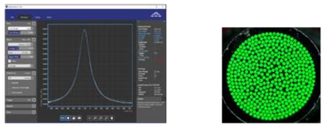
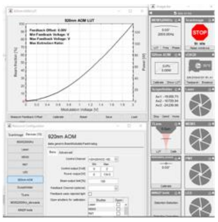
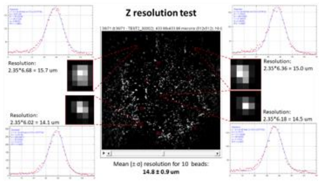
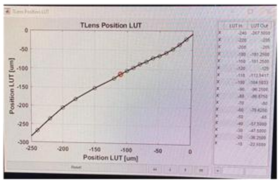
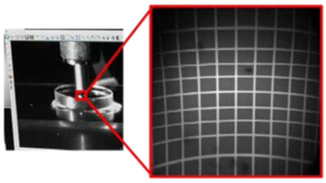
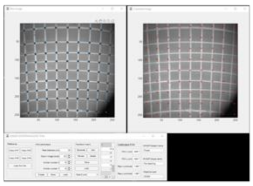
P5 - Performance Tests & Standard Testing Protocol

This protocol includes preliminary testing of a MINI2P system (Steps 1.1-1.5), laser calibration after the scope´s objective (Step 2) and four other steps for miniscope calibration (Steps 3-6). An overview of these steps is shown below.

Overview of key steps

Step1: Preliminary testing: system reports 	Step2: Laser power calibration 	Step3: Measure Z-resolution 
Step4: Tlens calibration 	Step5: Estimate the FOV 	Step6: Distortion detection and correction 

Reagents and other equipment

Component Name	**Supplier**	**Part Number/ #Item**
Assembled MINI2P 2022/2023 + Objective D0309*	LabMaker/self assembled	Several + #113*
Power meter and photodiode	Thorlabs	#g
Universal post holder	Thorlabs	UPHX/M** (#69)
Optical posts (x4)	Thorlabs	TRX/M** (#62)
Distortion grid	Thorlabs	#n
Fluorescent marker/highlighter	-	#n
Headbar for MEC (or CA1, V1)	3A prototype	#120
Glass plugs (x2)	Sunlight	**(new!) #121**
Cover slips	Sunlight	**(new!) #122**
FluoSpheres beads	ThermoFisher	**(new!) #123**
Distilled water or ethanol	-	-
Distortion grid setup		
Rail carriage	Thorlabs	XT95RC4/M (#57)
Kinematic rotation mount	Thorlabs	**(new!) KS1RS (#124)**
Beads setup «scope mounting»		
Rail carriage	Thorlabs	XT95RC4/M (#57)
Quick-connect goniometers (x2)	Thorlabs	**(new!) XRNG2/M (#125)**
Headbar holder	3A prototype	#57
M4 screws (x2)	-	-
M3 screws (x2)	-	-

*The miniscope calibration example can be adapted to air objectives too by just removing the cover glass placed on top of the distortion grid and bead sample. ** Variations of these items with different heights are suitable too (X=75, 100, …)

Step1. Preliminary testing: system report

Before starting the miniscope performance tests, ensure the system report requirements and designed parameters have been fulfilled according to Table 1. Each design parameter comes with examples of test-results and referenced to other protocols.

Table 1: System report designed parameters with acceptable values.

Step	Design parameters	Acceptable values/ Minimum Requirements	Test results (examples)
1.1	**HC-920 Coupling efficiency & output shape**	>70% after collimator Sharp, Gaussian-profile distribution in the center	Fig.1.1
1.2	**Pulse width & shape**	<130 fs (after the HC-920), Lorentz fitting, minimized side lobes, stable	Fig.1.2
1.3	**GFB efficency & dead cores**	> 70 % of green laser input (535nm) < 4 dead cores	Fig.1.3
1.4	**System wiring**	Breakoutboard to controlling box and other components Confirm MDF channels	Fig.1.4
1.5	**Connect MINI2P**	Insert HC-920-PM collimator Attach GFB/TFB Align collimator to MEMS mirror center	Fig.1.5

1.1 HC-920 coupling efficiency: defined as the ratio of the output power measured after the collimator, and the power at the exit of the coupling box before the coupling lens. It must be equal or higher than 70% (it typically does not exceed 80%).

Laser beam output: shape of the spot at the exit of the collimator holder must be sharp (not blurry) and with a Gaussian-like profile distribution in the center. The shape differs between different HC-920 fiber versions, as follows:

- K50-060-70: hexagonal-star shape or 4-pointed rounded symmetric star (Fig.1.1-a)
- K60-060-PM*: stretched oval shape (Fig.1.1-b)

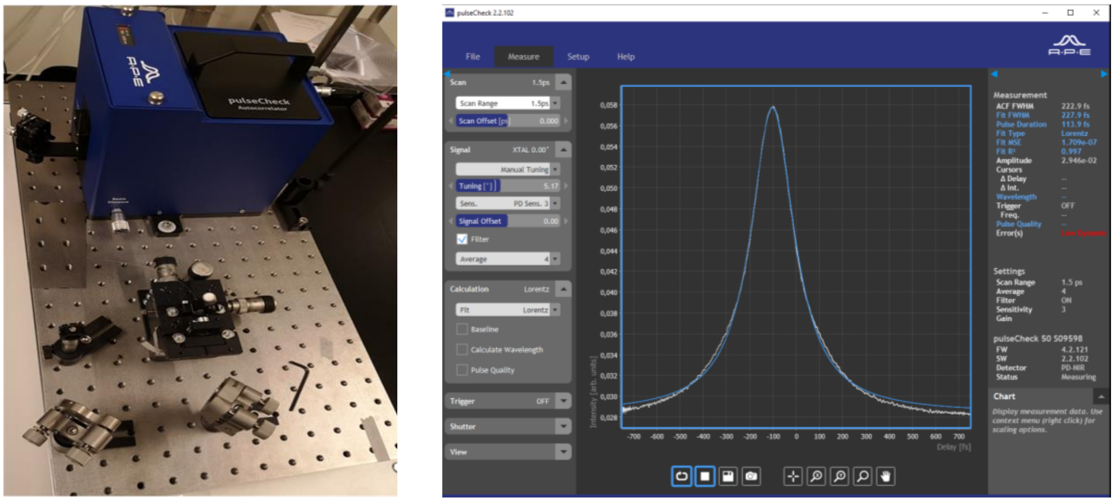
* HC-920-PM is the only fiber commercially available by NKT Photonics as of July 2023


a)


b)

Fig.1.1: *Collimated laser output shape after passing through 2.2-2.3-m long HC-920 fiber and collimator on a NIR detection card placed 2-3m away for two HC-920 fiber versions: (a) K50-060-70 and (b) K60-060-PM.*

1.2 **Pulse width** is also measured after the collimator. A set of mirrors are placed in front of the collimator with an angle to reflect the output laser beam to the autocorrelator entrance slit. With correct alignment, a light should be visible at the autocorrelator grid and centered in the cross.
Run APE Pulse Check as shown in video tutorial “HC-920 assembly” to measure the Pulse width by curve fitting a Lorentzian. Change the laser GDD (via Toptica/Sparklaser software) and rotate the half-wave plate (HWP) inside the coupling box such the side lobes and pulse width (<130 fs) are minimized.

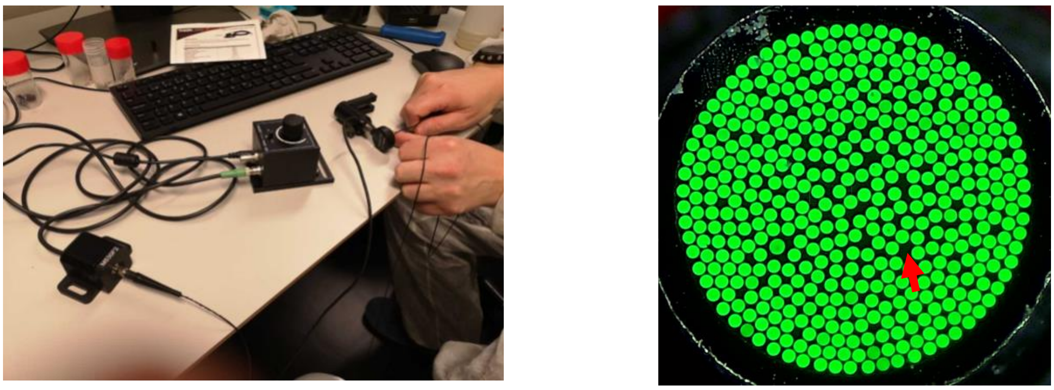


a) b)

Fig.1.2: *(a) Setup to measure pulse width; (b) Pulse plot measured by APE autocorrelator, with Lorentzian fitting and GDD for 2.2m HC-920-PM fiber.*

1.3 **GFB efficiency:** connect a green LED through one of the ends with the SMA connector (see Fig1.a) with fixed input power (around 30mW) and then measure the output power at the other end using a photodiode sensor connected to a power meter (select green laser wavelength ~ 530nm). GFB efficiency of 70% or higher is ideally recommended.
Efficiency lower than 60% does not meet the standard requirements for the MINI2P platforms.

The second test is to visualize the fiber cores under the microscope while shining green light with a green laser through the SMA end. An ideal GFB should not have more than 3-4 dead cores (see Fig.1.3-b). Both steps were described in GFB assembly tutorial protocol.



a) b)

Fig.1.3: *TFB/GFB standardized performance tests: (a) measuring efficiency with a 530 nm green laser; (b) fiber illuminated cores under a microscope (with 1 dead core).*

1.4 **System wiring**

This step is especially important to ensure all BNC cables are correctly connected from the breakout board channels to the controlling box, TLens driver, PMT shutter and laser, as illustrated in the schematics of Fig.1.4 and Table 2. The next steps are recommended as follows:

i. Load the Machine-data-file (MDF) suitable for the MEMS mirror (2000Hz for Slow or Large-FOV and 5600 Hz for Fast-FOV) when starting ScanImage. Load also Configuration settings given.
ii. Double check all connections, especially between breakout board (AO/D) channels and control box, but also to PMT shutter, laser controller and TLens driver.
iii. Open the different MDF tabs on *File>Machine configuration* to confirm the breakout board BNC connections match the selected AOX/DX in the ScanImage main tabs.

CAUTION! MEMS x-y axes and correspondent filters channels were swapped on ScanImage MDF to guarantee a more square-FOV. This can be reversed, just make sure to match the fast axis with its fast filter, and slow axis to its slow filter.

For a more comprehensive matching, please check MDFs available on GitHub for 2000Hz (slow MEMS, L-FOV) and for 5600Hz (fast MEMS, F-FOV).

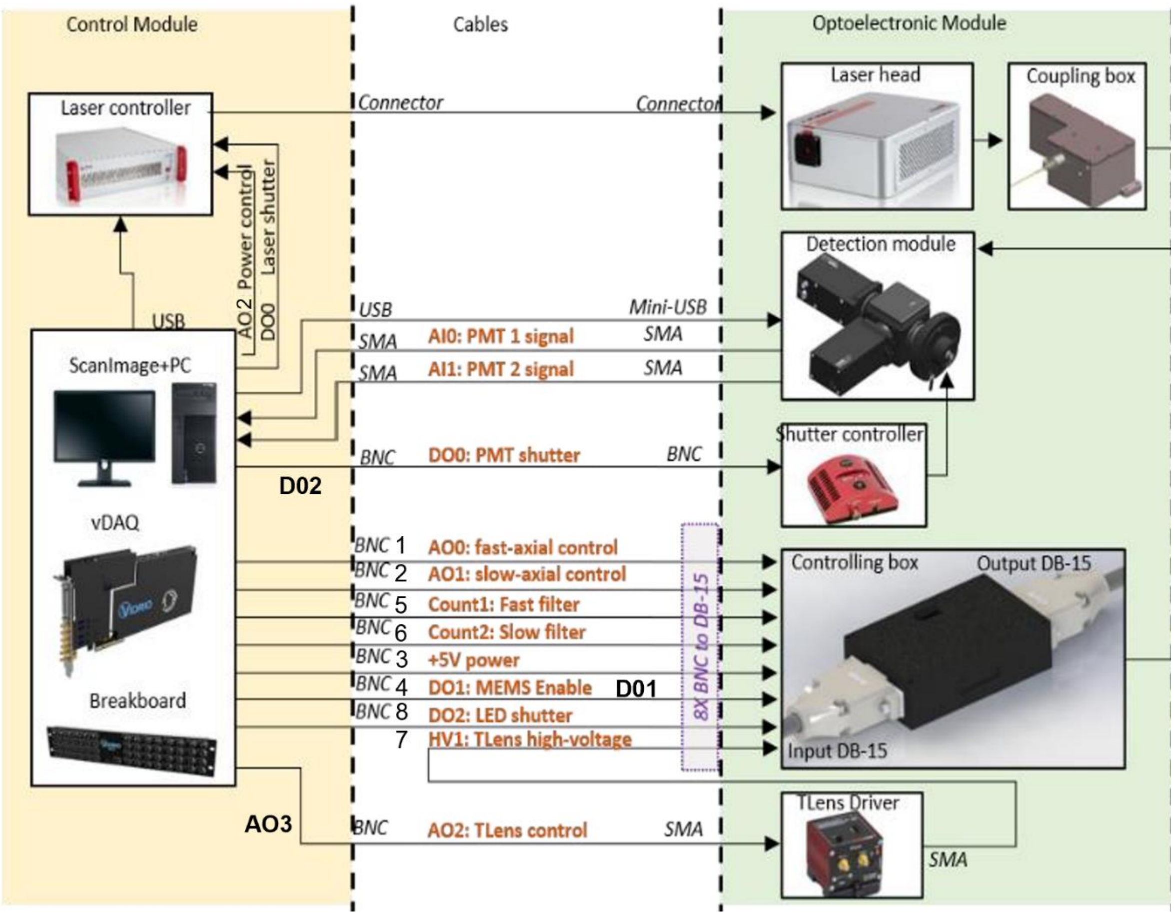


Fig.1.4.: System wiring with focus on connections from the vDAQ breakout board to the control box, laser, TLens and PMTs.

Controlling box input, BNC connections (1 to 8) is illustrated in Fig.1.4. All BNCs are directly connected to breakout board channels (see Table 2), except BNC7 which is connected to TLens driver. Digital shutters are also shown for laser (D0.0), PMT (D0.2) and MEMS enable (D0.1).

Analogue output channels, AOX are displayed for TLens driver (AO3), laser controller (AO2) and MEMS fast and slow axes (AO0-AO1).

*Table2: Correspondence between breakout board channels connected to controlbox (BNC 1 to 6) and driver (BNC 7) to MDF tabs in ScanImage for fast MEMS (5600Hz).*Axes were swapped for a more square FOV, yet filter clocks must match the swap otherwise MEMS can break.*

Breakout board channels	**Control box (BNC 1 to 8)**	**MDF tab for Large FOV (5600Hz)**
AO0*	BNC1 - "fast axis"	**MEMS5600Hz_slow axis*** dabs.generic.GalvoPureAnalog
AO1*	BNC2 - "slow axis"	**MEMS5600Hz*** dabs.mirrorcle.Resonant Axis Zoom control channel: AO1
+5V	BNC3 - power	---
D0.1	BNC 4 – Enable	MEMS digital shutter
D3.1	BNC 5 - "fast filter"	Filter Clock of MEMS Y/…/D3.1 Freq. 500 000 Hz
D3.2	BNC 6 - "slow filter"	Filter Clock X: (D3.2) Freq. 100 000 Hz
BNC-SMA cable (piezocontroller)	BNC 7 - TLens High Voltage	---
X	BNC 8 - not in use (for LED)	X

1.5 **Connect MINI2P to system**

i. Insert collimator holder all the way into scopebody P1 slit.

ii. Fixate HC-920 fiber collimator in an optimal position: beam hits the center of the MEMS mirror, and the reflected beam output should look oval and symmetric as in Fig.1.5-b.

Caution! No MEMS circuits should be visible on the sides in the reflected beam output (Fig.1.5-b).

Tips! If collimator is loose, screw a M1.2 to fixate it.

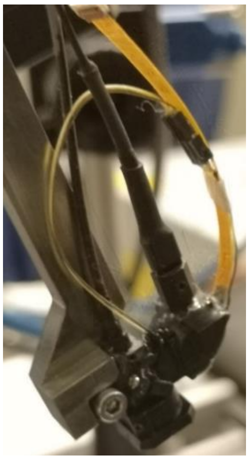
iii. Insert GFB/TFB into scopebody P3 slit until there is some resistance.

Caution! Do not push too hard in as it can break the dichroic mirror.

Tips! Instead of a screw to fixate it, it is possible to add some tack-it around it.

iv. Connect the MEMS flex cable (PCB) to the 6-wires connector (black end facing clip)

Caution! Before attaching the MEMS flex cable to the MEMS 6-wires connector please remember to disconnect the controlling module power (5V) and to disable the piezocontroller HV.



a) *MINI2P connected to system*

b) *laser output after objective with ScanImage in PARK*

Fig.1.5: *MINI2P mounted to system (a) and beam output after miniscope's objective after being reflected on the MEMS mirror (b) centered on the MEMS mirror. If non-centered, the MEMS circuits are visible on the edges of the beam output. ScanImage is on PARK and laser power is ~ 30%.*

Step2. Power laser calibration

2.1 Calibrate laser look-up-table (LUT) by measuring power output with a photodiode (item #g) placed in front of the objective (see Step 18 of Protocol P4, part 2).

2.2 Update normalized values to 920 AOM.beamlut located in the same folder as the MDF (e.g. C:\SI settings\2022\...).

Tips! Measuring in steps of 0.2V from 0 to 2V is typically enough range for Ca^{2+}imaging.

Note! Normalized output power (Fig.2) is the ratio of measured output power after the objective by the maximum power at 2V, i.e., in the illustrated case all power values were divided by 134.5mW.

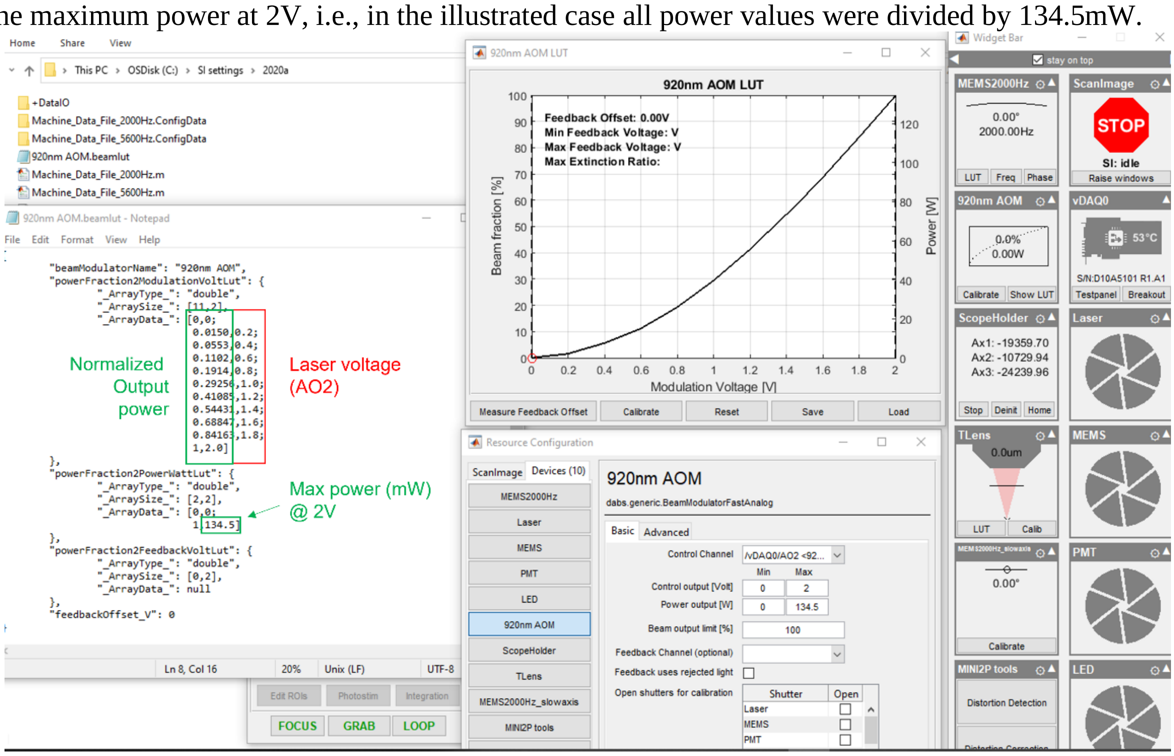


Fig.2: 920nm AOM beamLUT file of laser calibrated from 0-2V in steps of 0.2V with maximum power of 135mW (left); ScanImage laser LUT of beam fraction (in %, left vertical axis) and power (in mW, right vertical axis) as function of laser AO voltage (top bottom); MDF AOM tab delimitating voltage and power.

920nm AOM.beamlut file is available on GitHub under MINI2P_toolbox/Software/SI settings/

Miniscope calibration (steps 3-6)

Table2: Designed parameters with acceptable requirements for a high standard miniscope.

<table>
<tr><th>Step</th><th>Design parameters</th><th>Acceptable values/ Minimum Requirements</th><th>Test results</th></tr>
<tr><td>3.</td><td>Z resolution</td><td><16-17 μm
with objective D0277 / D0329</td><td rowspan="7">Report results in this column and/or add location of saved data (Z-stack for Res, MDFs with TLens LUT, 3D Transf. Matrix)</td></tr>
<tr><td>4.</td><td>Tlens calib LUT</td><td>Steps of 10μm along the Z axis
Typically, 20-25 datapoints</td></tr>
<tr><td>5.</td><td>Estimate FOV</td><td>>450 μm x 450 μm, MINI2P-L
>350 μm x 350 μm, MINI2P-F
At 0 μm plane (after correction)</td></tr>
<tr><td>6.</td><td>3D Transformation Matrix</td><td>Distortion detection at every 10μm along the Z axis
Total 20-25 matrices</td></tr>
<tr><td></td><td>Speed</td><td>15Hz (MINI2P-L, 256 line)
40Hz (MINI2P-F, 256 line)</td></tr>
<tr><td></td><td>Weight</td><td><2.5 g (without holder and baseplate)</td></tr>
<tr><td></td><td>Z-scanning</td><td>Range: >200 µm (up to 240μm)</td></tr>
</table>

In this part, the main four standard performance steps (steps 3-6) are provided to calibrate a miniscope (valid for either version, MINI2P 2022 and MINI2P 2023):
Step3. Measure Z resolution
Step4. Calibrate TLens look-up-table (LUT)
Step5. Estimate FOV using MINI2P SI device
Step6. Distortion detection and correction: 3D Transformation Matrix

Before measuring the resolution (Step 3), it is recommended to place a prepared distortion grid (see steps 4-b, 5-6) to check how the FOV looks like with higher zoom. Start with zoom 2.0, and then decrease slowly up to 1.0 (CAUTION! Always stop focus before changing the Zoom, otherwise the MEMS can break). If the FOV looks uniform and the MEMS slow and fast axes settings are adequate, proceed to step 3. Otherwise, open MDF configuration from File Menu, and decrease the applied voltage and/or angle on MEMS tabs until the FOV looks stable and uniform.

For **steps 3-4**, it is recommended to use a bead sample with 1 $\boldsymbol{\mu}$m diameter beads with implant as one would normally do for a surgery yet adapt it for miniscope calibration: the surface of the implant that usually faces the imaging plane is covered with fluorescent beads.

Bead sample preparation

i. Prepare headbar (e.g. for V1): glue a cover glass (Ø4.6mm, 1.5mm thick) to the inner diameter of your headbar, with the top flushed
   - Alternatively, use MEC assembly with prism or another suitable implantation.

ii. Flip it and spread a small 1μL drop of a diluted bead mix over the cover glass
   - FluoSpheres beads (item 123) to distilled water ratio: 1:200 to 1:500, depending on desired concentration.
     Caution! Spread the thin drop with a pipette to make a very thin layer

iii. Let it dry for 2h, and only then add a medium gel (index matching the brain) over the beads and fixate a cover slip (item 122) to protect and keep the beads in place.

iv. Place the prepared bead sample holder with M3 screws (See Fig.3)

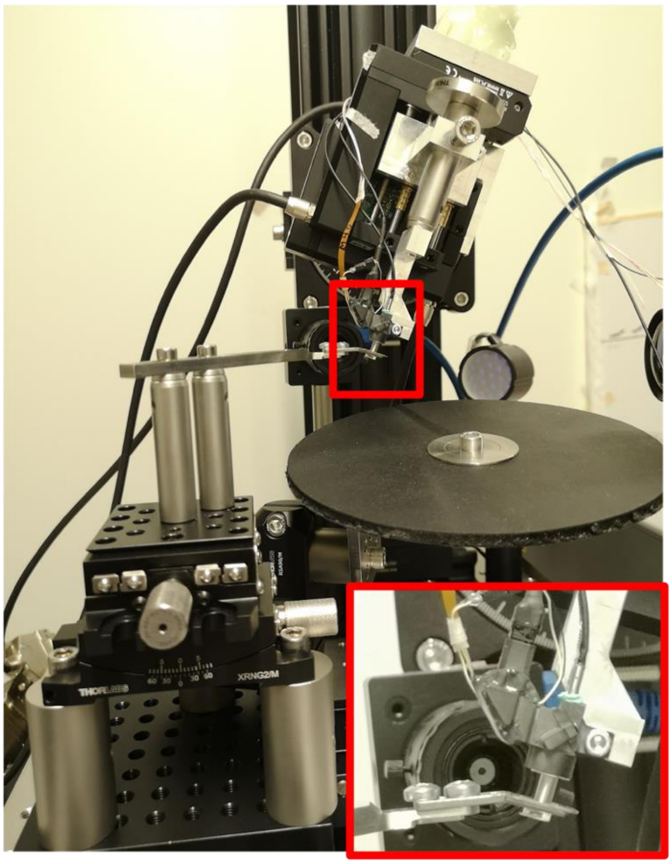


Fig.3 Example setups for scope mounting module with a bead sample mounted onto a headbar for D0309 objective and zoom-in of scope-to-beads alignment.

Step3. Measure Z-resolution

For resolution tests and TLens calibration, it is recommended to mimic the implantation configuration in-vivo. Therefore, fix your beads sample into the same headbar type (MEC, CA1 or V1) + prism or glass-plug/cover slit you use during surgery as described before.

To determine the resolution of the miniscope, measure the axial bead intensity distribution by recording a Z-stack of the prepared beads sample around its focus plane. Then select multiple beads across the FOV, and for each individual bead, plot its Gaussian distribution to find the **axial resolution, Res_Z** which can be approximated as:

$$Res_Z = 2\sqrt{2\ln 2}\ d \approx 2.35\ \mathrm{d}\ \text{(Eq1)}$$

Where d is the standard deviation of the Gaussian. To determine the lateral resolution, Res_{XY} correct first the imaged z-stacked by applying the transformation matrix determined at the focus plane. Fig.3.1 resumes the image acquisition approach to measure the Z-resolution.

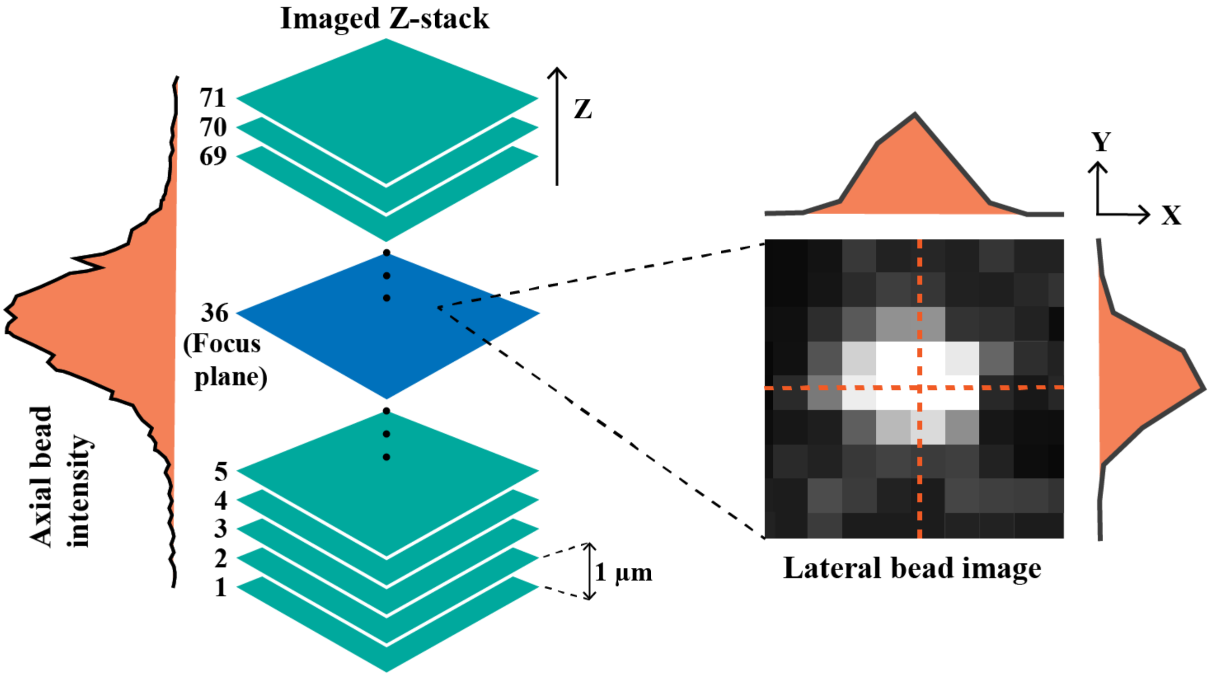


Fig. 3.1: Schematic depicting the image acquisition approach for determining the resolution of the miniscope. Herein, a bead in the sample will have a three-dimensional PSF, making it possible to plot its intensity along all three axes: X, Y and Z.

Before determining the resolution, first align the beads image to the objective plane as follows:

3.1. Place the prepared bead setup under the miniscope

3.2. Move the stages until you find the focal plane. Optimize the power (typically 15-20mW for a bead solution as the diluted above)

3.3. Align bead sample to scope light output. Manually move x-y tilt goniometers (items #125) placed under headbar holder until there is no clear shift of light.

3.4. Measure a Z-stack of N planes (N-odd number) as described in Fig.3.1.
Tips! To check alignment, a Z-stack with less planes is preferred.

3.5. Open the measured Z-Stack on Fiji and select four outermost regions of interest (RO1 to RO4), as in Fig.3.2. The bead sample is aligned to the scope light output if Z-profile has centered (focus planes) within ± 1 pixel.

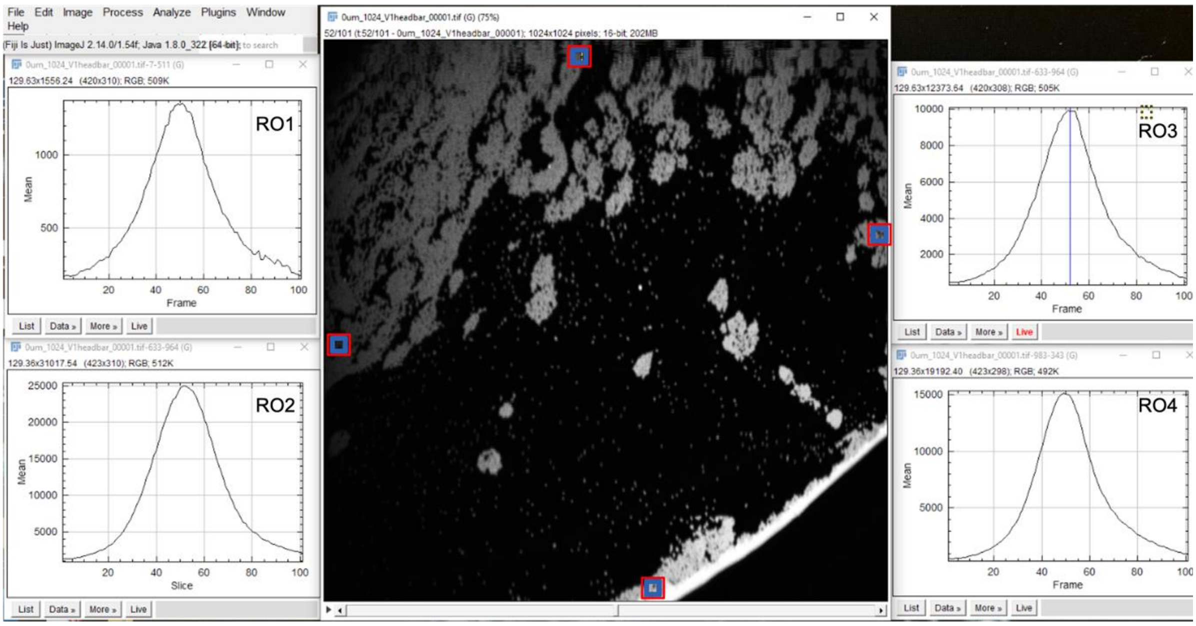
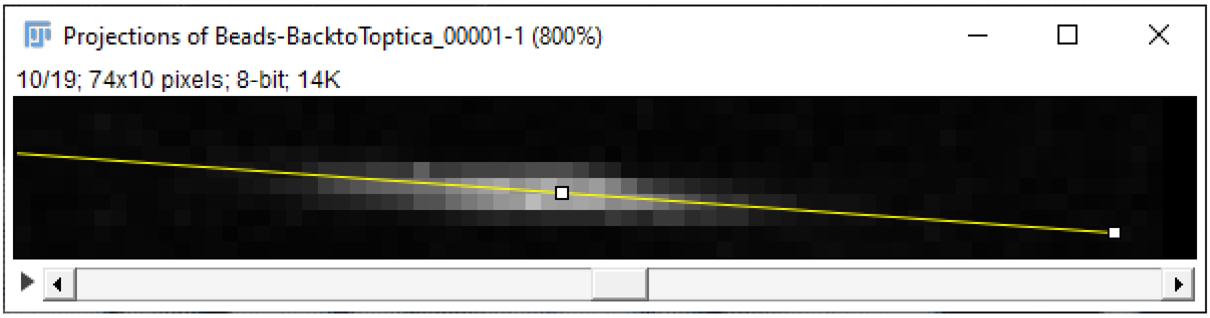


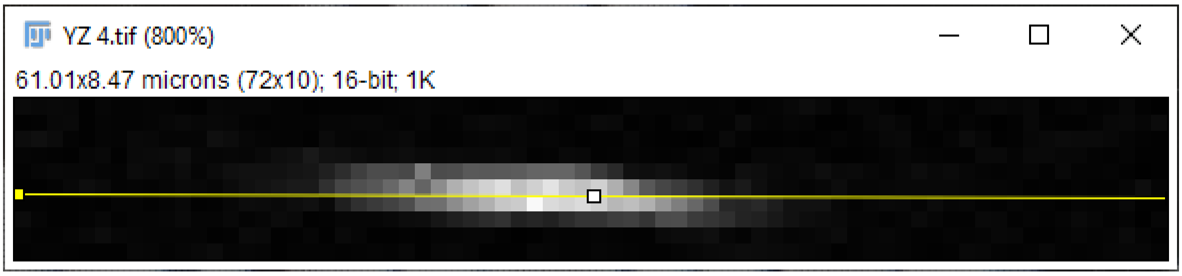
Fig.3.2: Z-stack of bead sample (consisting of 101 planes) and plot 4 regions of interest (ROIs) (top, bottom, left and right) across the FOV, which have central plane around frame 51± 1 indicating that the headbar beads sample is placed parallel to the miniscope's imaging plane.

3.6. Around the central plane, image a Z-stack of N planes at 512- or 1024-pixel image size, N being an odd number to guarantee the same number of planes above and below the central focus plane.
Note! This step should only be done after the bead sample is aligned as in Step 3.5 and Fig.3.2.
Tips! Check if number of planes selected is sufficient such there is complete dark images at the bottom and top planes. An example is given below in Figs. 3.4.
Tips! In examples given a stack of 71 (up to 101) slices, with 10-20 frames per slice was sufficient.

3.7. When ready, click "ENABLE" stack, and average by the number of frames per plane (typically around 10). Click GRAB to record.
3.8. Open the recorded Z-stack with Fiji. Select a single bead with the square cursor, and right click to duplicate stack for this single bead.
3.9. Plot Z-profile of single bead to find brightest plane. Move stack to that plane, adjust contrast in Image>Adjust>Brightness/contrast and press "Reset" at the brightest plane, and click "Apply" all.
3.10. In Image>Stacks plot **3D projection** (X or Y, 180 degrees) or **orthogonal views** to visualize the axial PSF.
3.11. Drag a line along the center of the PSFs as illustrated below.



3D projection of selected bead.



YZ orthogonal view of the same bead.

3.12. Plot the intensity values by clicking Analyze>Plot Profile. Copy the datapoints.

3.13. Fit the profile with a Gaussian curve by clicking Analyze>Tools>Curve fitting, paste the datapoints and select Gaussian. A similar profile as shown at the top-right of Fig.3.3 should be displayed.

3.14. Calculate the z-resolution using Eq (1)

3.15. Repeat steps 3.8-3.12 for at least 10 beads across the FOV.

3.16. To find the miniscope Z-resolution, average its values. A Z-resolution of $14.8 \pm 0.9 \mu m$ is shown in example of Fig.3.4

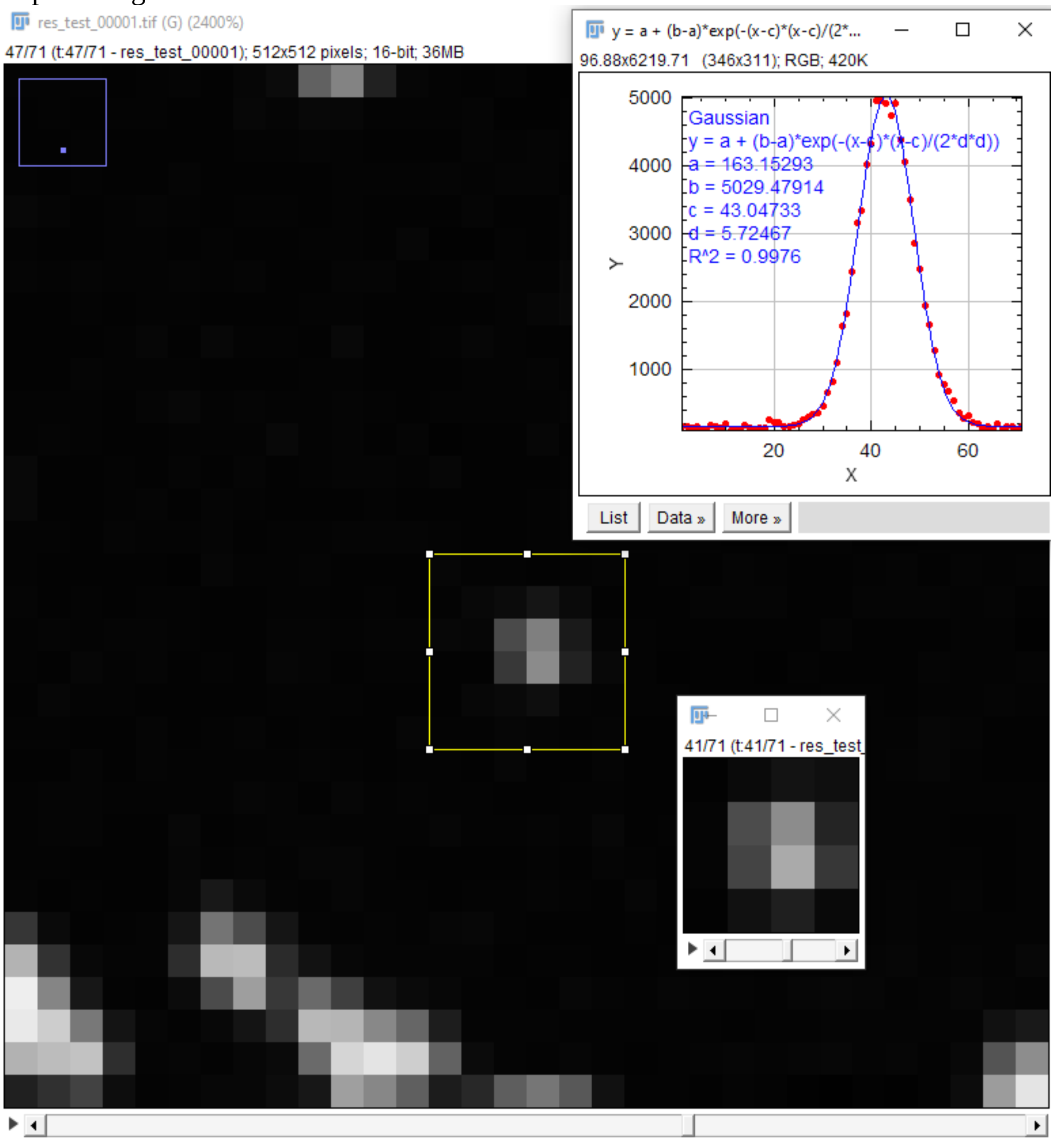


Fig. 3.3: Single bead zoomed-in of z-stack imaged using motors for 71 planes, 20 frames per plane at 512 pixels. Gaussian bead profile (top right corner) with d=5.72 and Z-Res of 13.4 µm.

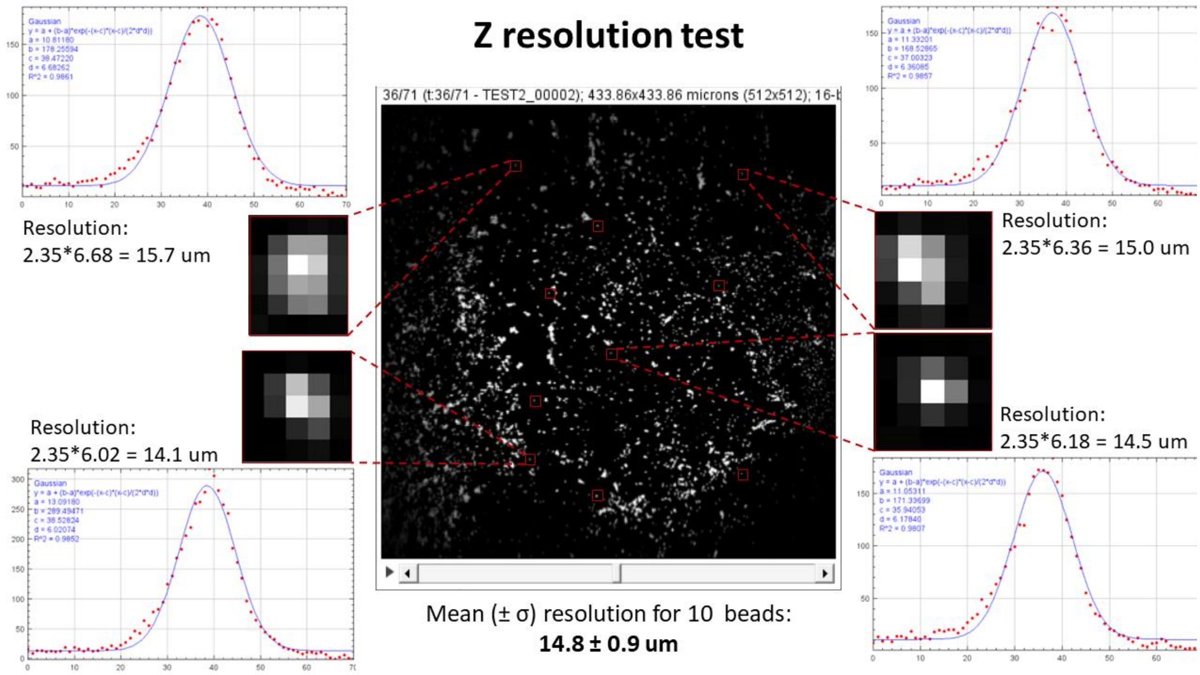


Fig. 3.4: Overview of Z resolution tests with example of gaussian profiles of 4 beads. It is recommended to measure Z-Res of 10 beads over the entire FOV and in this example.

Step4. TLens calibration

a) More precise TLens calibration method focusing on a centered bead

4.1 Open TLens LUT by pressing TLens “LUT” from the widget bar (see Fig. 4.1)
4.2 Choose a bead in the center to calibrate the TLens (see Fig. 4.2)
4.3 Move TLens from the deepest plane, 0um to -20μm which moves the focus up in z-direction. Move the linear stage motors down a 20μm step as in see Fig.4.1. This activates the next TLens LUT calibration point (in red, Fig. 4.1)
Tips! If the configuration has the scope 20 degrees rotated, correct x-y stage positions such selected bead is around the same position/ROI.

4.4 Record a stack of N plans, N being an odd number around this focus plane (Centered Stack)
Tips! In Stack controls, 31 slices, 10 frames per slice, spacing 1μm (using motors) should be sufficient. Otherwise, adjust the total number of slices such there are enough statistics for a clear Z-profile curve.

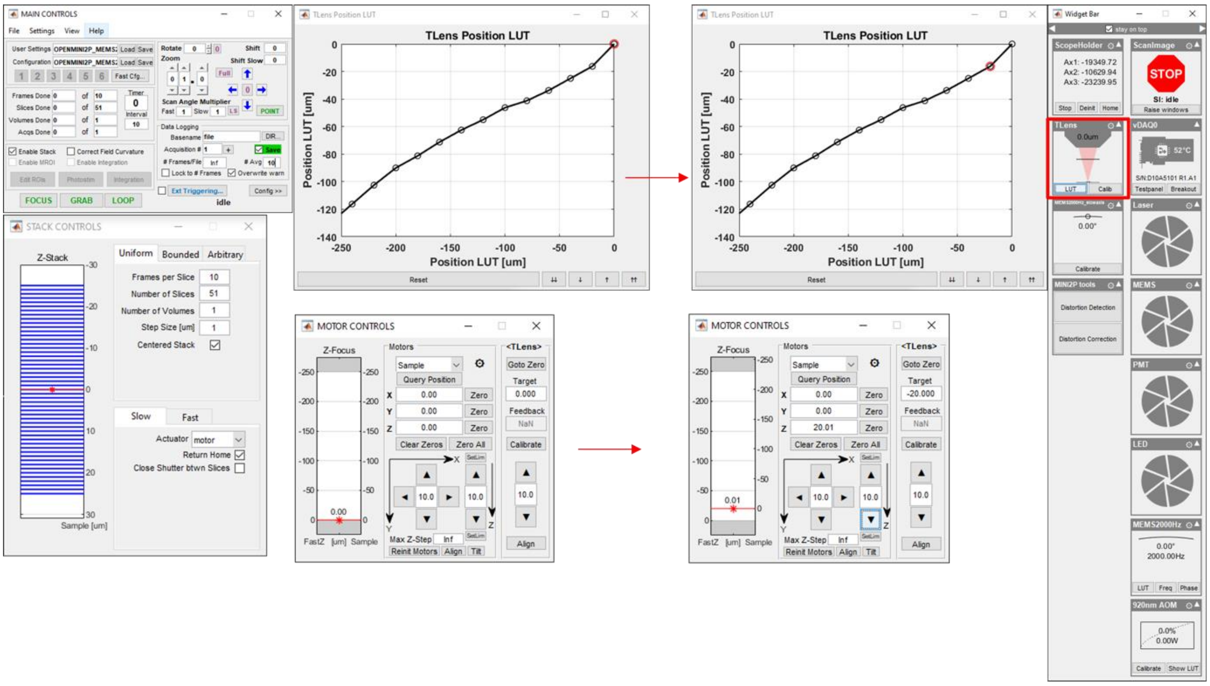


Fig.4.1: ScanImage overview of TLens position LUT, with emphasis of calibration showing transition between two datapoints at planes 0 and -20 µm in red, and motors controls tab where <TLens> and motors were moved 20 µm in opposite directions. To the left, example of Z-tack with 51 planes (10 frames per slice) to find Z-profile of single bead during TLens calibration.

4.5 Open the saved Z-stack in Fiji, select a region using a rectangle around the central bead chosen in step 4.1, save the ROI and plot its Z-profile (Image>Stack>Plot Z-axis profile).

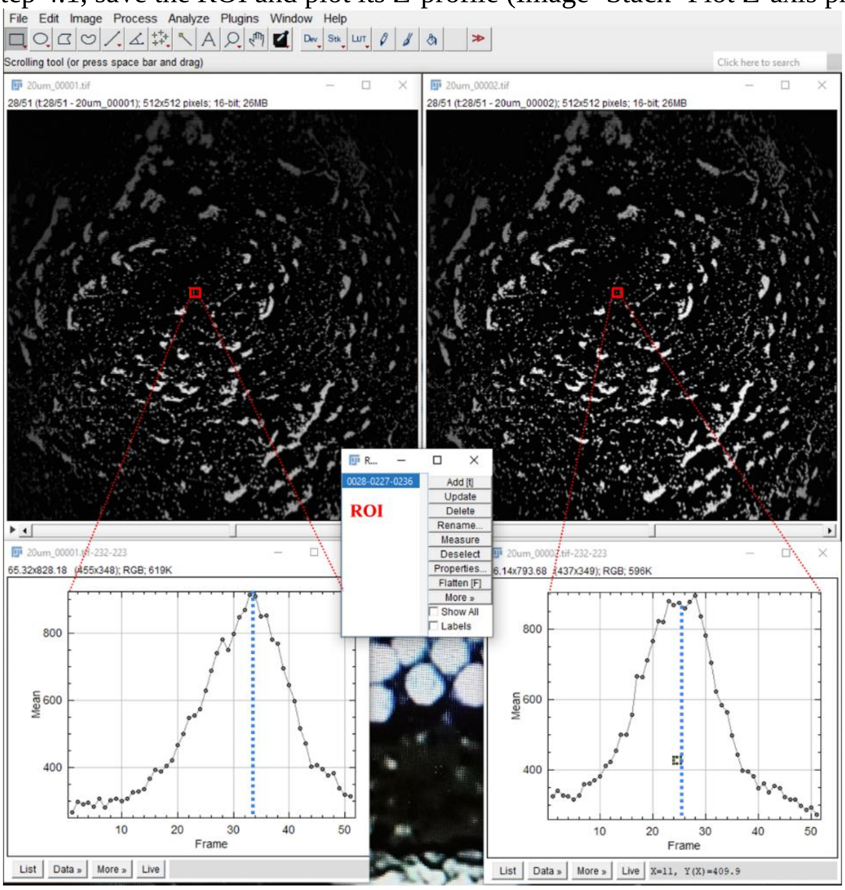
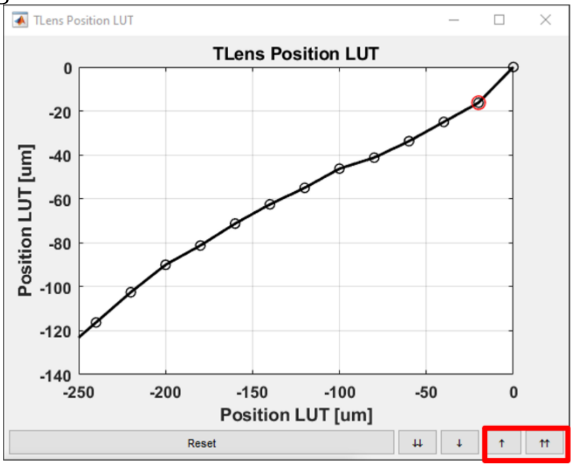


Fig.4.2: (top) Z-stack of beam sample (51 slices) for two different Tlens LUT values at -20µm plane (see Fig.4.1, right). (bottom) Z-profiles are displayed for the selected bead around the center of the FOV (red square), showing a shift towards the middle of the stack after correcting the TLens LUT datapoint by moving arrows ↑ or ↑↑.

4.6 If the bead distribution is not centered as shown Fig.4.2-left side, press the arrows pointing downwards ↓ and ↓↓ or upwards ↑ and ↑↑ on the **TLens position LUT** (see TLens position LUT below) depending on the desired shift direction.



Tips! By pressing on arrows pointing up, the focus plane of Z-profile moves to the left. For finer or cursor tuning, use one or two arrows, ↑ and ↑↑ respectively.

4.7 Record another Z-stack (as in Step 4.4) until the selected bead Z-profile is centered as in Fig.4.2-right.

4.8 Repeat TLens LUT calibration (steps 4.3-4.7) for the full working range, in steps of 20 μm (for finer calibration, in steps of 10 μm, so a total of 20-25 datapoints, -10, -20,-30, -40, …-240μm). An example of s-shape calibration curve is depicted in Fig.4.1.
Tips! Remember the TLens calibration must be done in one direction, otherwise a shift is expected due to hysteresis of the TLens piezo materials, as illustrated in Fig.4.3.

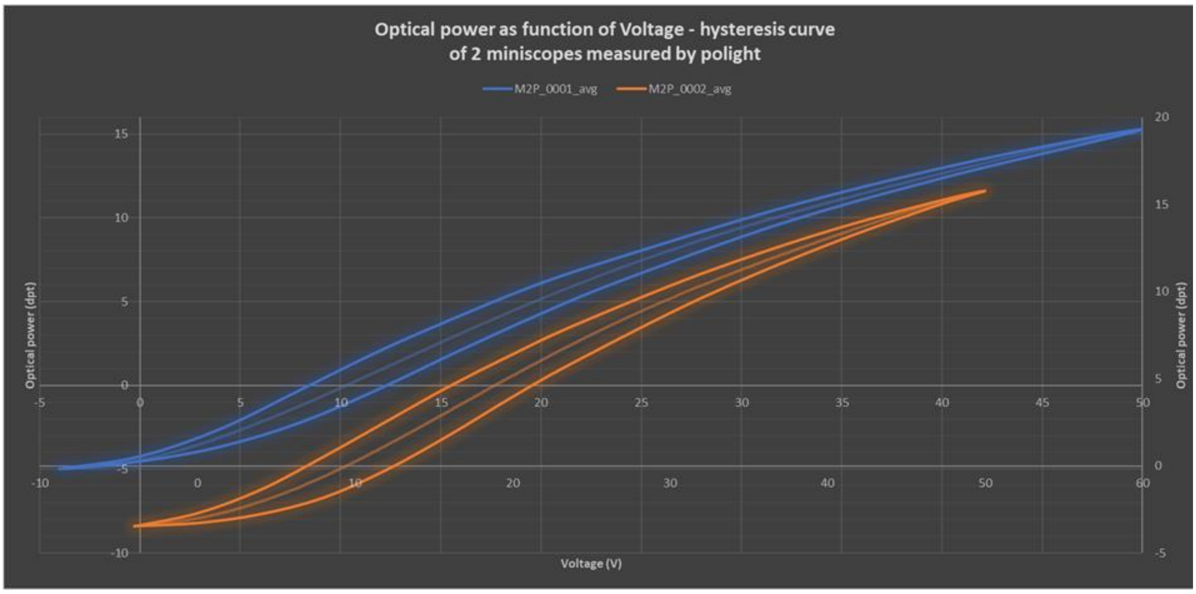


Fig.4.3: Hysteresis curve: optical power as function of voltage for a four-stacked TLens (average) when calibrated in different directions for two miniscopes. Measurements provided by poLight.

For Step 4b) and following steps 5-6, a distortion grid (item #m) is required, and its configuration must be adjusted to the type of objective (air/glass objective in this protocol: D0277 or lighter version D0309) and to the implantation configuration.

Distortion grid (item #m) preparation

- Add a thin layer of fluorescent marker over the second smallest grid, i.e. where the distance between closest parallel lines is 50um.
- Let it dry for 10min, then place a 1.5mm-thick cover glass on top of the marked grid with UV glue on the bottom edges of the glass and cure it to fixate it.
- Glue the grid sample to a rotator and adjust its position such it is parallel to the scope holder and miniscope (see Fig.4.4)

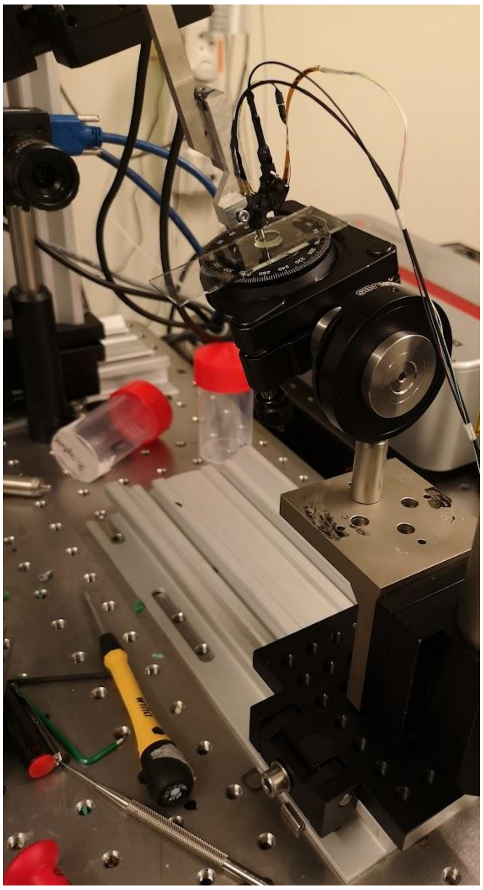


Fig.4.4: Example setup for distortion grid, with 20-degree angle suitable for MEC implantation.

Another method(s) to calibrate the TLens

b) Rough method using a distortion grid

i. Roughly align the distortion grid to the miniscope imaging plane.
Tips! When moving the motors up and down, the light should shift uniformly and not sideways or diagonally across the FOV.

ii. Find the focal plane (brightest image with light in the center) and align the distortion grid to the cursor as shown in Fig. 4.5. On Motors Controls tab, set Zero All.
Tips! Use a histogram by right clicking on the channel image to better visualize the pixel distribution.

iii. Open the TLens LUT from the widget bar. Move the TLens up from 0 to -10, and then move the z-motors down by 10μm. If the image is not yet as bright as its first, then drag the TLens arrows $\uparrow$ *or* $\downarrow$ from the TLens LUT tab until you find the same brightest focal plane.
Tips! To move the TLens position in bigger steps, use double arrows $\upuparrows$ *or* $\downdownarrows$.

iv. Continue moving the TLens up and the motors down in steps of 10μm, and for each plane correct the TLens LUT position as in Step3 for the whole working TLens range (typically -200/-240μm). The calibrated curve is typically an s-shaped curve, as in Fig.4.5.

v. When calibrating the TLens, always move in one direction, otherwise there will be a shift of the curve for the new position as shown in Fig.4.3 (hysteresis behavior of piezo materials).

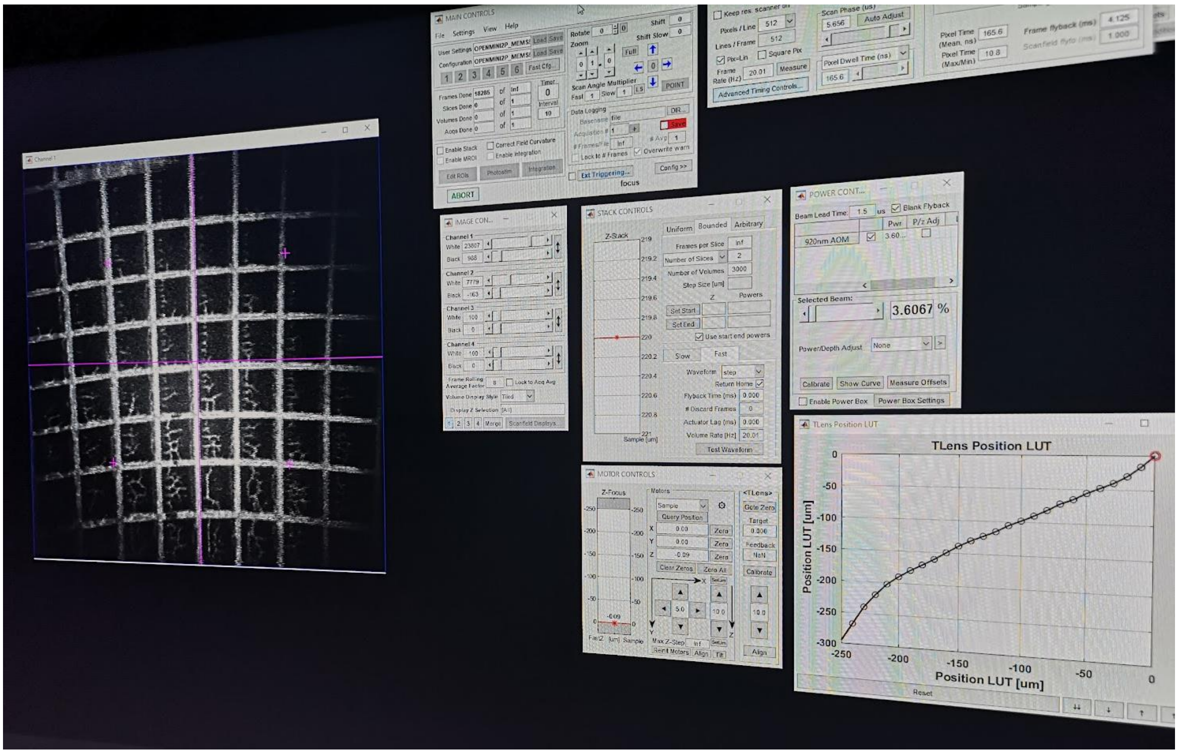


Fig.4.5: TLens calibration using a distortion grid (rough method) and typical s-shape LUT.

Step5. Estimate the FOV using MINI2P SI device

Goal: Optimize the MDF MEMS settings for a wider and stable Field-of-View (FOV) at focal plane 0μm using the distortion grid described previously (before Step 3). Then record and take the average image of about 50 frames and estimate the FOV using MINI2P SI device available on GitHub.

An estimation of the FOV is given (in µm) and Res_xy (in µm/pixel). After recording data, Distortion correction can be applied promptly.

5.1 Place the distortion grid under the microscope. Align it accordingly. Repeat steps 20-28 of Protocol P4 to prepare for imaging the grid sample.

Tip1! Start to move the miniscope in bigger steps (e.g. 200 μm). When the objective is approaching the grid (or glass), reduce to smaller z-step (e.g.50 μm) to prevent it from crashing against the glass/grid.

Tip2! While moving the scope holder down with z-Motor, always use ThorCam to better visualize the proximity to the grid (see left Fig.5.1).

Fig. 5.1: Image from ThorCam showing the Placement of the Distortion grid under the MINI2P objective Domilight D039. The grid was coated with a thin layer of fluorescence marker and, after dried, a cover glass was placed on top and cured with UV glue.

5.2 When nearly at the objective working distance, repeat steps 26-27 of Protocol P4 to start imaging. A typical power required is 3-5% to not saturate the image, and a PMT gain of 20.

5.3 Now move the motors in smaller steps (i.e. 2µm) in the z-direction until the image is focused.

5.4 While in Focus, select "show grid cursor" and align the 50 μm mid-lines to the vertical central and horizontal lines, by moving the stages in (x, y) directions as in Fig.5.2-b.
Tips! Use the rotator to overlap the distortion grid to the center of the grid cursor.

5.5 Prepare to record the **FOV at the 0 µm TLens plane** at the desired pixel size image (256 or 512), (see step 28 of Protocol P4) and make sure to take a reasonable number of frames (e.g. 20 to 50 frames) – see an example of a good quality FOV-image in Figs. (5.2).
Tips! Thick #Avg: 20-50 under Save in the Main Controls tab to record an image already averaged by the total number of frames and save as final FOV_avg.tiff

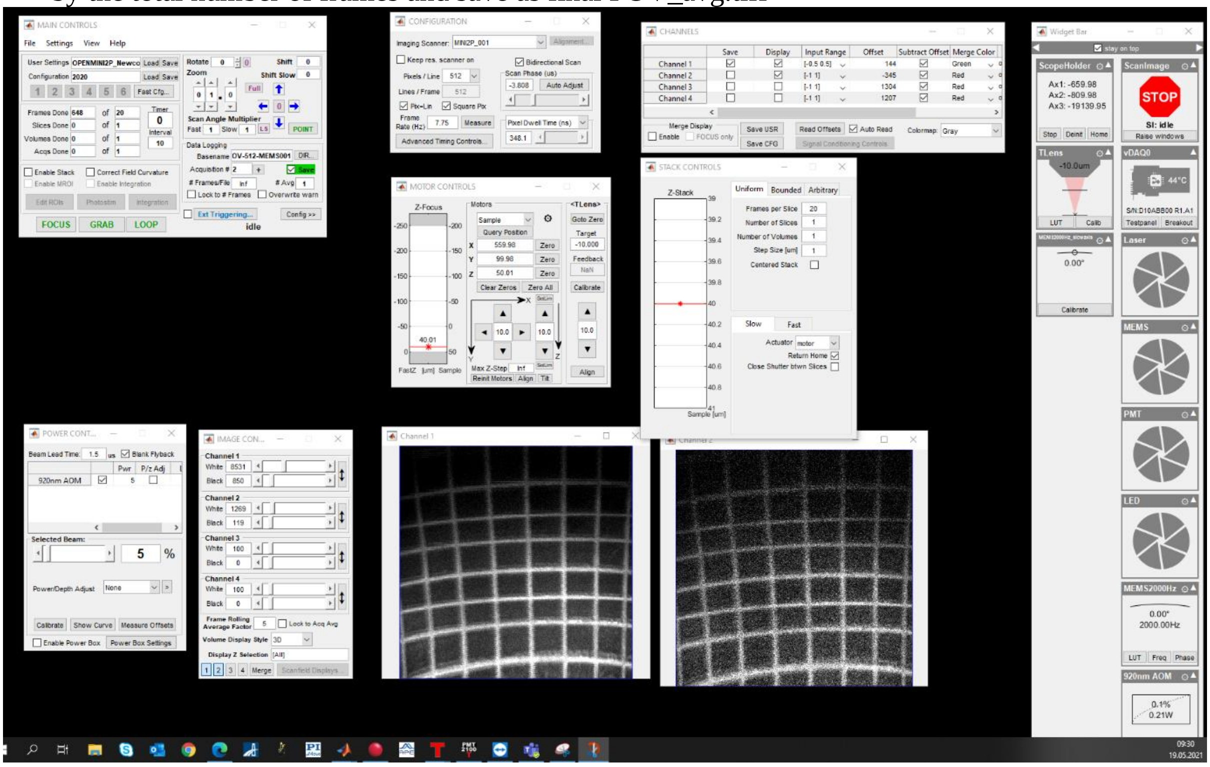


Fig.5.2(a): Arrangement of ScanImage main controls and channels display.

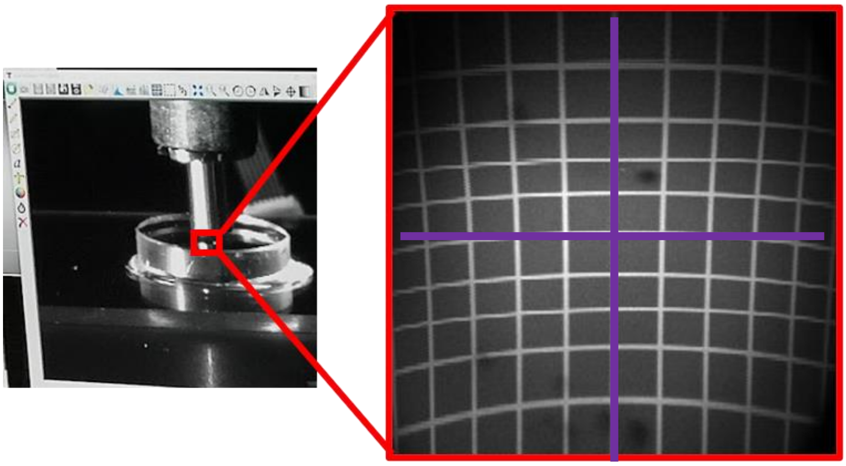


Fig.5.2(b): Zoom in of Fig.3.2 (a) showing the proximity of the objective to the prism and raw (as measured) FOV ≫ 500x500 um. Each square corresponds to 50um.

5.6 Run “Distortion Detection” by clicking on the widget bar
5.7 In the top left App Menu ‘Reference, click “COPY CH1” or “LOAD” FOVavg.tiff (see Fig.5.3).
5.8 Select number of anchor points (APs) X and Y and size in image (distance between APs) and press “Create” to visualize (50 μm real distance between lines) – to Step1 of Fig. 5.4.

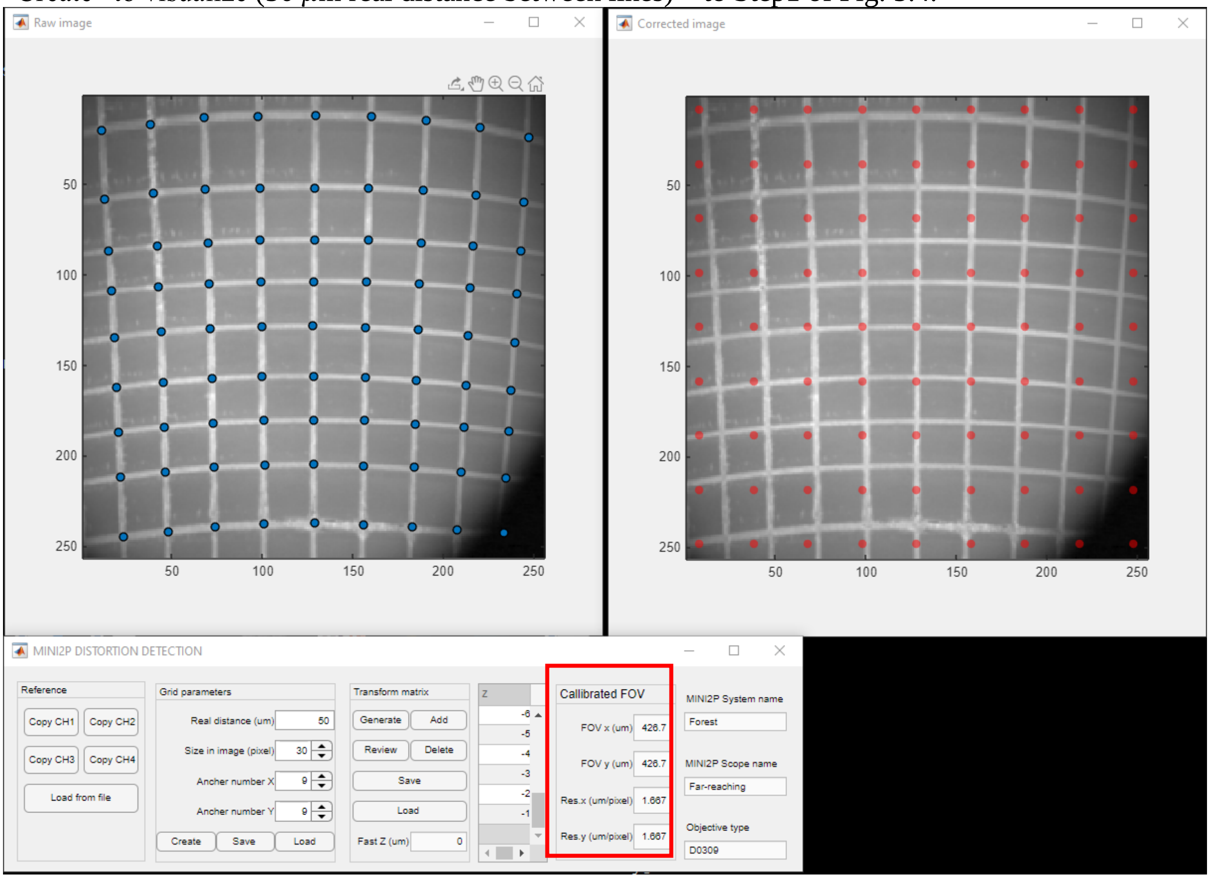


Fig. 5.3: Graphical user Interface of MINI2P SI device with estimated of FOV highlighted in red.

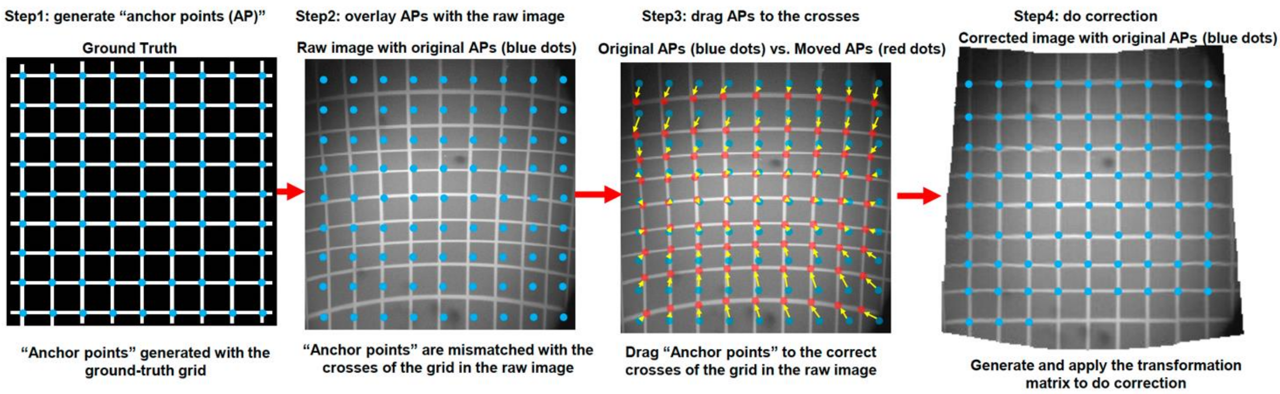


Fig. 5.4: Taken from Fig.S3(B) of Weijian Zong et al., 2022

5.9 Modify Anchor Point Pairs parameters such as grid numbers and starting points in both axes, as well as equally digital interval (pixels) such that the resulting APs overlap, as much as possible, the intersection points of the recorded grid (see APs of Fig.5.3-5.4). Notice typical mismatch of raw image with original APs (see step 2 of Fig. 5.4)

5.10 Drag, using your cursor, the original APs to the intersection points in the grid image, and click "Generate" to apply the transformation (Step 3 of Fig.5.4 and Fig.5.3)

5.11 Repeat Step 5.10 until the Corrected Grid (see Step 4 of Fig.5.4) looks evenly symmetric and all vertical and horizontal lines look orthogonal.

5.12 Press "Add" and click "Save" to generate Transformation Matrix. On the right side of MINI2P SI device, FOV in x and y values are displayed (in µm) and of Res-xy (in µm/pixel).

Step6. Distortion detection and correction: 3D Transformation Matrix

In this step, images of a fluorescence grid at different focal planes are recorded every 10 µm steps for the whole TLens range. MINI2P SI device is used to measure the distortion at each plane and generate a 3D Transformation Matrix.

Each intersection point of the grid is distanced by 50um from the next. The center of the grid must be aligned and overlap ScanImage horizontal and vertical cursors as much as possible, to facilitate the distortion detection, i.e., similar grid parameters can be used for different planes, with minor tweaks.

6.1 Download Readme_20220322.docx available under MINI2P_toolbox/Software/MINI2P_SI Device and follow the first pages to install the MINI2P SI device app.

6.2 Open GUI by pressing the button "Distortion Detection" on the widget bar as shown in Fig. 6.1

Steps for measuring distortion in different focal planes:

Note! To measure the distortion in different planes, you can either:

a) save all the FOVs at different planes beforehand (at 0um, -10µm, -20µm, … until -240µm) and load them, one at a time, by pressing **"Load from File"** under Reference.

b) display one FOV by clicking "FOCUS" on Main Controls, then open it on Raw Image GUI by pressing **"Copy CH1"** before moving to the next focal plane.

In either situation, please follow the next steps:

6.3 "Load from file" or "Copy CH1" at focus plane 0µm to display the reference image of the distortion grid on Raw image GUI" (See Fig.6.1)

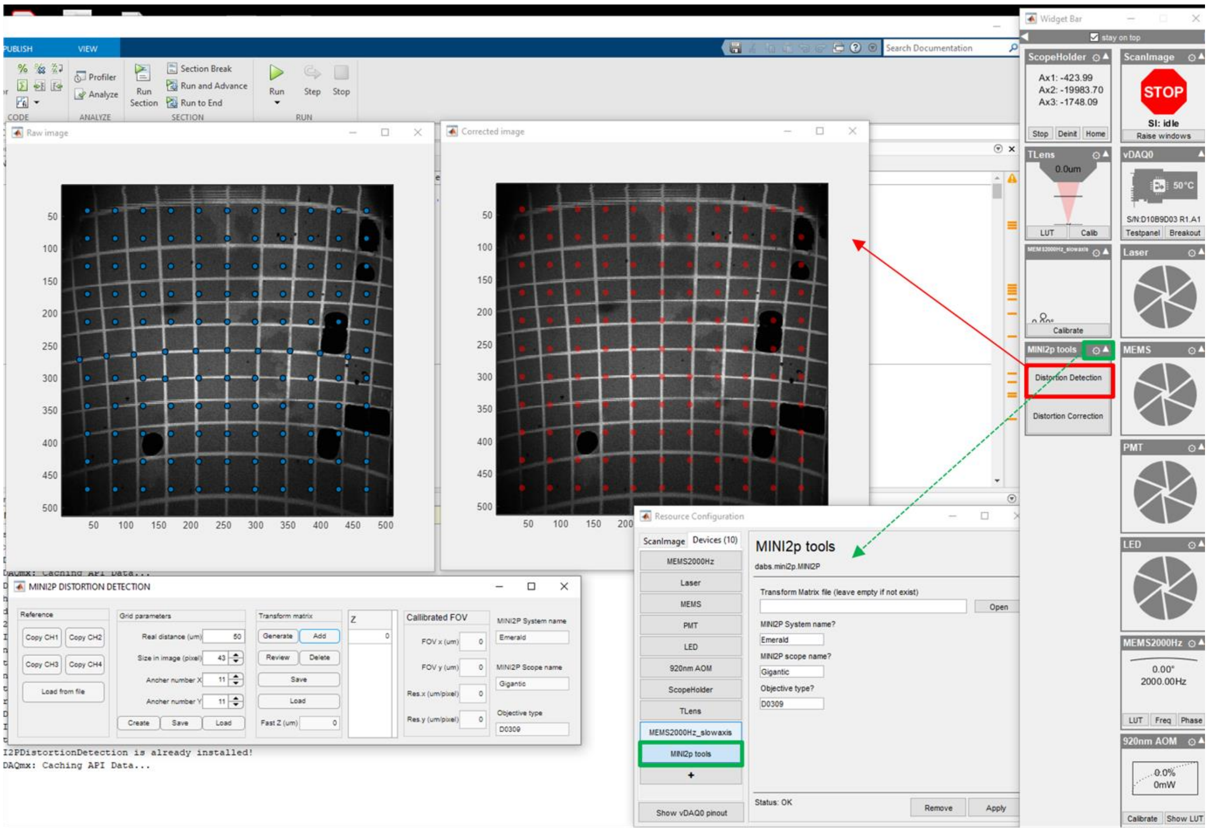


Fig.6.1: Overview of MINI2P device "Distortion Detection" GUI with reference grid raw image (left) and corrected image (right) after dragging anchor points to grid intersections, each distanced by 50 um.

6.4 On Grid Parameters, change size in image (pixel) and number of anchor points (in X and Y) such the middle anchor points overlap the horizontal and vertical grid intersections (or cursor). **Tips!** The higher the image size, the further apart are the anchor points

6.5 Drag all anchor points over the grid intersections, and then click "Generate" to display the corrected image on the right.

6.6 Adjust the anchor points in the raw image until all vertical and horizontal lines of the grid are orthogonal, not distorted in the corrected image.
Tips! If lines are tilted inwards for example, drag the anchor point in to move grid outwards.
Tips! Do not forget to press "Generate" to visualize the changes.

6.7 Press "Add" which will include a transformation matrix at focal plane 0µm. Click save if you wish to already keep this data, and a .mat file will be created in the same directory where ScanImage data is saved.

6.8 Repeat previous steps for remaining planes by moving the TLens up 10 μm up (and motors down) and apply the same procedure (up to the desired/whole TLens range, i.e. 200 to 240 μm)
IMPORTANT! After "Generating" the transformation matrix of a specific plane, before pressing "Add" (step 6.7), make sure the TLens is at the appropriate plane.

6.9 After finishing all the different focal planes, press "Save" the final 3D Transformation Matrix.

6.10 In the MDF MINI2P device-tab update the generated 3D Transformation Matrix path.

6.11 To correct .tiff files, click "Distortion correction" on the widget bar after recordings.