n = 1.33)
wavelength	Light-sheet 405 nm	Light-sheet & single-photon Bessel: 405 nm two-photon Bessel: 780 nm	Excitation: 488 nm Fluorescence: 522 nm

Fig. S1 Calculation conditions used in Fig. S2 and S3. (n: refractive index)



84

85 Fig. S2 Intensity distributions of activation light at the pupil of the objective lens,
 86 fluorescence emission on the xz and xy planes at the object, and the fluorescence signal
 87 on the image sensor calculated for the five imaging modes of (a) conventional SIM, light-
 88 sheet with single-photon-activation using NAs of (b) 0.67 and (c) 0.1, and Bessel sheet
 89 with (d) single- and (e) two-photon-activation using an NA of 0.67. A fluorescent sphere
 90 with a diameter of 10 μm was assumed as the object. The middle plane was imaged to
 91 evaluate the imaging property inside dense sample. The light sheet activation improves
 92 the contrast of the emission and the resultant image but brings about a trade-off between
 93 the axial resolution and field of activation. A scanned Bessel sheet with single-photon
 94 activation provides a large field of view, whereas the contrast improvement is not
 95 sufficient due to the sidelobes of the Bessel beam. Two-photon activation with a scanned
 96 Bessel sheet realizes the fluorescence distribution highly confined within the focal plane
 97 without sacrificing the field of view.

98

99

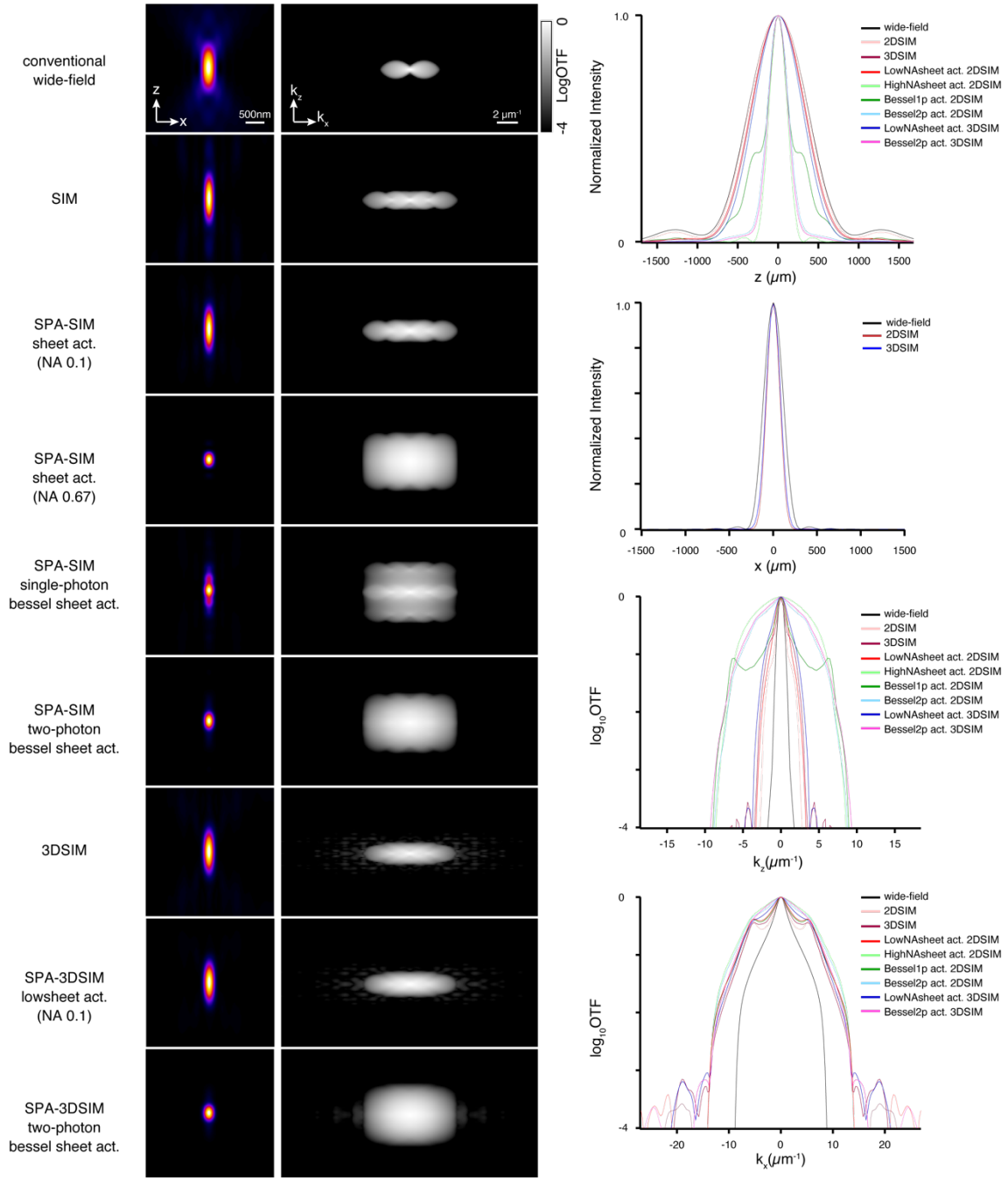


Fig. S3 Point spread functions and optical transfer functions of conventional wide-field microscopy, SIM, light sheet SPA-SIM with NAs of 0.1 and 0.67, Bessel light sheet for single- and two-photon activation in SPA-SIM with an NA of 0.67, 3DSIM, SPA-3DSIM with a light sheet and Bessel light sheet with two-photon activation. PSFs and OTFs were normalized by their individual maxima and plotted in linear and logarithmic scales, respectively. Intensity profiles at $x = 0$, $z = 0$, $k_x = 0$, and $k_z = 0$ were shown. We set the contrast of OTFs to a minimum value of -4 to eliminate calculation noise. We obtained PSFs by using vector calculation³.

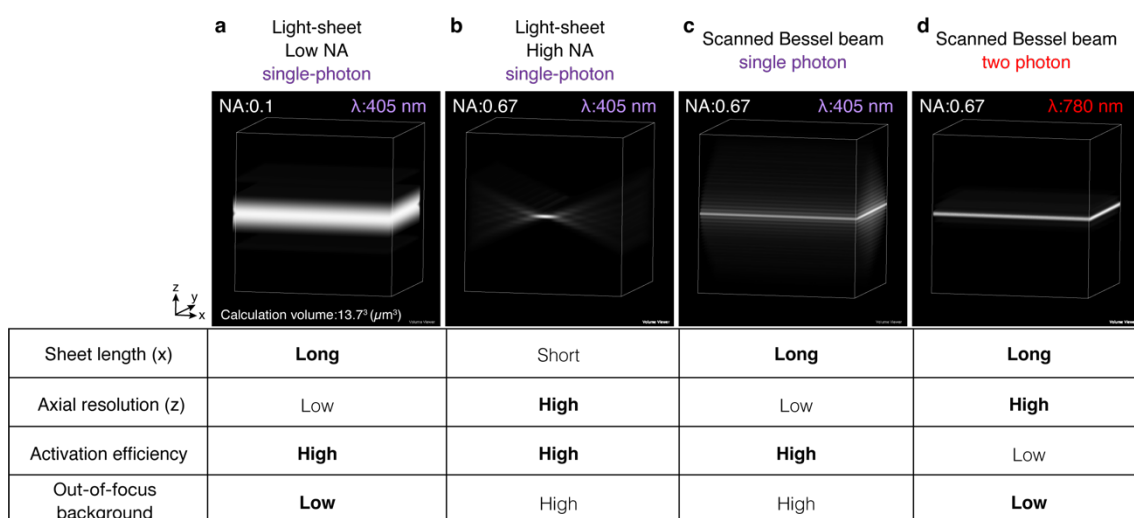


Fig. S4 Sheet length(x), axial resolution(z), activation efficiency and out-of-focus background with different activation modes. Spatial distributions of activation efficiency under single-photon light-sheet activation with NAs of (a) 0.1 and (b) 0.67 and scanned Bessel beam with (c) single-photon and (d) two-photon activation with an NA of 0.67 are shown.

There are several options for the activation modes that have their advantages and disadvantages. For observation of relatively thicker and larger samples compared to monolayer cells, the sheet length, axial resolution (sheet thickness), activation efficiency, and out-of-focus background are factors to be considered. Single-photon light-sheet activation with low NA (Fig. S4a) provides sufficient sheet length for FOV in SIM with high switching efficiency yet compromises the axial resolution. Increasing NA improves the axial resolution but increases out-of-focus background in a large area of FOV with reducing the sheet length (Fig. S4b). Scanned Bessel beam provides an enough sheet length with high NA objective lens, however the strong sidelobes produce background emission from out-of-focus planes (Fig. S4c). Two-photon activation mitigates sidelobes due to the nonlinear activation property (Fig. S4d), achieving large FOV and high axial resolution simultaneously, with compromised activation efficiency. Fig. 5 shows the experimental comparisons of spatial distribution of photoactivation.

In this research, we chose single-photon activation with a low-NA light-sheet (Fig. S4a) and two-photon activation with a high-NA scanned Bessel beam (Fig. S4d) because of low background nature. The single-photon activation mode is useful for increasing framerate (Fig. 2g,h). The two-photon activation mode realizes observations with higher axial resolution and lower background, which is useful for imaging dense and thick samples such as nuclei (Fig. 2p,r) and spheroids (Fig. 2u and Fig.3m-o), respectively.

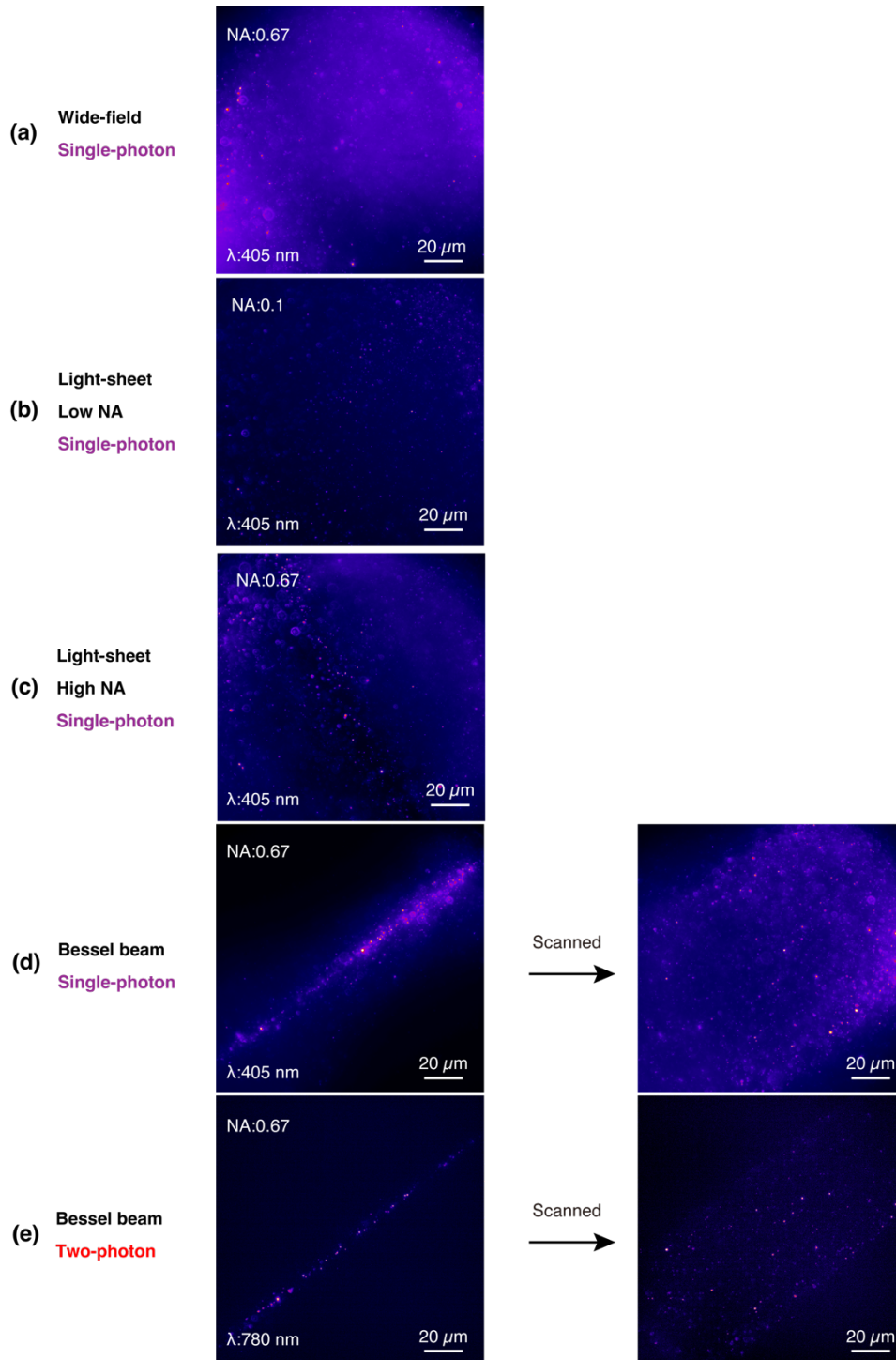


Fig. S5 Comparison of activation patterns using fluorescence imaging of 100nm beads embedded in agar gel. (a) Conventional widefield, light-sheet illumination with (b) an NA of 0.1 (thickness: 1.71 μ m) and (c) an NA of 0.67 (0.26 μ m), and scanned-Bessel beam with (d) single-photon excitation (1.25 μ m) and (e) two-photon excitation (0.30 μ m). The low-NA light-sheet illumination can cover the entire field of view (141.9 μ m)

141 $\times 141.9 \mu\text{m}$). The high-NA illumination can improve the depth discrimination
142 performance, however the sheet length for the propagation direction becomes shorter,
143 resulting in a smaller FOV. Using the Bessel beam, the length of plane illumination of
144 $140 \mu\text{m}$ can be achieved, and scanning the beam can cover a large area of FOV. With
145 single photon excitation using the Bessel beam, more out-of-focus fluorescence was
146 imaged, indicating the thicker excitation plane due to the strong sidelobes, which can be
147 significantly reduced using two-photon excitation.

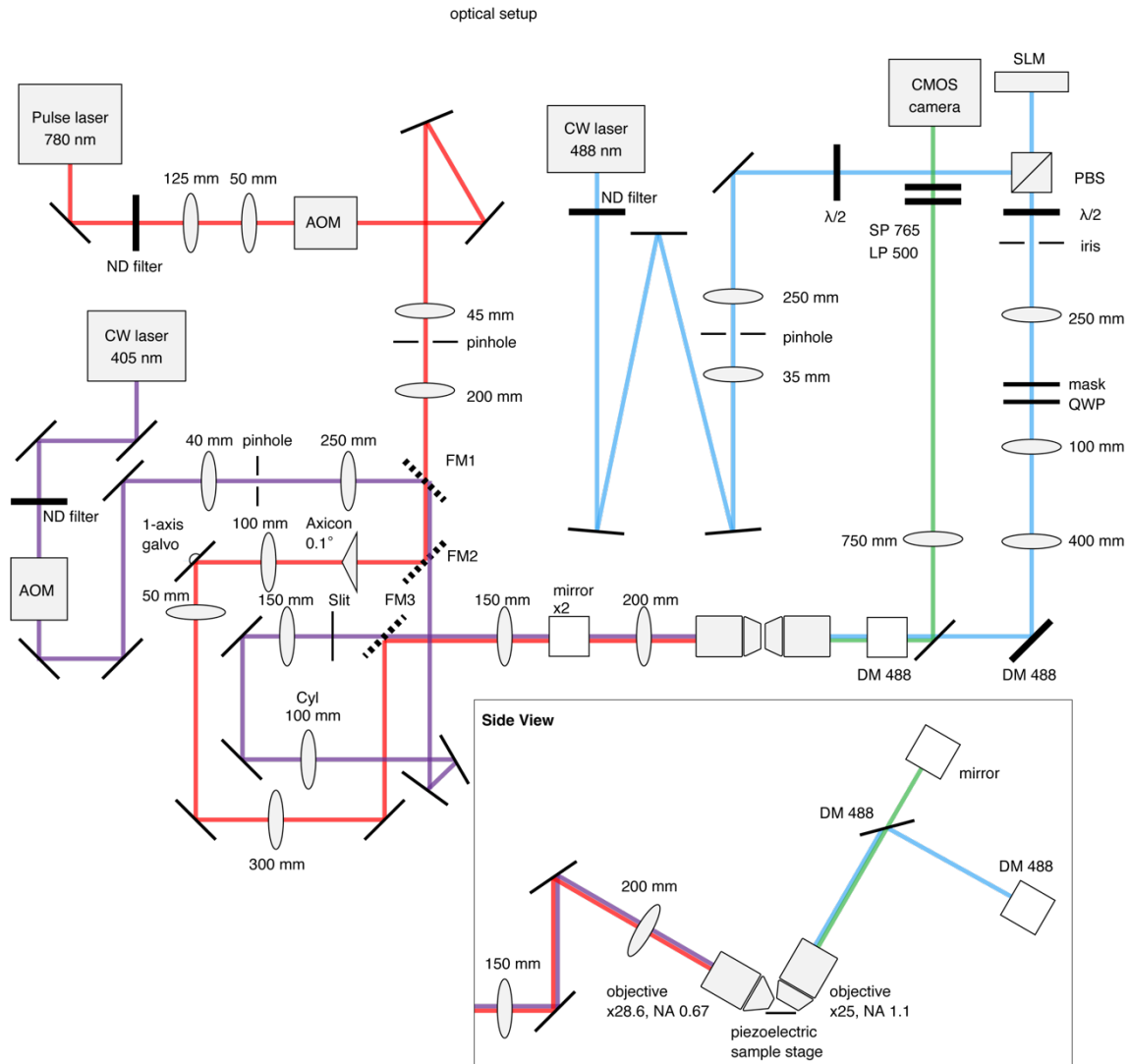


Fig. S6 optical setup.

The optical setup for SPA-SIM is composed by three sections: activation section, excitation section and detection section.

Activation

A continuous wave (CW) laser beam with a wavelength of 405 nm (Excelsior 405, Spectra Physics) or femtosecond pulse laser with a wavelength of 780 nm (MaiTai HP, Spectra Physics) is used for single- or two-photon activation. Neutral density (ND) filter is placed after the laser to attenuate the power of the light. The pulse width and the repetition rate are 100 fs and 80 MHz, respectively. The laser beam is passed through an acousto-optic modulator (AOM, ASM-802B8, IntraAction Corp.) for digital modulation of the irradiation power to the samples and is collimated by two lenses with a spatial filter pinhole. The beam is shaped in two ways: a cylindrical lens or an axicon lens and a

galvanometer mirror to form a light sheet on the sample plane. The laser beam was then focused using a water immersion objective lens (28.6 $\times$, NA0.67, working distance (WD) 3.74 mm, model 54-10-7, Special Optics) to activate the sample. An adjustable slit is placed at the intermediate pupil plane in the path of cylindrical lens to adjust the effective NA used for single-photon light-sheet activation. Flip mirrors (FMs) were used to change the activation modes for the comparison between wide-field and selective plane activation. We put FM1 out, and FM2, FM3 in for two-photon activation using a scanned Bessel beam. Wide-field activation was conducted with opposite procedure. We put FM1 in and FM2, FM3 out for single-photon light-sheet activation. Wide-field activation was conducted with the opposite. An axicon lens or a cylindrical lens was removed for each comparison.

Excitation

For excitation, a CW laser beam with a wavelength of 488 nm (Sapphire 488-500 LPX, Coherent) collimated by two lenses with a spatial filter pinhole is reflected by a polarized beam splitter (PBS, PBS251, Thorlabs) to a spatial light modulator (SLM, SXGA 3DM, Fourth Dimension Display) after passing through a $\lambda/2$ plate (WPH10M-488, Thorlabs). The SLM digitally modulates the beam pattern into sinusoidal patterns for structured illumination. After passing through PBS and a quarter-wave plate (QWP, WPQ-4880-4M, Sigma Koki) for circular polarization, the polarization conditions are adjusted for all structured illumination angles. 0th-order diffracted beam and other unwanted diffractions are blocked using a silver-coated mask in the 2DSIM mode. In the 3DSIM, a different patterned mask that can pass through a 0th-order beam is used. The beams passing through the mask are reflected by two long-pass dichroic mirrors (DM, Di03-R488-t3-25 $\times$ 36, Semrock) to compensate for the phase delay and were focused on the samples using a water objective lens for excitation (25 $\times$, NA1.1, WD 2.00 mm, CFI75, Nikon).

Detection

The Fluorescence emission from the samples is collected using the same objective lens, passed through the DM, and imaged using a sCMOS camera for detection (OLCA-Flash 4.0 v3, Hamamatsu Photonics). Long-pass (LP500, BLP01-488R-25, Semrock) and short-pass (SP765, FF01-790/SP-25, Semrock) filters are set in front of the camera to reject illumination and activation light. Nine or fifteen images are acquired with three angles and three or five phases of structured patterns for each super-resolution image. 1D piezoelectric stage (SFS-H40X, OptSigma) was used to move the samples for 3D imaging.

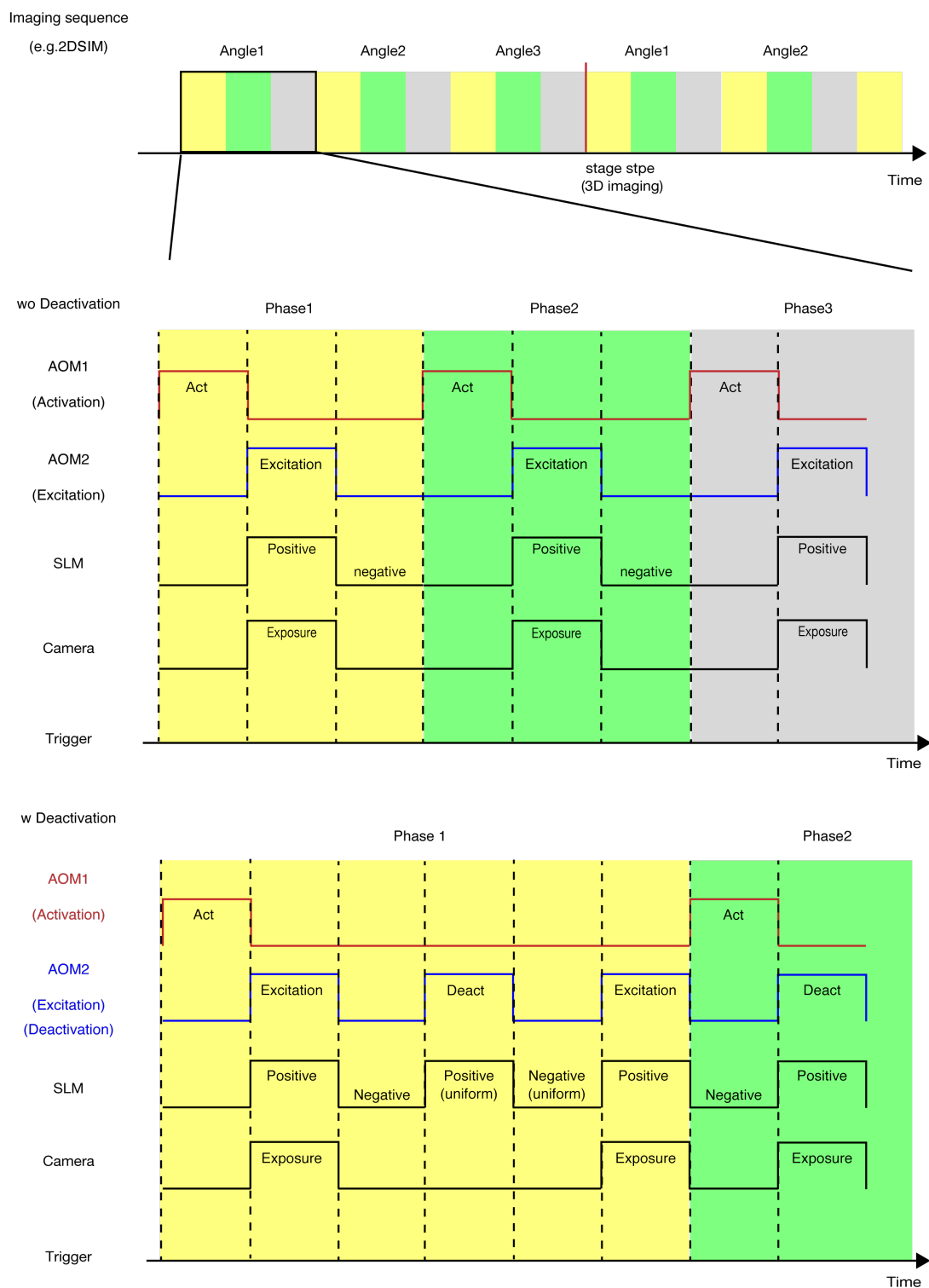


Fig. S7 Timing diagram of the measurement system. The top row shows the brief imaging procedure of SIM. Nine images with structured illumination of three angles by three phases are obtained to reconstruct super-resolution images. 3DSIM imaging requires five phases for each angle. For time-lapse imaging, the same process is repeated to fulfill the

designated time slot. For 3D imaging, the sample stage moves a single step after a single SIM-imaging process. Detailed diagrams in cases of with and without deactivation are shown below. AOMs for excitation and activation, spatial light modulator, and camera are triggered by a PC, which is indicated by black dotted lines. The imaging process without deactivation, which is used in time-lapse or 3D imaging, consists of activation, excitation, and relaxation for SLM. Activation is separated from the excitation process to avoid the temporal crosstalk of fluorescence excited by activation light and excitation light. Excitation, SLM, and camera are triggered at the same time to obtain the fluorescence images under structured illumination. A negative pattern for structured illumination is applied to SLM for avoiding the burn-in. Deactivation with uniform illumination and another excitation step are added in another imaging mode to avoid the on-state proteins being left in the next measurement and observe the distribution of the fluorescence from off-state proteins.

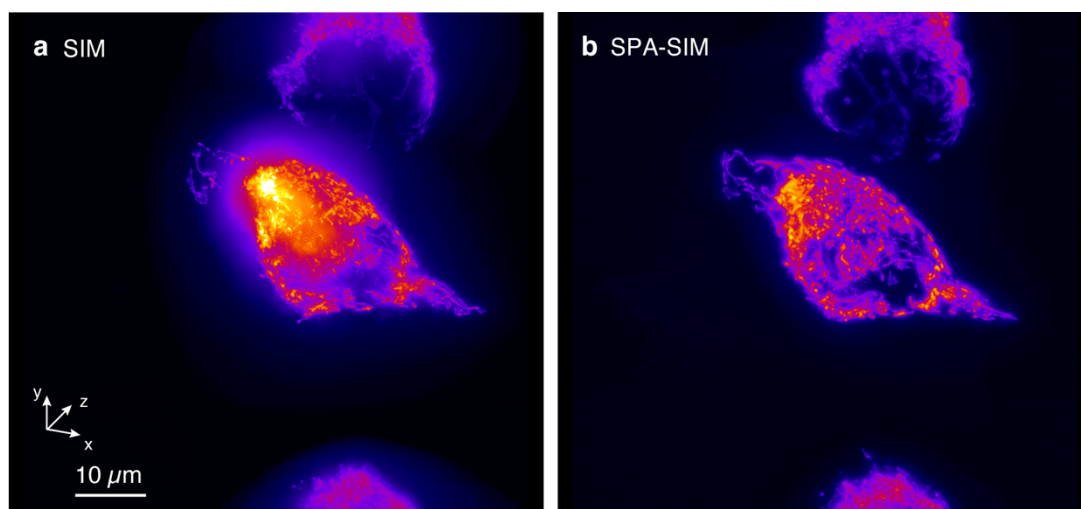


Fig. S8 3D images of mitochondria in living HeLa cells with (a) conventional SIM and (b) SPA-SIM with single-photon light sheet activation. The imaging volumes were $71 \times 71 \times 25 \mu\text{m}^3$. The experimental conditions are shown in Extended Fig. 2. SPA-SIM was used to visualize the mitochondrial components distributed inside the cells owing to out-of-focus rejection.

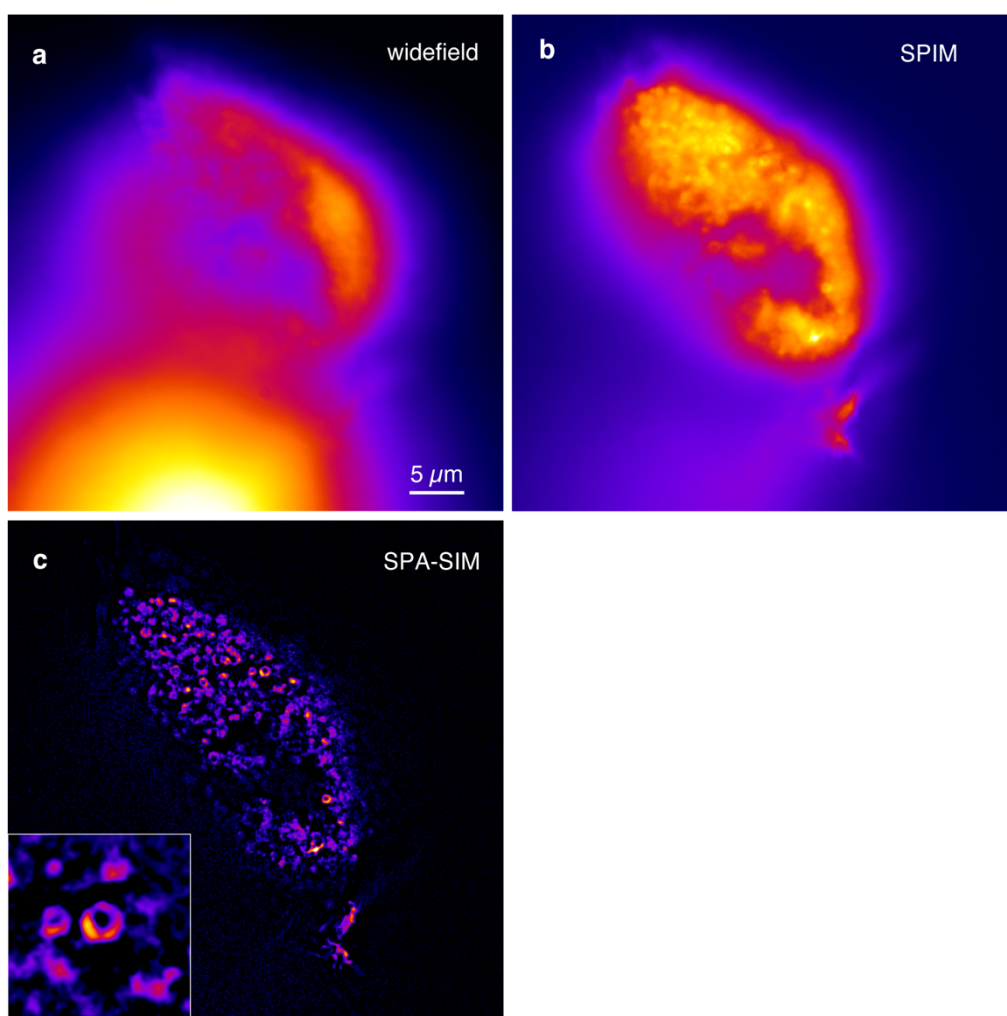


Fig. S9 Fluorescence images of the same MEF as Fig. 2k-l observed with (a) wide-field and (b) SPIM mode. (c) SPA-SIM image (Fig. 2k) is shown for comparison.

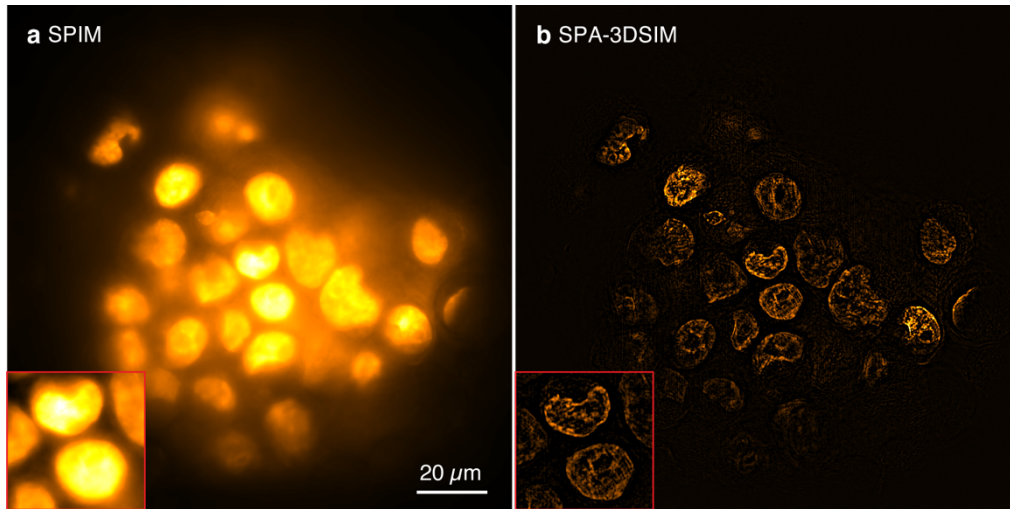


Fig. S10 Fluorescence images of the same cell spheroid sample as Fig. 2s-u observed with (a) SPIM. (b) SPA-3DSIM image (Fig. 2u) is shown for comparison. The reconstruction of the super-resolution image failed because the structured illumination pattern at the focus plane was not detected due to the high out-of-focus signal.

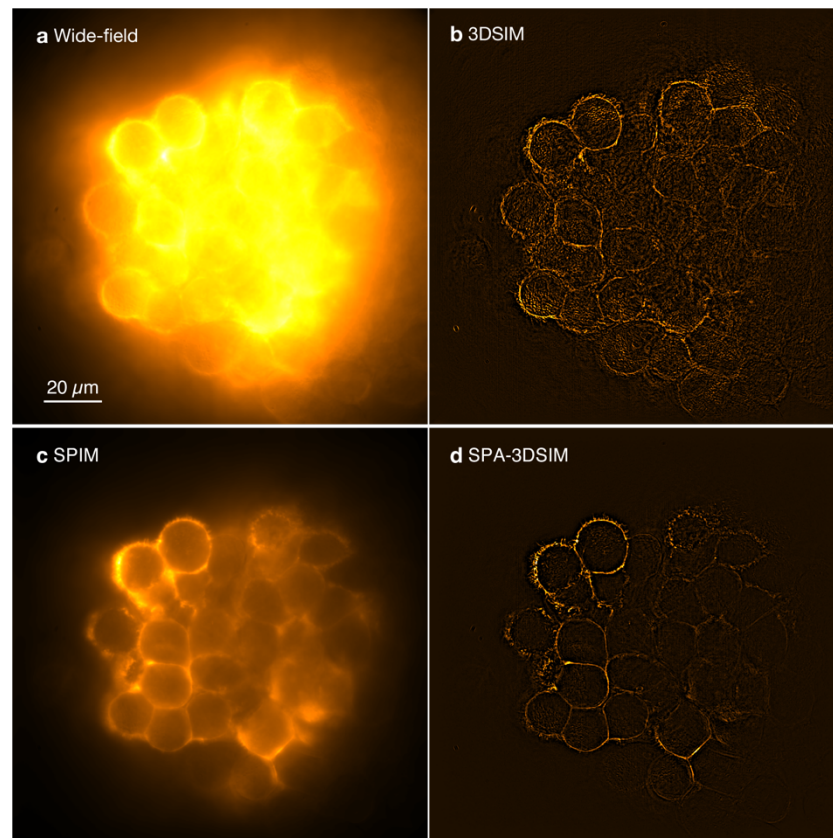


Fig. S11 Fluorescence images of actin filaments labeled by rsGamillus-S in a cell spheroid with a diameter of $\sim 200 \mu\text{m}$ at the depth of $69 \mu\text{m}$ observed with (a) wide-field, (b) 3DSIM, (c) SPIM, and (d) SPA-3DSIM with two-photon activation by a Bessel sheet.

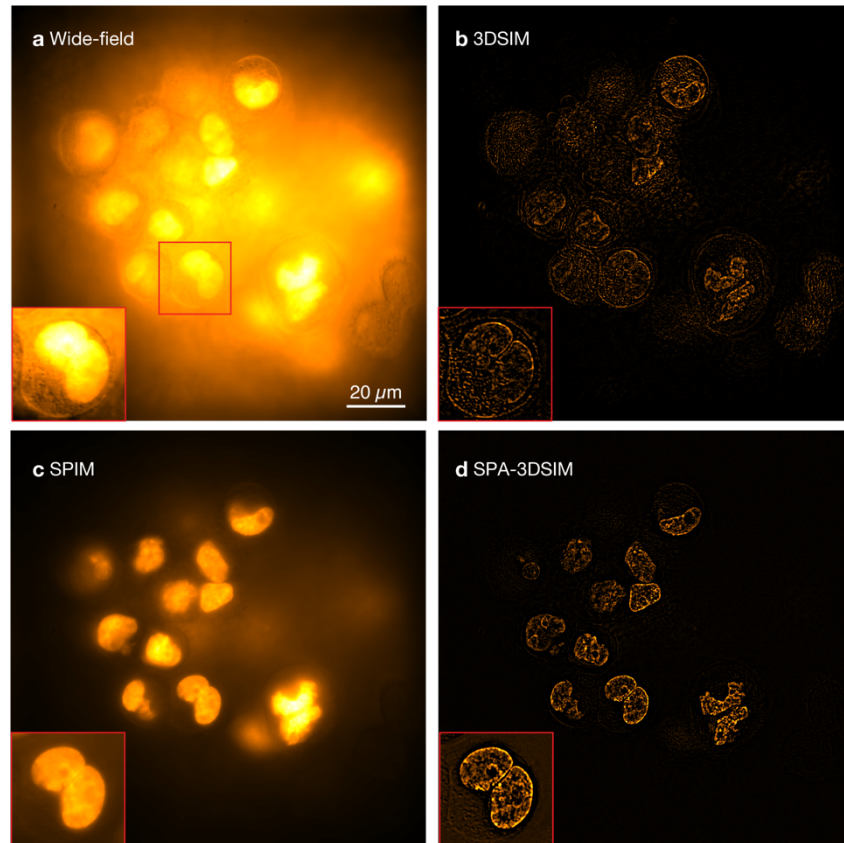


Fig. S12 Fluorescence images of nuclei labeled by rsGamillus-S in a cell spheroid with a diameter of $\sim 100 \mu\text{m}$ at the depth of $25 \mu\text{m}$ observed with (a) wide-field, (b) 3DSIM, (c) SPIM, and (d) SPA-3DSIM with two-photon activation by a Bessel sheet. Experimental conditions were the same as those shown in Fig. 2 (q)-(s).

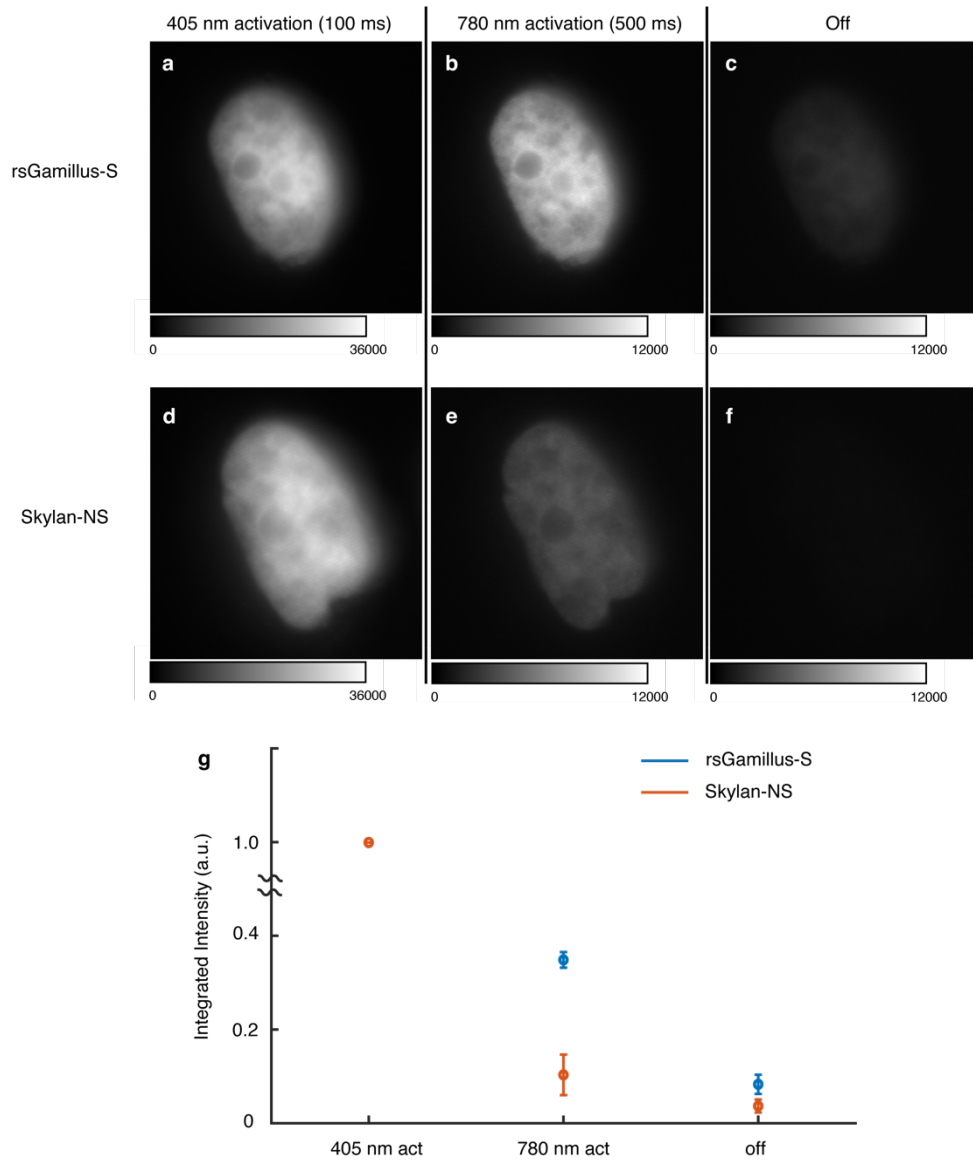


Fig. S13 Representative fluorescence images of histone H2B in a nucleus of a living HeLa cell labeled by rsGamillus-S and Skylan-NS. The images were captured after three different procedures as follows. (a,d) RSFPs were activated by 405 nm in 100 ms, switching almost all FPs to on-state. (b,e) Two-photon activation with Bessel sheet was applied in 500 ms which is same as the experimental conditions in the main text. (c,f) 488 nm light was irradiated for switching FPs to off-state. Activation laser (405 nm and 780 nm) powers were 248 μ W and 67 mW, respectively, and 488 nm excitation with 6.05 (W/cm^2) was used for imaging. (g) Averaged integrated intensity of 5 fluorescence images of a cell labeled by rsGamillus-S and Skylan-NS. Different cells in the same dish were measured. Integrated intensities under different activation conditions were normalized by the value of 405 nm activation. We confirmed rsGamillus-S was 3.37 times

260 more efficiently activated by two-photon Bessel sheet presumably due to the higher on-
261 switching rate, which is suitable for SPA-SIM imaging. Central circles and error bars
262 represent the mean and the s.d., respectively (rsGamillus-S, n = 5 cells; Skylan-NS, n =
263 5, $P < 0.0001$; Welch's *t*-test).
264

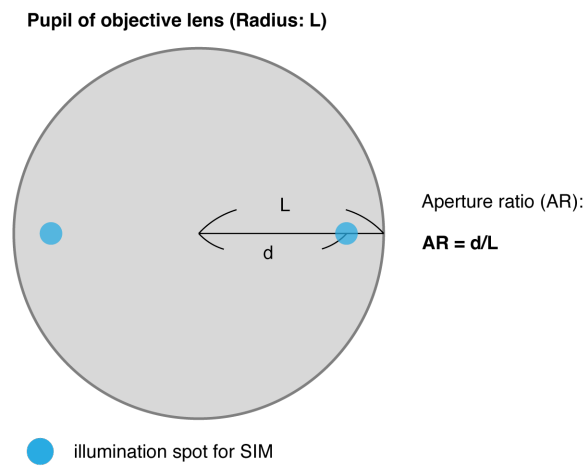


Fig. S14 Definition of aperture ratio (AR). L is the radius of pupil in an objective lens. d is the distance of the spot for structured illumination from center of the pupil.

Supplementary Video 1

Time-lapse images of actin filament movement in living a HeLa cell labeled with Skylan-NS observed by SPIM and SPA-SIM shown in Fig. 2 (g). The acquisition rate was 0.6 frame/s with an interval of 7 s to visualize slow actin motion.

Supplementary Video 2

Time-lapse images of mitochondria in a living HeLa cell labeled with Skylan-NS observed by SPIM and SPA-SIM at 1 fps with an interval of 4 s. Several images at different time points and enlarged views are shown in Fig. 2 (h).

Supplementary Video 3

3D images of mitochondria in a living HeLa cell labeled with Skylan-NS obtained using 3DSIM and SPA-SIM. The images from a single viewpoint are shown in Fig. 2 (i,j). The imaging volumes were $71 \times 71 \times 25 \mu\text{m}^3$.

Supplementary Video 4

3D images of mitochondria in living HeLa cells labeled with Skylan-NS obtained using conventional SIM and SPA-SIM. The images from a single viewpoint are shown in Fig. S8. The imaging volumes were $71 \times 71 \times 25 \mu\text{m}^3$.

References

1. Gustafsson, M. G. L., Agard, D. A. & Sedat, J. W. Doubling the lateral resolution of wide-field fluorescence microscopy using structured illumination. *Three-Dimensional Multidimens. Microsc. Image Acquis. Process. VII* **3919**, 141–150 (2000).
2. Gustafsson, M. G. L. *et al.* Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. *Biophys. J.* **94**, 4957–4970 (2008).
3. B.Richards & E.Wolf. Electromagnetic diffraction in optical systems, II. Structure of the image field in an aplanatic system. *Proc. R. Soc. London. Ser. A. Math. Phys. Sci.* **253**, 358–379 (1959).