Optical alignment procedures of descSPIM

(ver. 230520)

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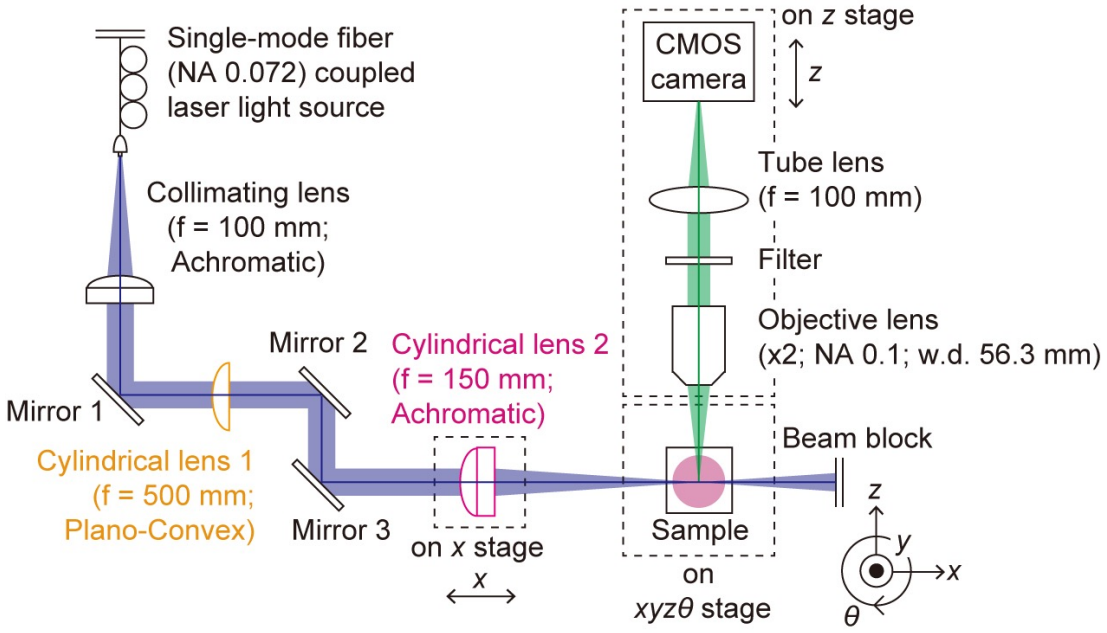
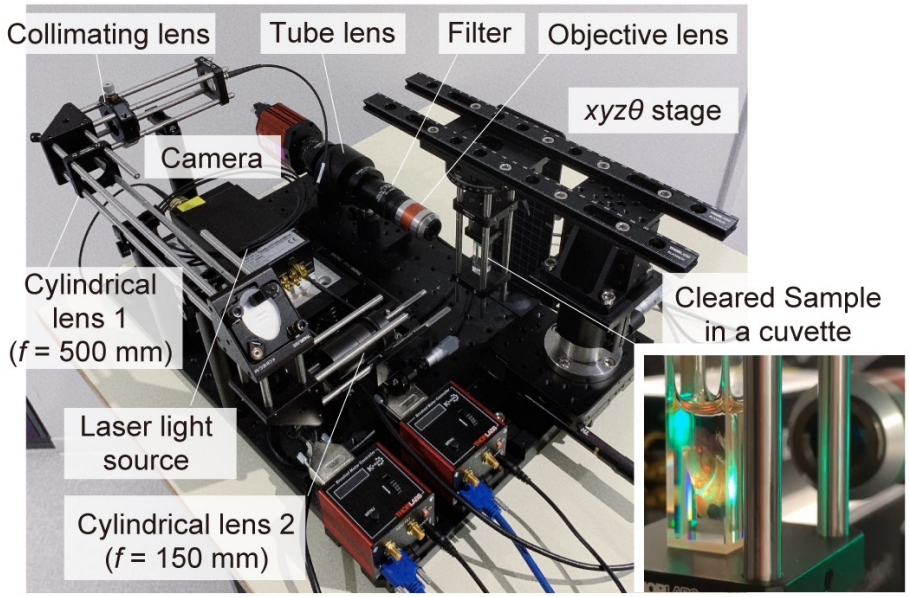
Optical setup (bioRxiv 2023; Fig. 1b, d)



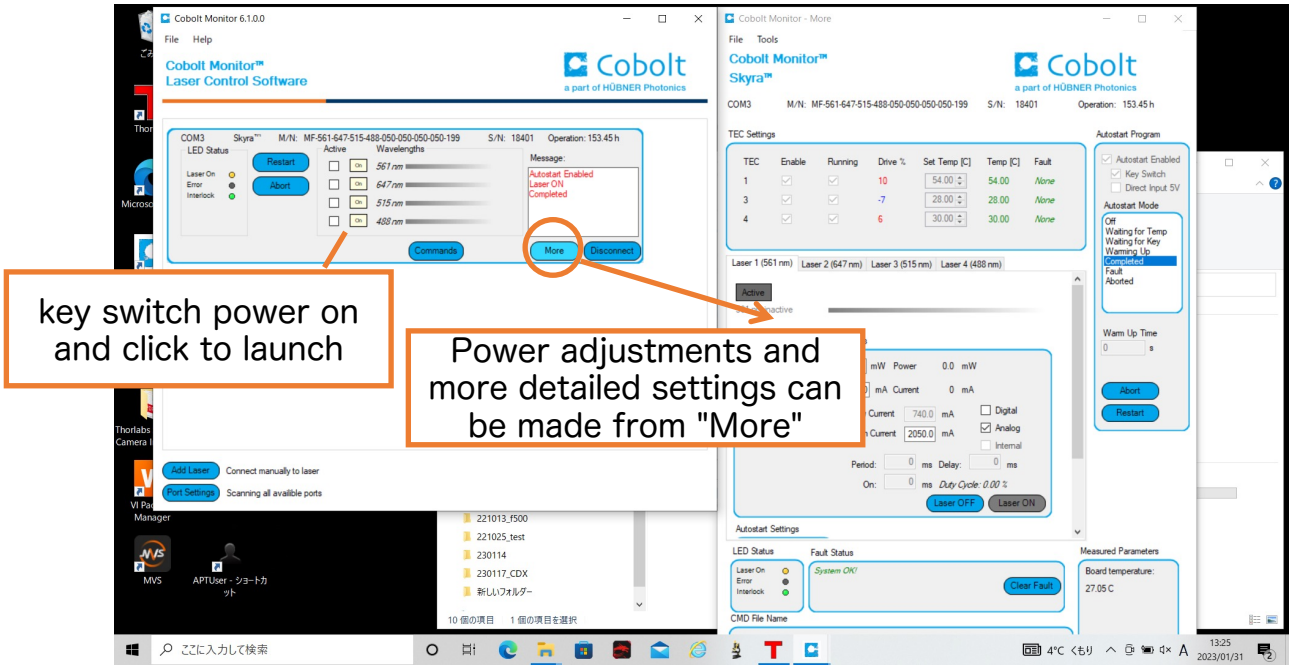


To do next before 3D imaging:

1. Laser light beam check and collimation (Collimating lens)
2. Laser light beam alignments (Collimating lens and Mirror 1, 2 and 3)
3. Cylindrical lens installation (Cylindrical lens 1 or 2 with x-stage)
4. Actuators and camera setup (Sample stage and detection optics)
5. Fluorescence signal check (Sample stage and detection optics)
6. Follow-up alignments (Cylindrical lens and detection optics; if required)

1. Laser light beam check and collimation

If Cobolt laser light sources, download the Cobolt Monitor™ software from the HÜBNER Photonics GmbH website.

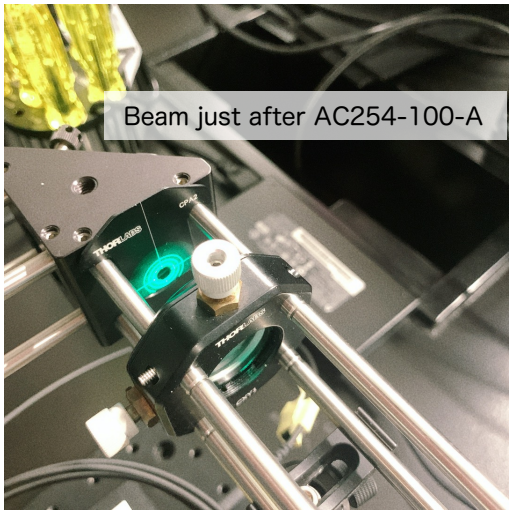


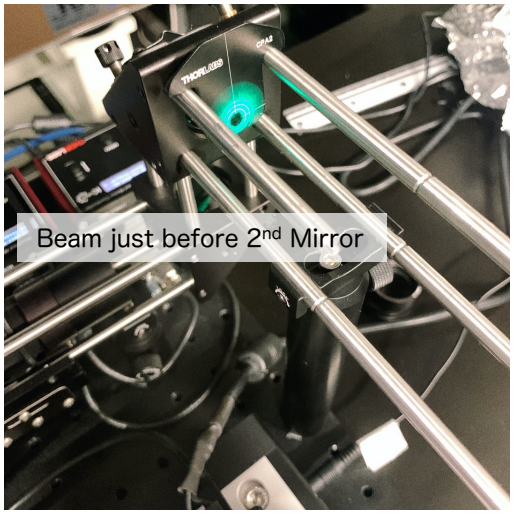
＊Emit the laser light beam with low-power (1-2 mW) initially, and gradually increase to the required intensity while making sure that the beam does not propagate to an unexpected direction.

Beam alignment Plate (CPA2) for 30 mm cage systems

Laser light beam alignment and collimation (parallel beam)

Check that the beam diameter just after the collimating lens (AC254-100-A) and the diameter of the beam propagated far enough (e.g. just before 2nd mirror) are almost the same size, and that there is no focusing point between them. If the size of the beam is different, adjust the position of the fiber port or the collimating lens.





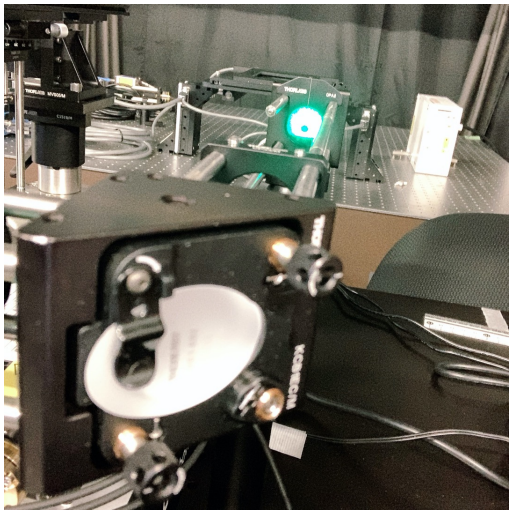
2. Laser light beam alignments

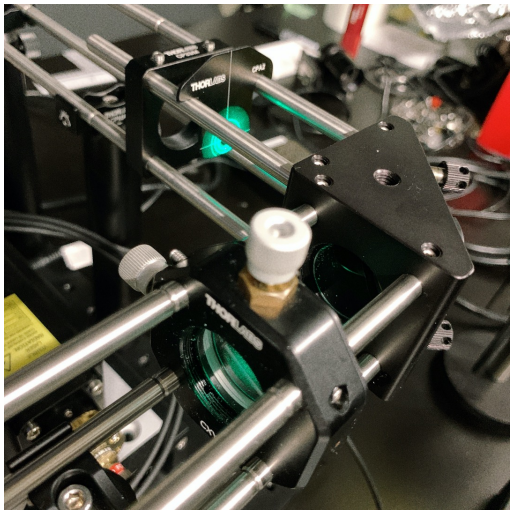
Optical axis alignment 1

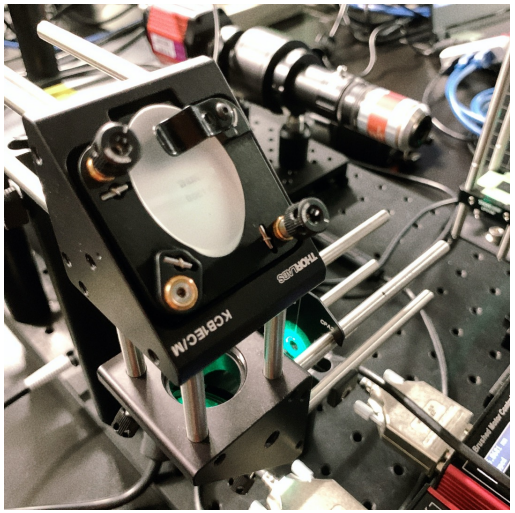
↓Place the alignment plate just before the second mirror holder and adjust the screws of the mirror 1 holder so that the beam is centered on the plate.

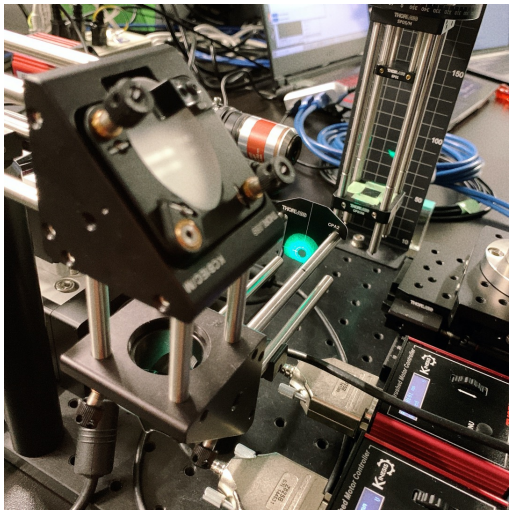
↓Place the alignment plate just after the first mirror holder, and if the beam is not centered, adjust the xy of the collimating lens holder.

↓Repeat the above two operations so that the beam is centered in both positions.









Optical axis alignment 2

↓Place the alignment plate just after the third mirror holder and adjust the screws of the mirror 2 holder so that the beam is centered on the plate.

↓Place the alignment plate at the end of the cage system and adjust the screw of the mirror 3 holder so that the beam is centered on the plate.

↓Repeat the above two operations so that the beam is centered in both positions.

＊By using two movable optics as described above, the beam can be moved to any optical path.

☞ "How to align a laser beam to different optical paths using two mirrors."

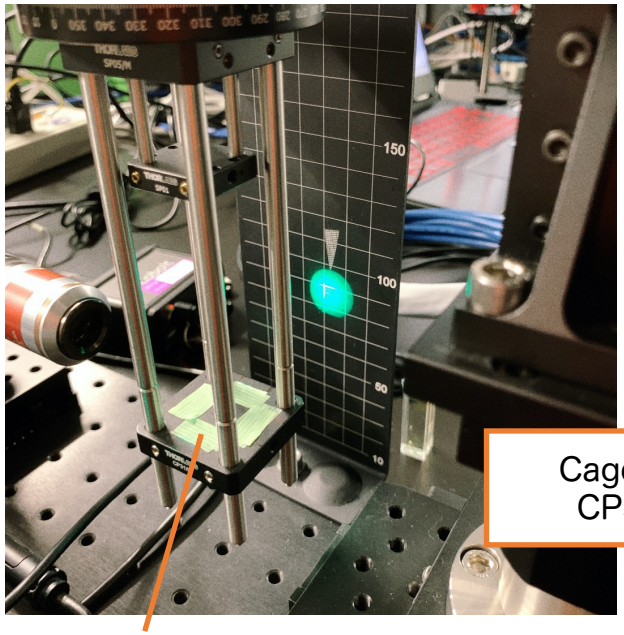
Thorlabs website; Insghts-tips (https://www.thorlabs.co.jp/newgrouppage9.cfm?objectgroup_id=14221)

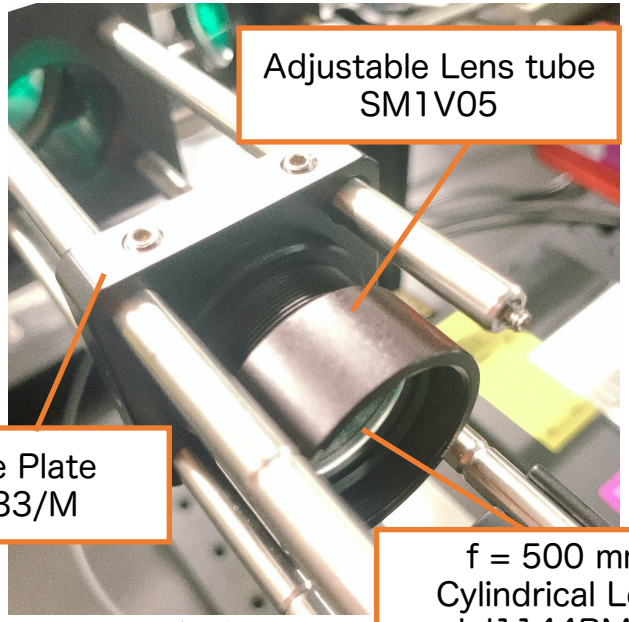
3. Cylindrical lens installation

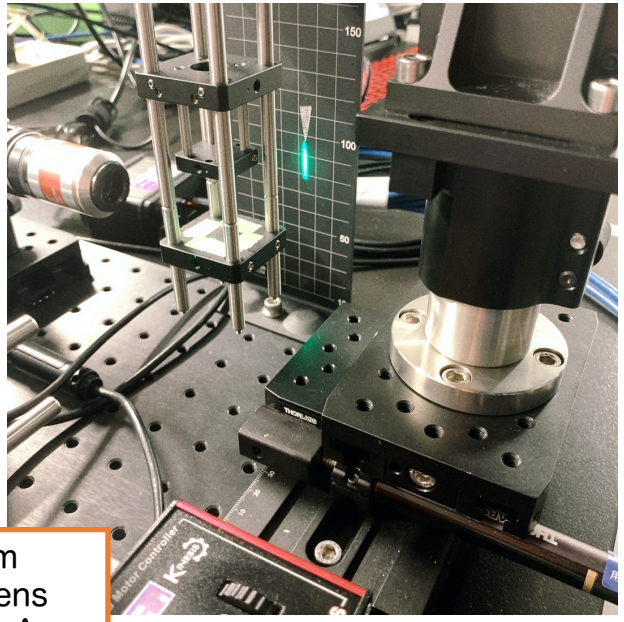
f = 500 mm cylindrical lens (Full-FOV (FF) mode)

↓Mark the irradiation spot (center along detection optical path; z-axis) on the safety screen

↓ Mount the f = 500 mm cylindrical lens on the cage plate and fasten it at the angle where the major axis is pararel to the y direction on the safety screen

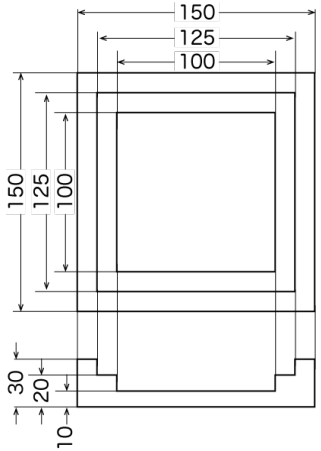






*The bottom of the sample holder indicated with curing tape to ensure reproducibility for placing the cuvette.

☞ One idea for reproductivity is to create the part shown on the left with a 3D printer.



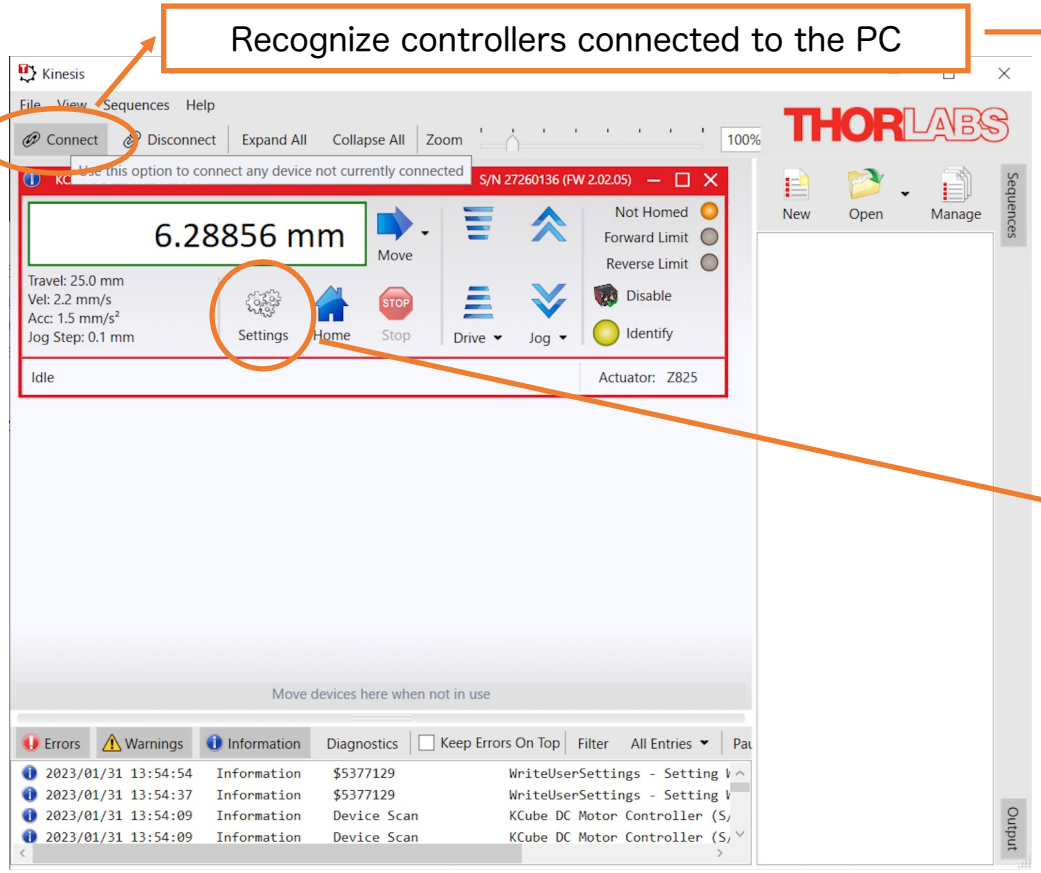
＊The cylindrical lens position is finely adjusted while capturing the actual fluorescent image (5 and 6).

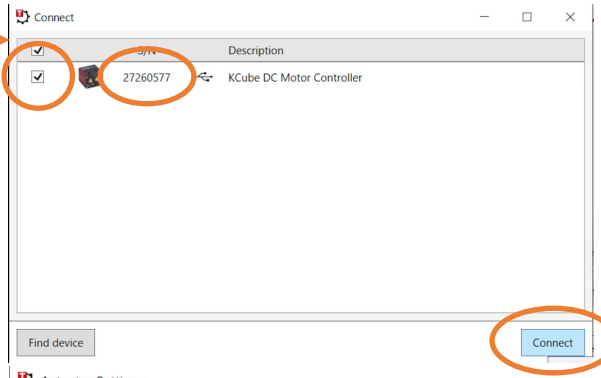
＊f = 150 mm cylindrical lens installation is mostly same (details are described in appendix of 5)

4. Actuators and camera setup

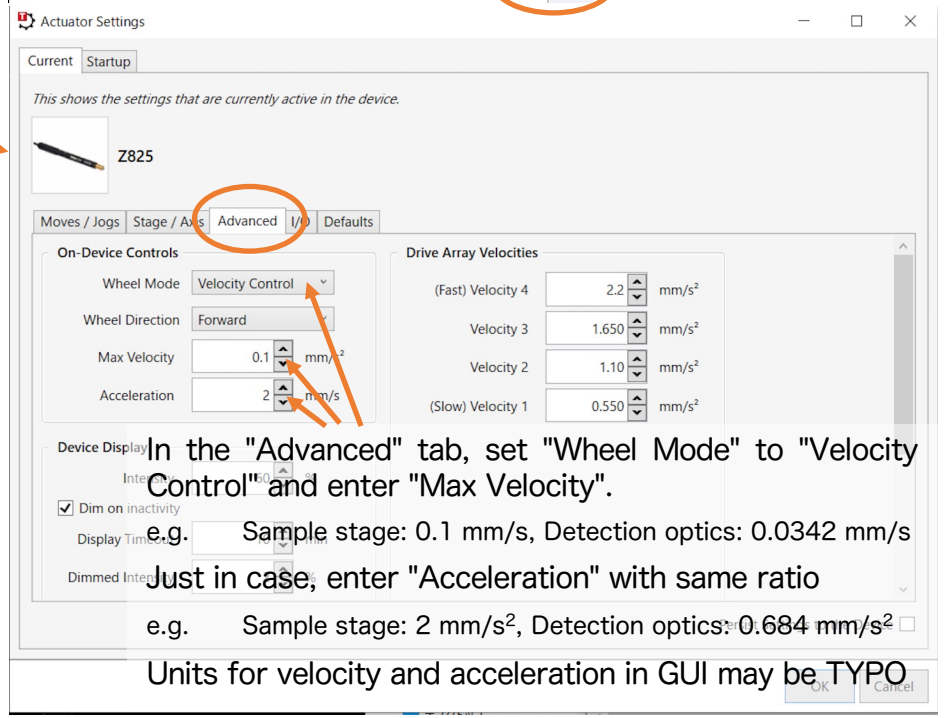
Download the software Kinesis™ from Thorlabs website (APT™ software is also available)

Recognize controllers connected to the PC





✓→Connect



In the "Advanced" tab, set "Wheel Mode" to "Velocity Control" and enter "Max Velocity".

e.g. Sample stage: 0.1 mm/s, Detection optics: 0.0342 mm/s

Just in case, enter "Acceleration" with same ratio

e.g. Sample stage: 2 mm/s², Detection optics: 0.684 mm/s²

Units for velocity and acceleration in GUI may be TYPO

Speed wheel

MENU button

Sample Stage Side

Detection optics side

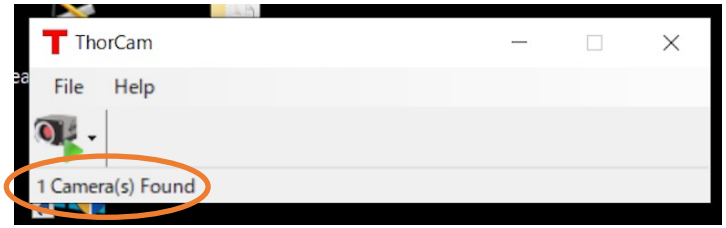
Setting is also possible with direct input to KDC101

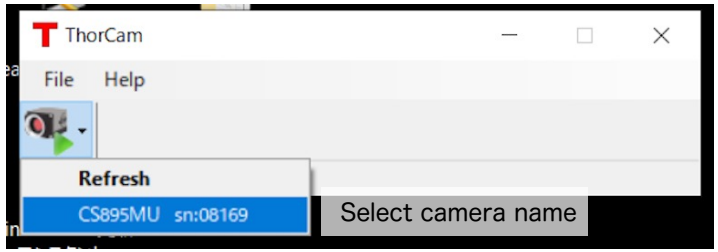
As shown on Supplementary Fig. 2 and Methods "General Imaging Procedure" in bioRxiv 2023, in the case of CUBIC-R (n = 1.52)

$z_{stage} = 0.342\ z_{detect}$ ($v_{stage} = 0.342\ v_{detect}$)

5. Fluorescence signal check

If you are using a Thorlabs camera, download the software ThorCam™ from the website





The camera connected to the PC is found.

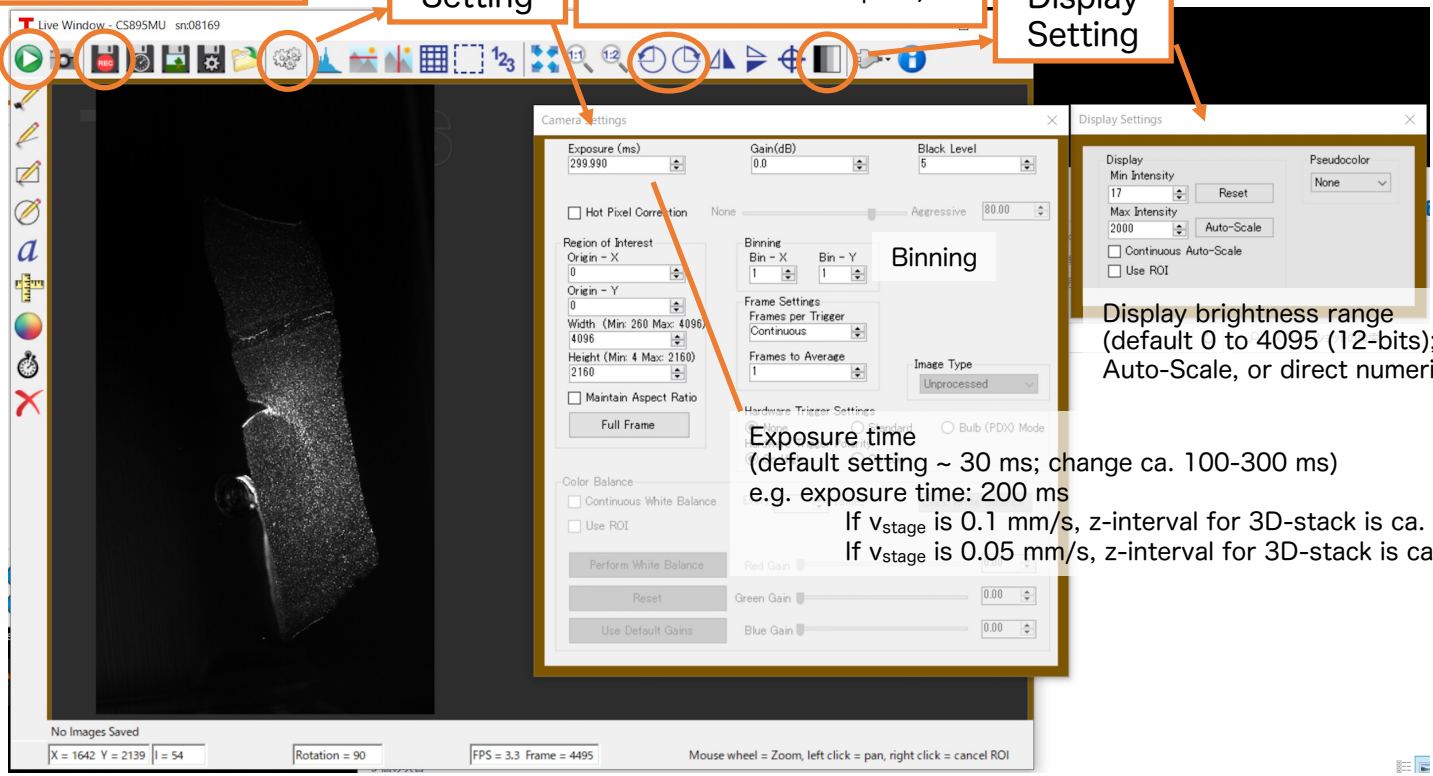
Video Rec
(used for 3D imaging)

Camera Setting

Camera angle adjustment
(90 degree rotation is required to reflect the real space)

Display Setting

Live



Binning

Display brightness range
(default 0 to 4095 (12-bits); adjust using Auto-Scale, or direct numeric input)

Exposure time
(default setting ~ 30 ms; change ca. 100-300 ms)
e.g. exposure time: 200 ms
If v_{stage} is 0.1 mm/s, z-interval for 3D-stack is ca. 20 µm.
If v_{stage} is 0.05 mm/s, z-interval for 3D-stack is ca. 10 µm.

5. Fluorescence signal check

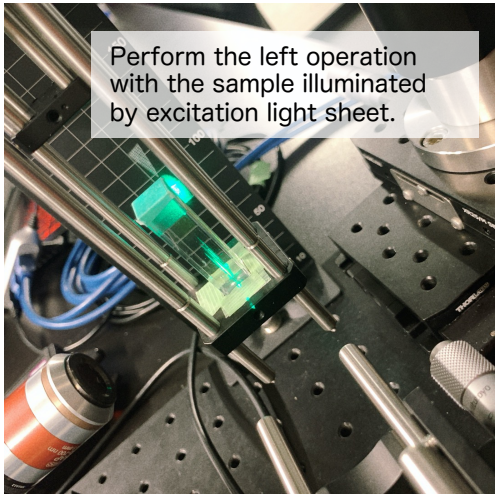
↓Place a test sample (nucleus-stained clerared tissue; fluorescent bead-embedded gel) in the sample holder, irradiate with excitation light sheet, and adjust the θ-stage so that the reflected light from the cuvette surface returns straight to the optical path.

↓Shade the sample with a blackout curtain and check if fluorescence is acquired by live playback of the camera.

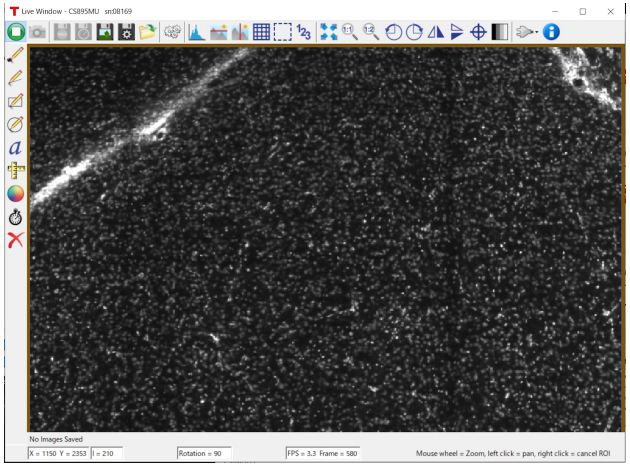
↓If fluorescence is not visible, find the focus by adjusting the position of the detection optics (by loosening the lens tube clamp (SM1TC) and moving it back and forth manually or by using a motorized actuator).

↓If fluorescence is visible (fluorescence signal should disappear when the excitation light is covered by obstacles; if not, it may be stray), find the focus by using a motorized actuator equipped on the detection optics

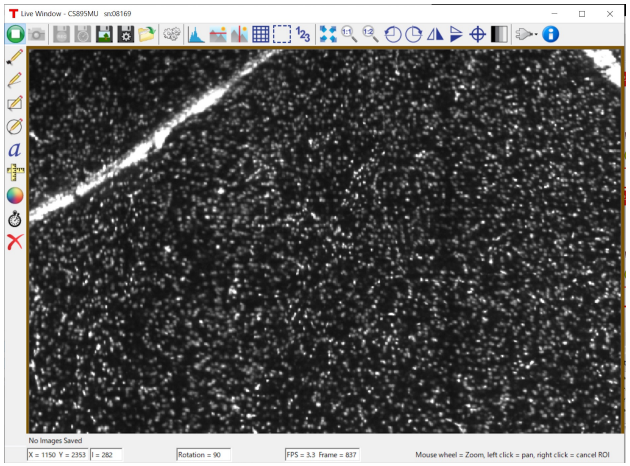
↓Adjust the position of the cylindrical lens on the excitation light to find the place where the background is lowest in the center of the field of view, and fix the lens.



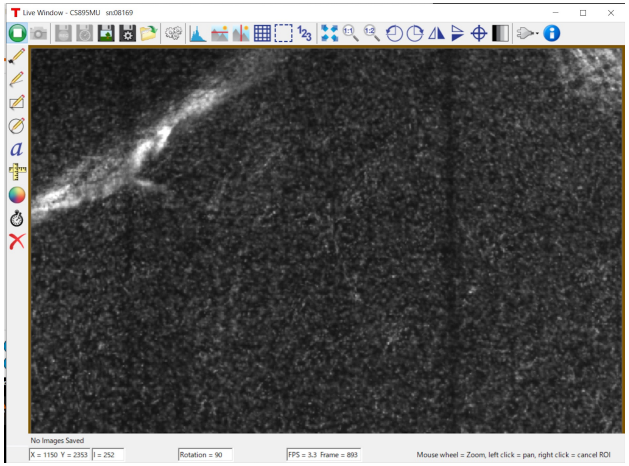
Cylindrical lens position along one-direction (x-axis for sample)



Not yet



Identical position between focus of cylindrical lens and the center of FOV



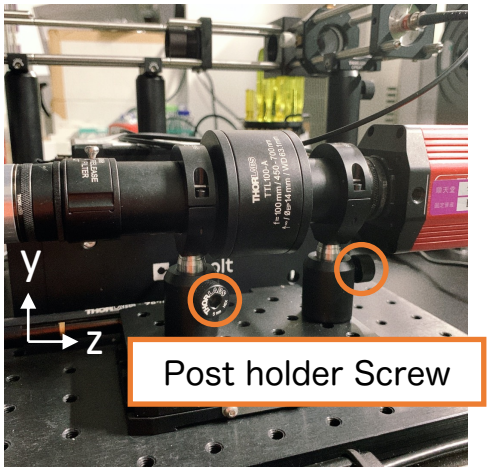
Too much

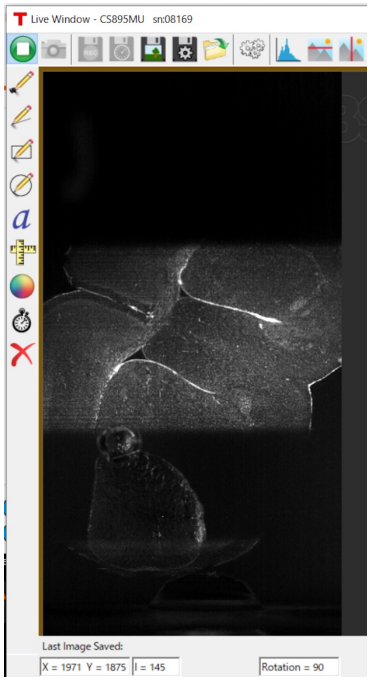
5. Fluorescence signal check

↓Place an alignment plate (with a ϕ5 mm hole) at the end of the cage system in the excitation light path and perform imaging.

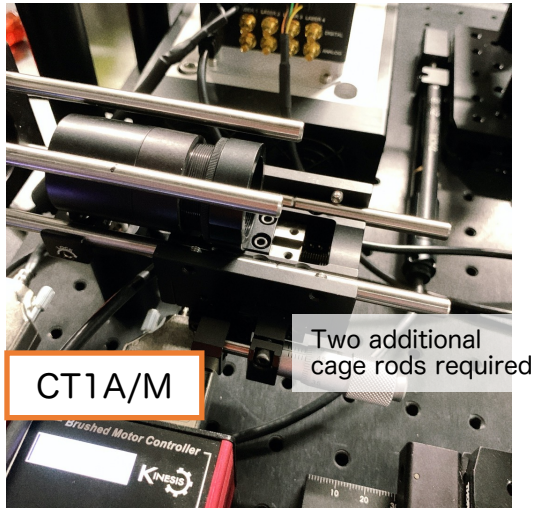
↓If the image is as the right figure, the center of the excitation light coincides with the center of the camera's field of view along the y-axis. ☞ OK

↓If it is not aligned with the, loosen the screws of the post holder of the detection optical system and adjust the vertical position.





Basic installing operations are the same for f = 150 mm cylindrical lens (1, 2, and 4 can be omitted once performed). However, since the effective FOV range along x-axis is narrower than f = 500 mm (1/10 or less), it is difficult to adjust only by hand without any micrometers. Thus, we recommend the installation with z-axis translation stage for cage system. While a z-axis translation stage (CT1A/M; travel 13 mm) is included in parts list, a z-axis translation mount (SM1ZA; travel 2 mm) is acceptable.



6. Follow-up alignments (if required)

nature methods

Perspective

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Practical considerations for quantitative light sheet fluorescence microscopy

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Check for updates

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Fluorescence microscopy has evolved from a purely observational tool to a platform for quantitative, hypothesis-driven research. As such, the demand for faster and less phototoxic imaging modalities has spurred a rapid growth in light sheet fluorescence microscopy (LSFM). By restricting the excitation to a thin plane, LSFM reduces the overall light dose to a specimen while simultaneously improving image contrast. However, the defining characteristics of light sheet microscopes subsequently warrant unique considerations in their use for quantitative experiments. In this Perspective, we outline many of the pitfalls in LSFM that can compromise analysis and confound interpretation. Moreover, we offer guidance in addressing these caveats when possible. In doing so, we hope to provide a useful resource for life scientists seeking to adopt LSFM to quantitatively address complex biological hypotheses.

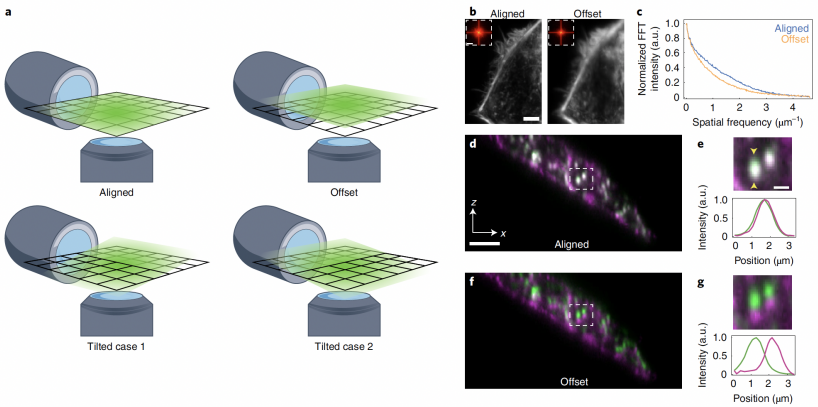
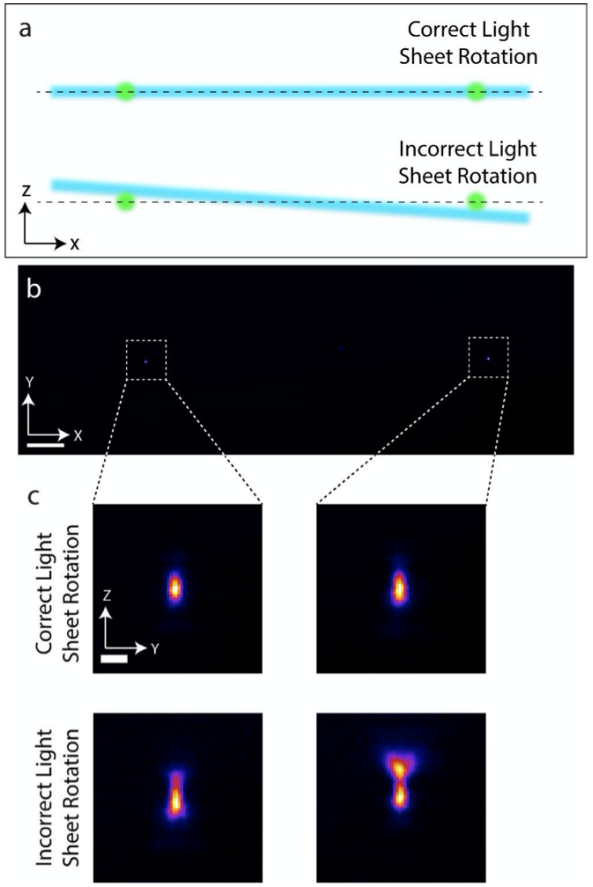


Fig. 2 | Misalignment of light sheet and focal plane compromises feature detection and axial localization. a, Schematic representation of misalignments between the light sheet and the image plane. **b**, MIPs from a volumetric image of a HeLa cell fixed and labeled with Alexa Fluor 488 Phalloidin. Images of the same cell with and without a 1 μm offset between the focal plane and the light sheet are shown, displaying degraded resolution and contrast. Insets show a fast Fourier transform (FFT) of the MIPs, scale bar, 2 μm^{-1}. Images were acquired using a modified lattice light sheet microscope. Scale bar, 5 μm. **c**, Radial profile plot of the normalized intensity from the FFT images shown in **b**. The offset profile shows a loss of high spatial frequency features compared to the aligned profile. **d**–**g**, Volumetric images of HeLa cells fixed and stained for LAMP1 (green) and transferrin (magenta) were acquired on a lattice light sheet microscope. **d**,**f**, A single *xz* cross section of the same cell is shown wherein the 488 and 561 nm light sheets are coaligned to the focal plane of the detection objective (**d**) and misaligned from the focal plane of the detection objective (**f**) by ±500 nm, respectively. Scale bar, 5 μm This light sheet offset causes an apparent axial shift of transferrin outside the lysosomes. **e**,**g**, An inset of the white dashed box in **d** and **f** as well as a line trace through the left-most lysosome (between yellow arrows) is shown for the coaligned (**e**) and misaligned (**g**) cases. Scale bar, 1 μm.



Supplementary Figure 3: **The effect of light sheet rotation across the field of view (FOV).** (a) Schematic diagram showing correct and incorrect light sheet (blue line) rotation with respect to the focal plane (black dashed line) and fluorescent beads (green circles). (b) Maximum intensity projection of two 200 nm fluorescent beads at opposite ends of the FOV. Scale bar = 10 μm. (c) Y-Z projections of the fluorescent beads at each end of the field of view both with and without proper rotation of the light sheet. A 1° rotation of the light sheet dramatically skews the PSF in opposite directions at each edge of the FOV. Scale bar = 1 μm. Images were acquired on a modified lattice light sheet microscope.

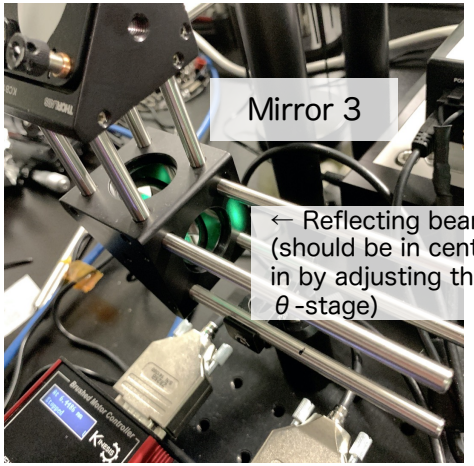
If the angle between the excitation and detection optical path is not perpendicular (>1 degree), focal spot is elongated and in case doubled along z-axis.

6. Follow-up alignments (if required)

Same procedure as 13

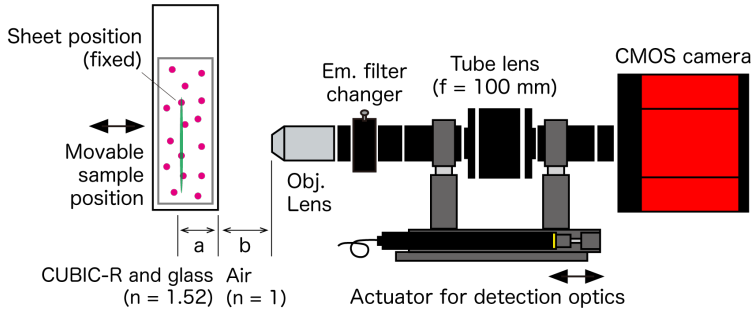
1. Place a test sample (CUBIC-R gel-embedded fluorescent beads) in the sample chamber.

2. Adjust the θ of the sample stage so that the reflected light from the surface of the cuvette returns to the center of mirror 3 (Right upper figure: the beam seen on the right side of the mirror).

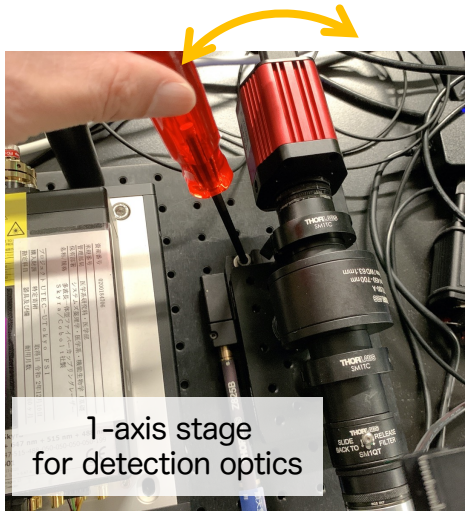


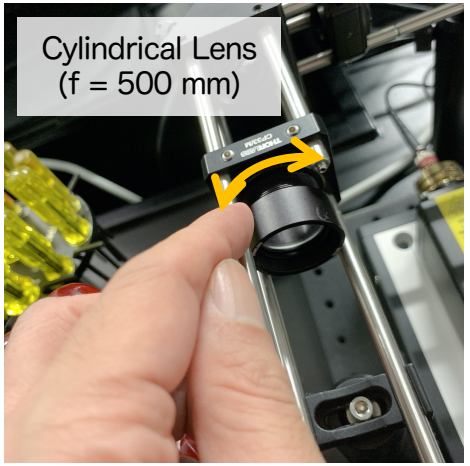
3. Adjust the position of the sample stage and detection optics to find the focal plane where the fluorescent image of the test sample can be acquired, and adjust the position of the cylindrical lens so that the focus of the light sheet is near the center of the field of view.

4. move the detection optics back and forth while capturing the bead image, and confirm the appearance and disappearance of the focal region in the field of view.



The heterogeneous appearance and disappearance of the focal region reflects the fact that the light sheet and the detecting plane of the camera are twisted (next page). Make the following alignments until the focal spot appears and disappears uniformly in the field of view.



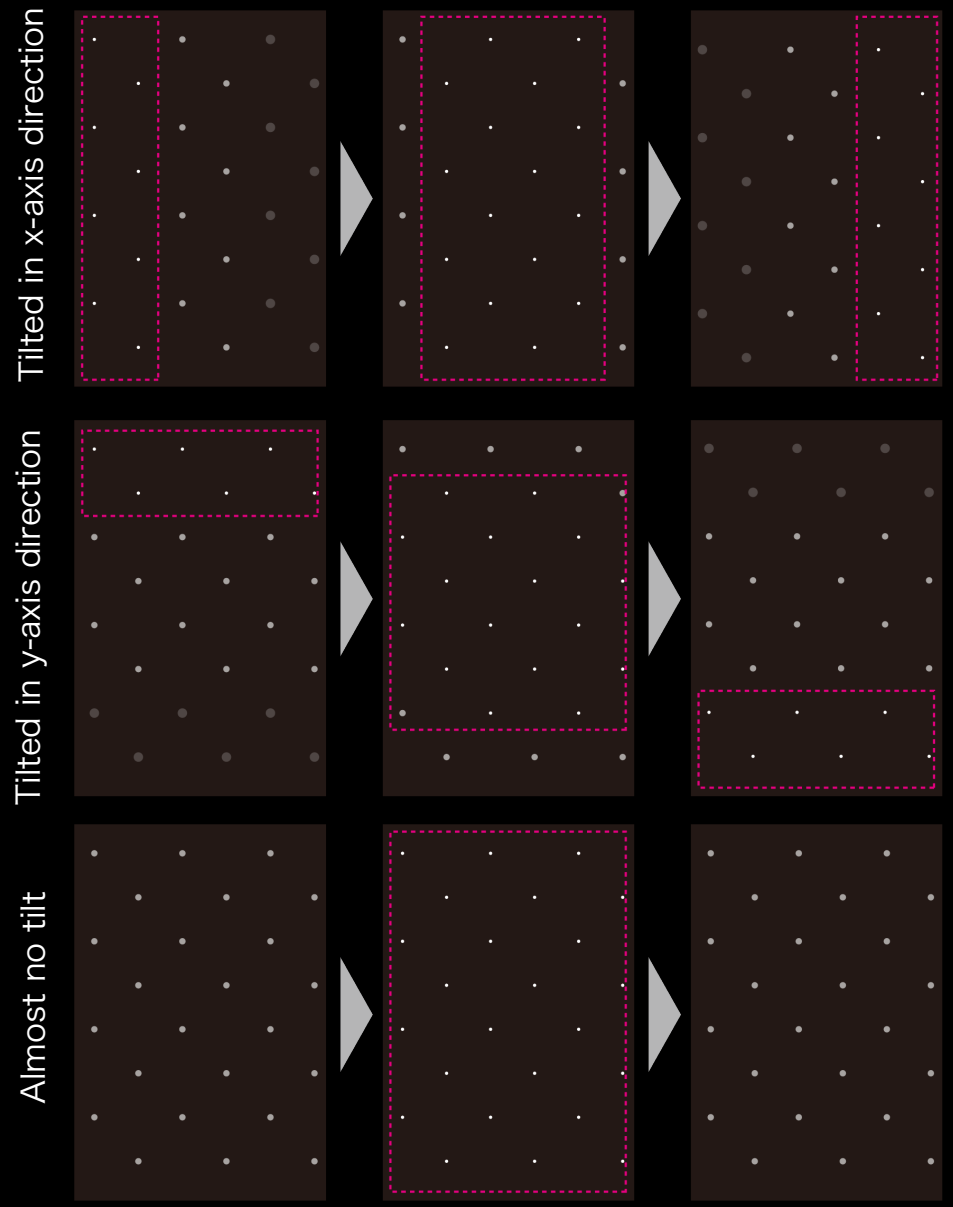


x-axis: Loosen the M6 screw between the 1-axis stage for detection optics and the breadboard, and adjust the tilt of the detection optics relative to the z-axis.

y-axis: Adjust the direction of the cylindrical lens.

5. Evaluate the PSF by measuring the fluorescent beads image while moving both the sample stage and the detection optics. Confirm that there are no two cobs in z-direction near the focal area of cylindrical lens, and that the area near the center of the field of view has excellent light collection.

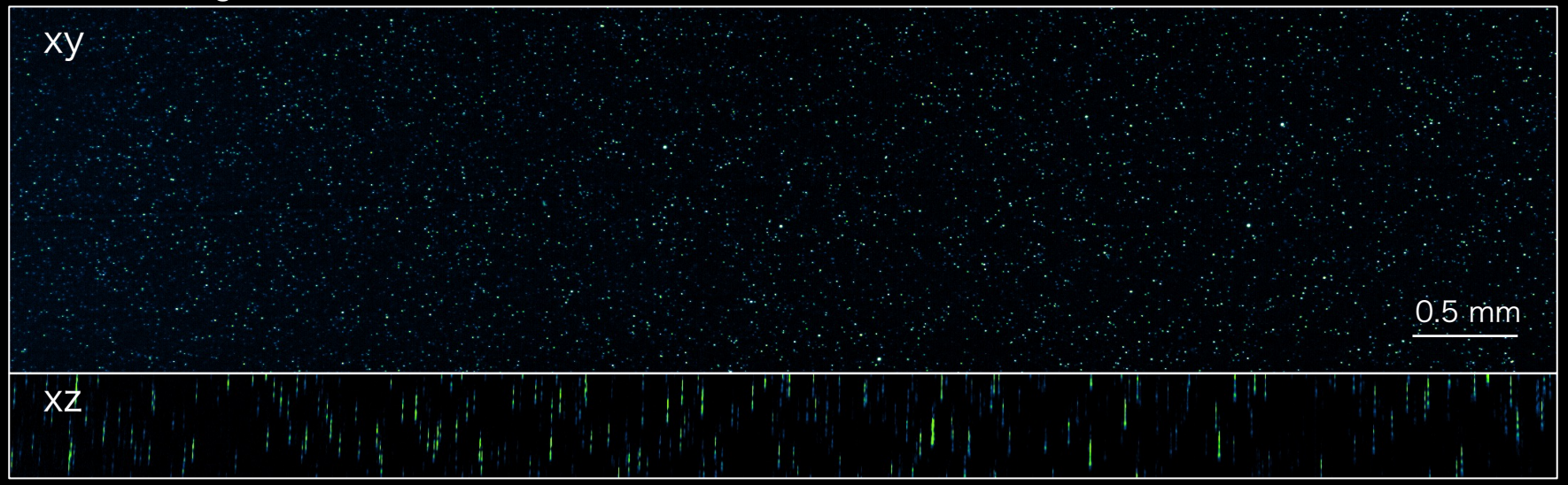
Assumed focal descSPIM images with moving detection optics



descSPIM images of gel-embedded ϕ1 um fluorescent beads

f = 500 mm cylindrical lens; Exposure time: 150 ms/frame; v_{stage} 0.033 mm/s, v_{detect} 0.011 mm/s
z-interval: 5 μm, 100 frames, z-range: 500 μm; xy: 7,452 μm × 1,760 μm

● Before alignment



● After alignment