Part I: Setting up the reference

1. Use a ruler to check the height of excitation laser beam
2. Setup a collimated low power green reference laser at one end of the optical table using Polaris mount. Make sure the center of the beam matches the height of excitation laser
3. Using at least 2 Iris along the beam path to make sure it is parallel in all 3 axes
4. Setup the manual rail at the desired position, leave 3 stages on it. Mount an Iris on the middle stage
5. Position the middle stage so it’s located at the center of reference laser. Replace the Iris with turning mirror and position it until it’s perfect 90 degree
6. Mount the galvo mirror at the end of manual rail. Position the height and rotation angle so the laser hits its center, and the angle is at perfect 90 degree
7. Confirm the height of reference beam at far away position
8. Setup O1 and O2 with their respective tube lens using cage system (see video on how to setup O1 with its cage system so it can be swapped in and out)
9. Input a secondary reference laser from the front end of objective lens. Adjust the position of tube lens until the output beam is collimated. These 2 cage systems can be set aside for later usage

Part II: Fluorescent light pathway until zoom lens

1. Setup the 70mm lens onto the 3rd stage on the rail and position it until laser remains on its reference pathway
2. Adjust the position of the stage until the laser is focused onto the galvo mirror. May need protection eyeglass for more accurate inspection
3. Setup a 50mm lens (or shorter focal distance) before the turning-mirror using a small cage system. Make sure the lens is mounted within a lens tube so it can be screwed in/out easily
4. Adjust the position of the lens so the laser remians on its reference pathway
5. Adjust the position of 50mm lens within the cage until the output laser after galvo is collimated. Then unscrew the lens tube
6. Setup the 88.9mm lens after galvo using a small cage system, adjust the position until the laser remains on its reference pathway
7. Adjust the position of 88.9mm lens within the cage until the output laser is collimated

Part III: Zoom lens: (find details in <https://amsikking.github.io/any_immersion_remote_refocus_microscopy/appendix.html#zoom_lens>)

Note: May need to machine several right-angle brackets that matches the desired height, guiding rail (check the video) as well as spacers

1. Setup all 3 P, N, P lens and position each linear stage on the plate at its desired position
2. Mount the plate onto the main optical table 2 holes after the 88.9mm lens
3. Place an Iris in between the plate and 88.9mm lens and another Iris far away along the reference laser pathway
4. Run the python code to set the stage at “132.5mm” position
5. Setup the first positive lens group on stage 1, adjust until the laser beam passes the center of faraway Iris (2 axes plus 2 rotation).
6. Close the Iris at the back to its minimum and make sure the back reflection hits the center of Iris in the back. Be gentle when fixing the position as over tightening may affect the alignment
7. Setup the negative lens group on stage 2 following the procedure in step 5 and 6. The 50mm lens may need to be swapped in/out to maintain desired laser spot size at faraway position
8. Setup the second positive lens group on stage 3 following the procedure in step 5 and 6. Make sure the back reflection light is clean and centered at the back Iris
9. Gently loosen the negative lens tube, use the spacer to find the appropriate position with respect to positive lens on stage 3. While fixing the position of negative lens tube, the spacer should be lightly held by the 2 lens tubes. Falling may indicate the imperfect alignment
10. Gently loosen the positive lens tube on stage 1 and follow the procedure in step 9 to find its appropriate position
11. Set up the guiding rail by placing them tightly against the side of the plate. Swap in the 50mm lens
12. Unscrew the plate and move it along the guiding rail until the output beam is collimated. Remove the 50mm lens
13. Setup a diffuser lens (can be made by scratching glass slide using sandpaper) and a small black screen
14. Position the diffuser lens at the focal point of the zoom lens, run the python code that position the zoom lens within its designed range. The speckle pattern observed on black screen can be used as a qualitative check for alignment
15. Remove the diffuser lens and black screen

Part IV: Fluorescent light pathway until O2:

Note: depending on the choice of dichroic mirror and O2, relay lens pair may not be necessary. The exact focal distance of relay lens pair does not particularly matter as long as they produce 1:1 magnification

1. Setup the dichroic mirror right after the zoom lens and position it until the mirror produces a perfect 90 degree reflection
2. Set up another manual rail and put in the 60mm lens. Follow step 4 and 5 in Part II to align the 60mm lens
3. Swap in the 50mm lens and unscrew the O2 from its small cage system
4. Align the second 60mm lens in a cage system following step 4 and 5 in Part II
5. Screw back in the O2 and remove 50mm lens. Fine adjust the alignment of the small cage system

Part V: Tilted alignment between O2 & O3

Note: Following steps involves compromise due to limited working space, ideally the position of O2 is never moved once been fixed in Part IV

1. Use 2 holdfast to mark the position of the stage where O2 is mounted. Then move the stage backwards until enough space is created so that O2 & O3 can be placed face-to-face at 180 degrees
2. Mount O3 with a turning mirror and position it onto a manual z-stage. The entire setup is mounted altogether onto a separate small plate for easier maneuver
3. Place a cage compatible Iris after O3 and place O3 in front of O2
4. Roughly adjust its position until the light spot passes through the center of Iris
5. Fine adjust and fix the height using z-stage and leave all other axes roughly aligned. Move the O2 back to its marked position
6. Start tilting the O3 incrementally, move the plate around after each tilt until the laser spot passes through the center of Iris
7. Depending on the O2 & O3 used, a tilting angle between 40-50 degrees should be aimed. Circular laser spot will start to be clipped into an eclipse/olive as the tilting angle increases
8. After the desired angle is reached (by eyeball, no need to be precise), fine adjust the position of the plate until the output beam is collimated and centered at the Iris
9. Gently fix the position of the plate using holdfast and insert emission filter and another turning mirror to turn the beam upwards
10. Mount camera with the commercial zoom lens and mount them altogether vertically onto a large z-translation stage (see in video)
11. Position the large z-stage until the laser beam roughly hits the center of commercial zoom lens

Part VI: O1 and bright-field imaging system

1. Change the angle of removable turning mirror in Part I so the laser is pointed towards the direction where O1 should be (the turning mirror is set up using separable cube so the base will ensure the angle is fixed once the mirror is inserted)
2. Place the cage system containing O1 onto the stage and mount a cage compatible Iris
3. Position the cage system until the laser spot passes through the center of the Iris
4. Remove O1 from the cage system and insert a secondary reference laser
5. Remove turning mirror and leave that cube empty. Swap in a dichroic mirror that will reflect the reference laser
6. Use a shear plate to check the collimation of beam reflected from the dichroic, adjust the position of O1 cage system until the beam is collimated
7. Bring back the turning mirror and make sure the laser is directed to the bright-field setup
8. Replace 50mm lens with a 100mm lens and adjust its position following step 4 and 5 in Part II
9. Mount the bright-field camera with its camera lens using a cage system and place a cage compatible Iris in front
10. Adjust the position of camera until the laser passes through the center of the Iris. There is no need to maintain 2f condition between the 100mm lens and camera lens, as this will not affect the performance of bright-field imaging system
11. Remove the Iris and secondary reference laser. Swap back O1

Part VII: Light-sheet formation and conditioning

1. Mount Powell lens and cylindrical lenses into rotatable cage plate. Powell lens may need a customized adapter depending on its shape
2. Mount Powell lens and the 50mm cylindrical lens using a cage system, then remove the cage plate containing 50mm cylindrical lens
3. Turn on the excitation laser and set at a low power (~ 3mW) and adjust the height of Powell lens so a vertical light sheet with uniform intensity distribution is formed. The vertical light sheet should be checked using a slit
4. Place the cage plate containing the 50mm cylindrical lens back into the cage system as well as a slit. Adjust the rotational angle of 50mm cylindrical lens until a perfect vertical light sheet is formed
5. Adjust the position of the 50mm cylindrical lens until a collimated light sheet is formed. If a collimated light sheet cannot be formed, it indicates the angle of cylindrical lens is 90 degrees off
6. Place the cage system in front of the excitation laser and using both reflected light and slit to confirm it’s placed at the center of the laser pathway
7. Set up the 75mm cylindrical lens and turning mirror onto a 2-axis manual stage (see in video) and using cage system to ensure it is positioned at 90 degrees
8. Place the 2-axis manual stage so it roughly aims at the center of dichroic mirror
9. Adjust the rotational angle until a beam converging on y-axis is formed (collimated on x-axis)
10. Adjust the y-axis knob on the manual stage until the output light sheet from 75mm cylindrical lens passes through the center of slit
11. Adjust the x-axis knob on the manual stage until the light sheet passes through the center of each following spherical lens. This doesn’t need to be precise
12. Prepare a glass of fluorescein solution and immerse the tip of O1 to visualize the light sheet
13. Looking from the size of the objective lens, check if the light sheet is emitted from the center of the tip
14. If not, carefully adjust the height of the cage system mounted on the s-axis manual stage until the light sheet is emitted from the center of the tip
15. Lossen the 75mm lens and adjust its position until the light sheet forms a focused spot onto the center of the galvo mirror
16. Remove the glass of fluorescein solution and clean the tip of O1

Part VIII: Tilt angle fine turning and quality check

1. Prepare droplets of 1% agarose gel containing 200nm fluorescent beads following the protocol
2. Place the sample under O1 and open MicroManager for live viewing
3. Set laser power at 20 mW (488nm) and adjust the intensity upper limit on MicroManager to around 500
4. Adjust the height of sample using the 4D stage and a focused thin line with PSF should be observed
5. If no PSF can be observed at all no matter what, it indicates the poor alignment during previous steps
6. Adjust the x-axis knob on the manual stage setup in Part VII (make sure toward the right direction as a reversely tilted light sheet won’t do anything) and the sharp region should start to grow as the tilt angle increases
7. Adjust the knob until the optimal angle is found that matches the tilting angle between O2 & O3. It is likely not the entire aperture will be sharp
8. If the PSF is blurry, it is usually caused by defocusing. Attempts can be made by adjusting the focusing ring on the commercial zoom lens but not suggested. Ideally, re-alignment between O2 & O3 should be performed until sharp PSF can be observed without adjusting the commercial zoom lens