An integrated imaging and staining platform for cubic millimeter scale array tomography

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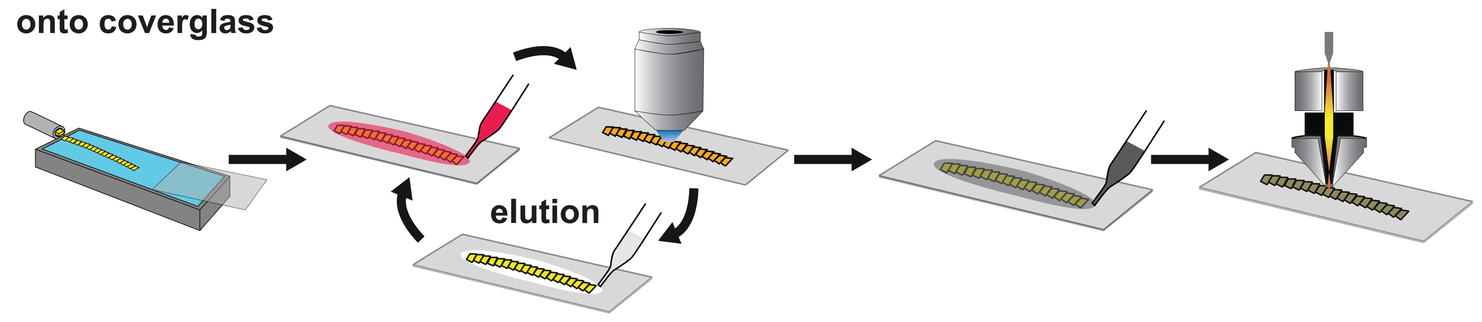
ABSTRACT

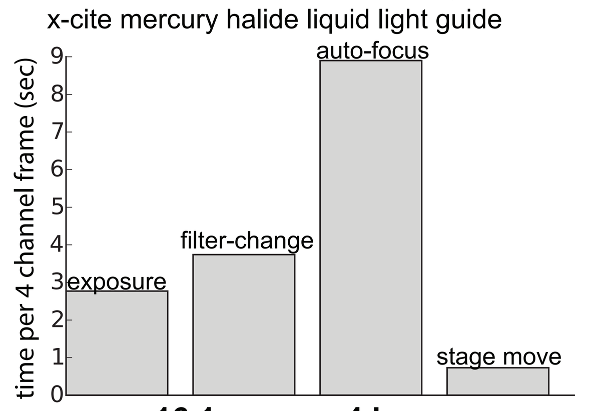
Array tomography (AT) involves reconstruction of images acquired from arrays of serial ultrathin sections, which can be imaged by fluorescence microscopy (FM-AT) for proteometric analysis, or by scanning electron microscopy (EM-AT) for higher resolution. Axial resolution is defined by the thickness of physical sections (50-200 nm); the lateral resolution of FM imaging is optimal given the direct adhesion of sections to an optical coverslip. However, the throughput of data acquisition is slowed by the image acquisition time, and the need for human intervention to stain and set up samples on the microscope. The first AT systems acquired data at a throughput of ~16 seconds per 4 channel image (Micheva 2007). Recent work incorporated a hardware-based autofocus technology that improved throughput to 5-12 sec per 4 channel image (Rah 2013), achieving 0.1 mm^3 in 878 imaging hours with 200 nm-thick sections. Data acquisition throughput is further impaired by the overhead involved in staining and setting the sample up for imaging. As part of the Open Synaptome Project (http://opensynapto.me) effort to improve all aspects of ATomo based synaptic analysis, we are developing a next-generation system to achieve imaging throughput on the order of $0.2mm^3$ per day per microscope. We will describe the design of the imaging system, which include hardware-based autofocus, large format sCMOS sensors, motionless high intensity wide-field laser illumination, and a custom open-source software solution. We will also describe our efforts to construct a robofluidic staining solution fully integrated with the microscope. Our goal is to enable continual staining and imaging without human intervention, thus increasing both throughput and consistency of results

ARRAY TOMOGRAPHY IMAGING



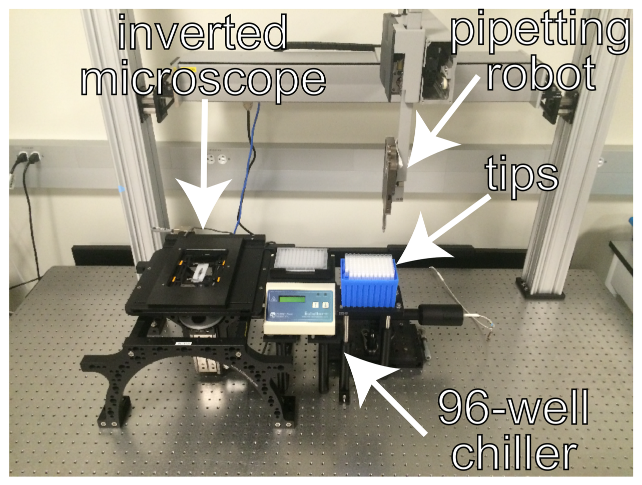
IMAGING SPEED IMPROVEMENTS

Standard Upright Automated Microscope
Zeiss Observer Z.1, 4 filter cubes,
x-cite mercury halide liquid light guide



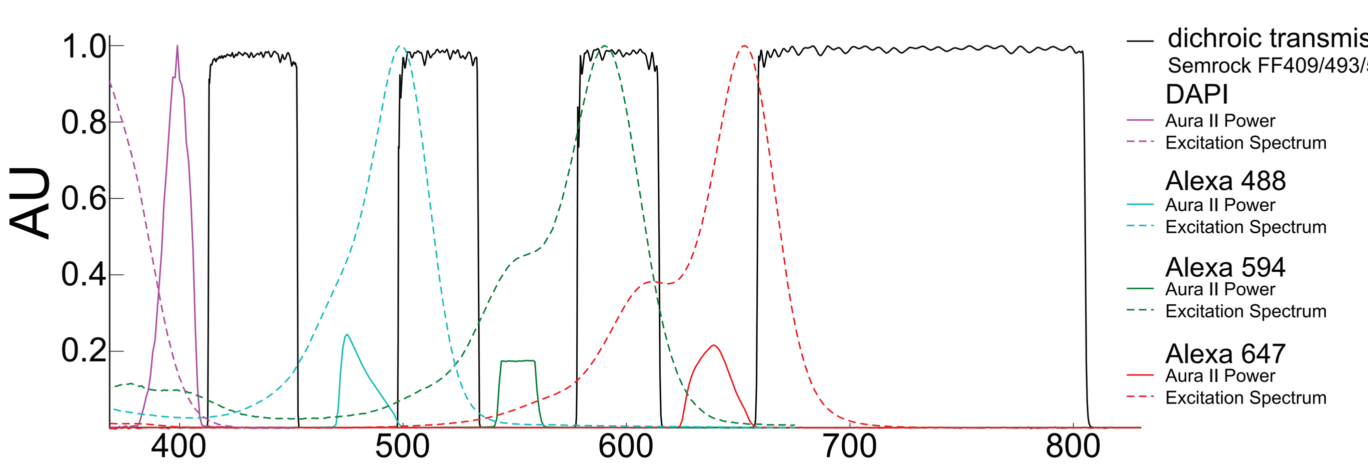
16.1 sec per 4 images
892 images/hour

Improvement	Estimated Speed Increase
hardware auto-focus (Rah et al. 2015)	100-400ms focus time ~2000 images/hour
quad band dichroic illumination switching	~10-50 ms change time ~4000 images/hour
laser illumination	~5x power density ~12,000 images/hour
sCMOS field size	4.2 vs 1.4 megapixels effectively 2.9x faster
NET IMPROVEMENT	**~40x faster net imaging**



Components

Tecan Omni Pipetting Robot
ASI RAMM Frame
ASI XY stage, Linear encoders
ASI Z stage on Z axis for large motion
ASI Piezo Z stage top for autofocus/fine motion
ASI Crisp autofocus
Illumination options:
Lumencor Aura II LED Illumination - Kohler
Andor ILE Laser launch, coupled into
Borealis Widefiled 175 mm square fiber
Andor Zyla sCMOS USB 3.0 camera

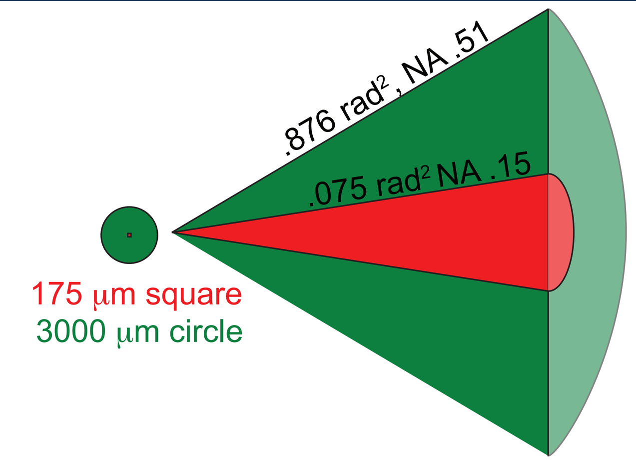


Quad Band Dichroic/Emitter enables, DAPI, Alexa 488, Alexa 594, Alexa 647 imaging with solid state switching between excitation spectra.

LASER ILLUMINATION

Laser Illumination has lower Na d,
much higher intrinsic brightness,
and higher coupling efficiency into objective.

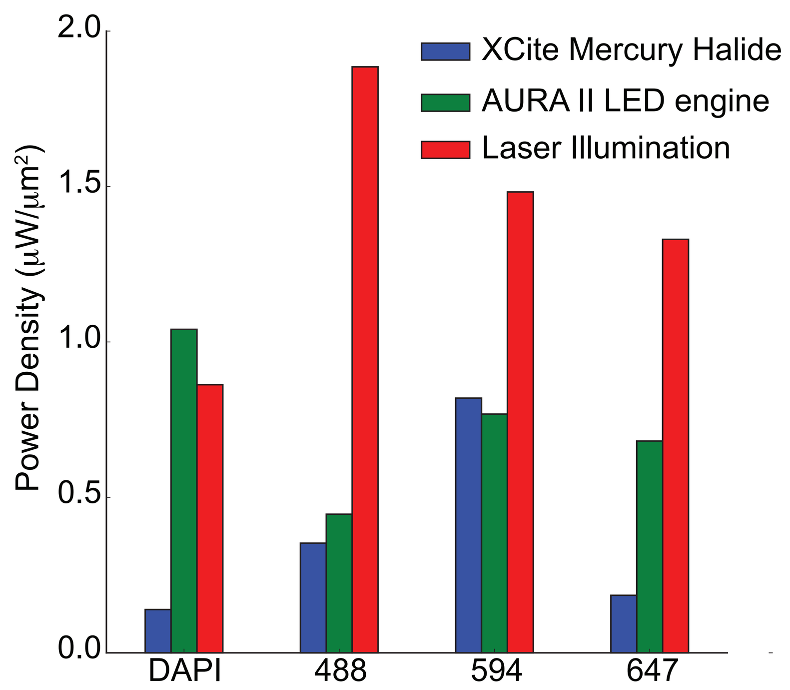
15% for 3mm NA .51 liquid light guide into 63x oil
100% for 175 um NA .15 fiber
(source: ASI documentation)



Xcite Mercury Halid,e, liquid light guide coupled
Field stop to 2,269 μm^2 with 63x 1.4nA Zeiss objective
Power measured in back focal plane
after DAPI, FITC, Cy5, Cy7 filter sets

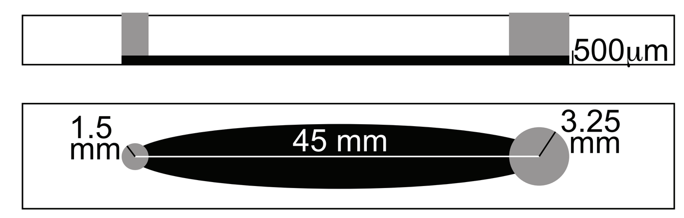
Aura II LED Light Engine, liquid light guide coupled
Field stop to 24,000 μm^2 with 60x 1.4nA Nikon obj.
Power measured in back focal plane after quad band

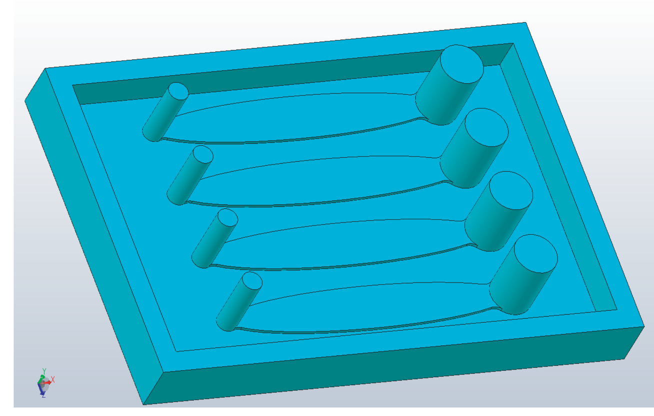
Laser Illumination - Borealis Widefield (Andor)
405 - 83 mW, 488 - 120 mW,594- 92mW, 647 - 83mW
Power measured in back focal plane after quad band
tip covers 40,000 μm^2 with 60x 1.4 nA Nikon obj.

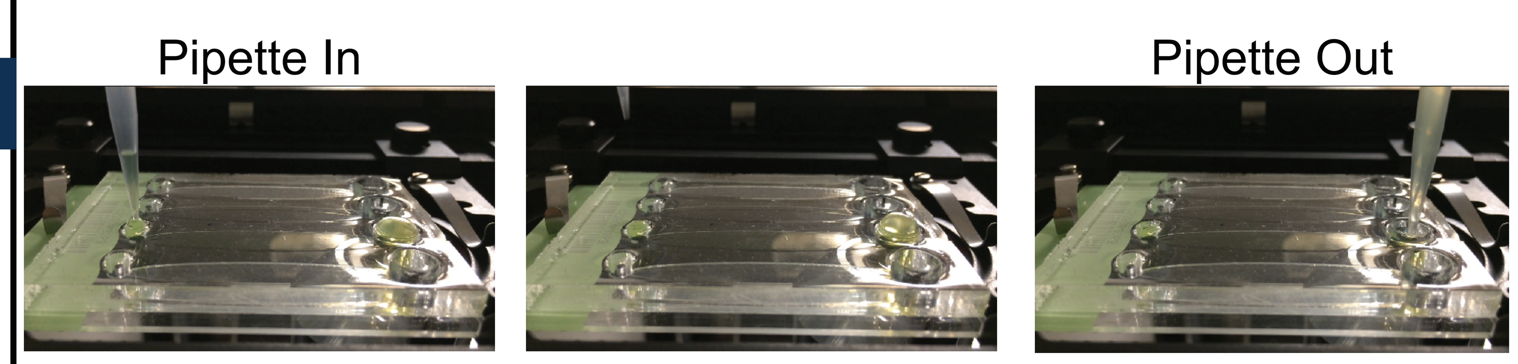


PASSIVE PUMPING FLOW CELL PROTOTYPE

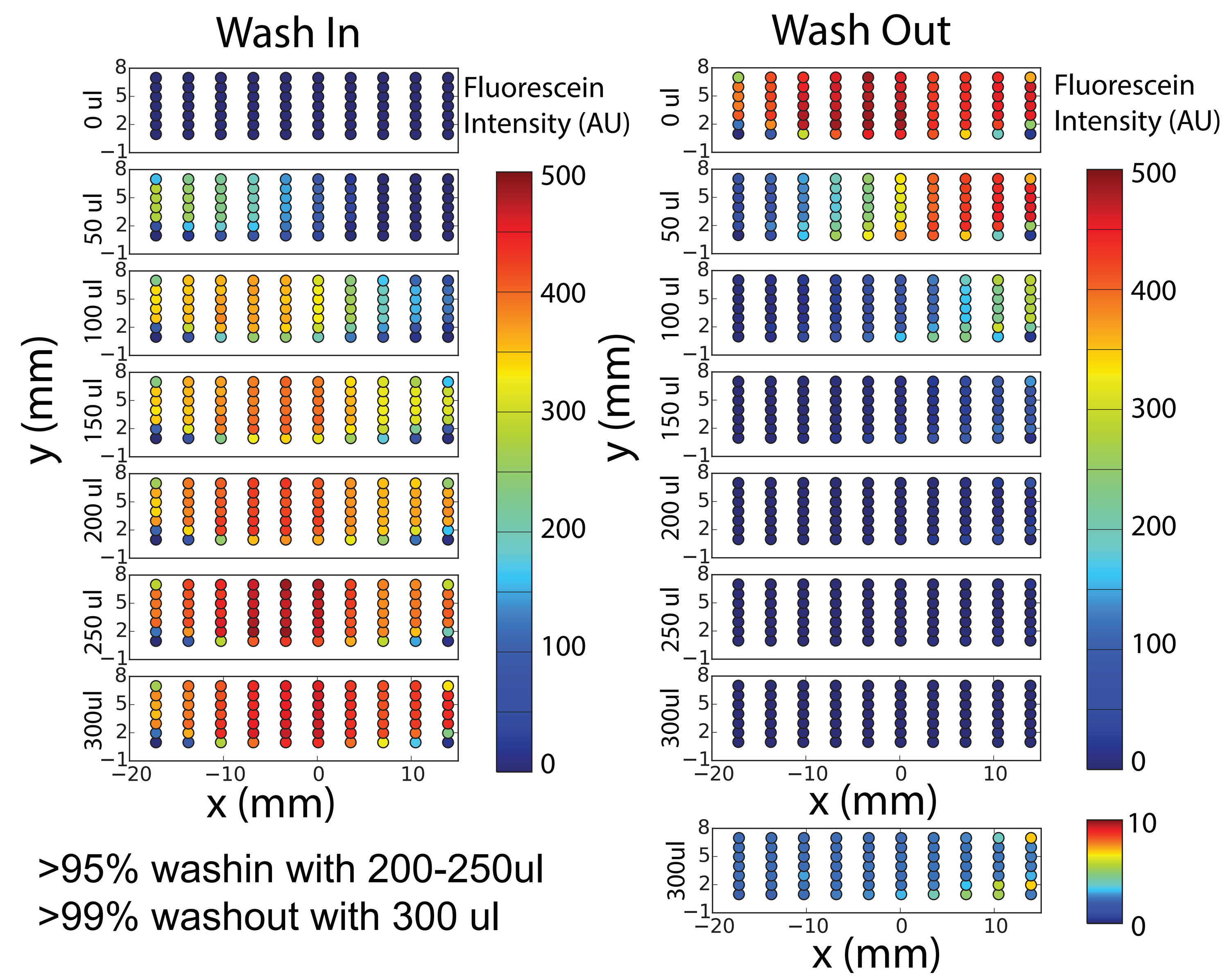
3D printed mold
PDMS (Sylgard) poured mold
Passive pumping design allows simple unpowered flow-cell compatible with vertical pipetting robot (Walker 2002)







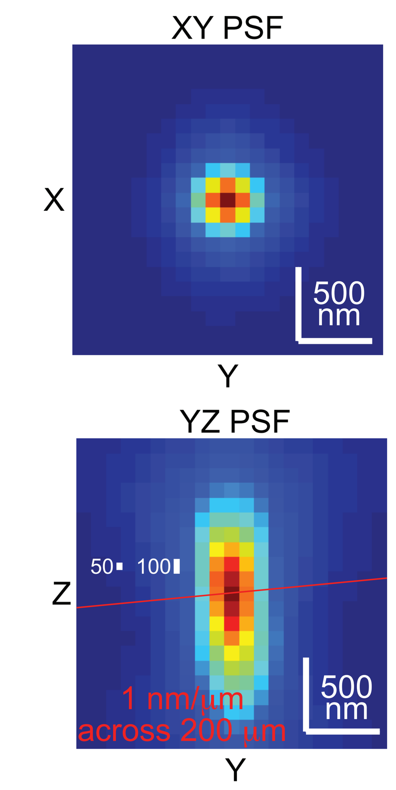
Non-saturating/quenching concentration of fluoroscein
10x air objective, median intensity of field, 50μl increments
Fully automated pipetting and image acquisition across x,y grid

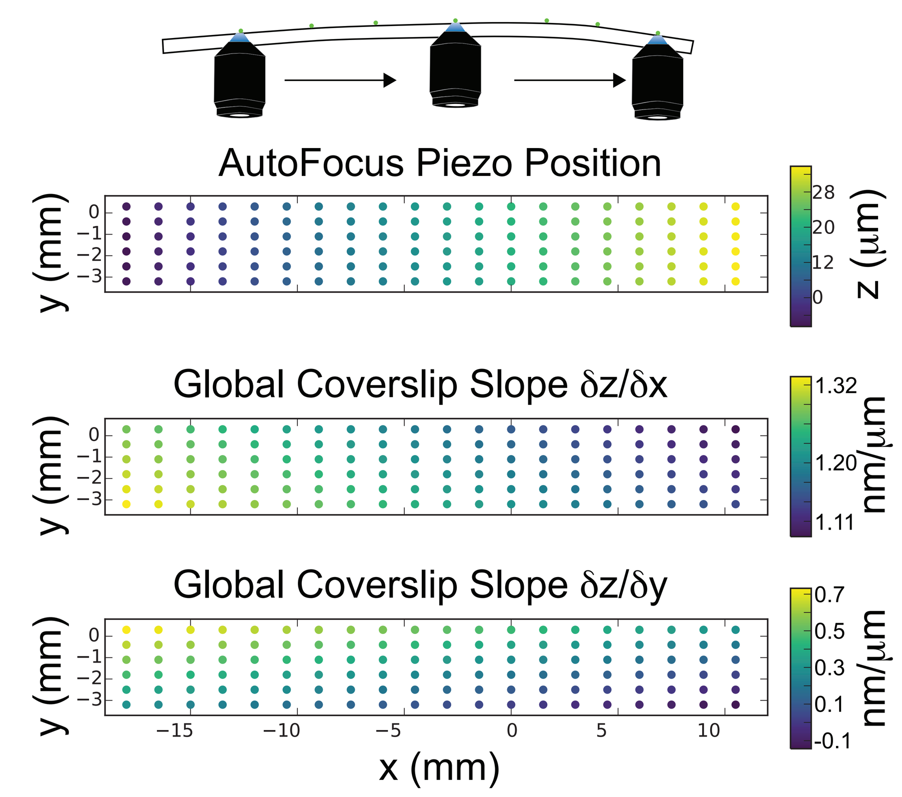


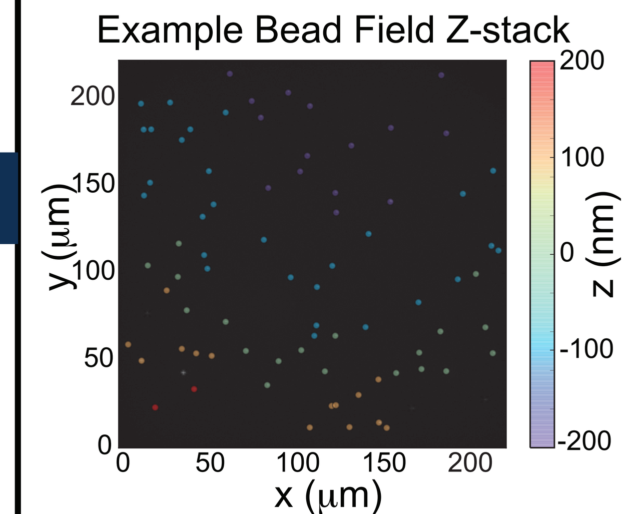
>95% washin with 200-250ul
>99% washout with 300 ul

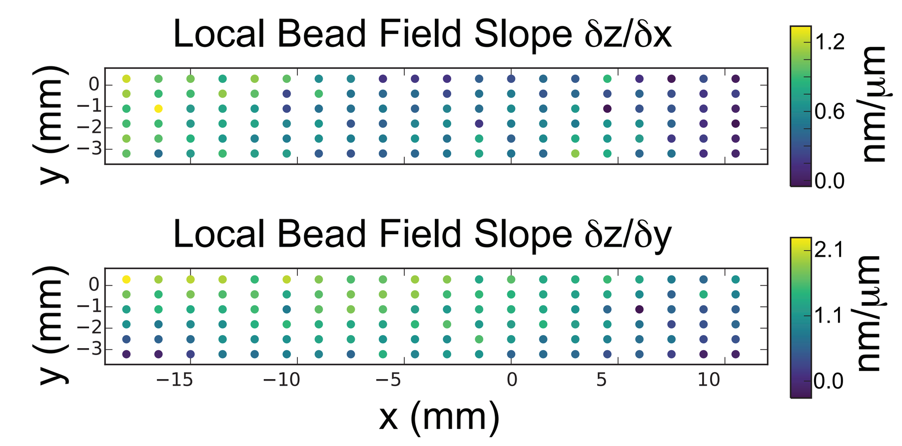
COVERSLIP FLATNESS

Reflection based autofocus used to measure Z position of coverslip relative to plane of motion of the stage. Z stack of bead field on coverslip surface taken at each position within a grid like pattern.



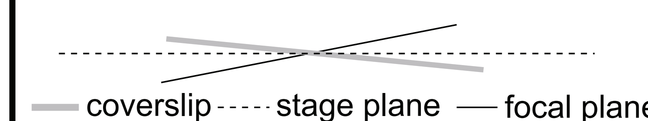


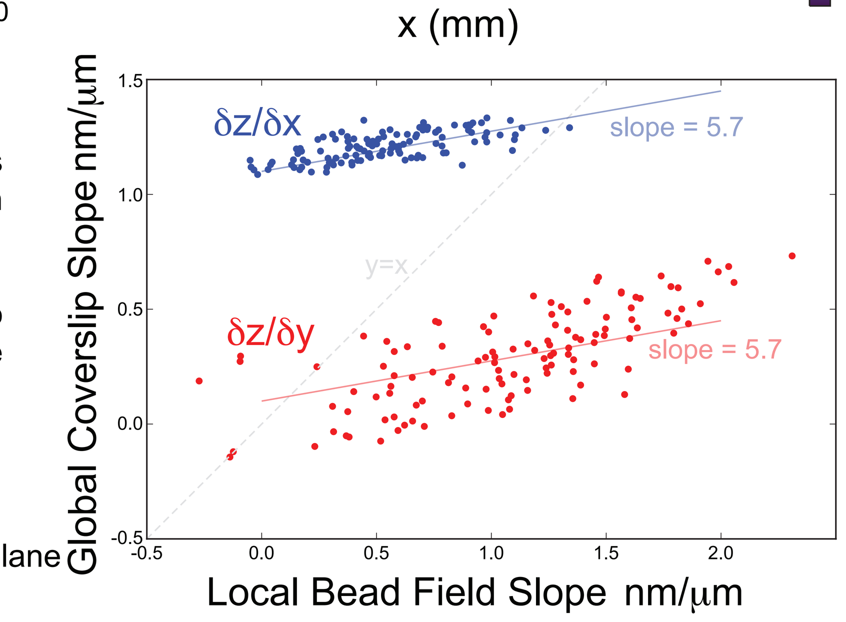




Slope of the bead field stack correlates with the global coverslip slope, but with non-unity slope. and non-zero offset.

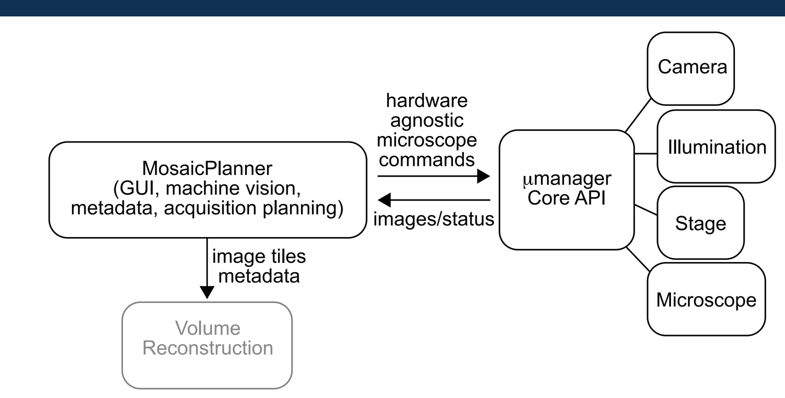
Non-zero offset corresponds to misalignment of focal plane and stage plane, of size ~1 mrad.

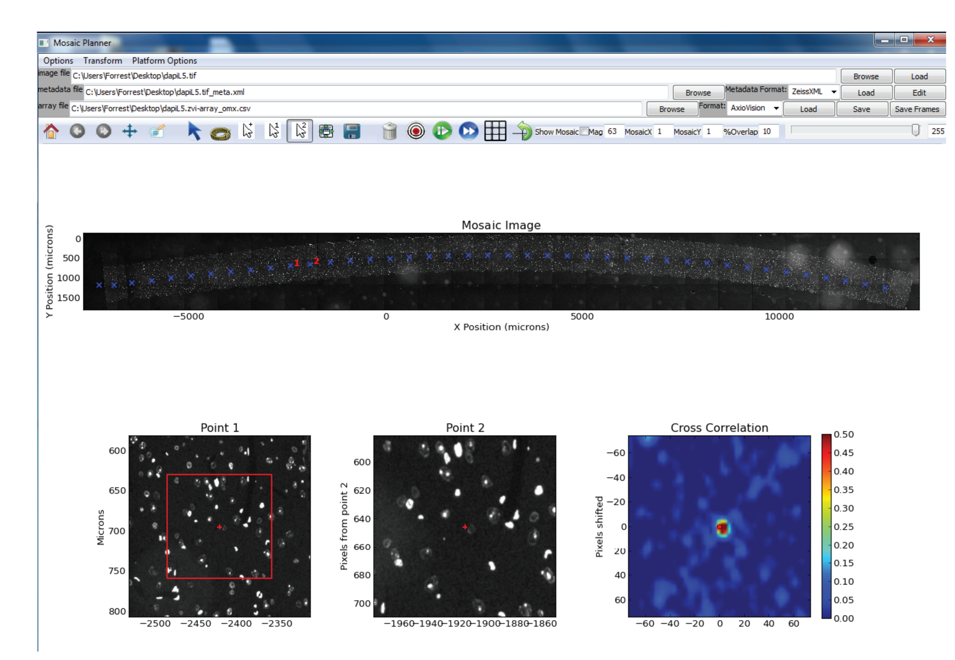




MOSAIC PLANNER - OPEN SOURCE ARRAY TOMOGRAPHY SOFTWARE

Goal: Enable acquisition of Array Tomography light microscopy datasets, by providing a software interface equiped with proper computer vision and user interface optimized for acquisition of AT data on any uManager compatible microscope hardware with sufficent automation capability. (motorized XY stage, motorized channel switching, motorized focus mechanism, digital camera).





REFERENCES AND GRANT SUPPORT

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SUPPORT

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