Construction and initial alignment of the dOPM detection and illumination optics

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Disclaimer

Laser safety

This alignment procedure involves the use of a Class IV laser system. It assumes that the laser has been registered and that all users of the system have been trained appropriately and registered according to local rules. All local laser safety rules and procedures must be followed at all times.

To allow safe alignment without the use of laser safety eyewear, the visible laser beams must be attenuated to a power below 1 mW. This is the exposure limit value (ELV) for a visible beam for an accidental exposure of 0.25 s, which is the standard assumed blink (natural aversion) time.

It is essential that only one laser is switched on at any time during alignment. The keys in the key switches for all other laser lines must be removed. We suggest two alternative methods to limit the power to below 1 mW for each individual line:

1) A fixed neutral density is placed at the output of the Omicron LightHUB where the transmission of the filter is chosen so that when the highest power laser line is operated at maximum power the maximum transmitted beam power remains below 1 mW. The neutral density filter must be visually checked to be in place before operating the laser. A calibrated power meter must be used to validate that the maximum beam power transmitted through the neutral density filter is less than 1 mW.
2) A calibrated power meter is placed at the output of the Omicron LightHUB and the power adjusted in the Omicron Control Centre software to be less than 1 mW for the laser line in use. Ensure that all other laser lines are switched off in software as an additional precaution. Then close the Omicron Control Centre software to prevent any accidental change to the laser power setting.

Detection optics

Align 60 mm Thorlabs rail system to the optical axis of beam emitted from lefthand port of microscope frame

1) Remove condenser lens from the microscope illumination pillar, close down the aperture stop at the top of the illumination pillar to a small diameter. Add a pinhole into the microscope objective turret of the microscope frame. The axial position of the pinhole should be positioned so it matches the back focal plane of the objective normally used. This is achieved using the appropriate custom-cut Thorlabs SM1 lens tube. Turn on the microscope transilluminator lamp and direct to the open side port of the microscope frame. This forms a pencil of rays on the optical axis.

2) Manually position the 60 mm Thorlabs cage system on the optical table so that the rays go through the centre of two T-shirt pinholes on the 60 mm rail. Secure the cage system.

Position TL2 so that it forms a 2f system with TL1

3) Use the longest set of Thorlabs rails to set up the longest possible temporary Thorlabs cage rail system. Mount the USB-powered laser operating at a wavelength of 532 nm at one end. Adjust the laser x/y mount to ensure that the laser beam is parallel to the rails and goes through the centre of Thorlabs alignment t-shirts both near to the laser and at the end of the rail system.
4) Then mount the USB-powered laser pointer in the nosepiece of the microscope and ensure that it runs down the optical axis of 60 mm cage system. Mount TL2 in the 60 mm xy-translation mount and adjust axial position using shear plate to ensure the beam is collimated after passing through TL1 (inside microscope frame) and TL2. Use the XY-translation of TL2 to roughly centre it on the optical axis. Lock axial position of TL2 cage mount and remove TL2 from XY-translation mount.

Position 30 mm rail system on the optical axis

5) Remove the USB-powered laser pointer and revert to transillumination configuration with pinholes at top of illumination pilar and on microscope objective turret of microscope frame. Attach 30mm cage system to 60mm cage system (including the quarter waveplate (QWP), mounted at an angle in between shims to avoid back-reflections, PBS and periscope) and check alignment of rays through approx. O2 back-focal plane at the end of the cage rails. Secure the full cage system.
6) Temporarily replace TL2. An image of the iris on the microscope objective turret of microscope frame (that is in the back focal plane (BFP) of O1) forms on the optical axis. Axially position the O2 cage plate mount (and thread lockrings) such that the BFP of O2 is coincident with this image. This will be optimised later. Remove TL2 again.

Set ¼ waveplate and periscope alignment

7) Place a flat mirror beyond the end of the 30 mm Thorlabs cage system. This can be the single flat ½" round mirror for 0° image rotation mounted on the PIMag assembly. Position the mirror roughly where the focal plane of O2 is expected to be.
8) Rotate the ¼ waveplate until max transmission of the reflected pencil of rays through the PBS is achieved. Use the flat mirror mount actuators to align this directly back along the optical axis.
9) Rotate the ¼ waveplate until max reflection in the PBS is achieved for green wavelengths (use dichroic in microscope to check just green wavelengths). Do not adjust ¼ waveplate again after this.
10) Align periscope mirror such that reflected rays propagate as close as possible to parallel along the camera cage system. Don't worry if beam is slightly off centre with respect to centre of cage system. It's most important that beam is parallel to cage system, which should be parallel to optical table and perpendicular to TL2-O2 optical axis. If a long way from optimal, consider remounting the PBS to be more central and oriented correctly in the PBS cube mount.

Mount TL3 and camera

11) Replace condenser in microscope frame and select O1 in microscope turret. Mount a USAF test chart in the sample holder with appropriate immersion media and coverslip. Place a diffuser on the top of the test chart. Use a drop of water between coverslip and test chart and between test chart and diffuser.
12) Focus on test chart with either eyepieces or widefield (WF) camera so that the test chart is in the focal plane of O1 (**Figure 1)**. NB: if eyepieces and WF focal plane are not equivalent, adjust the axial position of WF camera.
13) Mount both TL2 and O2 and the PIMag assembly in the "flat mirror configuration" (**Figure 2**).

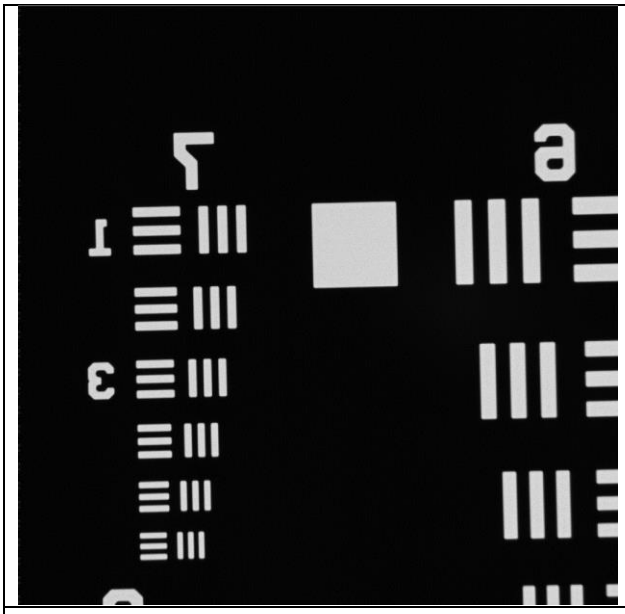


Figure 1: Part of Test chart as seen on the WF camera with 60x objective

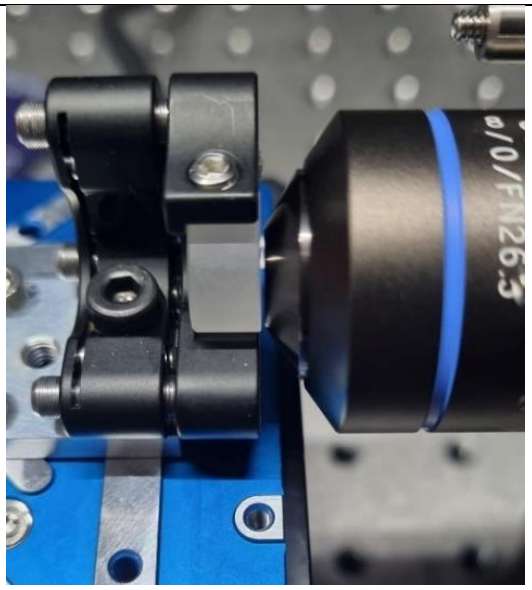


Figure 2: Side-view of the flat mirror mounted on the PIMag used to align the detection optics

14) Take TL3 and camera to a location from which ~infinity can be imaged, e.g. a distant building imaged out of the office window, and position TL3 on the camera with adjustable lens tube such that infinity is in focus (**Figures 3-4**).
15) Mount the camera and TL3 in the optical path after the periscope and translate the flat mirror until a focused image of the test chart appears in the camera.
16) Adjust the camera mount manually to the point where the image is as flat, centred and as correctly oriented as possible. Ideally, use the crosshair graticule in the eyepieces to place a distinct feature of the USAF test chart in the centre of O1's FOV. Then move camera so that the same feature in the centre of the camera. Fix the camera position securely.

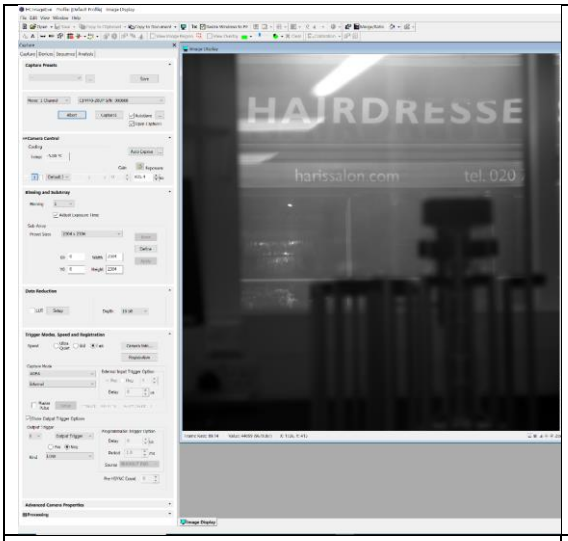


Figure 3: Image of shop across the street in focus on the camera, as viewed using HCImageLive software. Ideally, image an object even further away if possible.

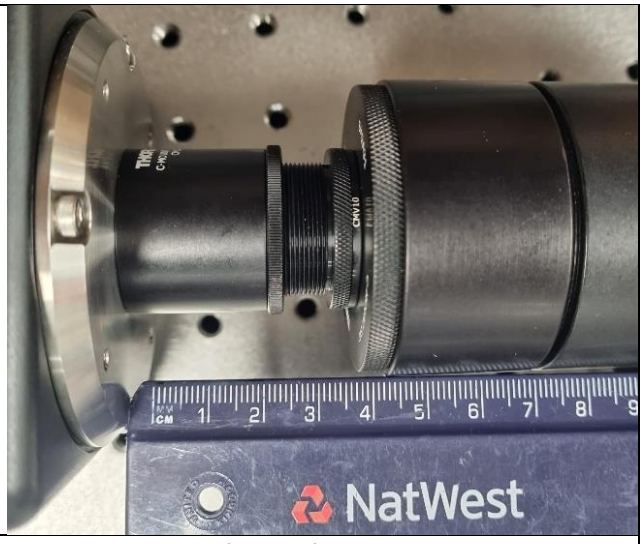


Figure 4: In order from left to right: camera, C-mount tube (CML25), locking ring, adjustable c-mount CMV10, c-mount to SM2 adaptor (SM2A31), TL100. Approximately 9-10 turns on the CMV10 thread remain exposed when the TL100 is at its focal distance from the camera sensor (imaging at infinity).

TL2 O2 Pupil alignment

17) Translate the USAF test chart so a square or a set of three bars are at the centre of the FOV.
18) Refocus O1 100 μm higher than the focal plane in sample space and bring the image back into focus by translating the flat mirror using the Newport stage in the PiMag assembly.
19) In the acquisition software, draw an ROI that identifies the outline and centre of the square
20) Refocus O1 100 μm below the focal plane in sample space and bring the image back into focus by translating the flat mirror using the Newport stage in the PIMag assembly.
21) Use the **x/y translation** of TL2 to move the square in the test chart so that it is aligned with the ROI previously drawn. NB: the magnification is unlikely to be the same at various refocused positions due to the fact the TL2 doublet separation and O2 position are yet to be optimised. Ignore the size and concentrate on position.
22) Repeat from step 18 until the square does not translate in x/y in the FOV when refocusing (**Figure 5**)

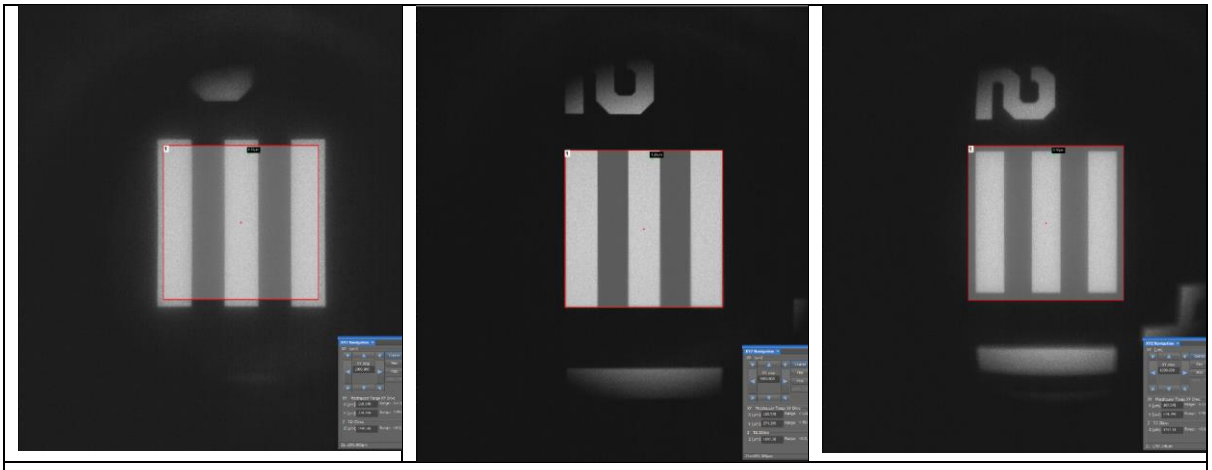


Figure 5: Test chart image at ±100 um (left and right) and 0 um (centre) remote refocus. Lack of lateral (x/y) translation with refocus indicates correct pupil alignment.

Achieving n1/n2 magnification for aberration-free remote refocus

Correct TL2 doublet lens separation

23) Locate a square or set of lines on the test chart and centre it on the FOV. Ensure it is in the focal plane using the eyepieces or WF camera. Measure its x and y extent in pixels and convert to microns using NISelements camera pixel calibration
24) Use the test target line width specifications to estimate effective lateral x/y magnification of image and record with doublet lens separation.
25) Iterate the TL2 doublet lens spacing until refocus space magnification is 1.33 at zero refocus.
26) Remove O2 and check the axial position of TL2 using the green laser and shear plate as per step 4.
27) Replace O2 and remount the test chart to reconfirm doublet lens spacing gives magnification of 1.33 at zero refocus, returning to step 24 until complete.

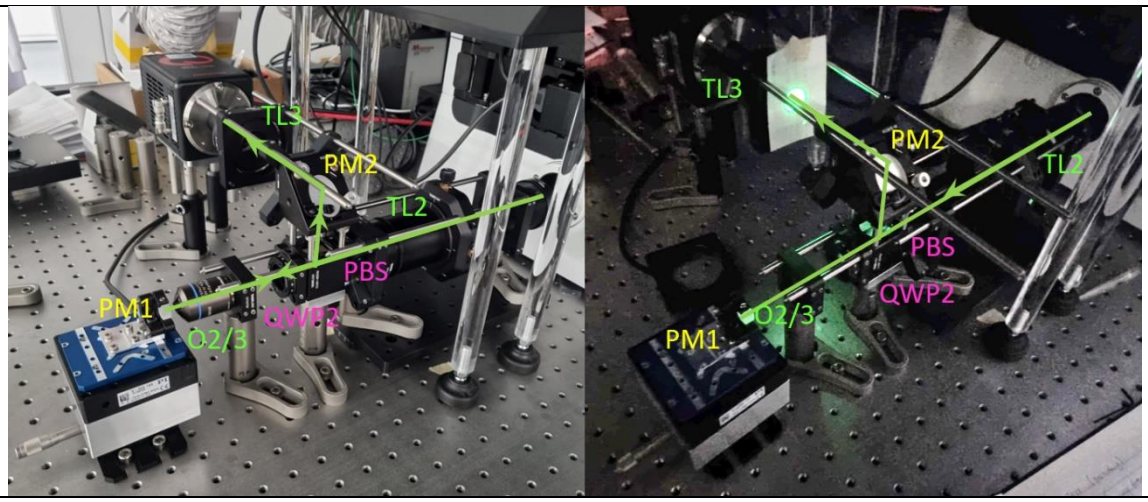
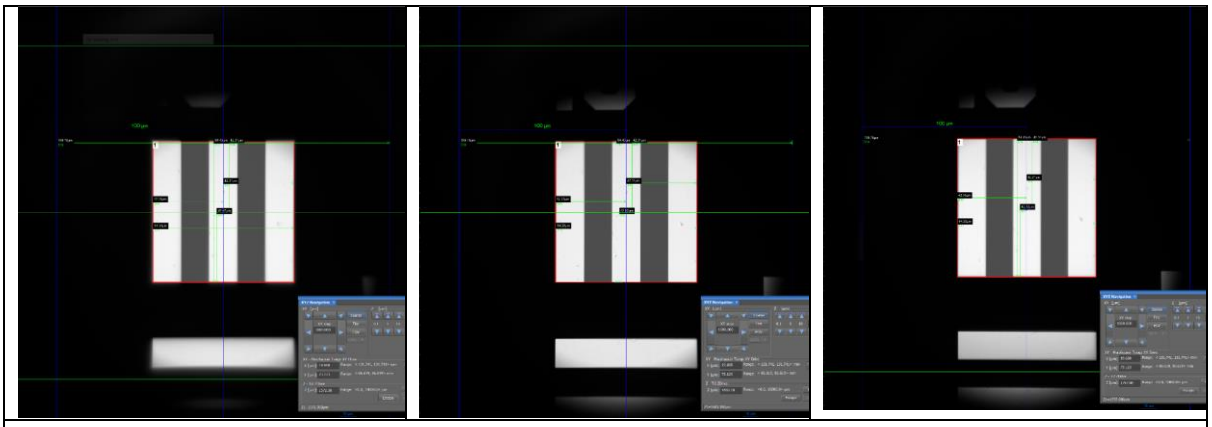


Figure 6: Remote-refocusing detection system constructed on the lefthand side of the microscope camera port, consisting of tube lens TL2, polarized beam splitter PBS, quarter waveplate QWP2, objective O2/3, flat mirror PM1 mounted on the PIMag assembly. After double pass through the remote-refocusing setup, the light from the microscope is reflected by the PBS into the periscope PM2, through tube lens TL3 and onto the camera. Image on the right shows how QWP angle is optimized for maximum transmission of green-filtered light towards to the camera

Correct O2 positioning along the optical axis

28) Locate a square on the USAF test chart and centre on the FOV and follow the steps from point 17 to align O1 and O2 pupils if needed.
29) Record the axial position of O2 relative to a fixed position on the optical rail.
30) Refocus O1 100 µm higher than the focal plane in sample space and bring the image back into focus by translating the flat mirror using the Newport stage in the PIMag assembly. Record the x/y magnification.
31) Refocus O1 100 µm lower than the focal plane in sample space and bring the image back into focus by translating the flat mirror using the Newport stage in the PIMag assembly. Record the x/y magnification
32) Using the results from above, determine the change in magnification as a function of O1 z-position. Further intermediate points can be taken for a more detailed characterisation if required.
33) Translate O2 on the optical axis and repeat from step 28 until the refocused magnification changes minimally from the 1.33 magnification at zero refocus set by the TL2 doublet lens separation (**Figure 7**).



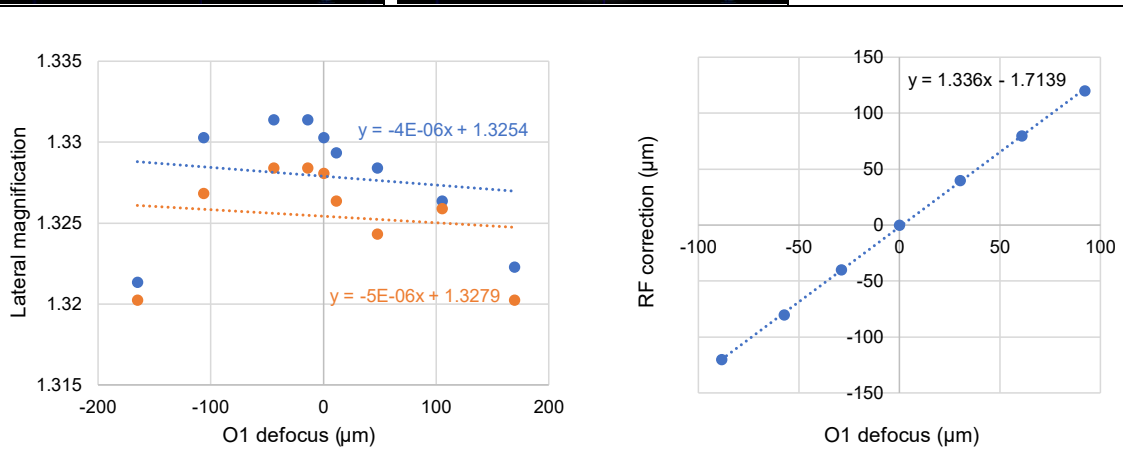


Figure 7: Top: Test chart image at ±100 um (left and right) and 0 um (centre) remote refocus, with 1.33 magnification maintained across the refocused range. Bottom left: Measured lateral x (blue) and y (orange) magnification as a function of O1 defocus. Note the gradient of the linear fit is $<10^5$, indicating low variation of magnification across the remote refocusing range. Bottom right: required remote refocus correction for O1 defocus, with the gradient of the linear fit reporting the axial magnification.

Define the illumination axis

34) Remove the entire PIMag assembly and O2. Extend the cage rods protruding from the O2 mounting cage plate. Then slide the Thorlabs CP30 90 degree cage system adapter onto the two cage rods protruding from one side of the O2 mounting cage plate.
35) Use vernier callipers to measure 22.9mm from the position of the flange of O2 to the closest face of the CP30 (this corresponds to the parfocal length of the objective, 45 mm, minus the outer face to midpoint distance of the CP30, 22.1mm).
36) Position cage rods from the CP30 to define the position of the Thorlabs 30 mm cage on which the illumination optics will be mounted at 90° to O2-TL2 cage axis.
37) Repeat from step 34 for the opposite illumination arm. Then place one continuous cage rod through both illumination arms to ensure that they are at the same axial position and to increase structural integrity.

Prism Alignment

38) Replace the PIMag assembly, again ensuring that it is mounted normal to the optical axis.
39) Retract the PIMag assembly using the Newport stage and replace the flat mirror with the prism mount and prisms.
40) Remove TL2, O2, condenser lens and O1. Close the aperture stop on microscope illumination pillar and add a pinhole into the objective turret. As per step 1, turn on transillumination to form a pencil of rays.
41) Translate the PIMag laterally (movement of prism perpendicular to O2-TL2 axis) so the tip of the prisms bisects the pencil of rays.
42) Place targets either side of the beam path on which the two halves of the pencil of rays reflected by each of the prisms can be observed. Measure the distance from the optical axis of the reflected beam – this distance should be the same on both sides if the prism mirrors are both at the same angle to the optical axis. (**Figure 8**).
43) To correct the angle of the prisms if needed, loosen the bolts holding the prism mount into the prism mount pedestal and make small adjustments to the angle of the prism mount by hand before resecuring.

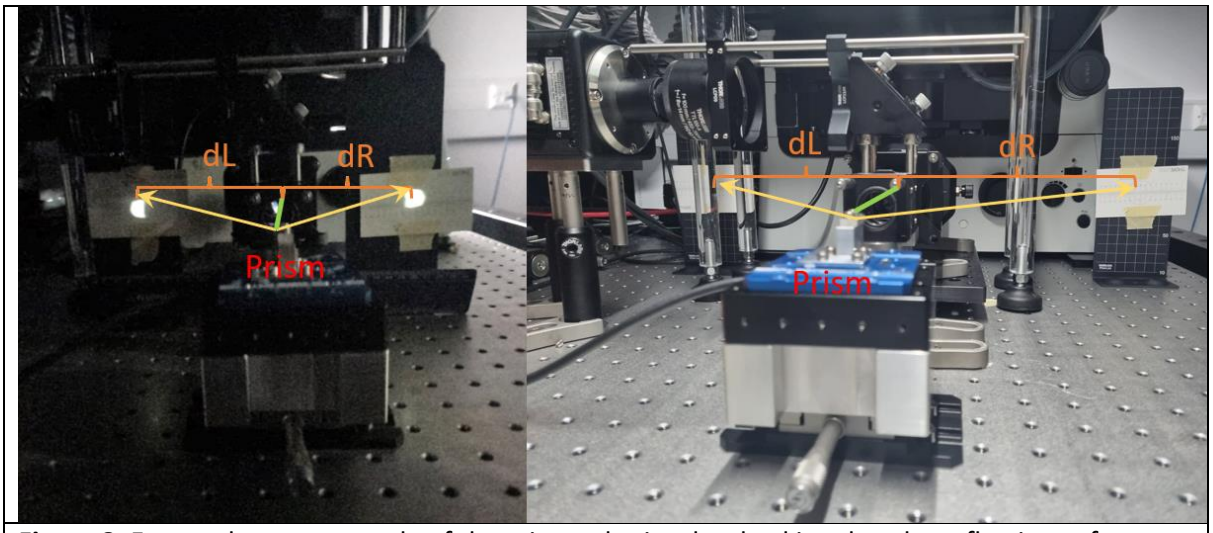


Figure 8. Ensure the correct angle of the mirrored prism by checking that the reflections of a pencil beam off each surface of the prism mirrors are at the same angle with respect to the optical axis by checking that their distances from the optical axis (dL and dR) are the same. Here, the two images show the targets at two positions, with the room lights off (left) and on (right)

Initial positioning of the mirror prism

44) Replace the lenses and mount USAF test chart in the sample plane of the microscope.
45) Position a phone camera or USB camera + telecentric lens above the front of O2. Focus the camera on the front of O2. Transilluminate the USAF test chart and position in the focal plane of O1 as determined by the eyepiece or WF camera.
46) While carefully monitoring the phone/USB camera image to prevent collisions of the prism tip with O2, bring the tip of the prism towards O2 (moving it axially) until an image of the test chart is formed on the OPM camera. NB: The image of the USAF testchart will be superimposed with the image of the rough surface from the metallic tip of the prism mount (Figure 7, centre). When in focus, record the position of the PIMag Newport stage axial micrometer**. This position is only 350 µm away from collision with the objective O2!**
47) Translate the PIMag slowly in the lateral direction to move the tip to either extent of the FOV of the OPM camera. Check that the image of the tip of the prism mount does not defocus. **If it does, the PIMag axis is not normal to the optical axis and this needs to be corrected!**
48) Move the prisms, for example by 150 µm, towards the objective.
49) Monitoring the camera feed to prevent collisions of the prism tip with the objective, translate the PIMag until the central feature in the test chart is in focus. Record the PIMag position for each of the two mirrors and update NIS Elements macros and microscope frame programable buttons (**Figure 8**, left and right).

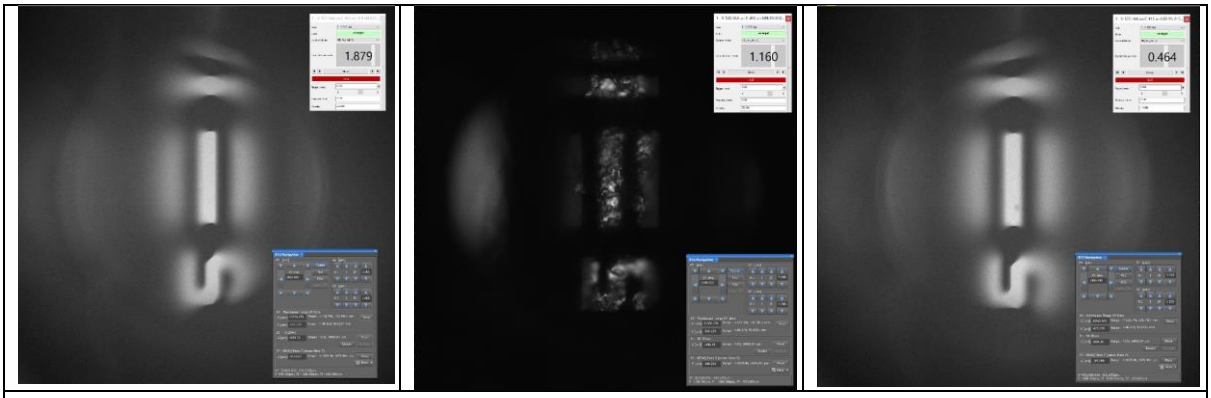


Figure 8: Image of test target on the OPM camera formed with the tilted prism mirrors (left and right) and reflected off the metallic tip of the prism mount (centre)*.

Illumination optics

Positioning of the light sheet focusing lenses

50) Measure using the callipers 45 mm from the O2-TL2 optical axis along the illumination arms and use this as the initial position for the flange of the 4x Olympus light sheet focusing objectives.
51) Measure 1.3 mm (back focal plane position) plus 150 mm (focal length of the cylindrical lens) behind the flange of the 4x objectives. This measurement will be via the face of the final motorized steering mirrors in the illumination arms and therefore approximate. This is the ideal position of the cylindrical lenses.
52) Reuse the CP30 and spare cage rods to ensure the beam path makes a 90 degree turn between the cylindrical lens and the focusing objective.

Build and align the first (shared) half of the illumination beam path

53) Use 2-3 mirrors, cage plates and rails to create a temporary long zig-zag shaped illumination path. Position a pinhole at the near end and a diffuser with centered pinhole at the far end (**Figure 9**).
54) Ensuring that the laser safety precautions for limiting the laser power to less than 1 mW outlined at the start of this document are followed, start at laser wavelength located at the front of the laser combiner (445nm), and working back to 642 nm, ensure that each laser line passes through the centre of two pinholes and the centre of the diffuse by adjusting the beam shifter wedge and beam combiner dichroic mirrors within the laser combiner. (Refer to LightHub Beam combiner manual for more details). Make sure to fix the final position of the beam combiner dichroics by tightening their fixing rings.

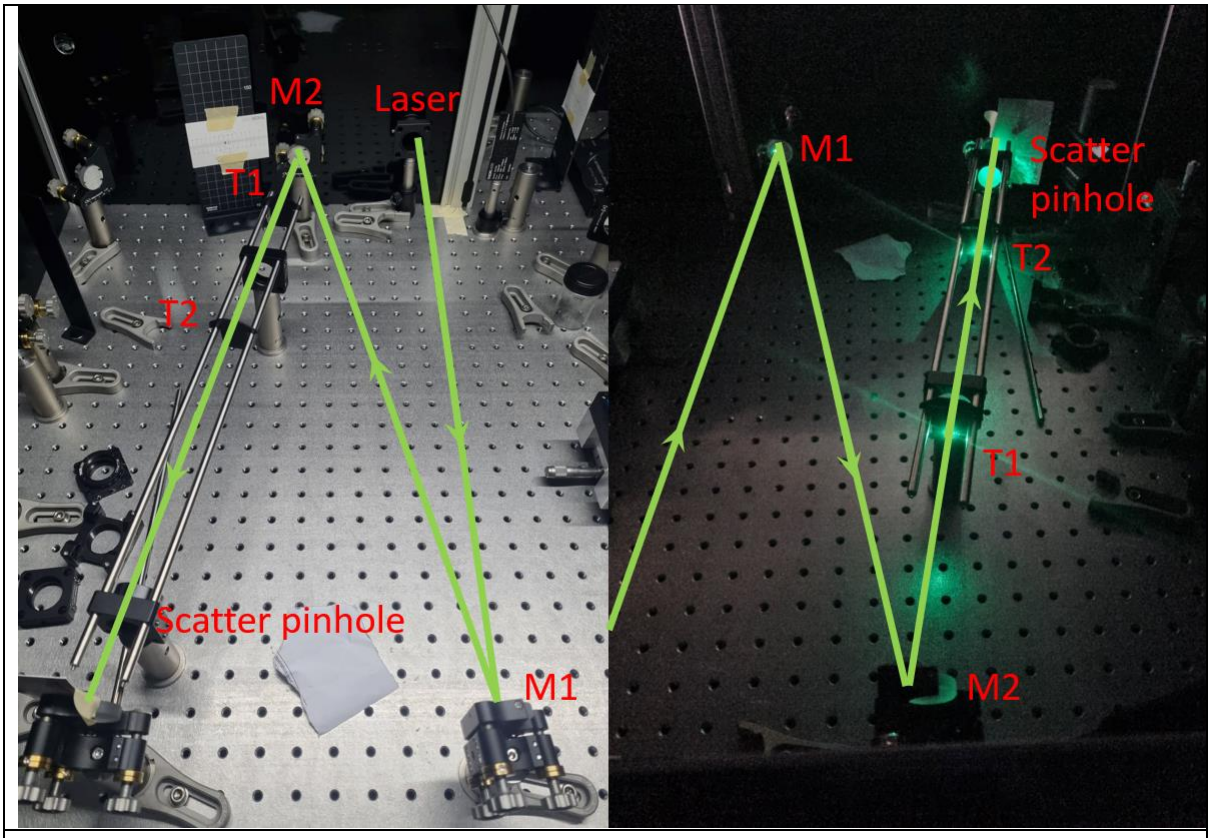


Figure 9: Mirror relay used to coalign the different wavelength, ensuring that each laser line passes through the centre of a pinhole placed close to the laser output aperture and through the centre of the diffuser pinhole at the end of the optical path.

55) Place mirror M1 at the output of the laser **(Figure 10)**. Next, set up the beam expander consisting of the 10x objective (L1) mounted in a x-y-adjustment cage plate, and the 150 mm lens (L2) using two cage plates and rails. First carefully position the rails (no lenses) using the t-shirts so that the beam passes through the centre of the rails. Insert objective L1 and adjust the objective's x-y position such that the beam emerges from L1 parallel to the rails (check using t-shirts).
56) Insert L2 and use a shear plate to set the separation between the objective L1 and the lens L2 such that the beam passing through the expander comes out collimated. Mount an adjustable pinhole (P1) after the lens and set it to approximately 6 mm diameter. Check that all wavelengths produce a centred beam spot on the pinhole.
57) Place mirror (M2) after the expander **(Figure 10)**. Position M3 to bring the beam approximately along the optical axis of O2-TL2. Insert half waveplate (HWP), a quarter waveplate (QWP) and a 50/50 beam splitter (BS).

Align the illumination arms for each view

58) Use mirrors in each illumination arm (mirror M4 in the path reflected by the BS, mirrors M5-6 in the path transmitted by the BS, and one motorized mirror in each path (MM1-2)) to direct the laser beam through the cylindrical lenses CL1-2 and illumination objectives L3-4 **(Figure 10).**
59) Retract the prisms and remove the cylindrical lenses and the 4X objectives
60) Turn on the green laser in the laser combiner. **NB: Verify power is < 1 mW and close the software so it cannot be increased accidentally.**

61) Using t-shirts along various points of the illumination path ensure that the two arms of the illumination pass through the centre of each lens mount, normal to the lens surfaces.
62) On a screen at the transmission exit of the 50/50 beam splitter in the return direction, collinearity of the beams around the clockwise and anticlockwise paths can be observed by the interference of the recombining return paths.
63) Turn off the laser, replace 4x objectives and turn lasers back on. Re-confirm alignment.
64) Turn off the laser, replace cylindrical lenses and turn lasers back on. Re-confirm alignment.

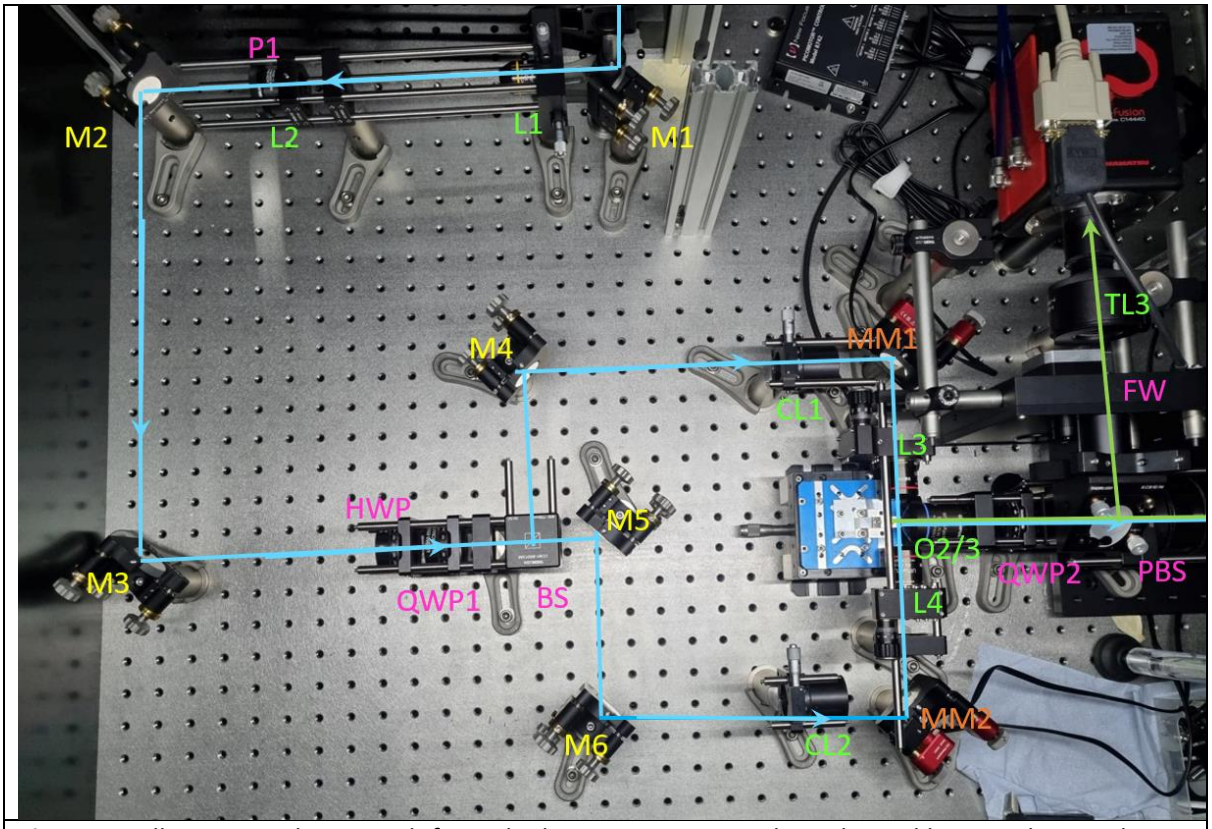


Figure 10: Illumination beam path from the laser output up to the polarized beam splitter cube (PBS) consisting of mirrors M1-6, lenses L1-4, pinhole P1, half waveplate HWP, quarter waveplates QWP1-2, beamsplitter cube BS, cylindrical lenses CL1-2, objective O2. FW denotes the filter wheel and TL3 is the third tube lens before the camera.

Couple the light sheet into the system

65) Turn off the laser and move the prism into the previously determined position in step 48.
66) Mount a fluorescent Chroma slide in the microscope. Use a coverslip, with a drop of water between coverslip and slide, and emersion media (water) between objective and coverslip.
67) Focus on the surface of the slide using epifluorescence and the eyepieces or WF camera.
68) Turn on the lasers and move the PIMag to one of the two positions determined previously in step 49.
69) In ideal circumstances, very small adjustments if any will now be needed to achieve coupling of the excitation light into the sample such that it excites fluorescence in the field of view being monitored by the OPM camera. Adjustments to try are, scanning the PIMag a short distance, moving the prism position with the Newport stage (apply caution to avoid crashing prism mirrors into O2), steer the beam with the final steering mirror (MM1 or MM2), move the 4x objectives in z on the illumination axis to focus the sheet.

70) Switch to the other illumination arm and attempt to couple the illumination light to the sample to excite fluorescence that can be observed on the OPM camera too (**Figures 11-12**).
71) Record the PIMag position to macros and programable buttons and note the Newport stage micrometer position.

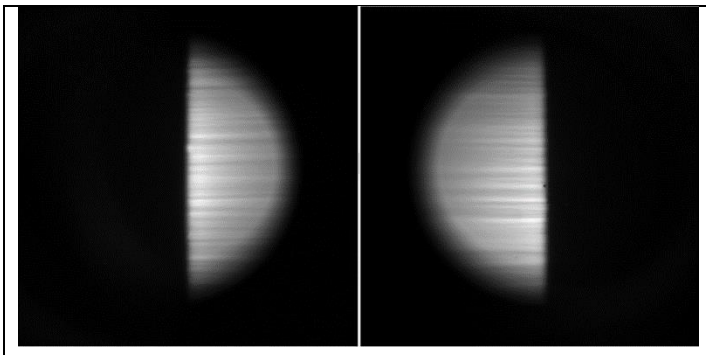


Figure 11: Image of the coverslip boundary of a fluorescent Chroma slide for the two views (left and right), after coupling and focusing of the light-sheet*. Sharp boundary of the coverslip-Chroma side interface over the full vertical extent and whole axial scan range indicates good co-alignment of the light-sheet and detection plane.

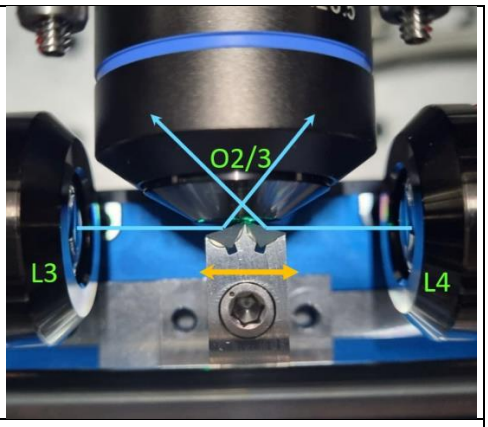


Figure 12: Close up of the PIMag-mounted mirrored prism, positioned at the focus of objective O2/3. Lateral translation of the prism switches between coupling of the light-sheets from illumination objectives L3 and L4.

Adjusting the waveplates in illumination path

72) Put the PIMag into the centre position (recorded in step 71) for one view.
73) Adjust the QWP and HWP in the illumination path to achieve the maximum transmission through the PBS and minimum reflection and record their angles.
74) Switch to the other view and minimise again.
75) Position QWP and HWP at average of orientations recorded in steps 73 and 74.

Focusing the light sheet illumination

76) Mount a thin fluorescent sheet. Place it in the focal plane of O1 using epifluorescence and the eyepieces or WF camera.
77) While imaging the sheet with OPM draw a line profile in NIS elements and display the peak and FWHM.
78) Using the final steering mirror (MM1 or MM2) angle and the 4x z-position, iterate to a maximum peak and minimum FWHM.
79) Ensure the cylindrical lens is oriented correctly by making sure the image of the fluorescent sheet is horizontal in the FOV.

Optimising the light sheet angle

80) Mount a 3D fluorescent bead sample (e.g. Tetraspeck 100-200 nm or Spherotech 300 nm beads suspended in agarose gel). Adjust focus of O1 to be approximately 50 µm above the top of the coverslip using epifluorescence.
81) While still in the WF mode, adjust the correction collar of O1 to minimize spherical aberration. When positive or spherical aberration is present, as the primary objective is scanned up or down the optical axis, the rings around all bead images will diverge or

converge consistently. When the spherical aberration has been corrected, scanning axially will result in bead images focusing, and defocusing, uniformly with positive and negative axial defocus, with less prominent rings (**Figure 13**). The correction collar will need to be adjusted when switching to samples with different thickness coverslips.

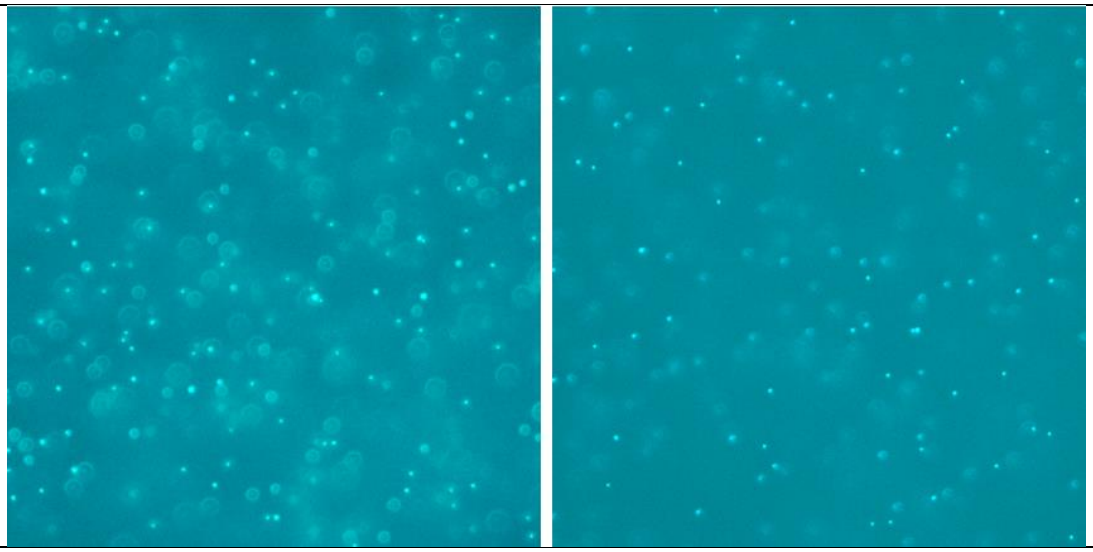


Figure 13: Widefield image of 300 nm fluorescent beads (cropped FOV) before (left) and after (right) adjusting the correction collar. The images show slightly different axial planes within the sample.

82) While imaging beads with light-sheet illumination and OPM camera, locate two beads, one that is in-focus (irrespective of brightness) that is close to the bottom of the imaging volume and another that is close to the top (in-focus irrespective of brightness). These beads are in the angled focal plane of the imaging system but the light sheet illumination is not necessarily co-planar with this focal plane.
83) Steer the light sheet in the focal volume using the two final mirrors (M4 and MM1 or M6 and MM2), this can be done by beam walking to keep the position of the light sheet in the back focal plane of the 4x constant but changing the angle. Aim to achieve homogenous illumination and sharpest + brightest images of beads over the full FOV **(Figure 14).**

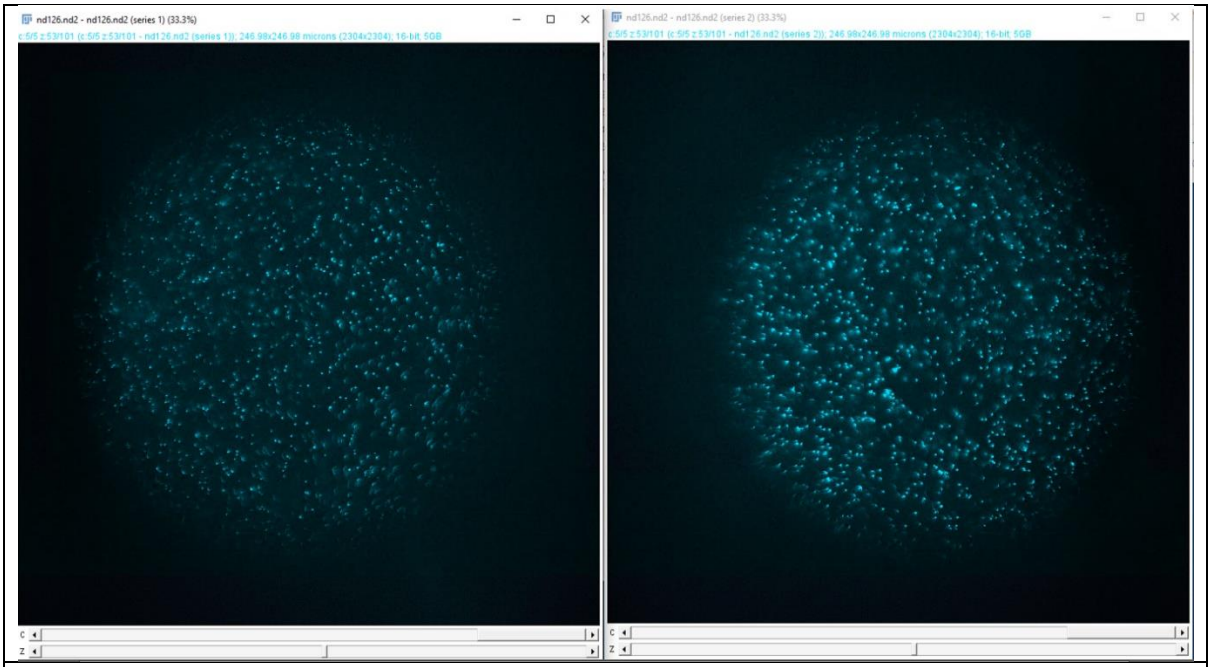


Figure 14: Image of 300 nm Spherotech rainbow bead volume in the left and right views over the full camera sensor FOV

*Note: The camera orientation was later flipped in NISelements such that the two view images flip vertically (about the horizontal axis), rather than horizontally.