

# Any immersion remote refocus (AIRR) microscopy

This project is maintained by amsikking in the York lab, and was funded by Calico Life Sciences LLC

## Appendix

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Note that this is a limited PDF or print version; animated and interactive figures are disabled. For the full version of this article, please visit one of the following links:

[https://amsikking.github.io/any\\_immersion\\_remote\\_refocus\\_microscopy](https://amsikking.github.io/any_immersion_remote_refocus_microscopy)

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## Any immersion remote refocus (AIRR) microscopy

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### Theory

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Here we present the equations used in the results section to evaluate the widefield and remote refocus microscope design options.

#### **Numerical aperture and resolution**

The numerical aperture (NA) is defined as:

$$NA = n_i \sin \theta_i \tag{a1}$$

where  $n_i$  is the refractive index of the immersion medium and  $\theta_i$  is the collection half-angle of the objective. In the case of a planar refractive index boundary like a coverslip the NA is conserved via Snell's law:

$$n_s \sin \theta_s = n_i \sin \theta_i \tag{a2}$$

where  $n_s$  is the refractive index of the sample and  $\theta_s$  is the collection half-angle in sample space. According to the Rayleigh criteria the smallest resolvable feature size  $r_{min}$  is then:

$$r_{min} = 0.61 \frac{\lambda}{NA} \quad (a3)$$

where  $\lambda$  is the wavelength of the collected light [Pawley 2006].

### Focal length, field of view and pixels

The objective lens focal length  $f$  is found with:

$$f = \frac{f_{TL}}{M} \quad (a4)$$

where  $f_{TL}$  is the tube lens focal length (200mm for Nikon) and  $M$  is the magnification of the objective. The sample space field of view (FOV) is then assumed to be:

$$FOV = \frac{FN}{M} \quad (a5)$$

where FN is the field number according to the manufacturer (20mm assumed for Nikon). When choosing an objective it can be useful to estimate the number of Nyquist pixels  $N_{px}$  the lens can deliver across the FOV for specifying a camera using:

$$N_{px} = 2 \frac{FOV}{r_{min}} \quad (a6)$$

Note: this can also serve as a measure of the *quality* of the objective, where more pixels usually indicates a more sophisticated lens design (similar in concept to *etendue*).

### Standard focus range

From Sheppard 1991, equation 18 proposes that in a medium of refractive index  $n_1$ , the maximum acceptable wavefront aberration (Rayleigh criterion) will not be exceeded if the thickness of a slab of dielectric  $t$  and refractive index  $n_2$  meets the following condition:

$$t \leq \frac{\lambda n_2^3}{2n_1^2(n_2^2 - n_1^2) \sin^4(\alpha/2)} \quad (a7)$$

where  $\alpha$  is the collection half-angle in the  $n_1$  space (i.e. the immersion). Originally evaluated for a phase error of  $\pi/2$ , equation (1) gives negative values for  $n_1 > n_2$ . If we allow  $n_1 > n_2$  and a phase error of  $\pm\pi/2$  then we can rewrite (1) in a slightly more convenient format to give the maximum depth of standard focus  $z_{sf\_max}$ :

$$z_{sf\_max} = \frac{\lambda}{2 \sin^4(\theta_i/2)} \left| \frac{n_s^3}{n_i^2(n_s^2 - n_i^2)} \right| \quad (a8)$$

### Remote refocus range

From [Botcherby 2007](#), equation 23 proposes that the Strehl ratio  $S$  of a remote refocus can be modelled by:

$$S = 1 - \frac{4n^2 k^2 z^4 (3 + 16 \cos \alpha + \cos 2\alpha) \sin^8(\alpha/2)}{75 f^2 (3 + 8 \cos \alpha + \cos 2\alpha)} \quad (\text{a9})$$

where  $n$  is the refractive index of the sample,  $k = 2\pi/\lambda$  is the wavenumber,  $z$  is the axial distance from the traditional focal plane,  $f$  is the focal length and  $\alpha$  is the collection half-angle of the objective. If we set  $S = 0.8$  (the traditional diffraction limit) and rearrange we can estimate the maximum remote refocus depth  $z_{rr, max}$ :

$$z_{rr\_max} = \pm \frac{1}{2 \sin^2(\theta_s/2)} \sqrt[4]{\frac{15\lambda^2 f^2 (3 + 8 \cos \theta_s + \cos 2\theta_s)}{\pi^2 n_s^2 (3 + 16 \cos \theta_s + \cos 2\theta_s)}} \quad (\text{a10})$$

Note: the substitution  $\alpha = \theta_s$  is subtle. In [Botcherby 2007](#) at the critical substitution of equation 8 ( $\sin \theta = \rho \sin \alpha$ ) the paper reads "In this expression  $\alpha$  is the semi-aperture acceptance angle of the lens" so one could assume  $\alpha = \theta_i$ . However the substitution is in reference to coordinates in *object space*.

### Combined range

For a microscope with remote refocus optics (e.g. Figure 2) we can now estimate the maximum focus range in the sample  $z_{max}$  as the sum of the standard and remote ranges:

$$z_{max} \approx z_{sf\_max} + |z_{rr\_max}| \quad (\text{a11})$$

Note: the *approximately* equals sign. The standard focus and remote refocus ranges use different definitions for the diffraction limit, and are approximate models that ignore higher order aberrations. There is also no consideration given to field effects, chromatic aberrations or design and manufacturing imperfections that can be expected in real objective lenses.

## Objective selection

In a SOLS microscope [Millet-Sikking 2019], as the angular range in the sample  $\theta_s$  decreases, the tilt of the last microscope  $\theta_{tilt}$  increases according to:

$$\theta_{tilt} = 90 - \theta_s \quad (deg) \quad (a13)$$

Currently the AMS-AGY v2.0 objective [Yang 2022] has the most extreme tilt range of any SOLS compatible objective, with maximum tilt of 55 degrees. So combining  $(a1)$ ,  $(a12)$  and  $(a13)$  the minimum acceptable sample space NA is:

$$NA_s \geq 1.33 \sin(90 - 55) = 0.76 \quad (\text{a14})$$

After applying the lower bound on NA of 0.76, the 'best' objectives for the standard immersions were selected in the following 2 categories:

- **Nikon objectives, highest NA:**

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Part #	M	NA	Imm.	WD ( $\mu\text{m}$ )	FOV ( $\mu\text{m}$ )	$r_{\min}$ ( $\mu\text{m}$ )	N_px
MRD70470	40x	0.95	air	210	500	0.342	2927
MRY10060	60x	1.27	water	180	333	0.256	2608
MRD73950	100x	1.35	silicone	310	200	0.240	1663
MRD71970	100x	1.45	oil	130	200	0.224	1787

- **Nikon objectives, most pixels:**

Part #	M	NA	Imm.	WD ( $\mu\text{m}$ )	FOV ( $\mu\text{m}$ )	$r_{\min}$ ( $\mu\text{m}$ )	N_px
MRD70270	20x	0.80	air	800	1000	0.406	4930
MRD77200	20x	0.95	water	990	1000	0.342	5854
MRD73250	25x	1.05	silicone	550	800	0.309	5176
MRH01401	40x	1.30	oil	240	500	0.250	4005

Note: Speciality objectives in the categories of TIRF and Multi-photon may not perform optimally for remote refocus and were avoided.

## Zoom lens

In our previous remote refocus designs we used static lenses to adjust the magnification between the sample and the remote space according to equation 2 [Millett-Sikking 2018, Millett-Sikking 2019]. Specifically we chose to modify the second tube lens in the optical train, often referred to as 'tube lens 2' or TL2. The TL2 location is convenient, as it leaves the first objective and tube lens pair in their traditional (stock) configuration, and allows the addition of a unity magnification scanning system. Here we take the same approach, and optimize our zoom optics at the TL2 location, specifying some of the upstream and downstream optics in order to produce a concrete design.

## Specifications

To produce an inexpensive zoom lens with stock parts we narrow the optical requirement to a specific RR system. We assume a widefield microscope at 40x magnification since this offers many attractive primary objective options (air, water, silicone and oil immersion at high NA from various manufacturers). We then assume a 5mm focal length air lens for the second RR objective (often referred to as 'objective 2' or O2). For example the Nikon 40x0.95 air lens (9.5mm back focal plane diameter) is a good high NA option, with a high enough collection cone ( $\sim 72^\circ$ ) and pixel count ( $\sim 2927$ ) for most RR designs. We target a RR magnification that covers the full biological refractive index range (1.33-1.51) by tuning the focal length of TL2 in the range 150-132.5mm (i.e. 30-26.5x for microscope 2). Finally we assume a standard sCMOS field of view ( $\sim 13.5\text{mm}$  width) and add the additional constraints of needing a constant track length and telecentric image.

## Design

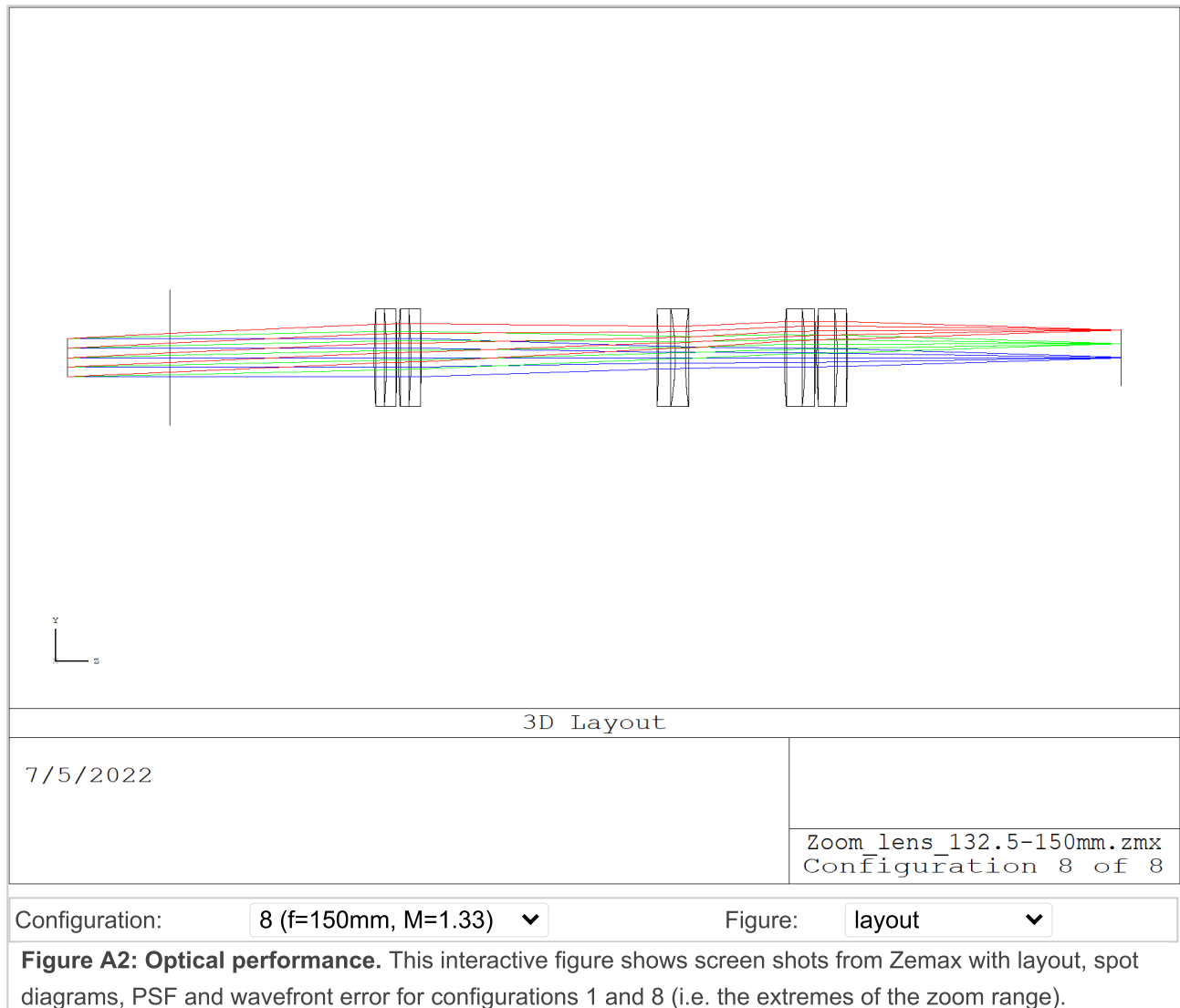
Using the above specifications, we adopt a simple 'positive-negative-positive' (PNP) zoom design and cycle through readily available stock parts. After some iteration we settle on a series of Edmund Optics achromats that satisfy our optical requirements, whilst also being mechanically compatible and inexpensive (see the [mechanics section](#) for parts). Our constraint of only using stock lenses forces a 5 achromat solution ([Figure A2](#)), where a 3 achromat custom design would suffice. We note that a 'standard' tube lens typically has 2 achromats, so a 3 lens solution would only add 1 achromat to the optical train (a minimal drawback for the system). However, we consider the small penalty on transmission efficiency ( $\sim 1.5\%$ ) from the 2 additional achromats in the design we present to be a good trade for avoiding bespoke glass.

## Performance

In [Figure A1](#) we see the spot diagrams as a function of field (centre to edge) and configuration (min to max focal length) across the visible spectrum (450-700nm). We can see that the system is mostly diffraction limited throughout the matrix, with some marginal degradation at the edge of the field for the shorter focal lengths (higher RR magnifications). In the interactive [Figure A2](#) we show detailed views of the performance at the extremes of the range with configurations 1 and 8 (i.e. magnifications of 1.33 and 1.51). See the zoom lens 'optics' folder for more details (including Zemax files).

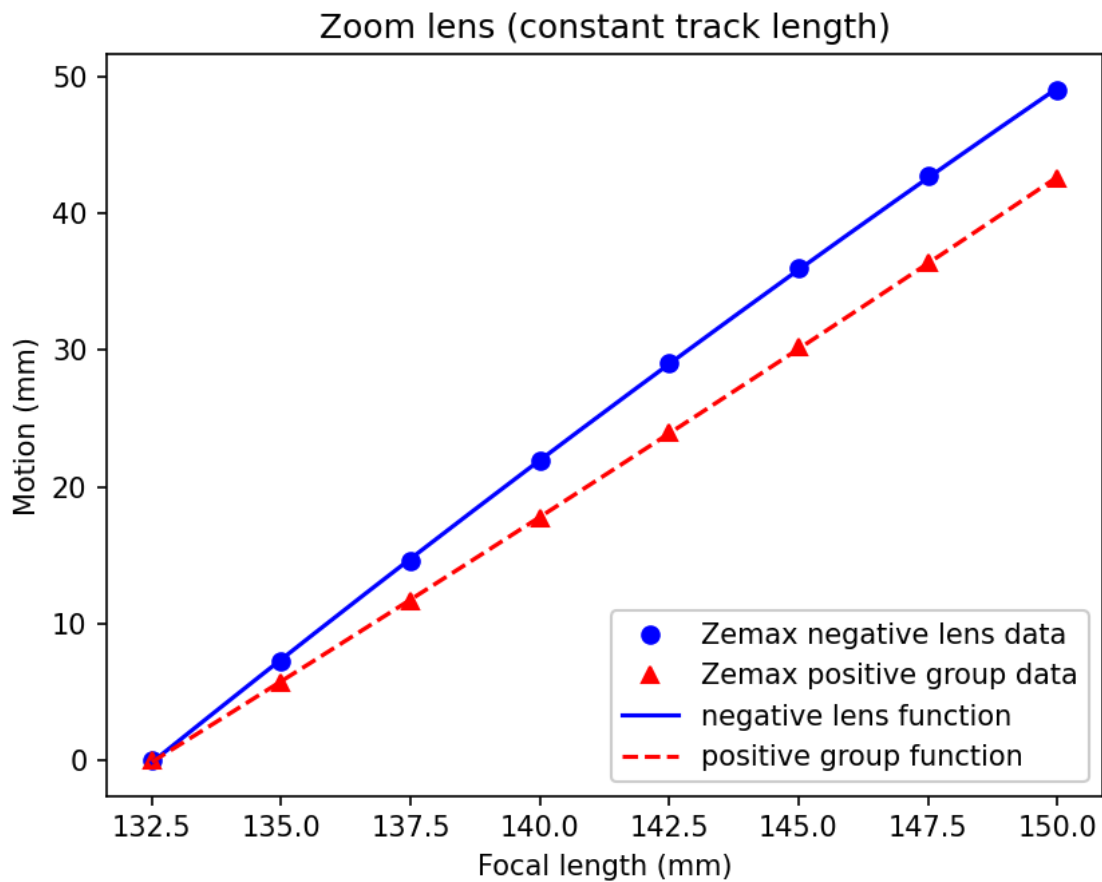


**Figure A1: Multi-configuration spot diagram.** This screen shot from Zemax shows the spot diagrams as a function of field (0, 1.3 and 2.6deg) and configurations (1-8) across the visible spectrum (450-700nm). Here the configurations 1 to 8 correspond to target focal lengths of 132.5mm to 150mm in steps of 2.5mm. Spots that appear within the Airy diameter (black ring) can be considered diffraction limited.



## Motion

Part of the design challenge in a zoom system is accomodating the motion of elements (or groups of elements). Here we adopt relatively large motions of 2 of the 3 lens groups from the PNP design. This has the drawback of being slower than small motions, but the benefit of loosening the opto-mechanical tolerances of the system, thereby allowing us to use stock optics and mechanics. In [Figure A3](#) we see how we need to move the lens groups from the 'zero position' with a focal length of 132.5mm to the 'limit position' with a focal length of 150mm. The negative lens (centre element in the design) moves the most by almost 50mm, while the positive group near the image move slightly less by around 42mm (the first lens group in the design is static).



**Figure A3: Zoom lens motion.** This plot shows how the negative lens (central element in the PNP design) and positive group (2 achromats near the image) should move to produce a given focal length for the zoom lens. The first lens group in the PNP design is static.

Below we provide the 'motion functions' that convert a requested focal length to a required movement of each lens or lens group (for driving linear stages or equivalent mechanics). Here's [an example](#) for a focal length of 140mm:

```
# Zoom_lens_132.5-150mm_motion.ods trendline polynomials
# Data from Zemax
def negative_lens_motion(f_mm):
    motion_mm = (- 0.012921858472509 * f_mm**2
                  + 6.467807910667240 * f_mm
                  - 630.2815284192340)
    return motion_mm

def positive_group_motion(f_mm):
    motion_mm = (+ 0.005518525287293 * f_mm**2
                  + 0.885914869404723 * f_mm
                  - 214.4010550854960)
    return motion_mm

# Input:
```

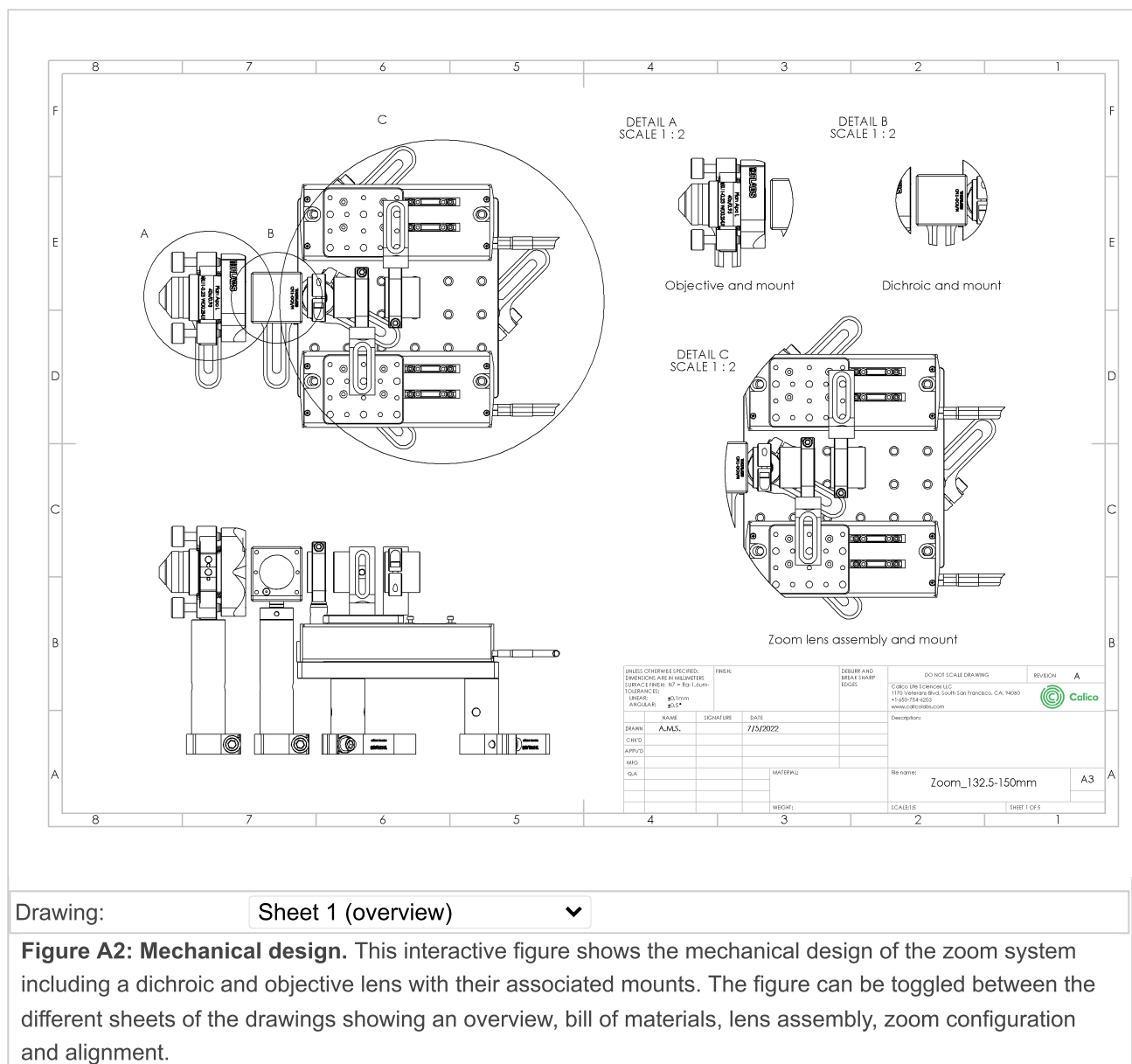
```
f_mm = 140
N_motion = negative_lens_motion(f_mm)
P_motion = positive_group_motion(f_mm)
print('requested focal length = %0.2f'%f_mm)
print('negative lens motion    = %0.2f'%N_motion)
print('positive group motion   = %0.2f'%P_motion)

# Output:
## requested focal length = 140.00
## negative lens motion    = 21.94
## positive group motion   = 17.79
```

## Mechanics

Here we offer a relatively simple mechanical design ([Figure A4](#)), that utilizes some fast 50mm linear stages from Thorlabs (DDS050). However, any mechanical solution that achieves the required alignment of the lenses and relative motions is acceptable. The solution we present combines the zoom optics into a single large mount that can (in principle) be aligned to an axis that is parallel to the optical table, and includes provision for an objective, a dichroic and an unhampered image plane. The DDS050 stages have a max speed of 500mm/s so we can expect a responsive zoom experience for tuning the magnification to different sample types. See the included [Zoom\\_lens\\_132.5-150mm.pdf](#) for a high quality reproduction, or the '[mechanics](#)' folder for more details (including CAD files).





## Large FOV SOLS

### Current prototype

On the road to building an 'any immersion' large FOV SOLS microscope we opted to build a 'static' configuration (i.e. no zoom lens) to show the potential of the new approach. The system was built to accommodate primary objectives with a 5mm effective focal length (i.e. the Nikon 40x family) with a remote refocus tuned for watery samples (i.e. a sample RI of 1.33). The transition from air to water immersion is one of the most extreme examples of an RI mismatch and so the Nikon 40x0.95 air and 40x1.15 water objectives were chosen as options for the primary objective. The last microscope (often referred to as microscope 3 in a SOLS design) was tilted to 50deg to accommodate the NA of both the air and water objectives. A simple cylindrical lens light-sheet excitation path was assembled, and combined with the following emission path optics:

Item	Supplier	Part	Description
S	Biologist	Sample	preferred refractive index: 1.33
O1	Nikon	MRD77410 or MRD00405	CFI Apo LWD Lambda S 40XC WI (NA=1.15) or CFI Plan Apo Lambda 40XC (NA=0.95)
TL1	Nikon	MXA22018	Tube lens, EFL=200 mm
SL1	Thorlabs	TTL100-A	Scan lens, EFL=100 mm
G1	Thorlabs	GVS211/M	1D Galvo system, $\varnothing$ 10 mm, E02 coated
SL2	Thorlabs	TTL100-A	Scan lens, EFL=100 mm
TL2	Thorlabs	AMS custom	Tube lens, EFL=300 mm
D	Chroma	ZT405/488/561/640rpcv2	Quad band dichroic
O2	Nikon	MRD00205	CFI Plan Apo Lambda 20XC (NA 0.75)
O2*	Carolina	633037	Cover Glasses, Circles, 25 mm, Thickness 0.13-0.17 mm
O3	ASI	AMS-AGY v2.0	Special glass tipped objective (a.k.a. KingSnout)
E	Chroma	ZET405/488/561/640m	Quad emission filter, $\varnothing$ 25 mm mounted
TL3	Thorlabs	TTL200MP	Tube lens, EFL=200 mm
C	PCO	edge 4.2 cl	sCMOS camera, 2048x2048 px, 6.5 $\mu$ m pitch

## Data

The Python scripts that were used to run the microscope and process the data are included in the [data folder](#). The microscope was aligned on a best effort basis, with an overexpanded Gaussian beam for the light-sheet input (wasteful but gave uniform illumination). The light-sheet was generated with a 1mm slit aperture just before the cylindrical lens which was mapped onto the O2 back focal plane with a 1:1 relay (so about 0.66mm at the O1 BFP in this configuration). The PSF data was processed with the same method as [here](#) (which includes sample data). To avoid bloat the raw data (~500GB) is not included here so the publication can be readily stored and circulated offline.

## Improved configuration

Some attractive upgrades to the current prototype include:

- Adding the zoom lens to allow continuous tuning of the remote refocus volume to the sample refractive index (which should also keep the light-sheet aligned).
- Swapping O2 from the Nikon 20x0.75 air objective to the 40x0.95 air objective. This gives improved collection from higher NA primary objectives, and the 40x has the correct effective focal length (5mm) to pair with the zoom assembly.

- Using a high quality coverslip at O2. [ASI](#) currently offers the Nikon 40x0.95 air objective with a high quality (AR coated) coverslip glued to the front.
- Adjusting the focal length of tube lens 3 for a good compromise on the Nyquist pixel size (something like a 250mm EFL).

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