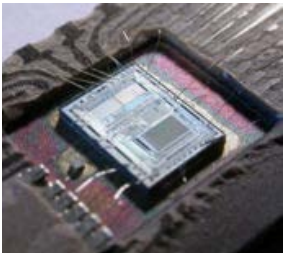


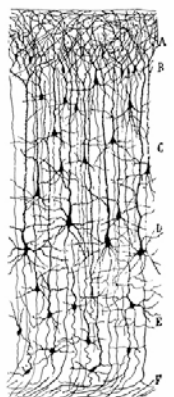
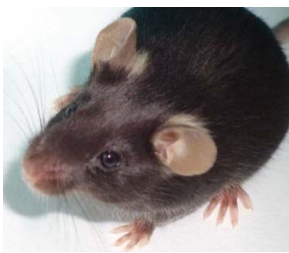
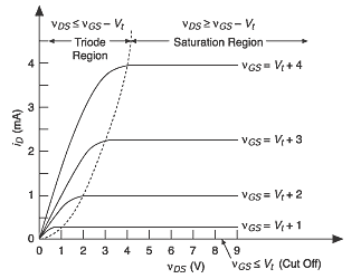
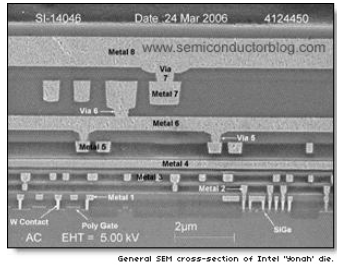
Modern 3D electron microscopy for mapping neuronal circuit connectivity

Kevin Briggman
National Institute of Neurological Disorders and Stroke
National Institutes of Health

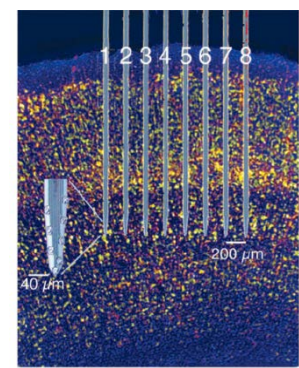
Reverse engineering neuronal circuits



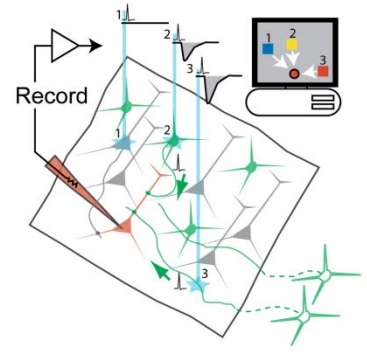
$2 + 2 = 4$



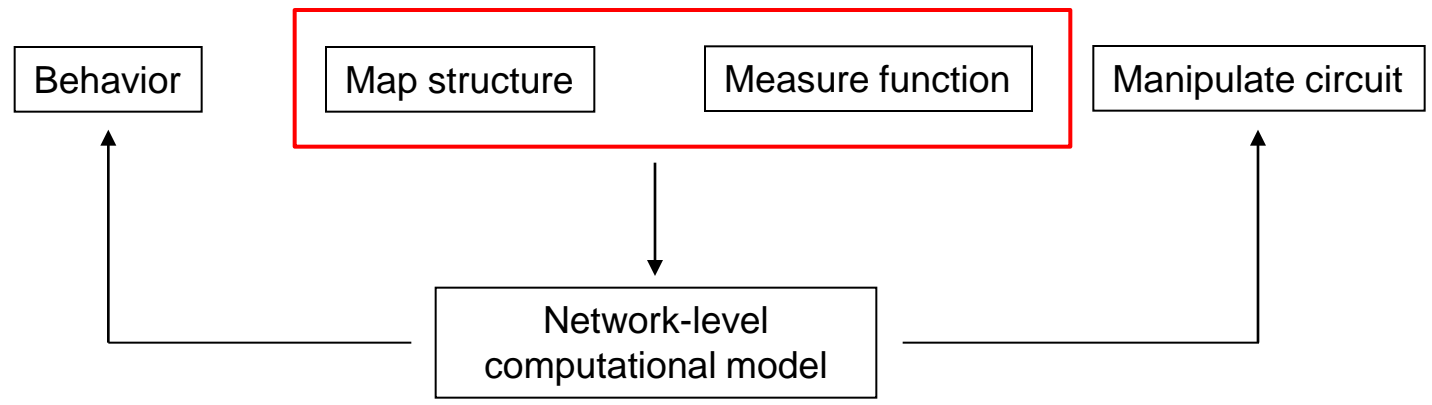
Cajal, 1904



Buszaki, 2004

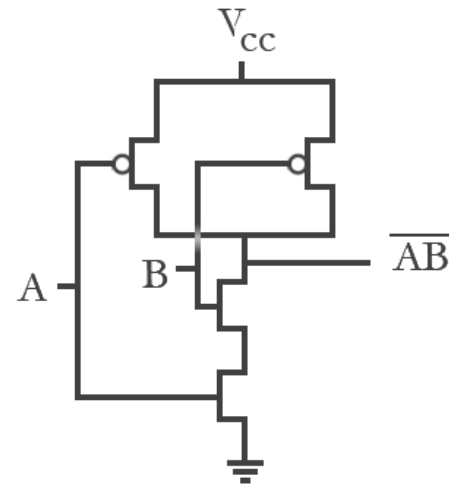
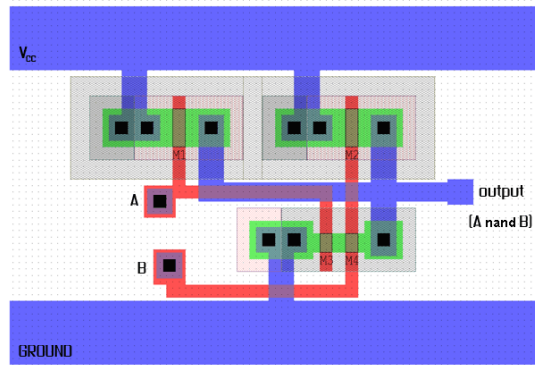
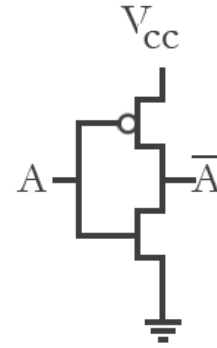
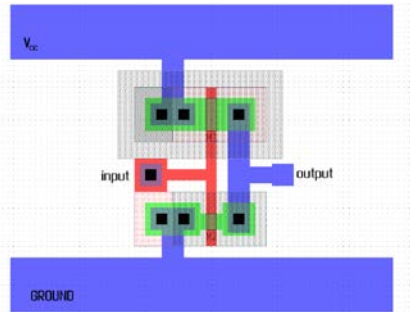


Luo et al 2009

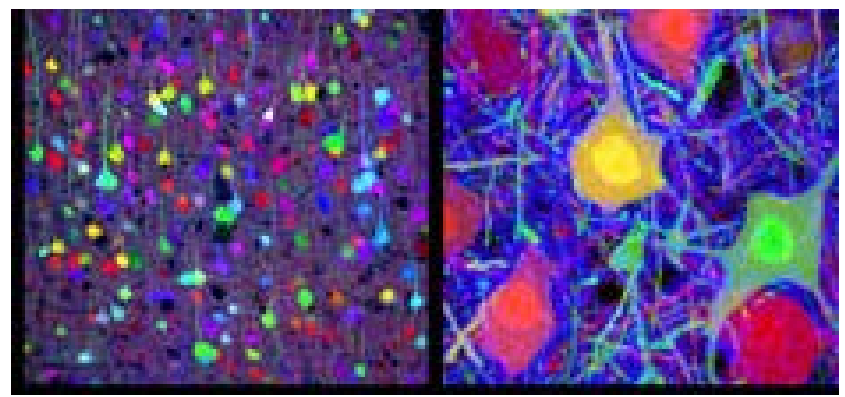
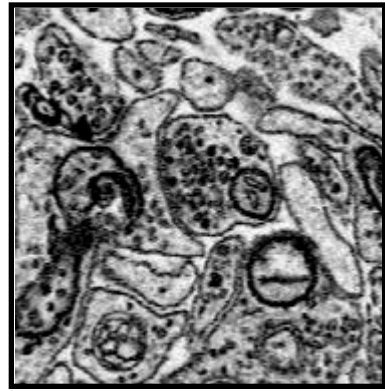
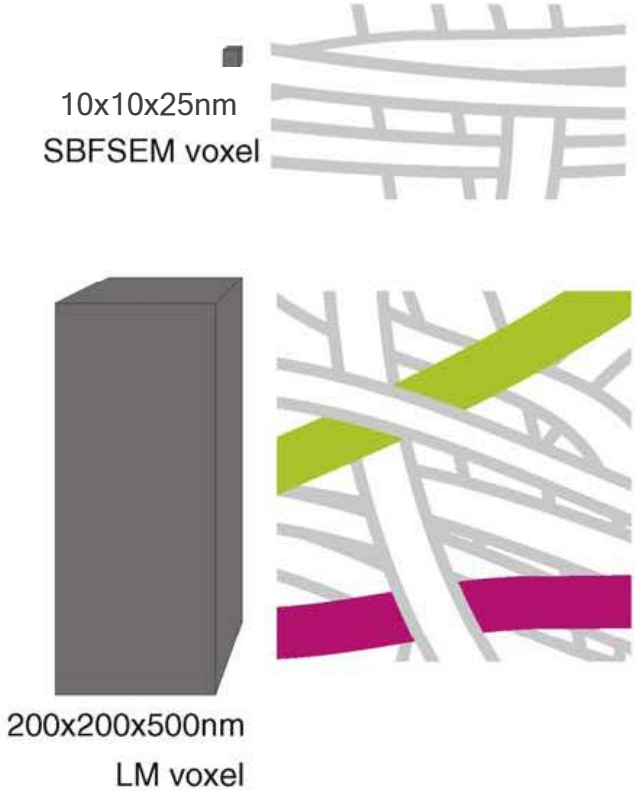


Synaptic connectivity is necessary, but not sufficient, to understand the neural basis of behavior.

But, the hope is that structure tells us something about function:



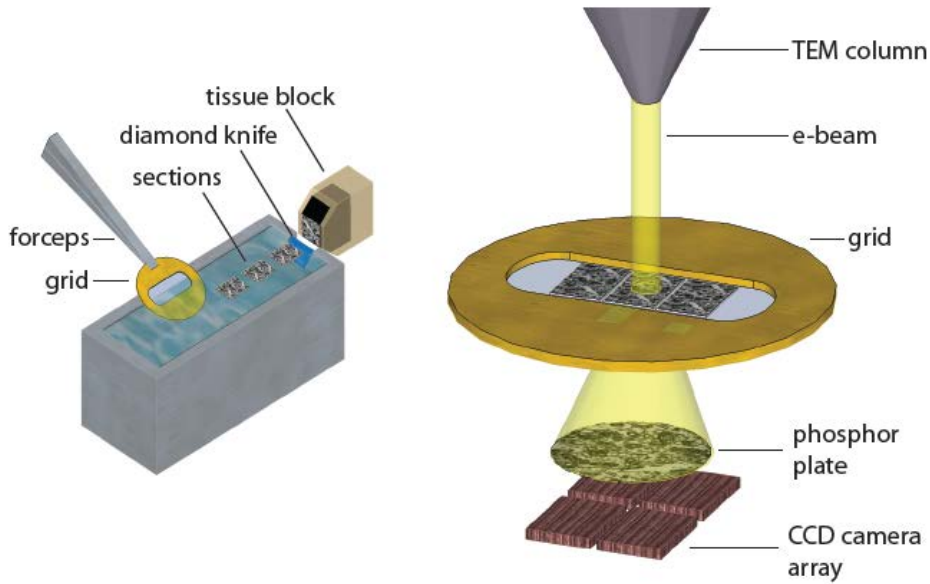
Electron microscopy is needed for dense synaptic reconstruction



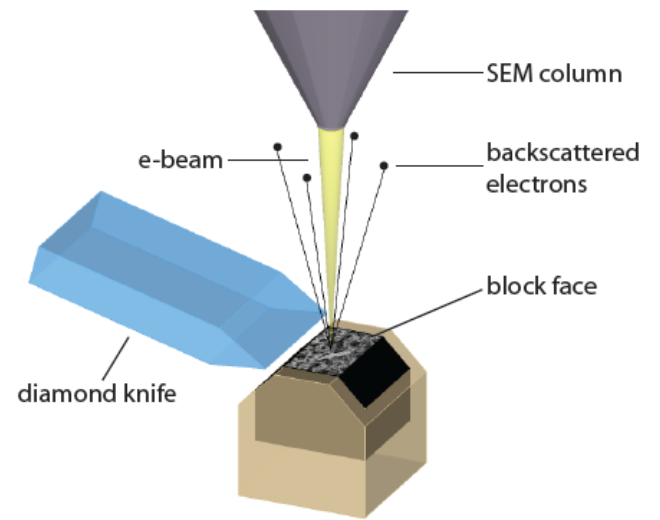
Livet et al 2007

3D electron microscopy techniques

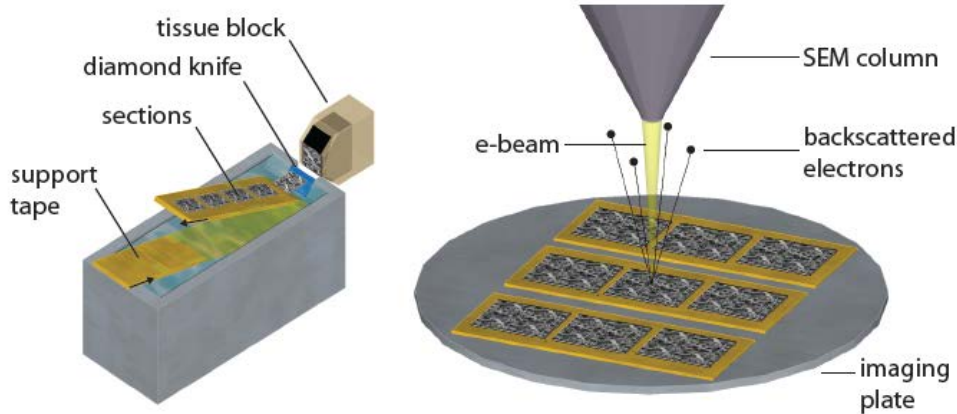
Serial section TEM (ssTEM)



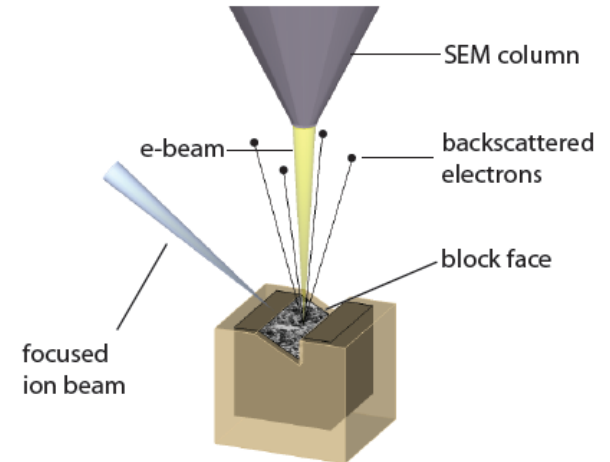
Serial block-face SEM (SBEM)

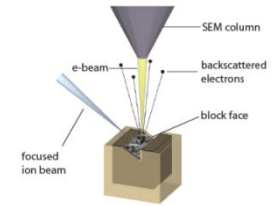
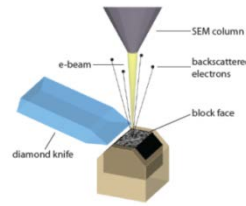
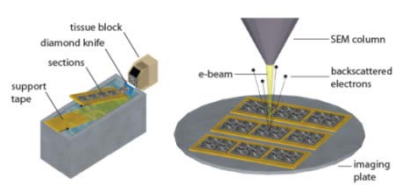
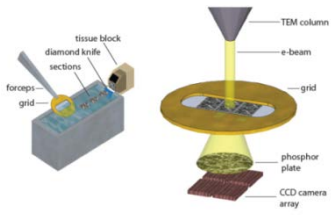


Automated tape collection ultramicrotomy (ATUM)



Focused ion beam SEM (FIBSEM)



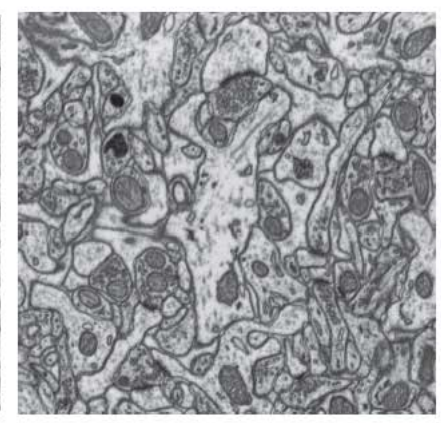
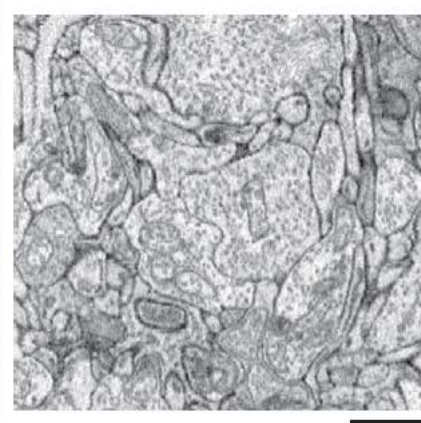
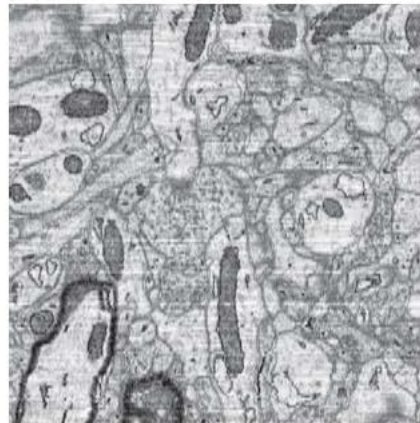
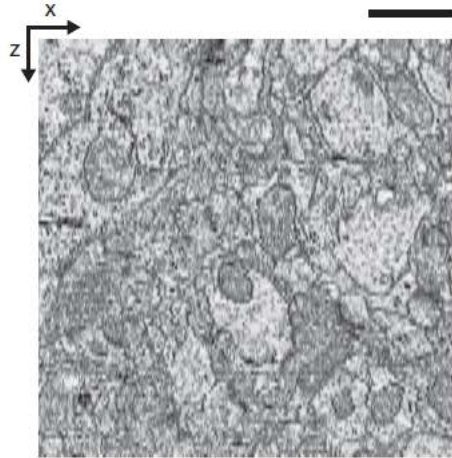
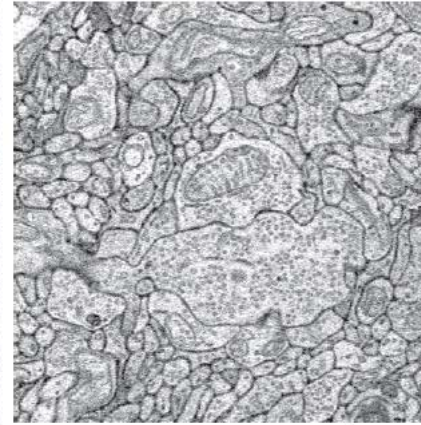
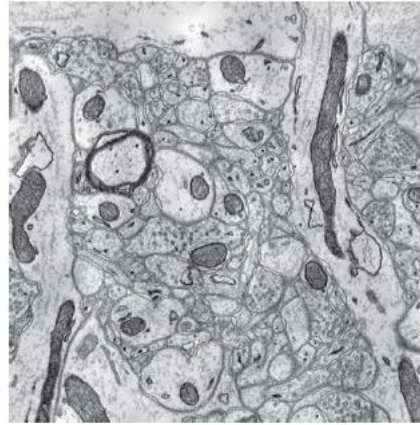
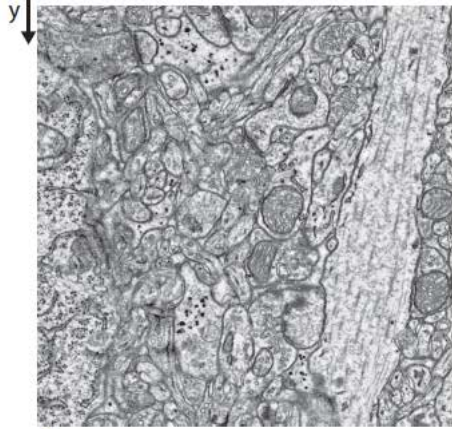


ssTEM (TEMCA)

ATUM-SEM

SBEM

FIB-SEM



Poor isotropic resolution
 Non-destructive
 Section distortions
 Large field of view

Good isotropic resolution
 Non-destructive
 Section distortions
 Large field of view

Good isotropic resolution
 Destructive
 No distortions
 Large field of view

Best isotropic resolution
 Destructive
 No distortions
 Limited field of view

Conventional heavy metal stains

hydrogen 1 H 1.0079																	helium 2 He 4.0026						
lithium 3 Li 6.941	beryllium 4 Be 9.0122																	boron 5 B 10.811	carbon 6 C 12.011	nitrogen 7 N 14.007	oxygen 8 O 15.999	fluorine 9 F 18.998	neon 10 Ne 20.180
sodium 11 Na 22.990	magnesium 12 Mg 24.305																	aluminum 13 Al 26.982	silicon 14 Si 28.086	phosphorus 15 P 30.974	sulfur 16 S 32.065	chlorine 17 Cl 35.453	argon 18 Ar 39.948
potassium 19 K 39.098	calcium 20 Ca 40.078	scandium 21 Sc 44.956	titanium 22 Ti 47.867	vanadium 23 V 50.942	chromium 24 Cr 51.996	manganese 25 Mn 54.938	iron 26 Fe 55.845	cobalt 27 Co 58.933	nickel 28 Ni 58.693	copper 29 Cu 63.546	zinc 30 Zn 65.39	gallium 31 Ga 69.723	germanium 32 Ge 72.61	arsenic 33 As 74.922	selenium 34 Se 78.96	bromine 35 Br 79.904	krypton 36 Kr 83.80						
rubidium 37 Rb 85.468	strontium 38 Sr 87.62	yttrium 39 Y 88.906	zirconium 40 Zr 91.224	niobium 41 Nb 92.906	molybdenum 42 Mo 95.94	technetium 43 Tc [98]	ruthenium 44 Ru 101.07	rhodium 45 Rh 102.91	palladium 46 Pd 106.42	silver 47 Ag 107.87	cadmium 48 Cd 112.41	indium 49 In 114.82	tin 50 Sn 118.71	antimony 51 Sb 121.76	tellurium 52 Te 127.60	iodine 53 I 126.90	xenon 54 Xe 131.29						
caesium 55 Cs 132.91	barium 56 Ba 137.33	57-70 *	lutetium 71 Lu 174.97	hafnium 72 Hf 178.49	tantalum 73 Ta 180.95	tungsten 74 W 183.84	rhenium 75 Re 186.21	osmium 76 Os 190.23	iridium 77 Ir 192.22	platinum 78 Pt 195.08	gold 79 Au 196.97	mercury 80 Hg 200.59	thallium 81 Tl 204.38	lead 82 Pb 207.2	bismuth 83 Bi 208.98	polonium 84 Po [209]	astatine 85 At [210]	radon 86 Rn [222]					
francium 87 Fr [223]	radium 88 Ra [226]	89-102 **	lawrencium 103 Lr [262]	rutherfordium 104 Rf [261]	dubnium 105 Db [262]	seaborgium 106 Sg [266]	bohrium 107 Bh [264]	hassium 108 Hs [269]	meitnerium 109 Mt [268]	ununillium 110 Uun [271]	unununium 111 Uuu [272]	ununbium 112 Uub [277]		ununquadium 114 Uuq [289]									

* Lanthanide series

** Actinide series

lanthanum 57 La 138.91	cerium 58 Ce 140.12	praseodymium 59 Pr 140.91	neodymium 60 Nd 144.24	promethium 61 Pm [145]	samarium 62 Sm 150.36	europium 63 Eu 151.96	gadolinium 64 Gd 157.25	terbium 65 Tb 158.93	dysprosium 66 Dy 162.50	holmium 67 Ho 164.93	erbium 68 Er 167.26	thulium 69 Tm 168.93	ytterbium 70 Yb 173.04
actinium 89 Ac [227]	thorium 90 Th 232.04	protactinium 91 Pa 231.04	uranium 92 U 238.03	neptunium 93 Np [237]	plutonium 94 Pu [244]	americium 95 Am [243]	curium 96 Cm [247]	berkelium 97 Bk [247]	californium 98 Cf [251]	einsteinium 99 Es [252]	fermium 100 Fm [257]	mendelevium 101 Md [258]	nobelium 102 No [259]

Contrast in block face SEM

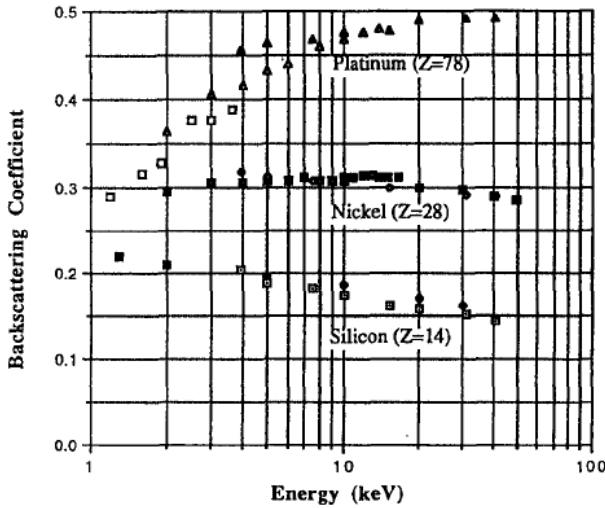
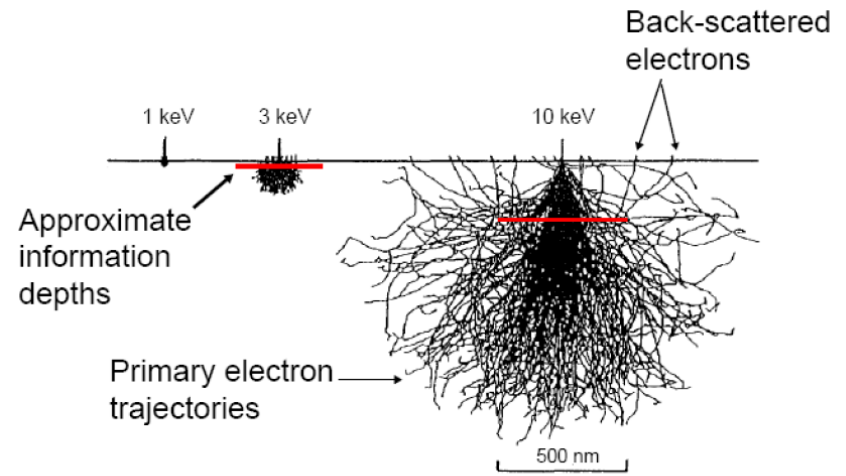
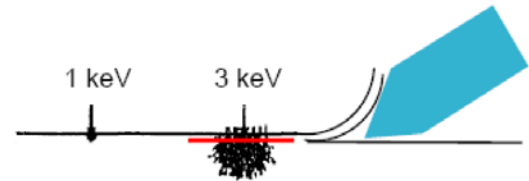
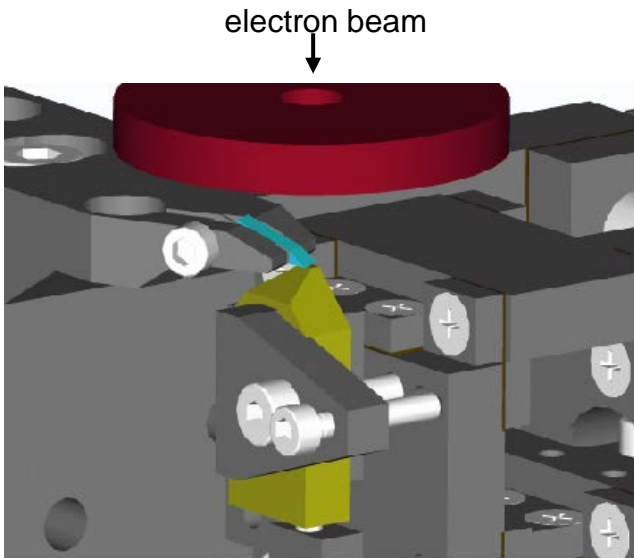


Fig. 6. Variation in the backscattering yield coefficient η for silicon, nickel and platinum as a function of the incident energy. Data taken from references in Joy (1995b).



Modified from Joy & Joy (1996)



Leighton, S. B. (1981). SEM images of block faces, cut by a miniature microtome within the SEM - a technical note. Scan Electron Microsc, (Pt 2): 73-6.
 Denk W, Horstmann H (2004) Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol 2(11): e329.

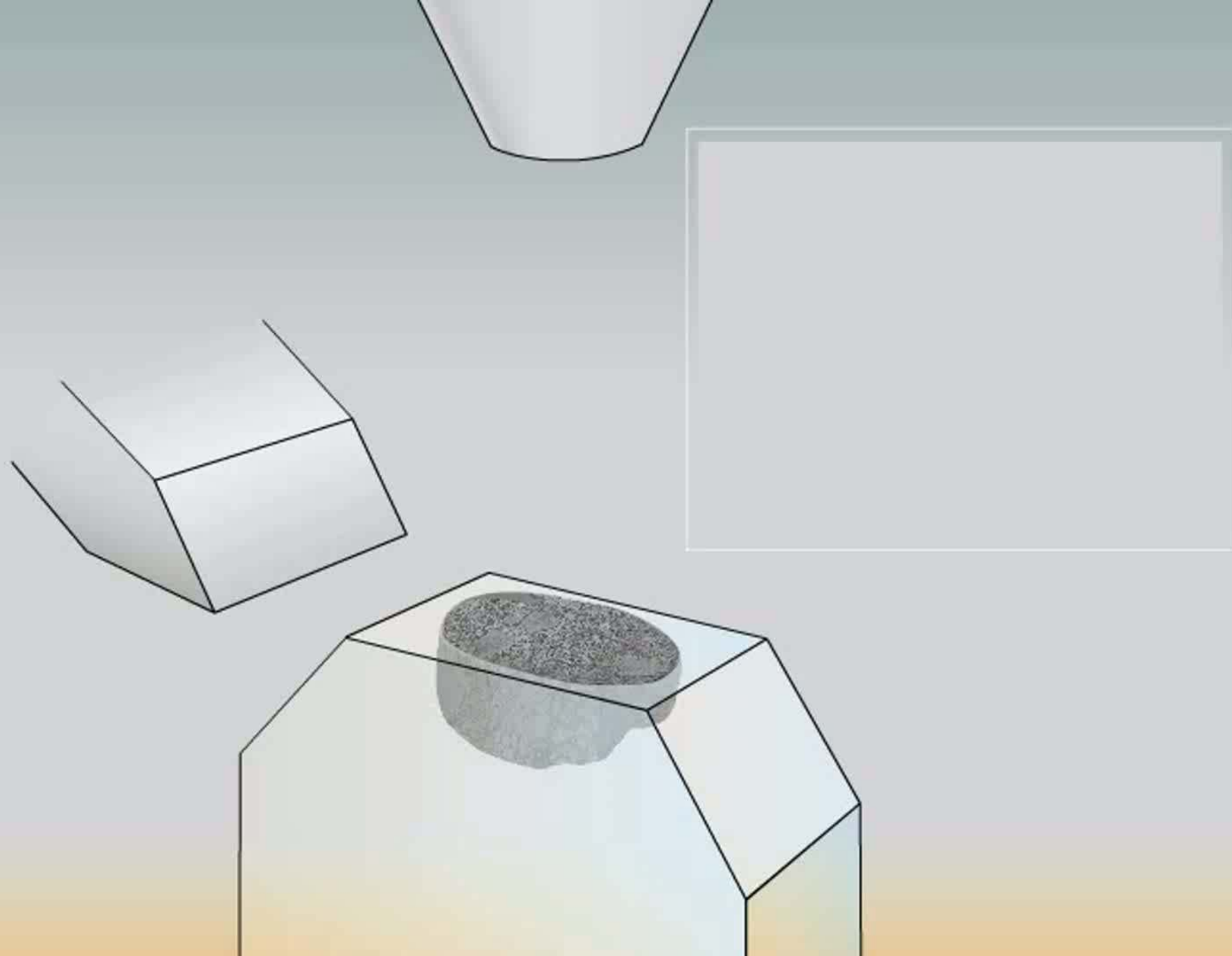
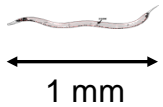


Illustration: Julia Kuhl

What SBEM volume is currently feasible?

C. elegans



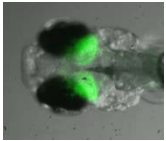
10^{-3} mm^3
 10^2 neurons

Retina



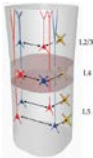
10^{-2} mm^3
 10^4 neurons

Fish brain

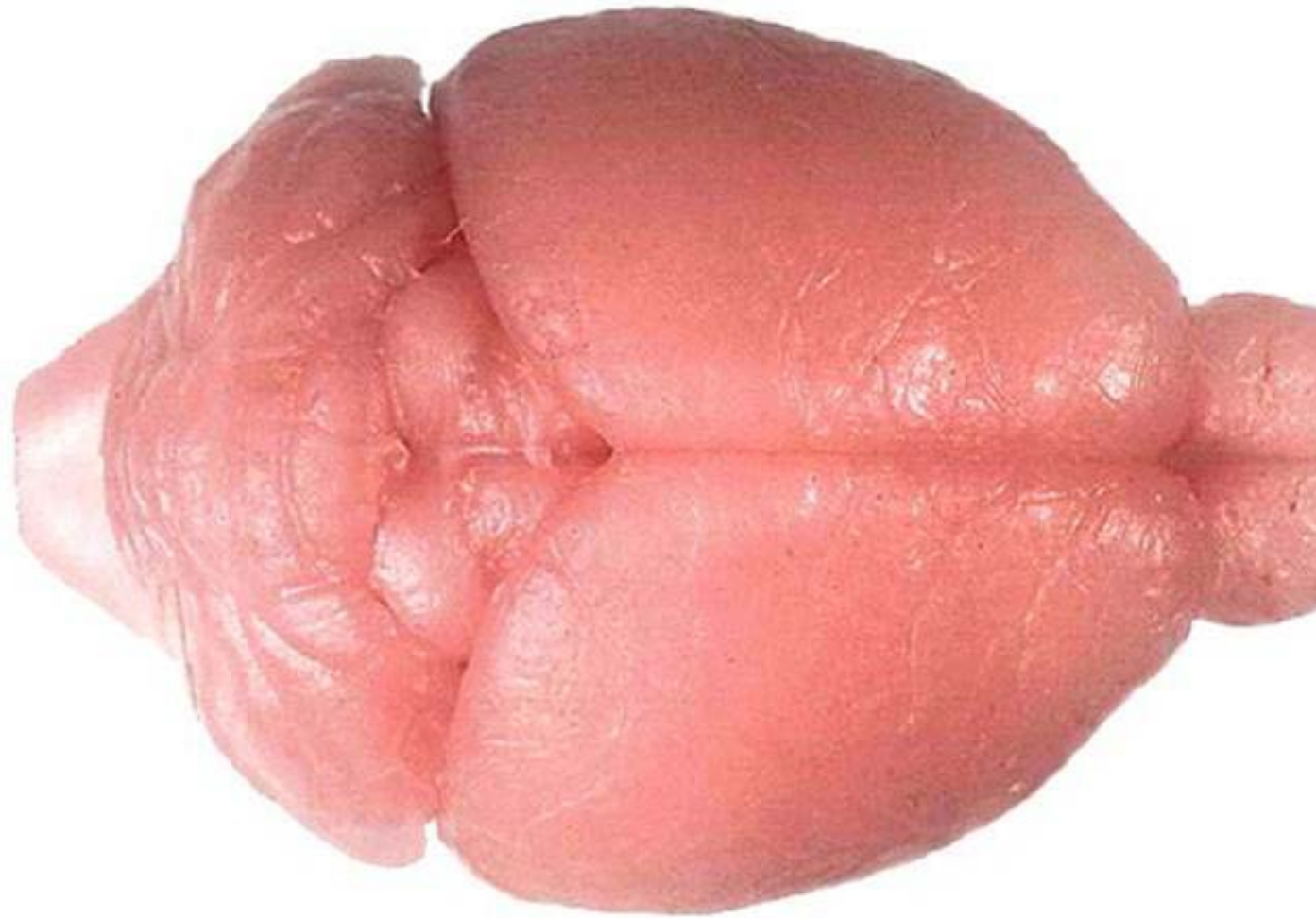


10^{-1} mm^3
 10^5 neurons

Mouse barrel column



10^{-2} mm^3
 10^4 neurons

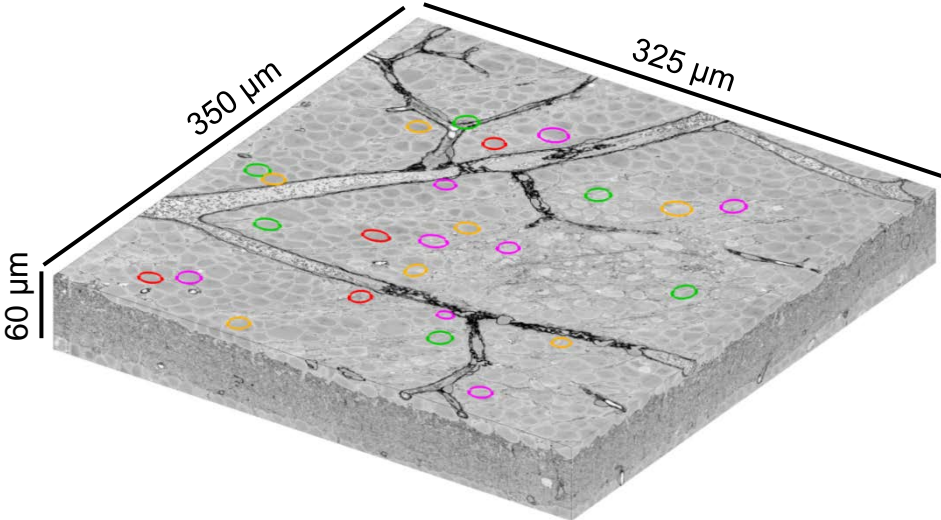


Mouse brain

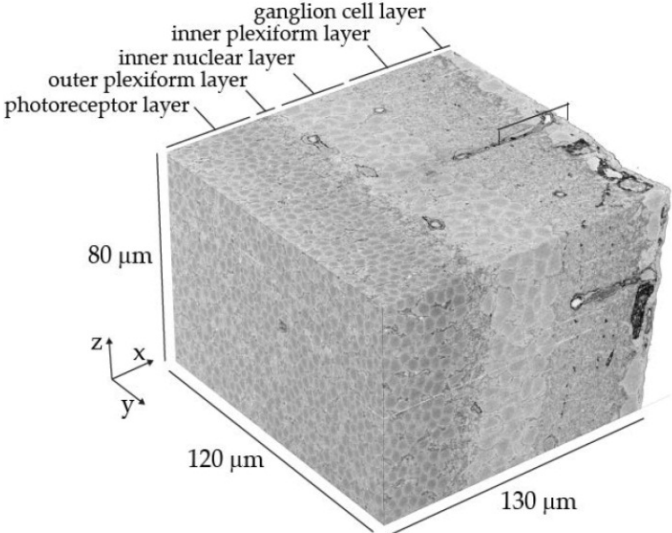
10^3 mm^3
 10^8 neurons

Neuronal circuit analysis of SBEM mouse retina data / are connectomes useful?

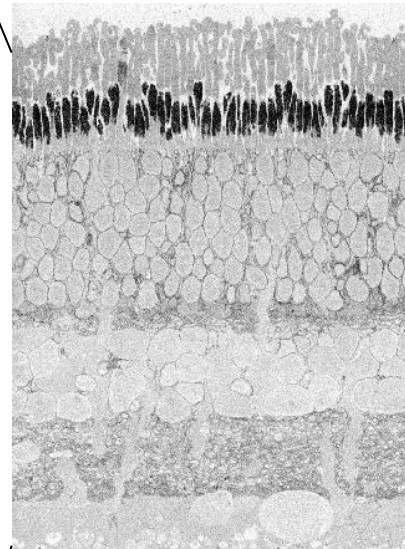
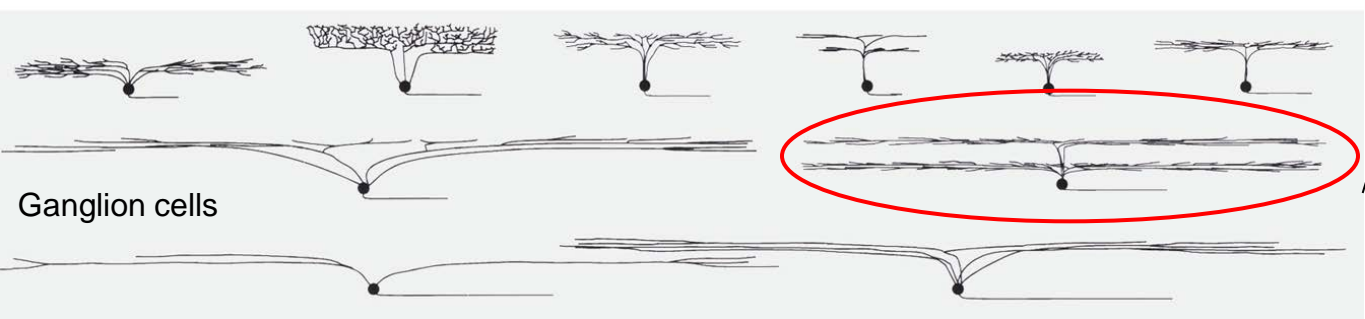
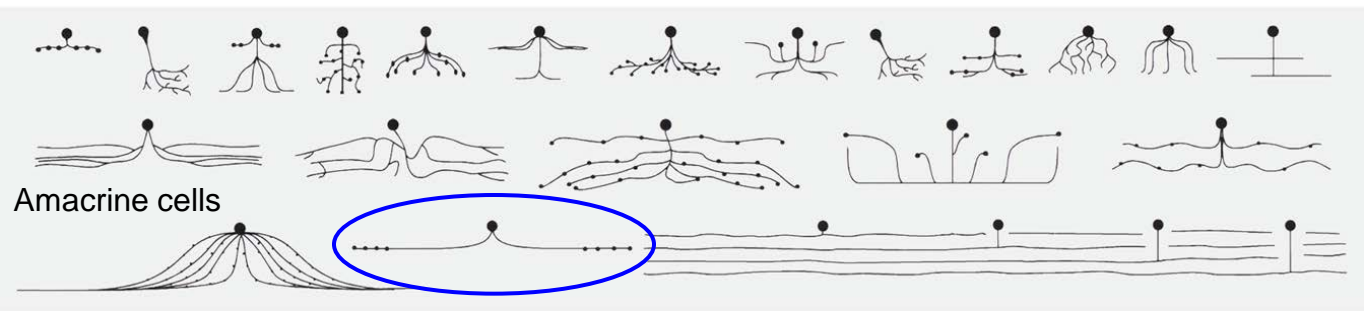
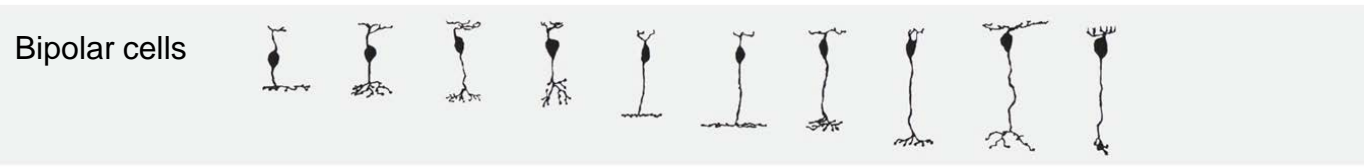
Sparse, targeted reconstruction following 2-photon calcium imaging



Dense, comprehensive reconstruction



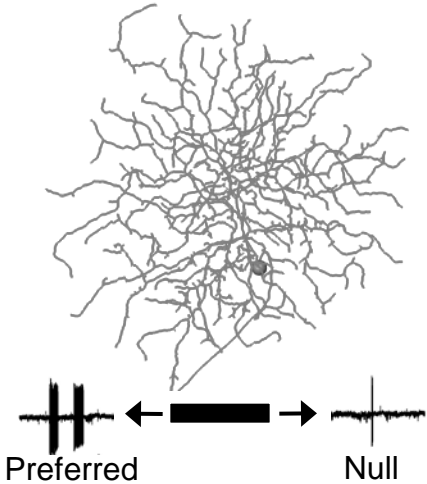
The mammalian retina



Masland (2001)

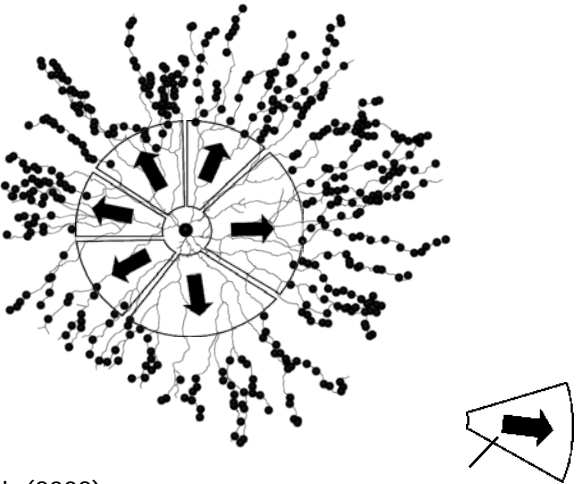
Sparse reconstruction of direction-selectivity circuitry

DS ganglion cell



Linear direction-of-motion detector

Starburst amacrine cell

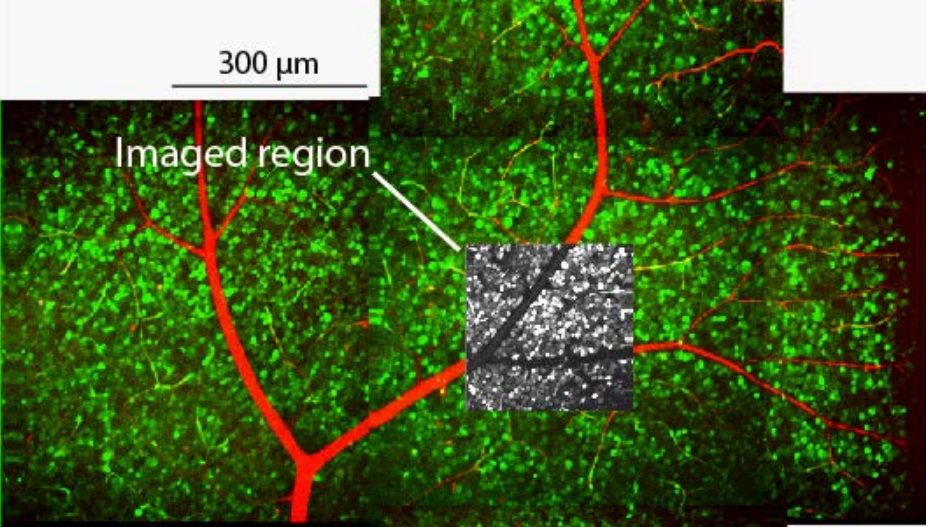
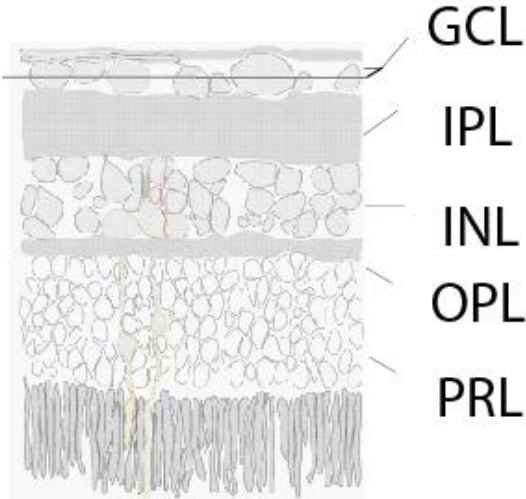
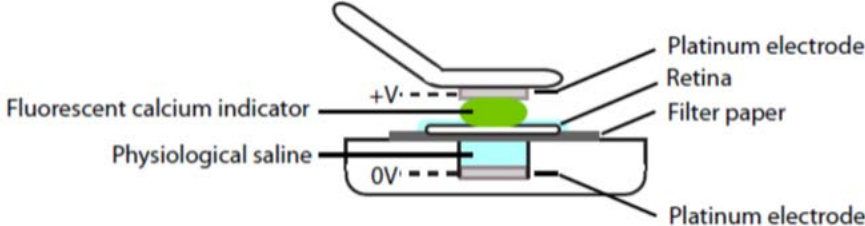


Radial direction-of-motion detector

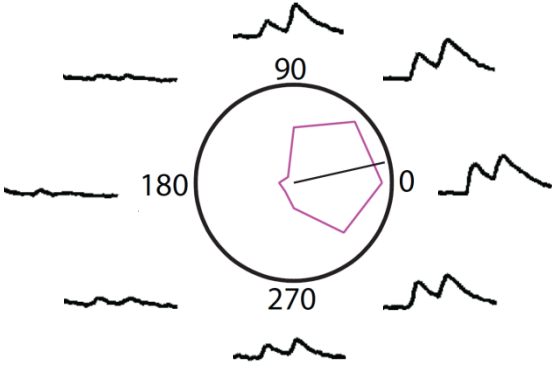
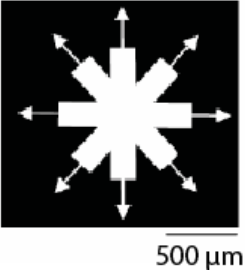
Euler et al. (2002)

Motion direction triggering
GABA and/or ACh release from SAC

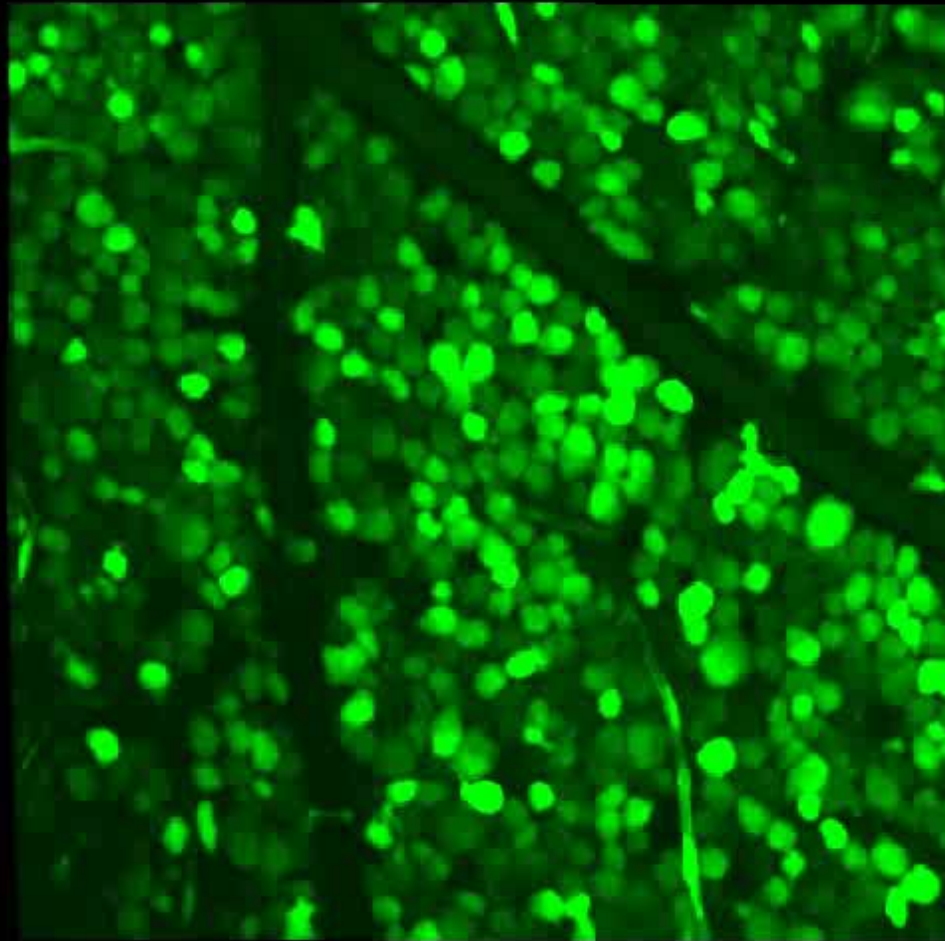
Bulk electroporation and 2P imaging of GCs



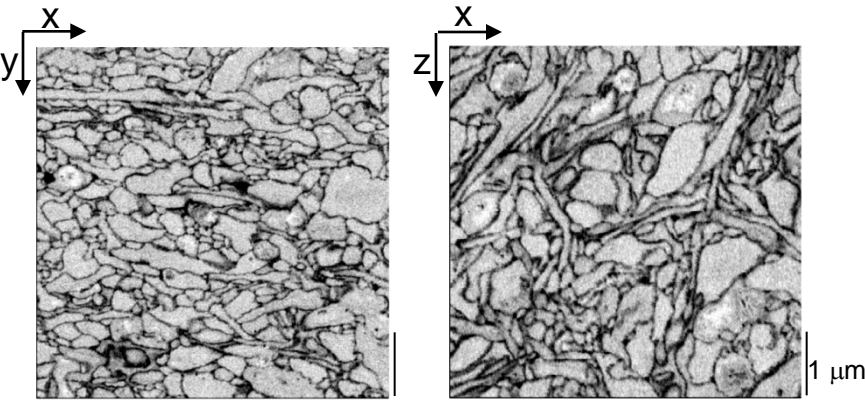
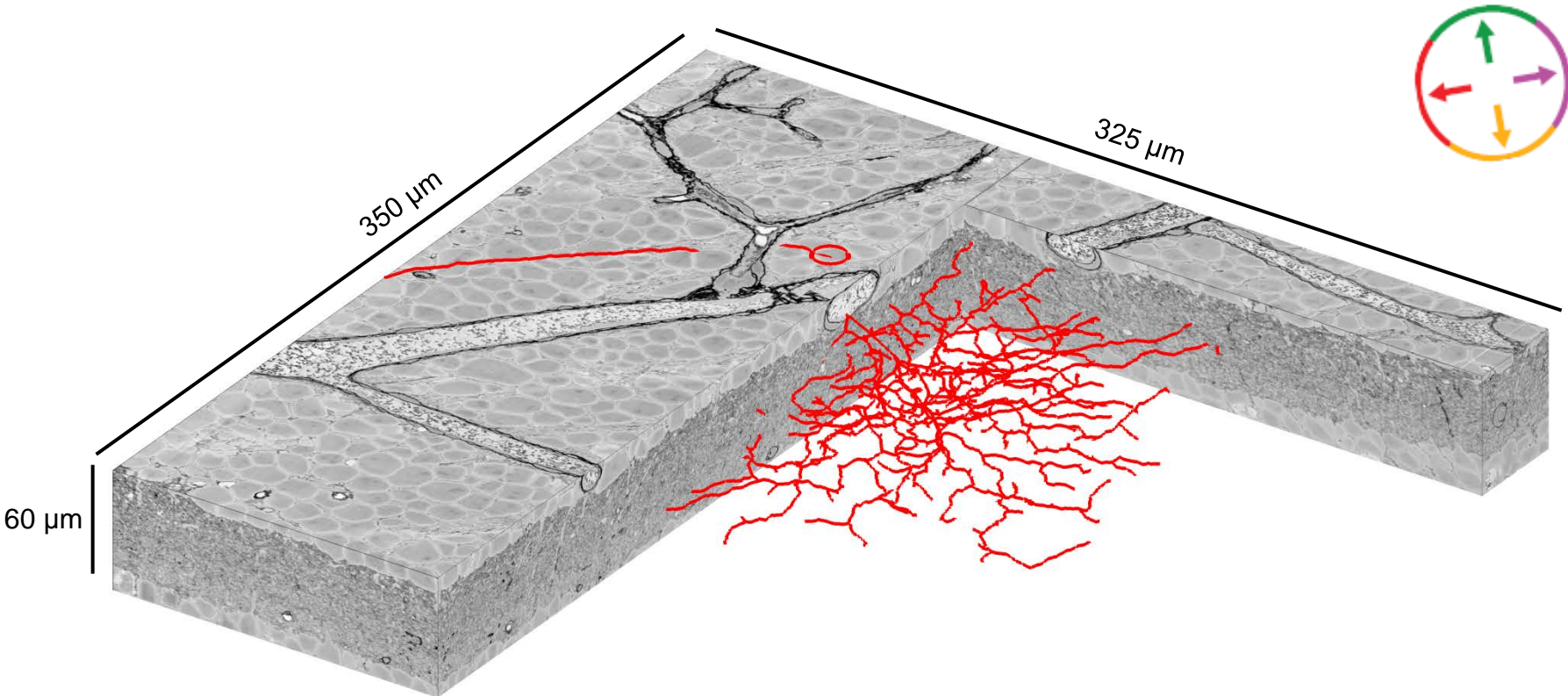
Oregon Green BAPTA-1
Sulforhodamine 101



Briggman KL & Euler T. (2011) Bulk electroporation and population calcium imaging of the adult mammalian retina. J Neurophys



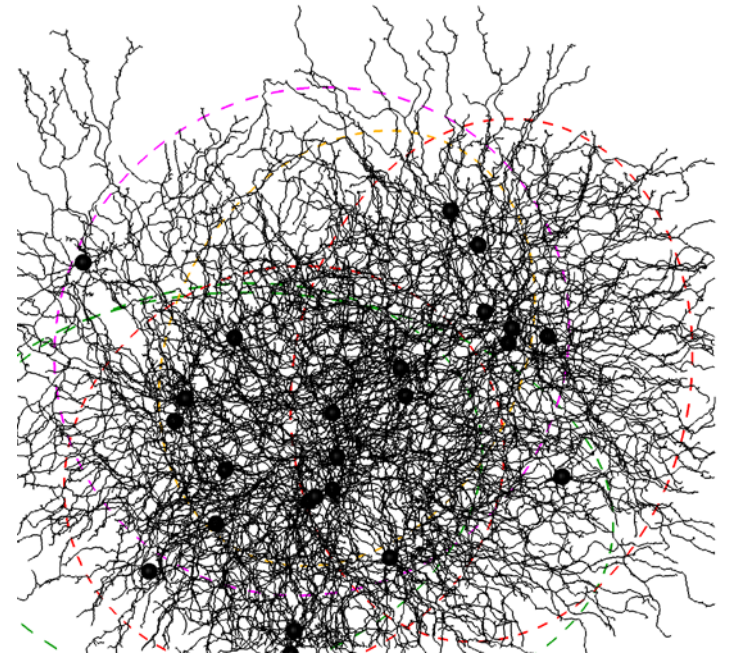
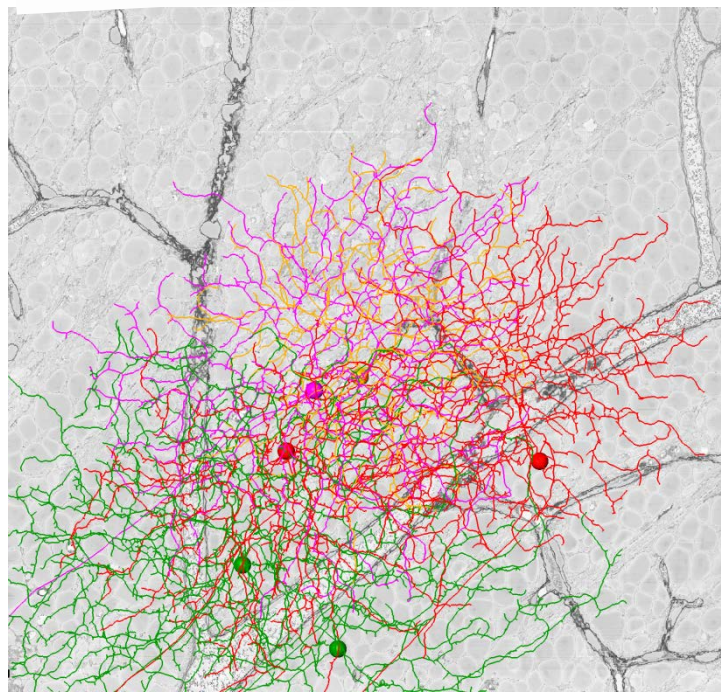
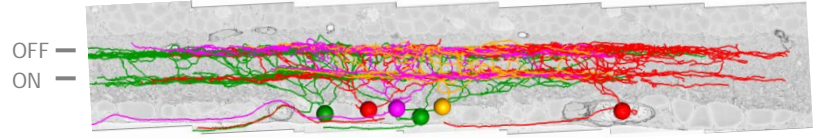
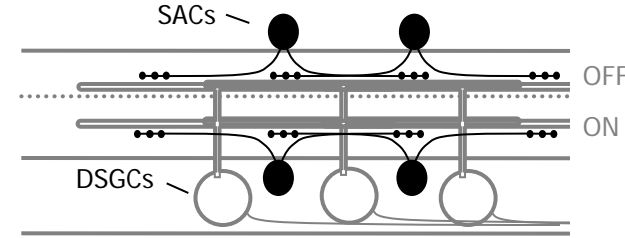
Structural identification of DSGCs



16.5 x 16.5 x 25 nm voxels
~ 1 TByte
~ 13,000 25nm sections
0.5 MHz scan rate
~ 7 week acquisition

Briggman KL, Helmstaedter M, Denk W. (2011) [Wiring specificity in the direction-selectivity circuit of the retina](#). Nature

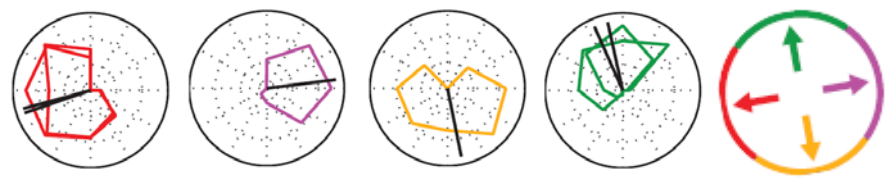
EM reconstruction of DSGCs and SACs



n = 6 DSGCs

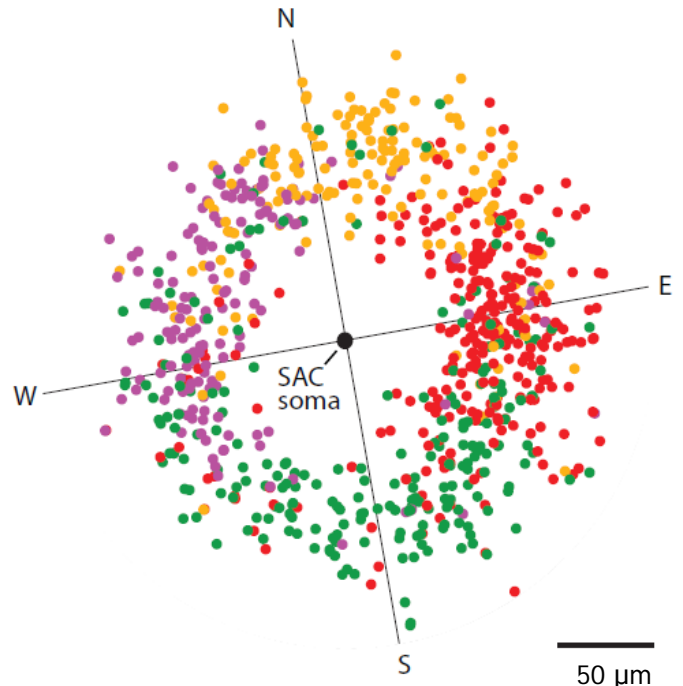
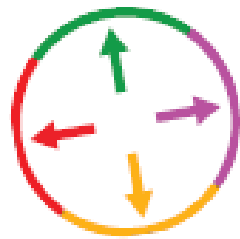
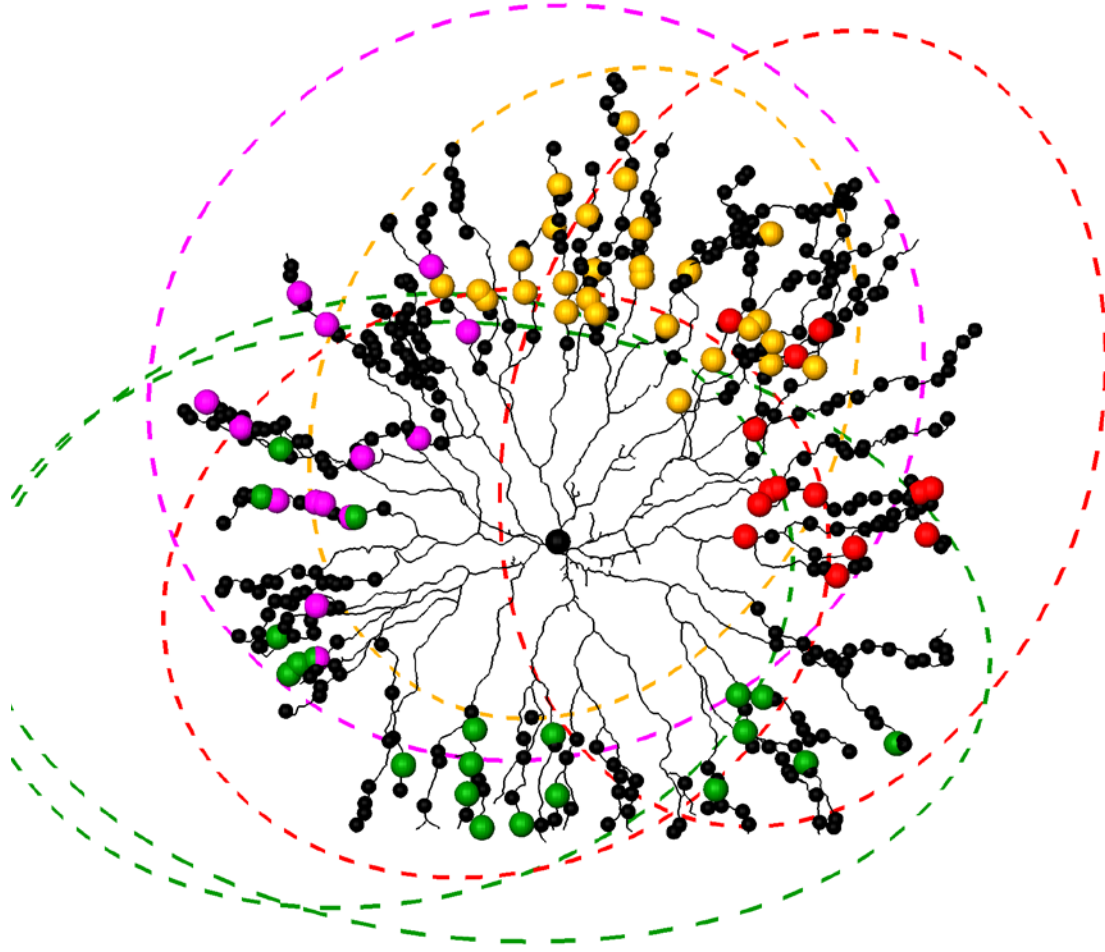
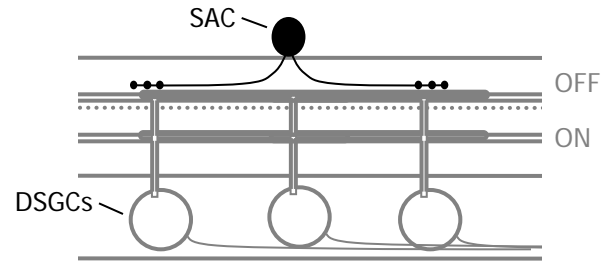
100 μm

n = 24 SACs (10%)



Briggman KL, Helmstaedter M, Denk W. (2011) [Wiring specificity in the direction-selectivity circuit of the retina](#). Nature

Specificity of SAC output synapses

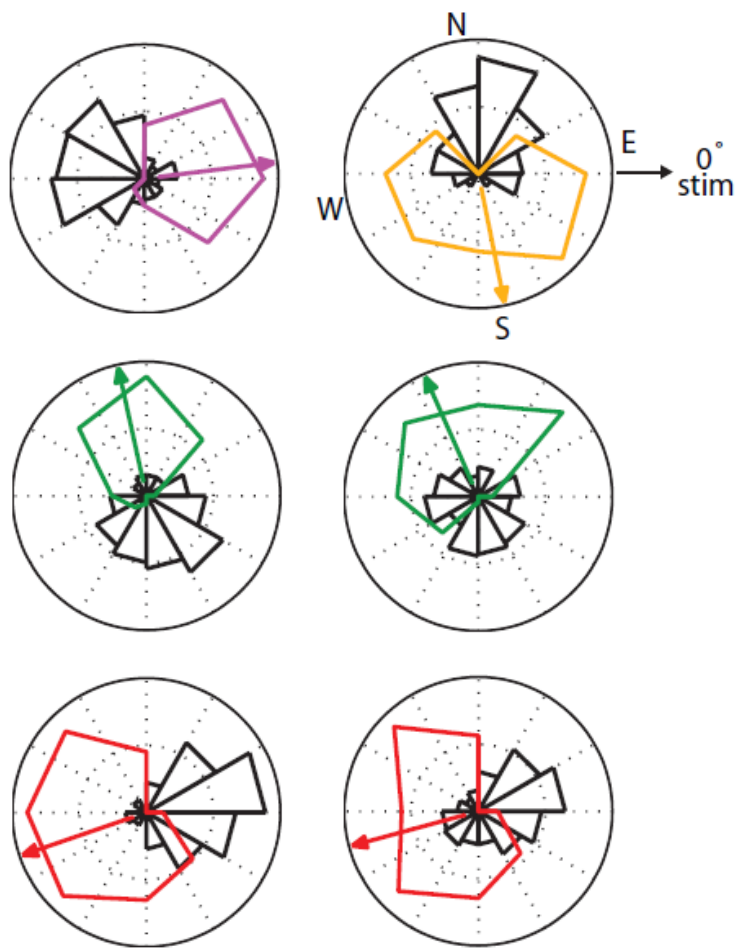
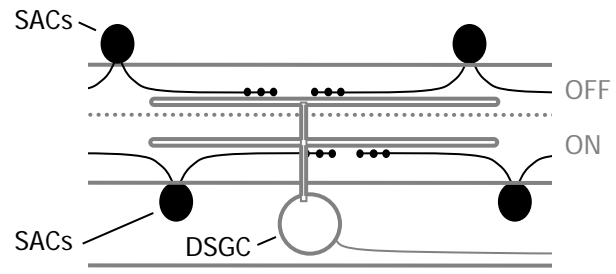
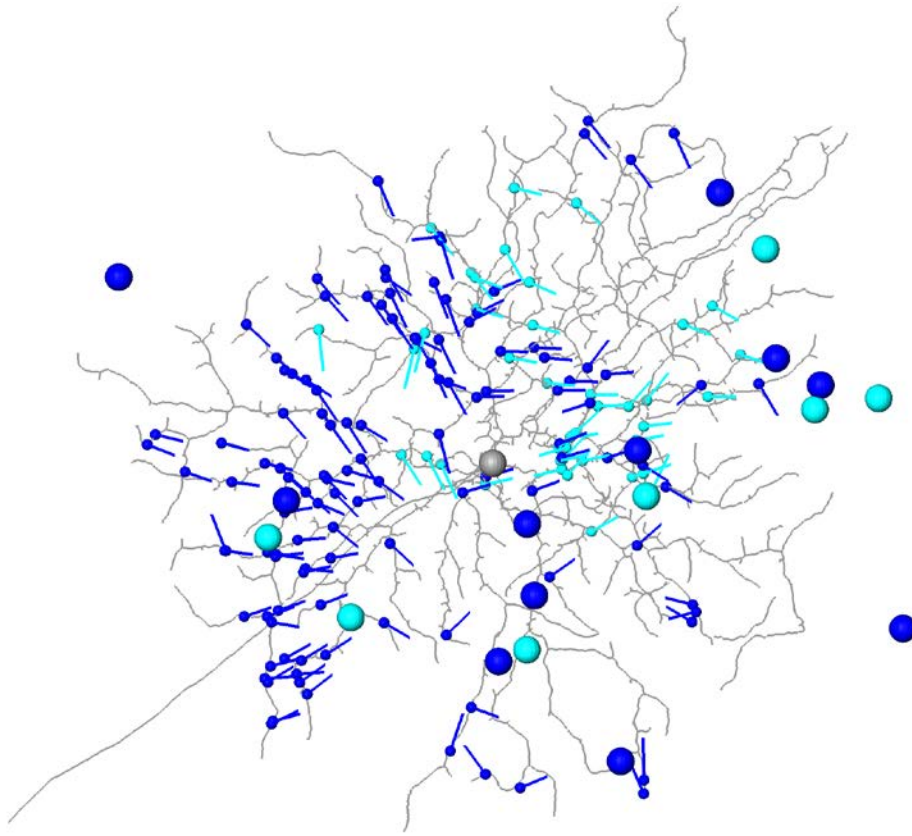


n = 831 synapses, 24 SACs

Briggman KL, Helmstaedter M, Denk W. (2011) [Wiring specificity in the direction-selectivity circuit of the retina.](#) Nature

Directional input to DSGCs

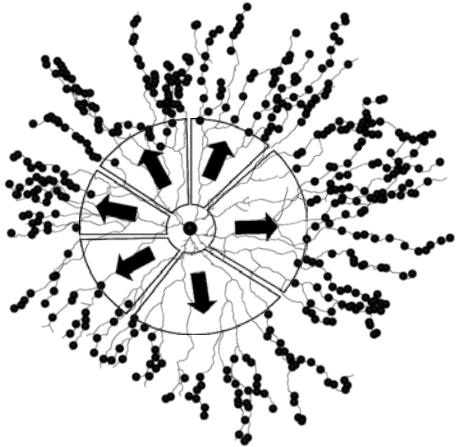
- DSGC
- On, Off SAC soma
- On, Off SAC synapse



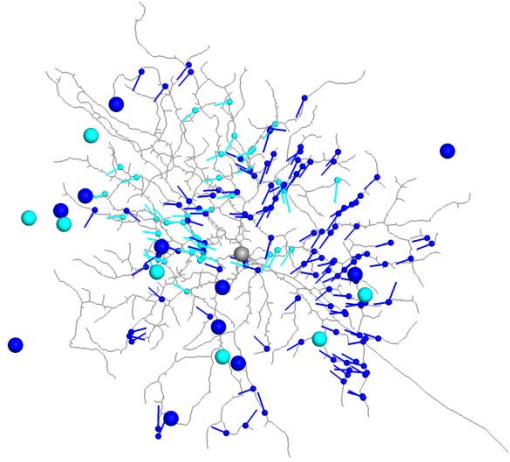
Briggman KL, Helmstaedter M, Denk W. (2011) Wiring specificity in the direction-selectivity circuit of the retina. Nature

Sparse reconstruction of direction-selectivity circuitry

Starburst amacrine cell

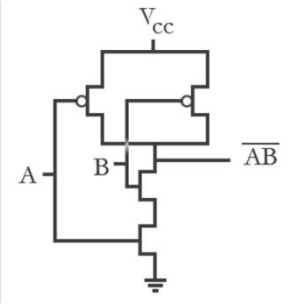
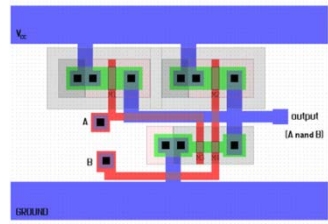
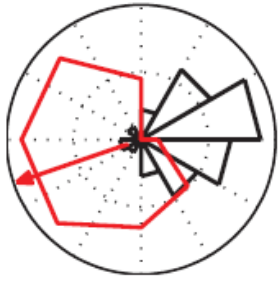
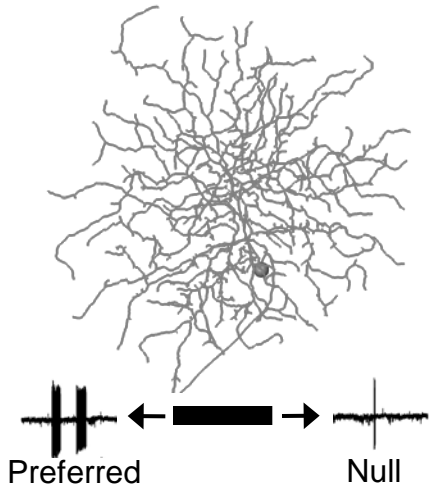


+

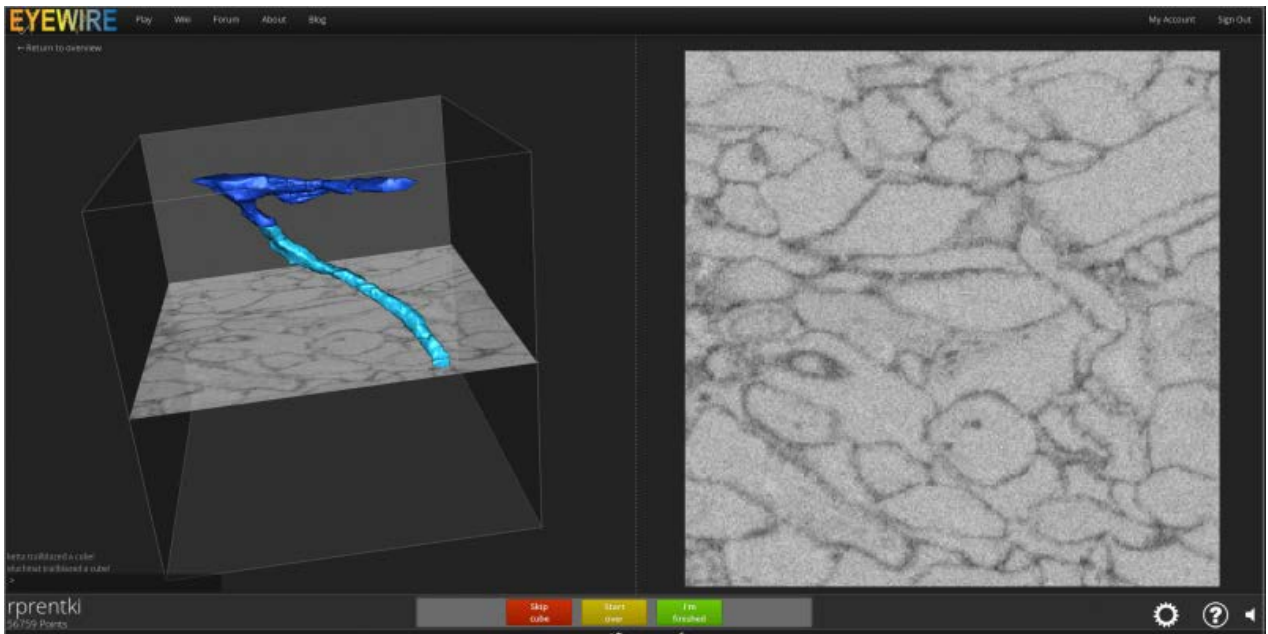
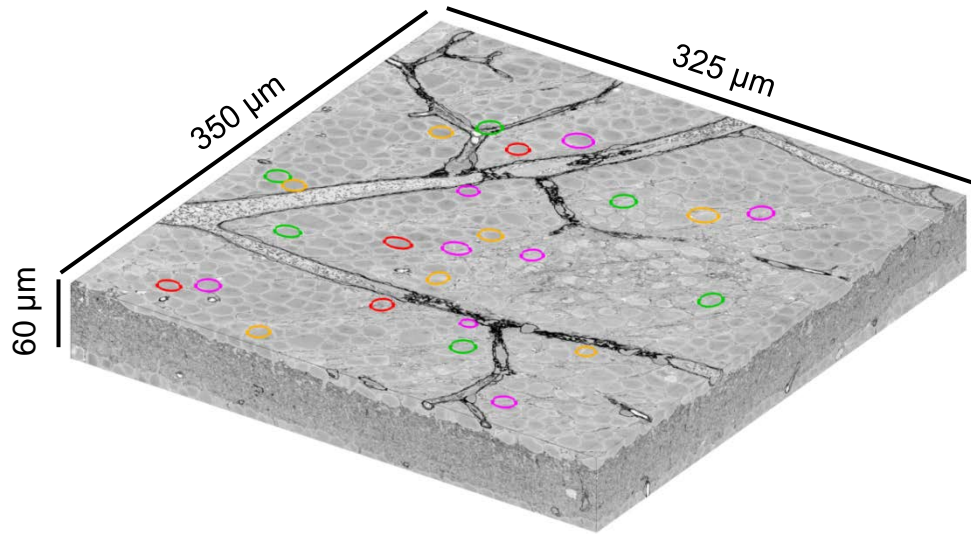


=

DS ganglion cell

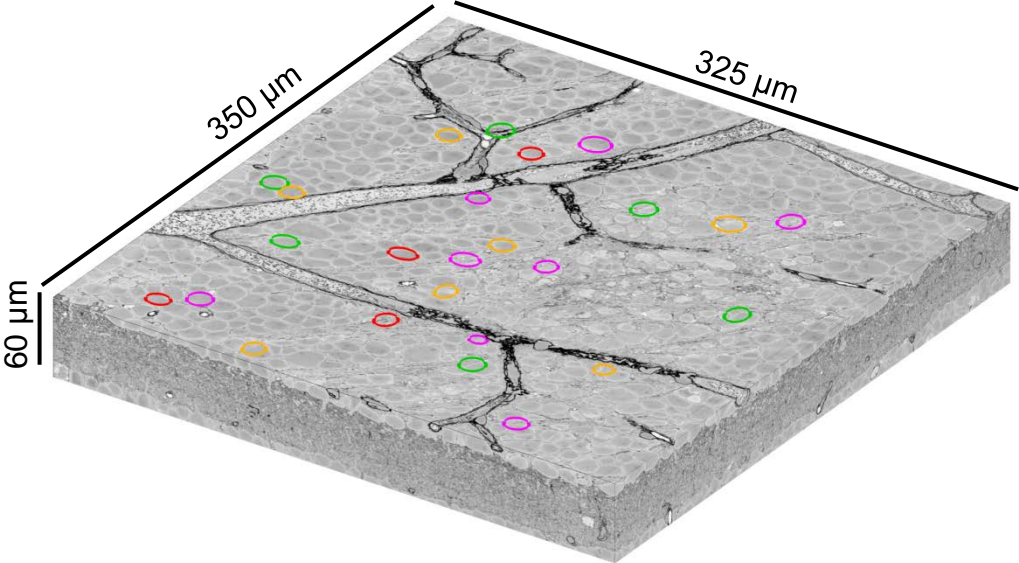


Data is difficult to collect, but ultimately yields rich datasets

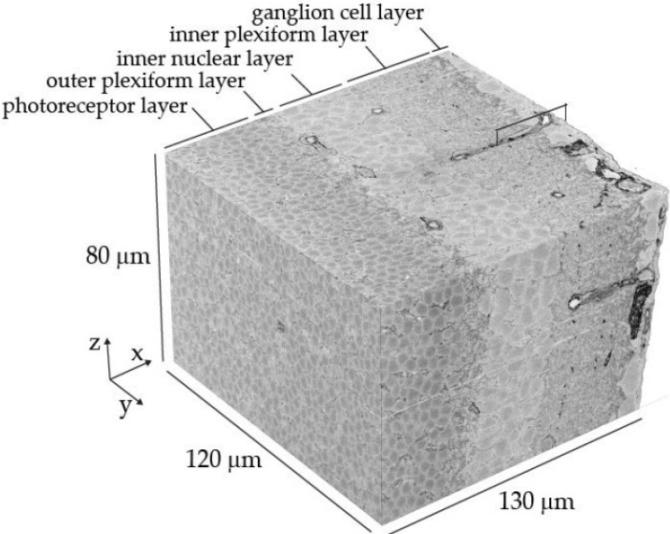


Neuronal circuit analysis of SBEM mouse retina data / are connectomes useful?

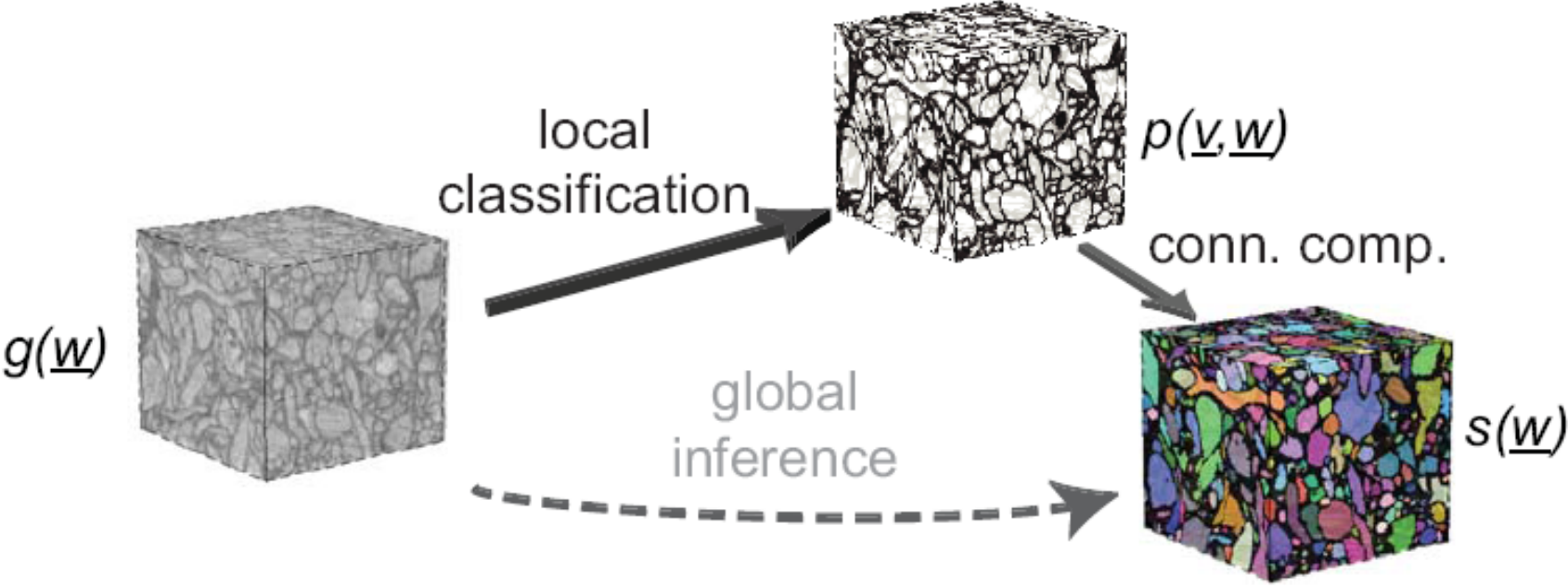
Sparse, targeted reconstruction following 2-photon calcium imaging



Dense, comprehensive reconstruction

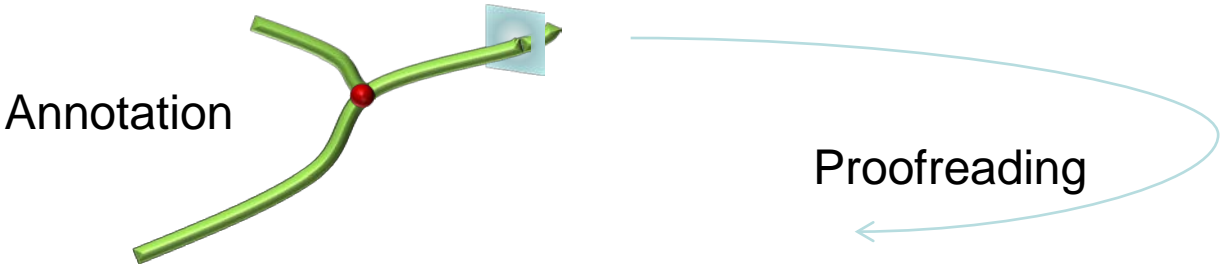


Automated image analysis?

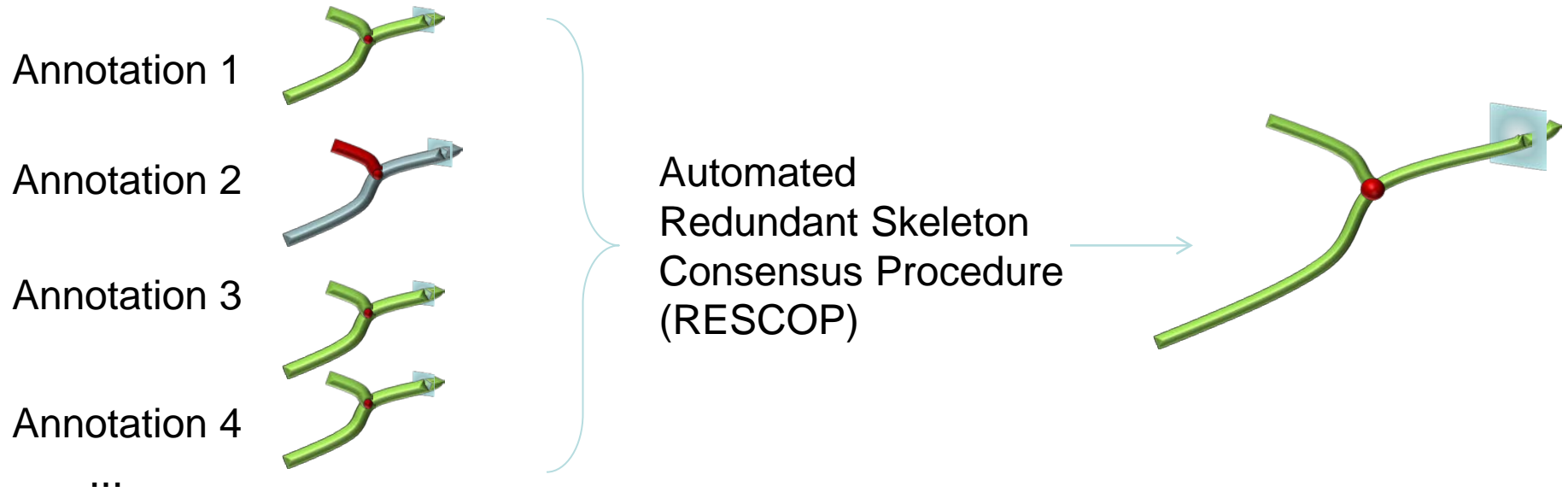


Forward-only annotation: efficiently scalable

(Iterated) proofreading

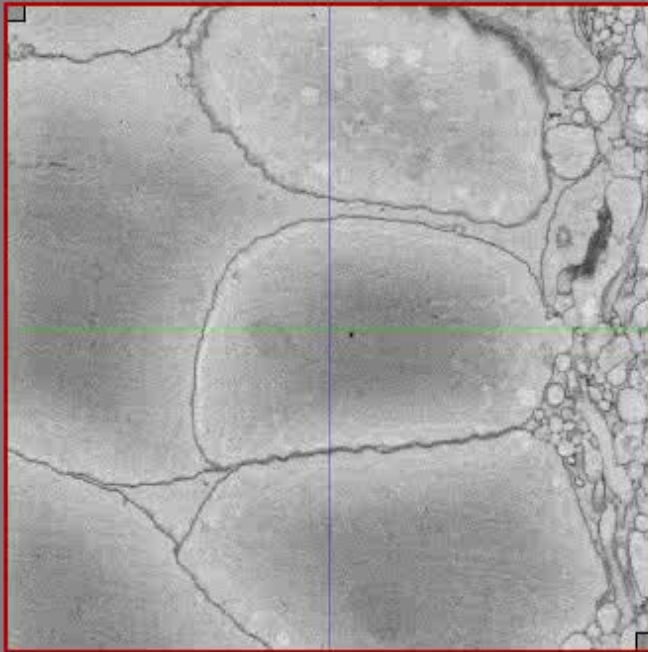


Forward-only annotation + consensus

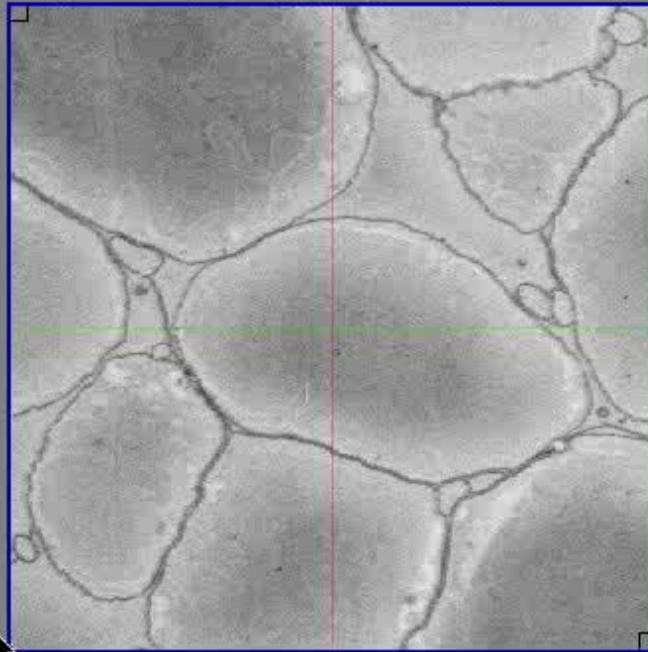


Helmstaedter M, Briggman KL, Denk W. (2011) High accuracy neurite tracing for high-throughput neuroanatomy. Nature Neurosci

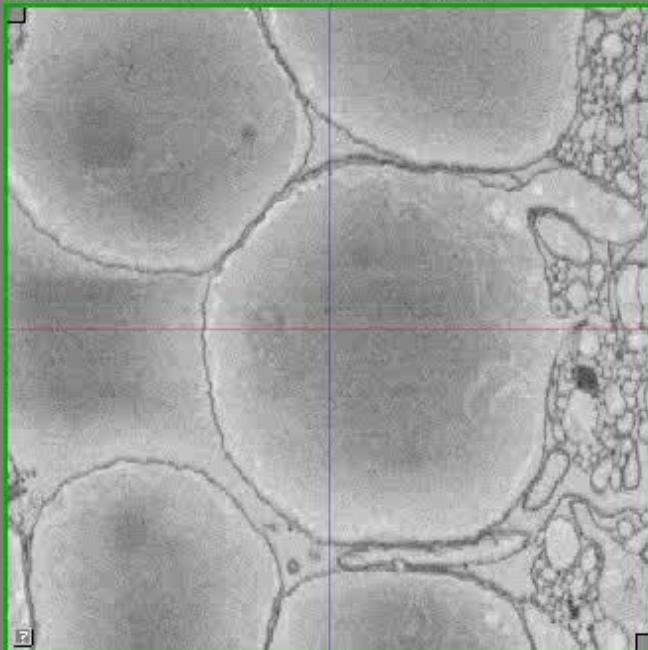
Viewport XY x length: 14.080[um] y length: 14.080[um]



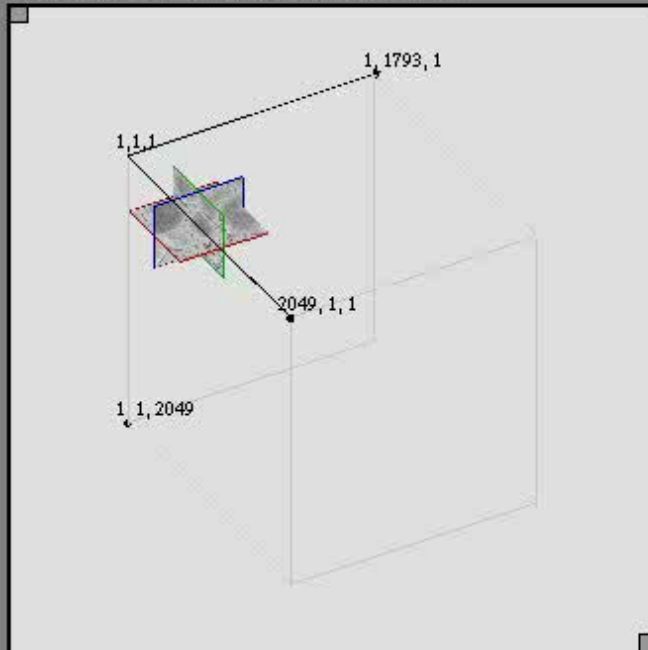
Viewport YZ x length: 14.040[um] y length: 14.080[um]



Viewport XZ x length: 14.080[um] y length: 14.040[um]



Viewport Skeleton #trees:0 #nodes:0 #segments:0



Helmstaedter
Briggman
Denk (2011)
Nature
Neuroscience

KNOSSOS
programmed by
Jörgen Kornfeld,
Fabian Svava

knossostool.org



The mammalian retina

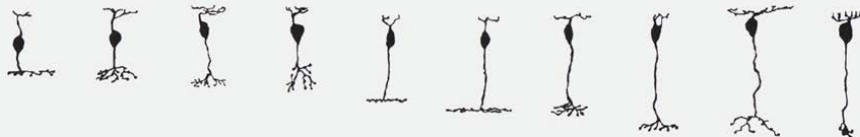
Photoreceptor cells



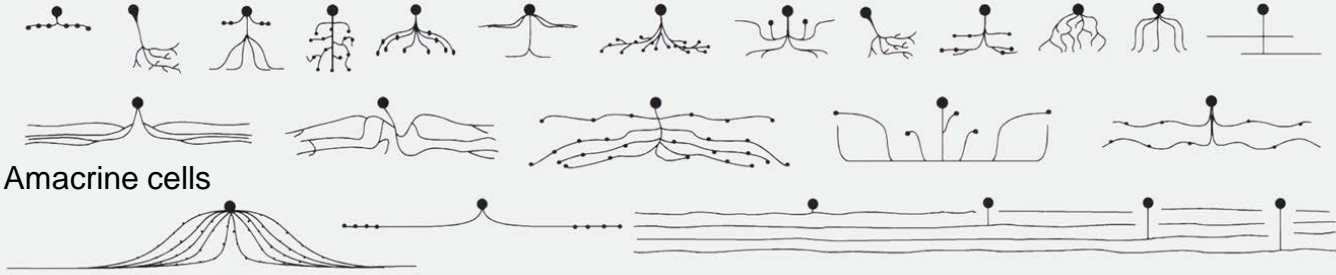
Horizontal cells



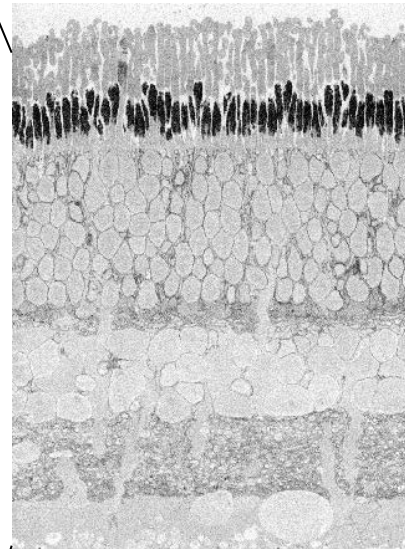
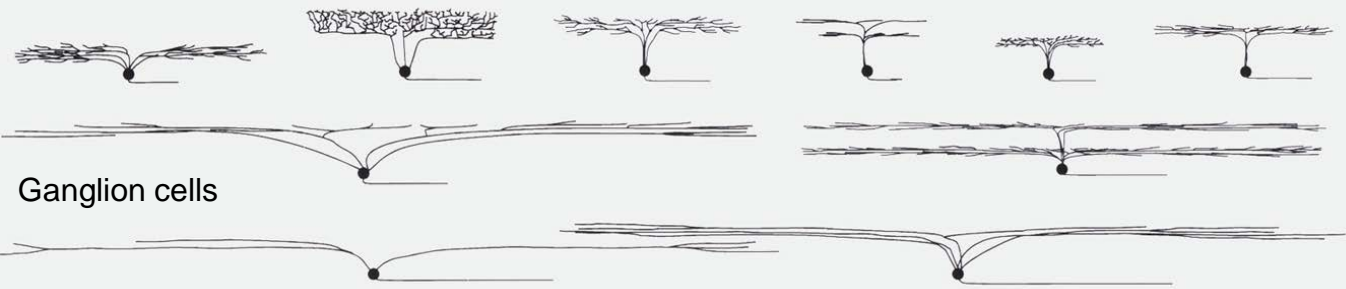
Bipolar cells

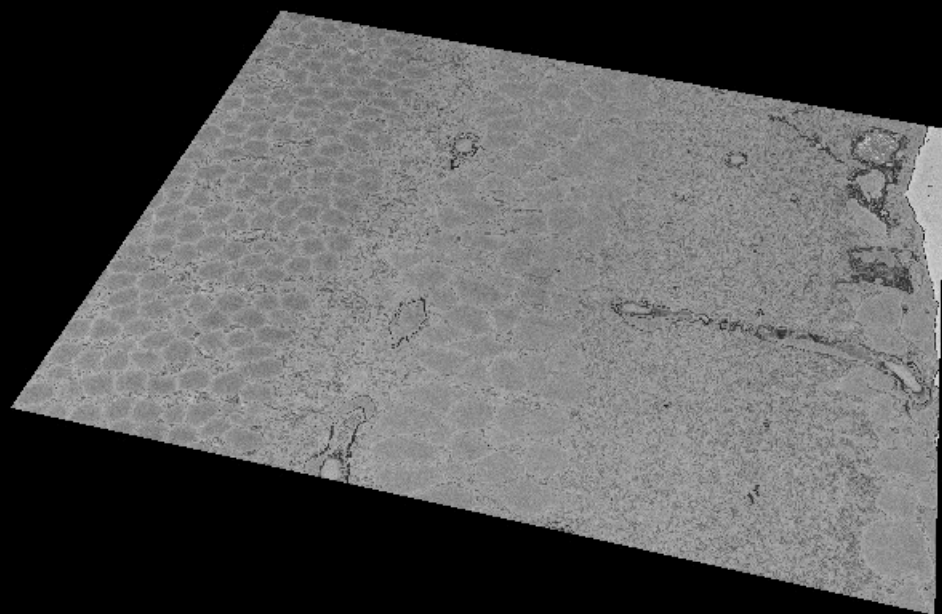


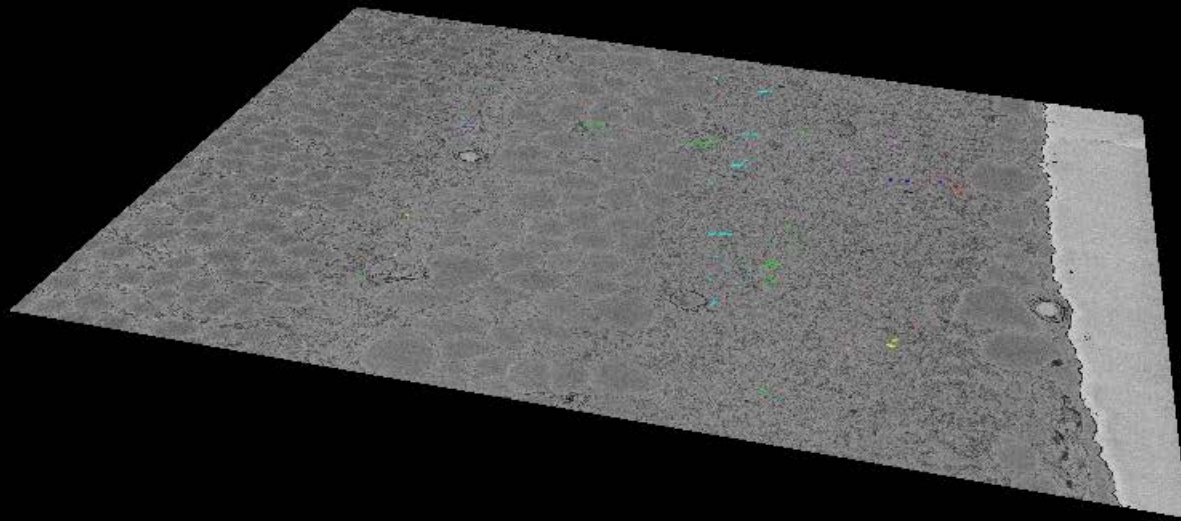
Amacrine cells



Ganglion cells



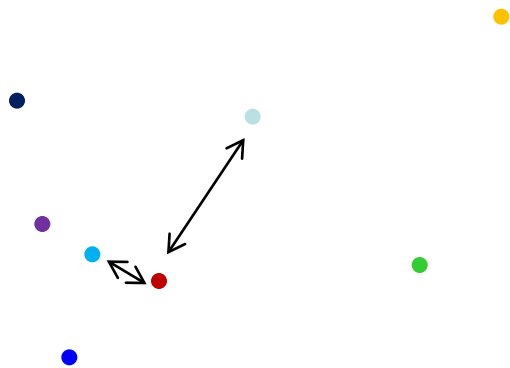




Skeleton annotation is insufficient for contact detection

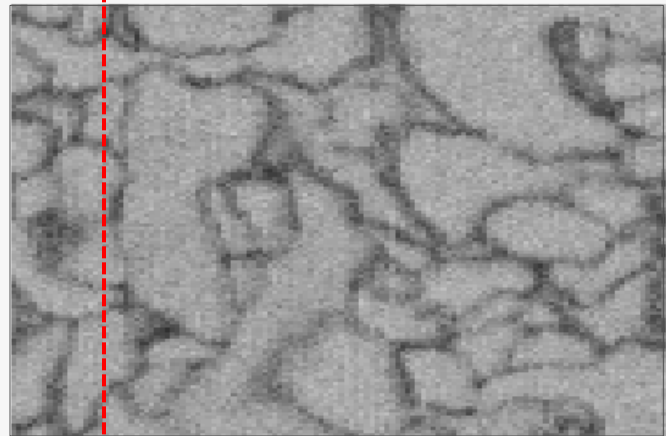
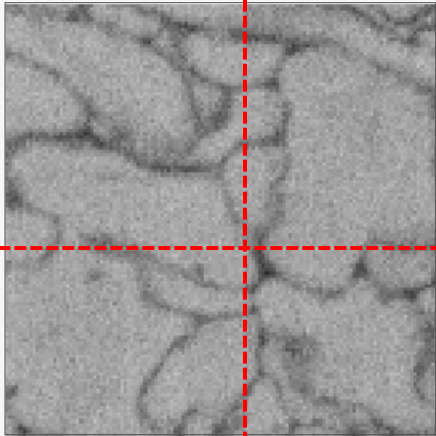


Skeleton annotation is insufficient for contact detection

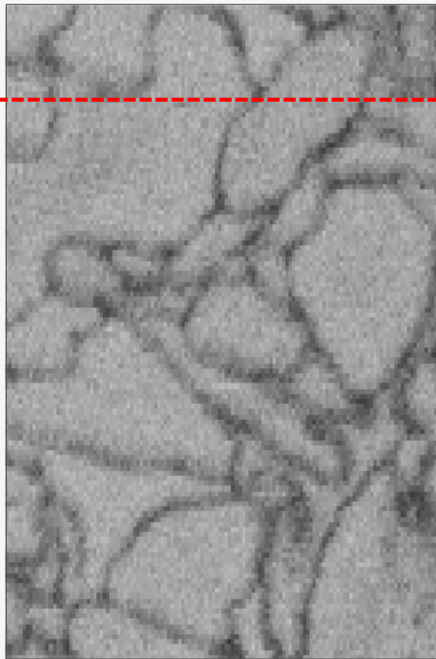


Skeleton annotation insufficient for contact detection:
“growing out” volumes required

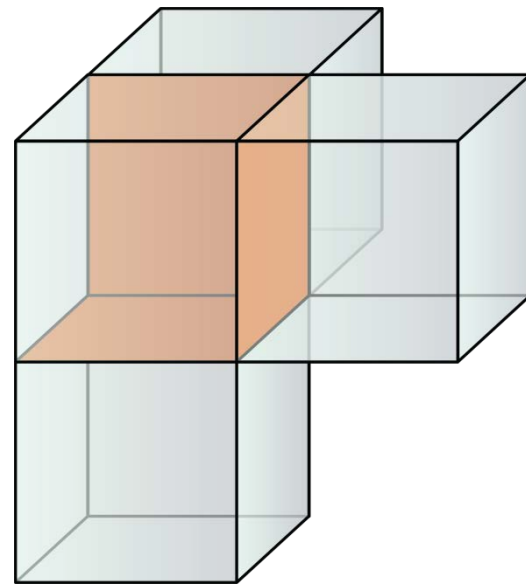
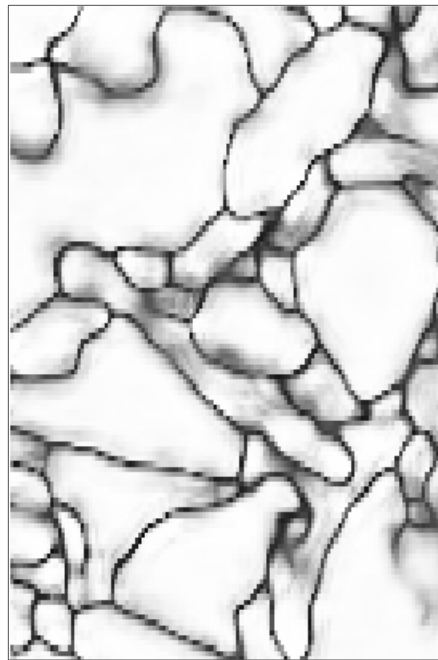
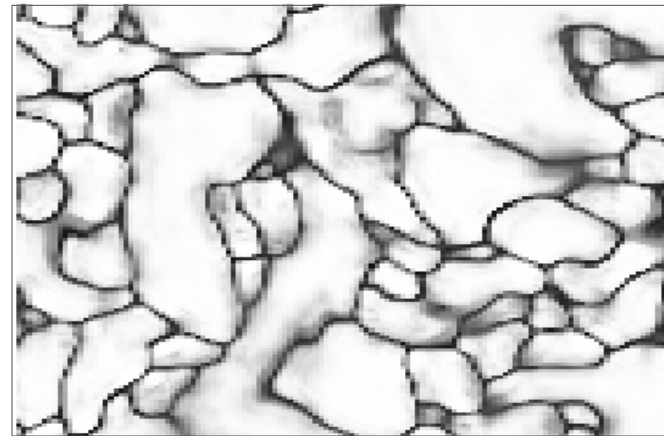
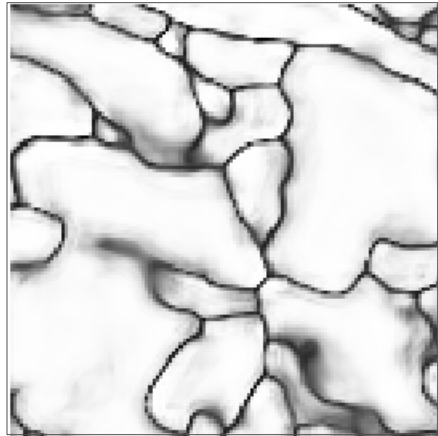




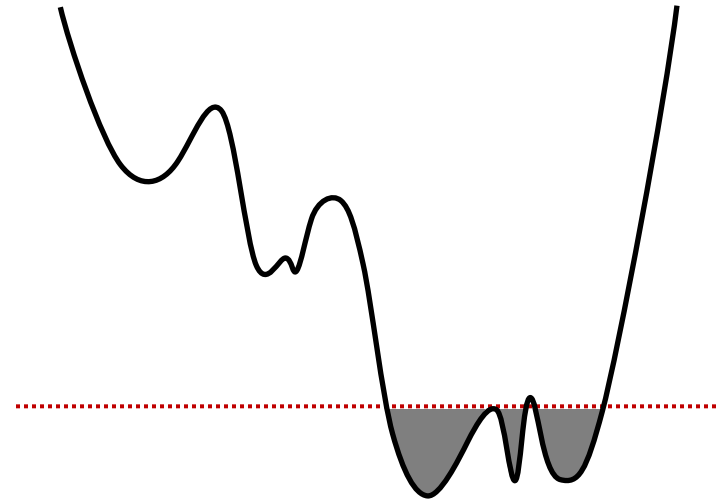
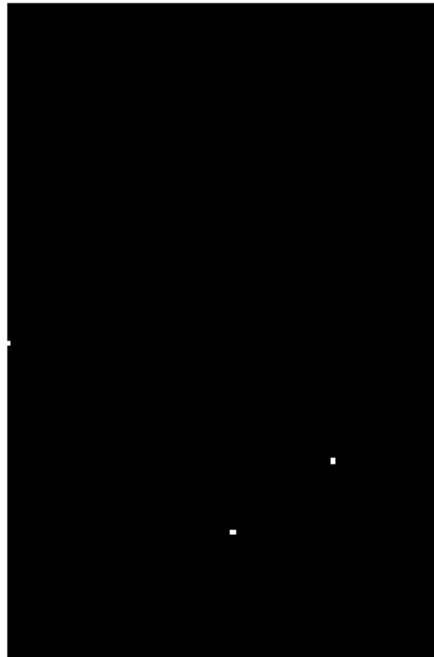
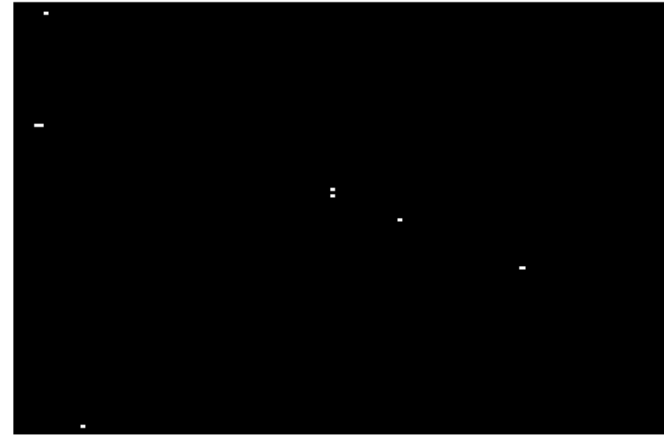
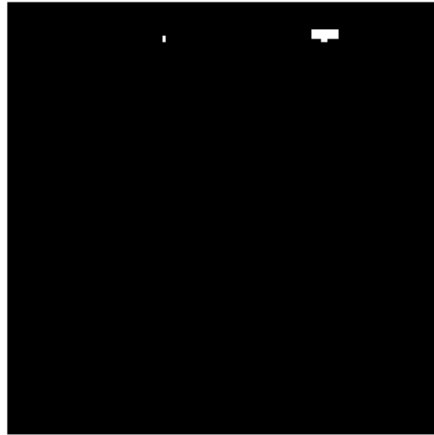
500 nm



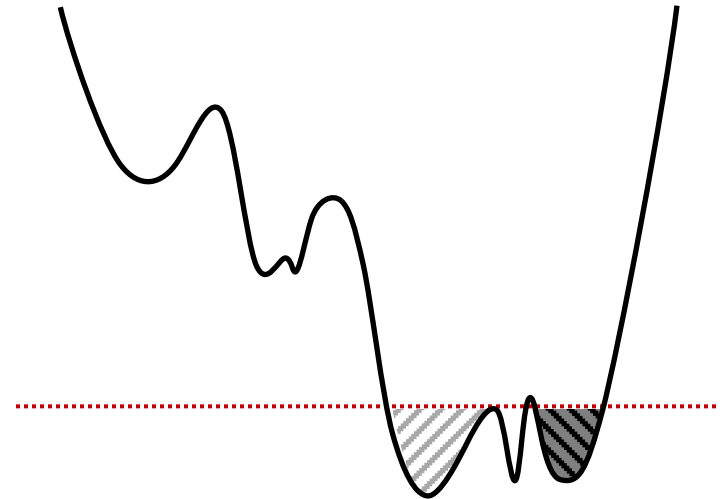
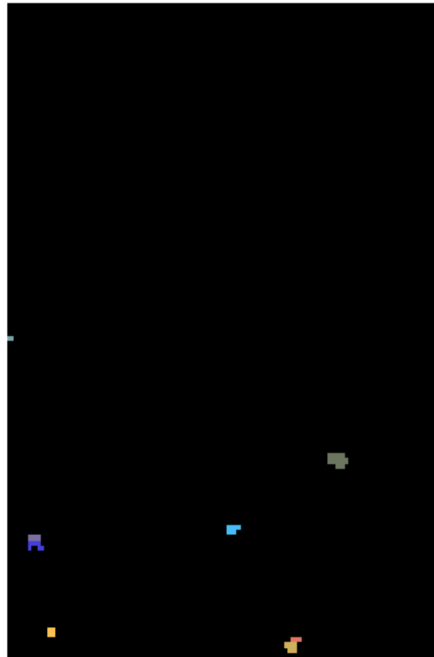
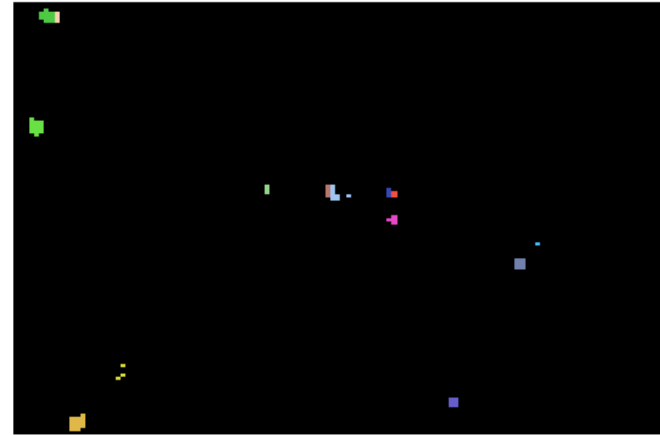
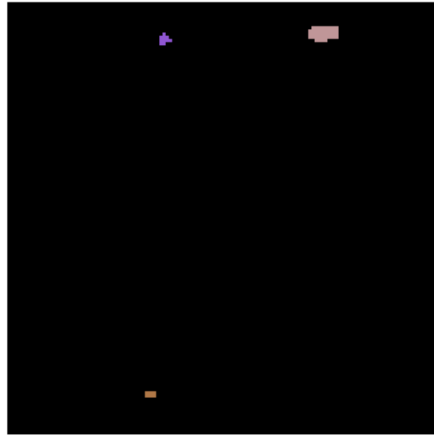
Convolutional-Neural-Network Voxel-Connectivity Classifier



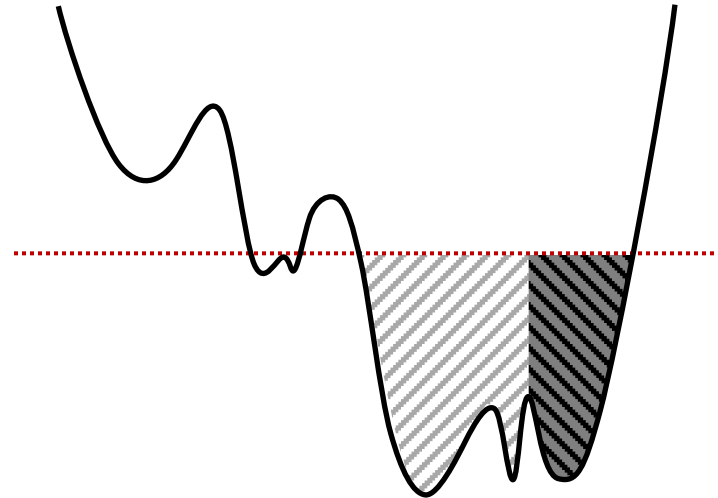
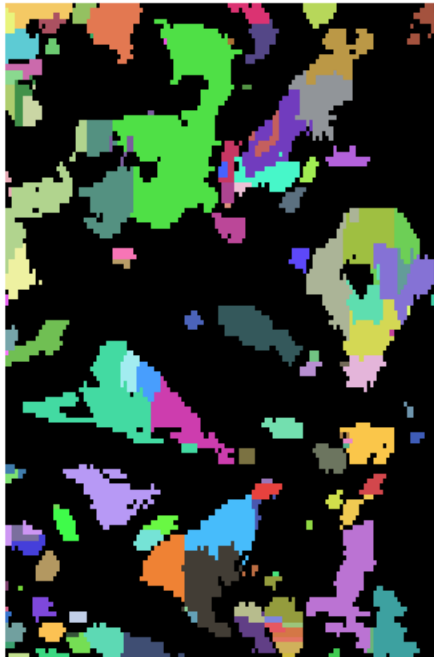
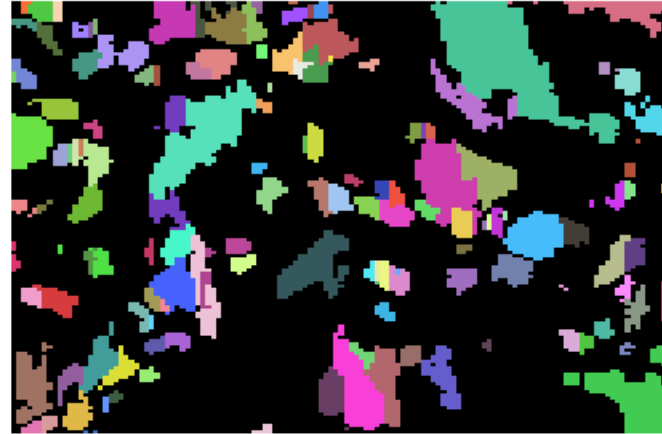
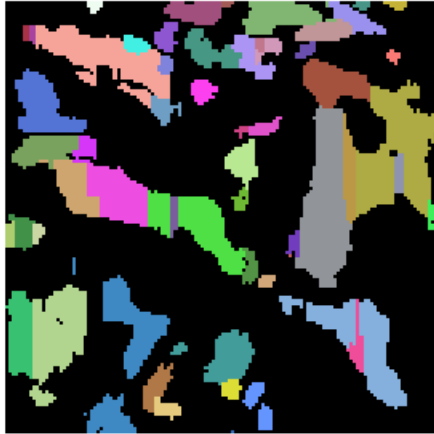
Initial seed generation at a high threshold



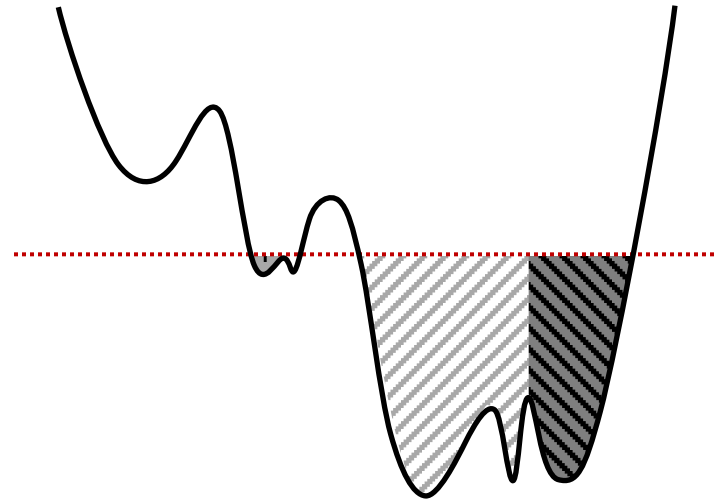
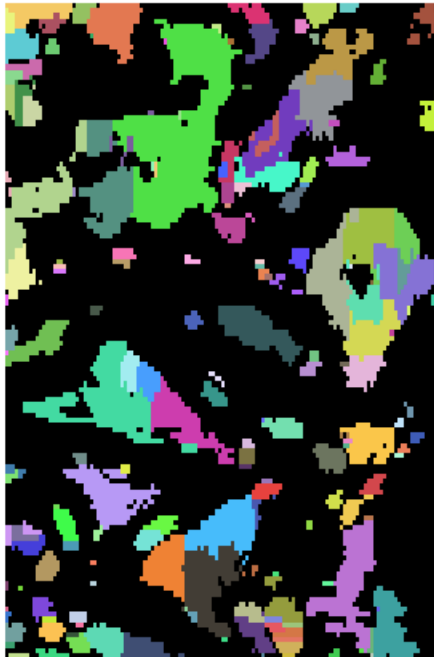
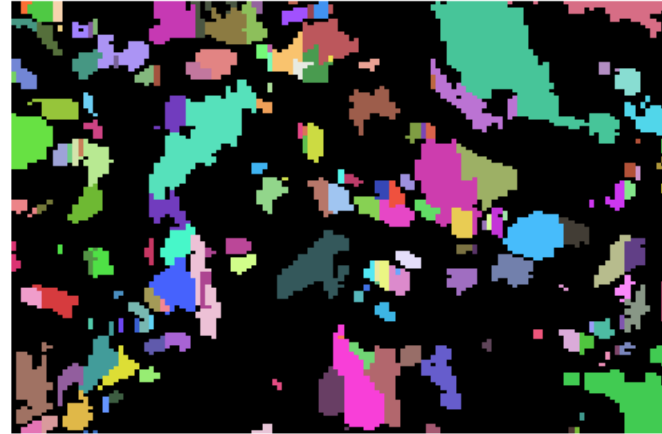
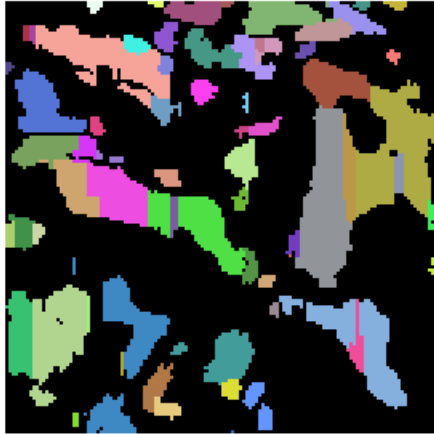
Initial seed generation at a high threshold



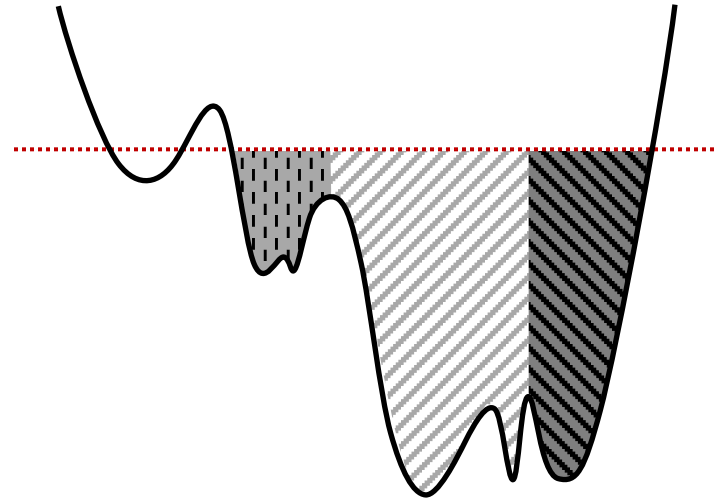
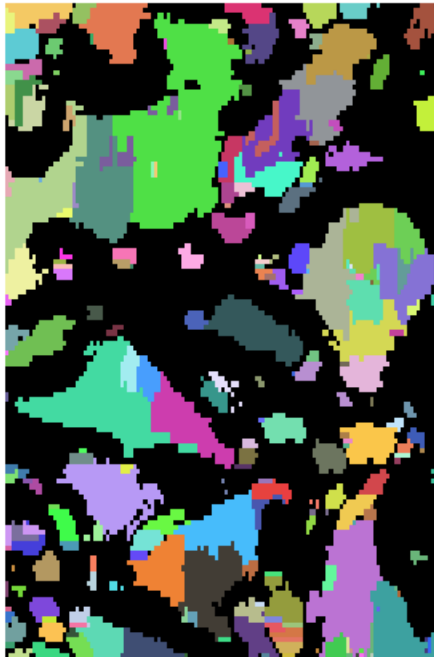
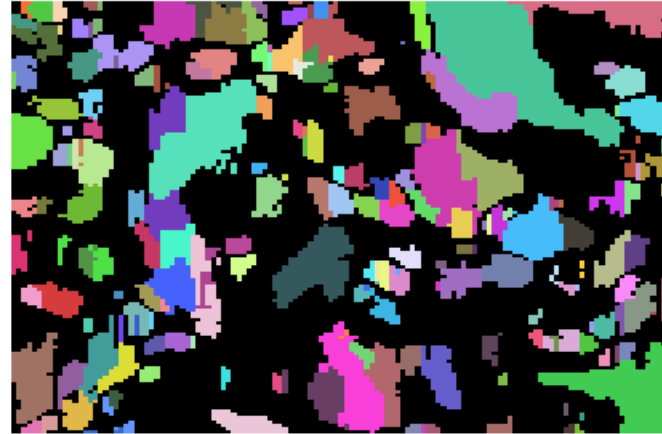
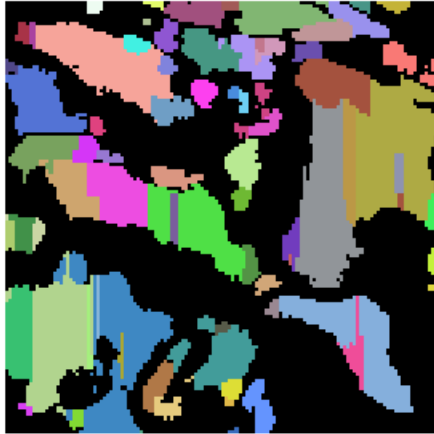
Growing objects



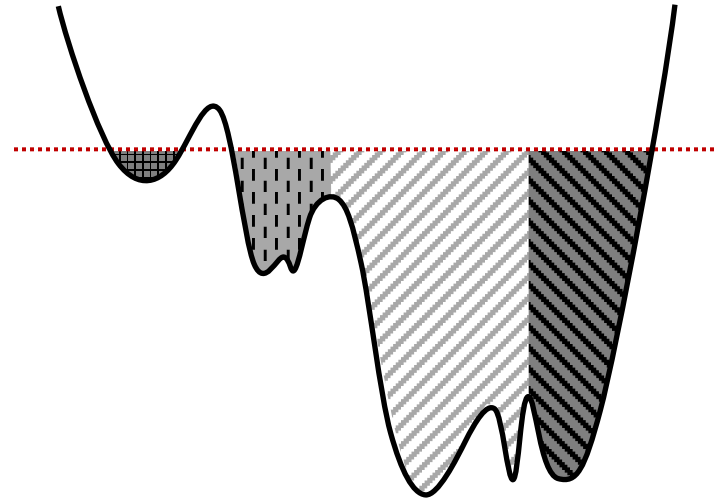
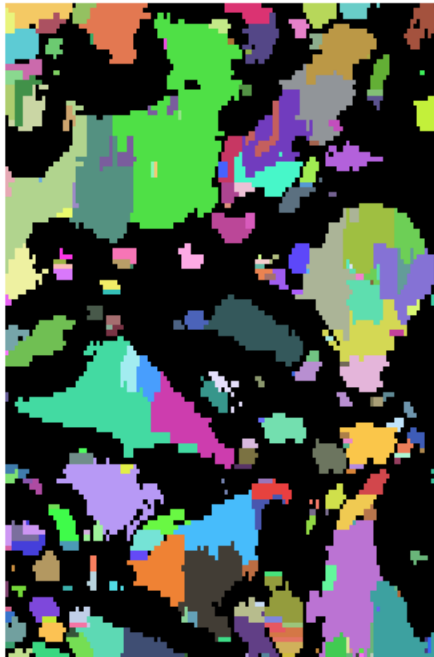
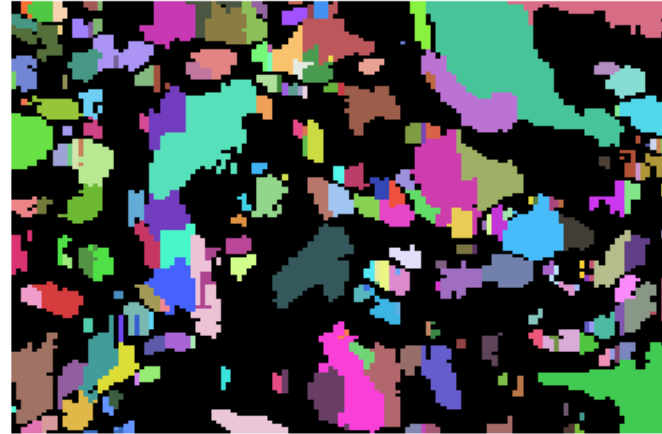
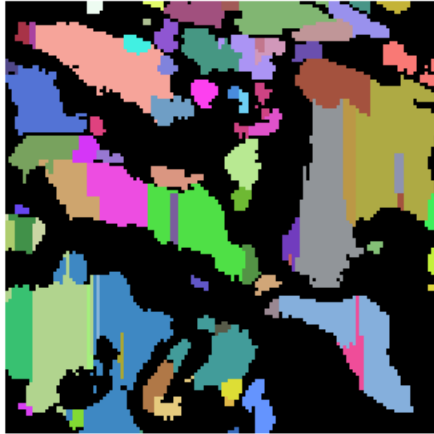
Re-seeding



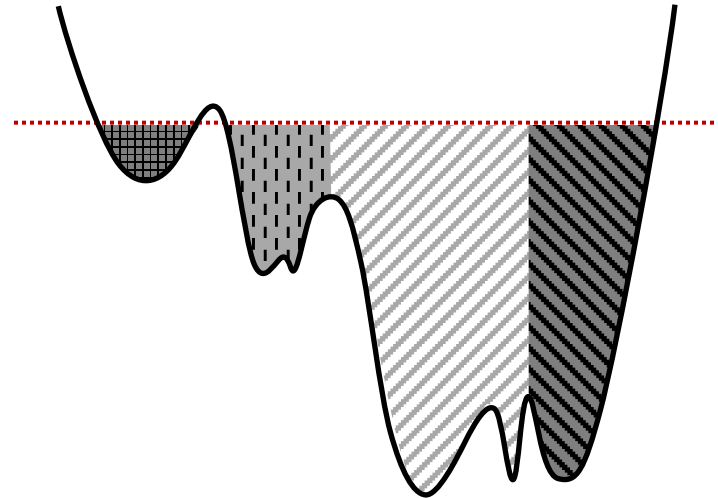
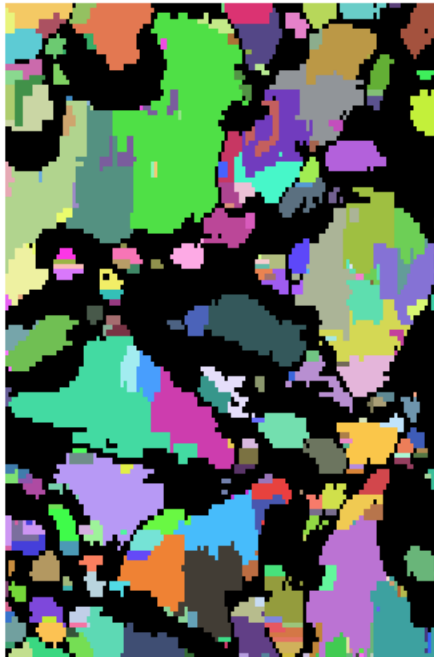
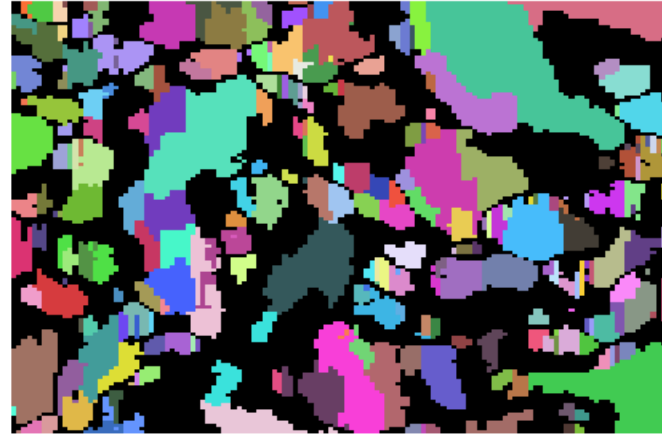
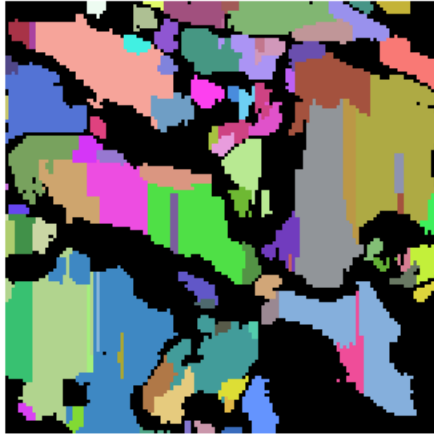
...Growing...



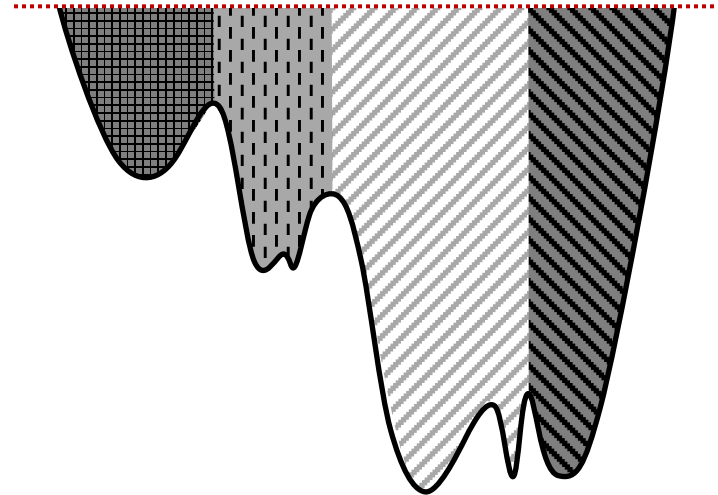
Re-seeding

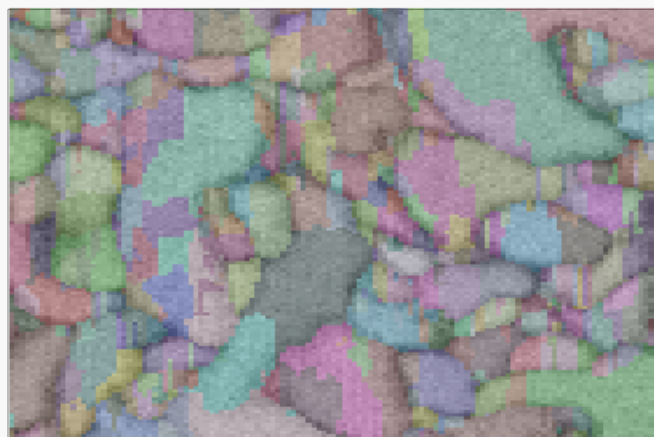
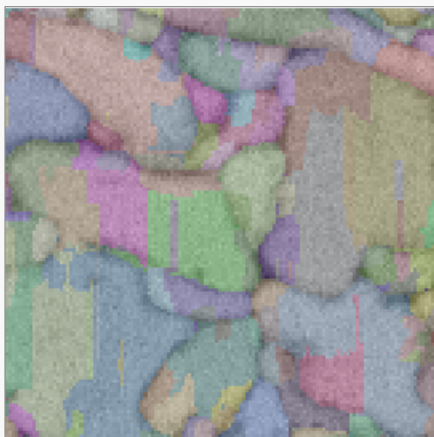


...Growing...

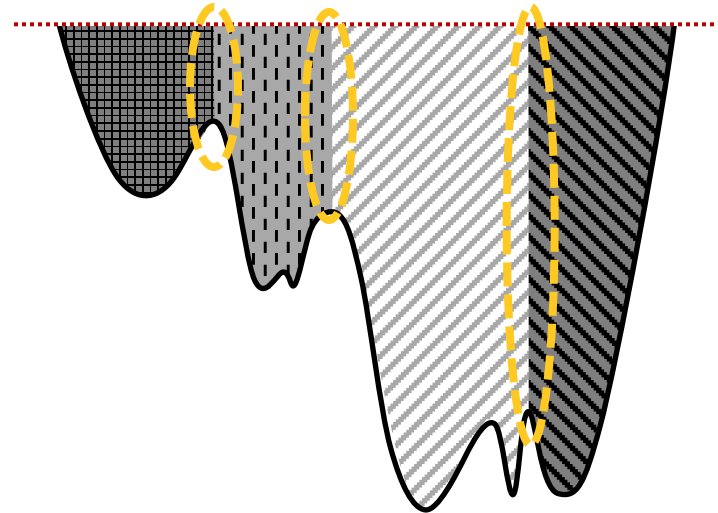


Grown to confluence

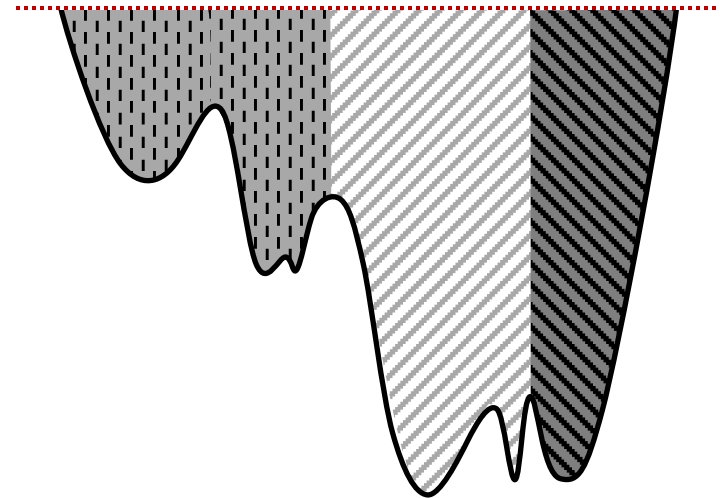
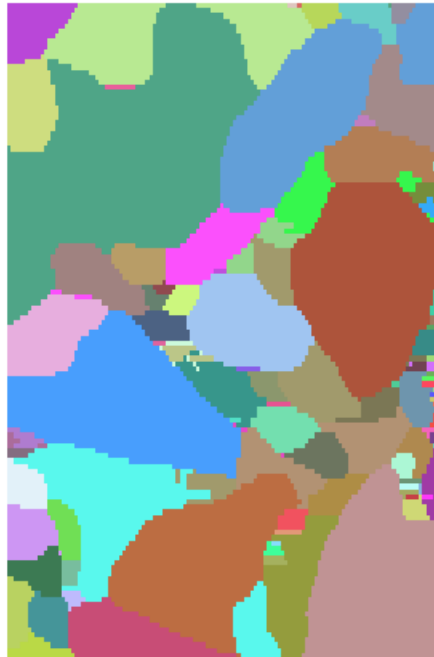
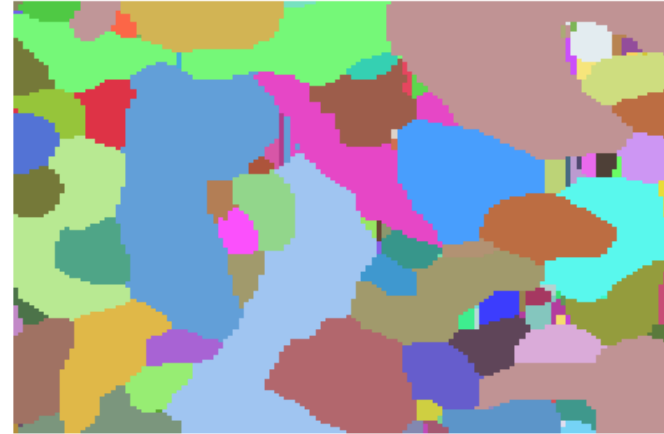


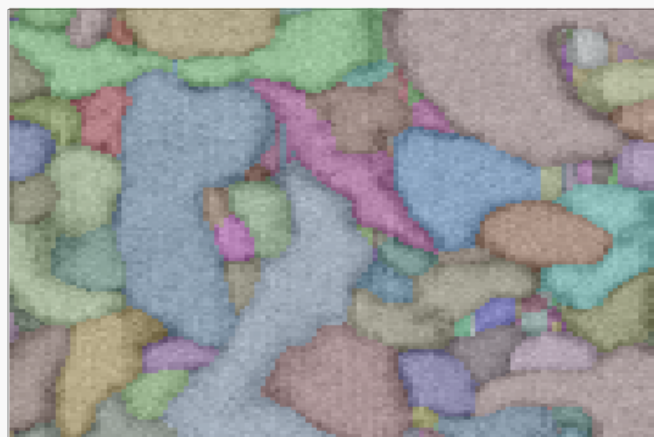
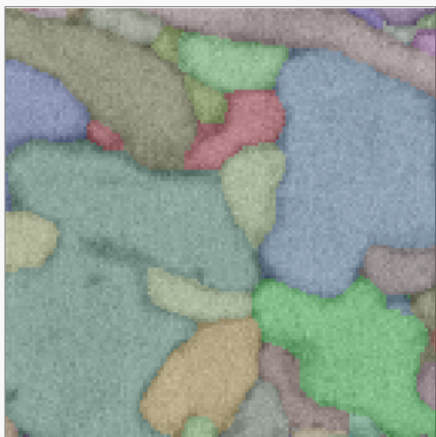


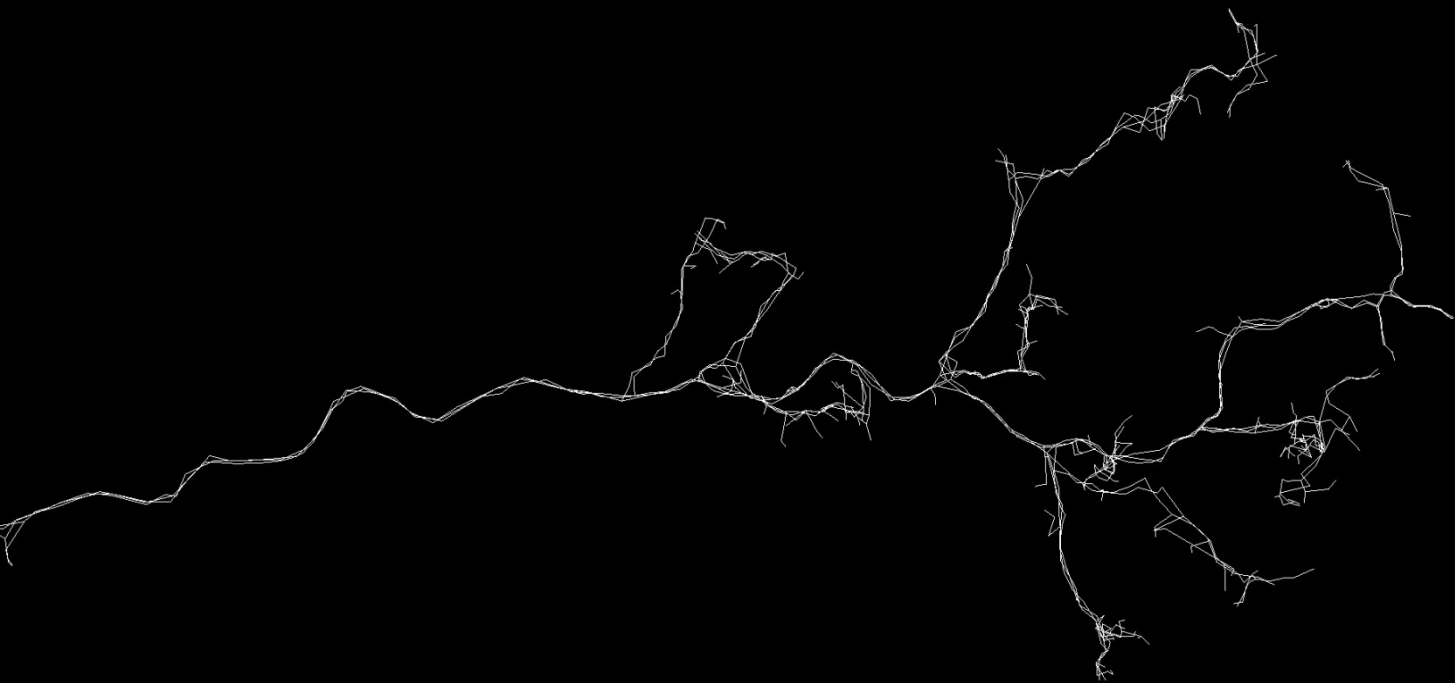
Examine contact areas

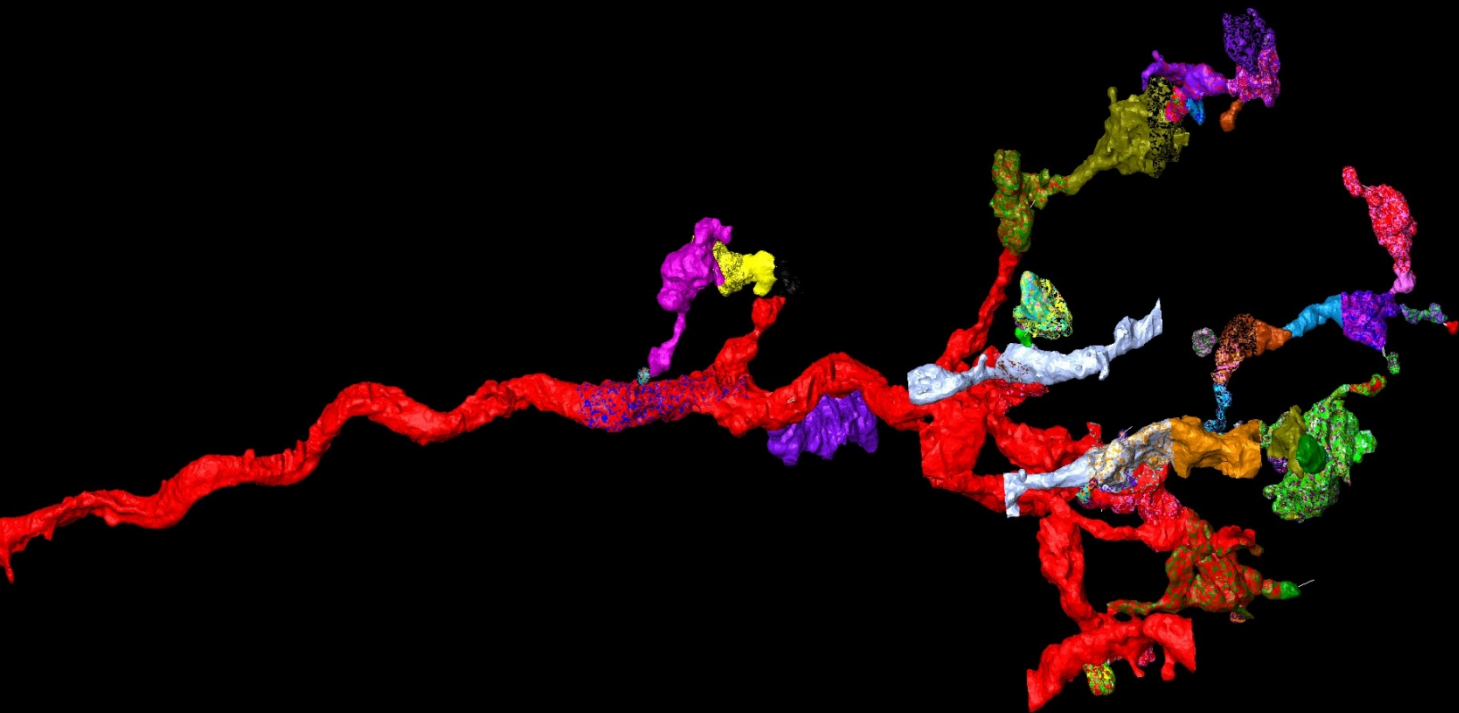


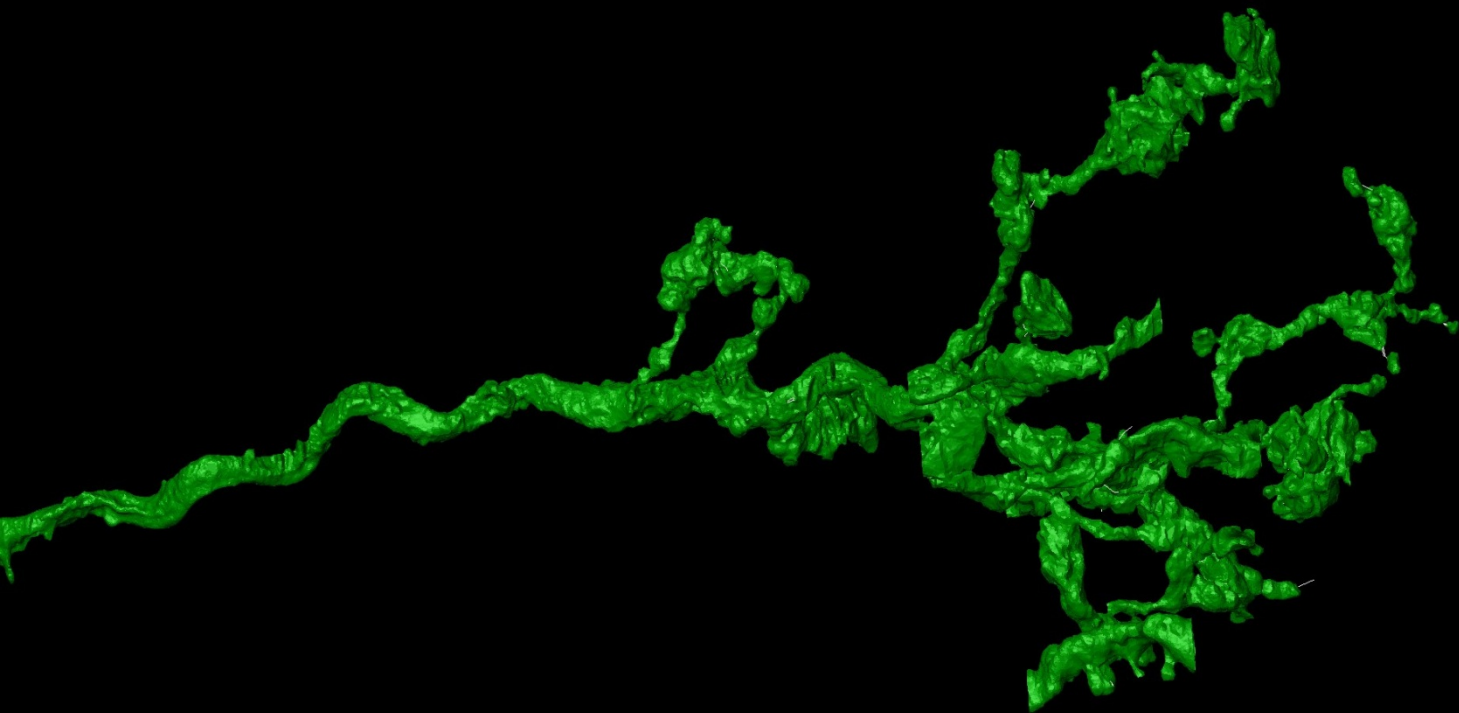
Merging (several iterations with different rules)

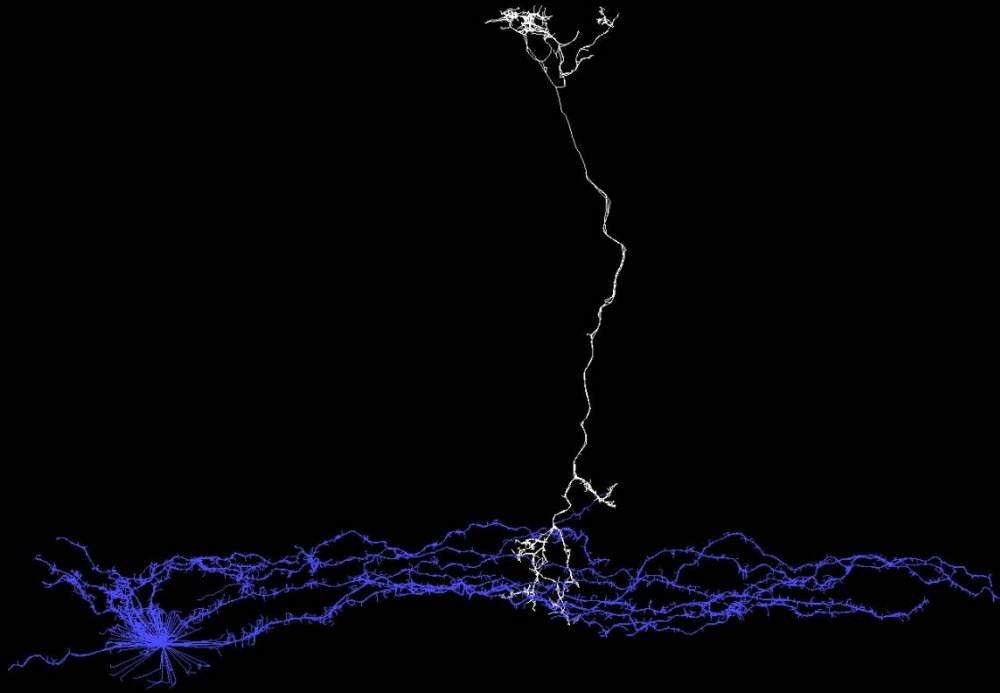


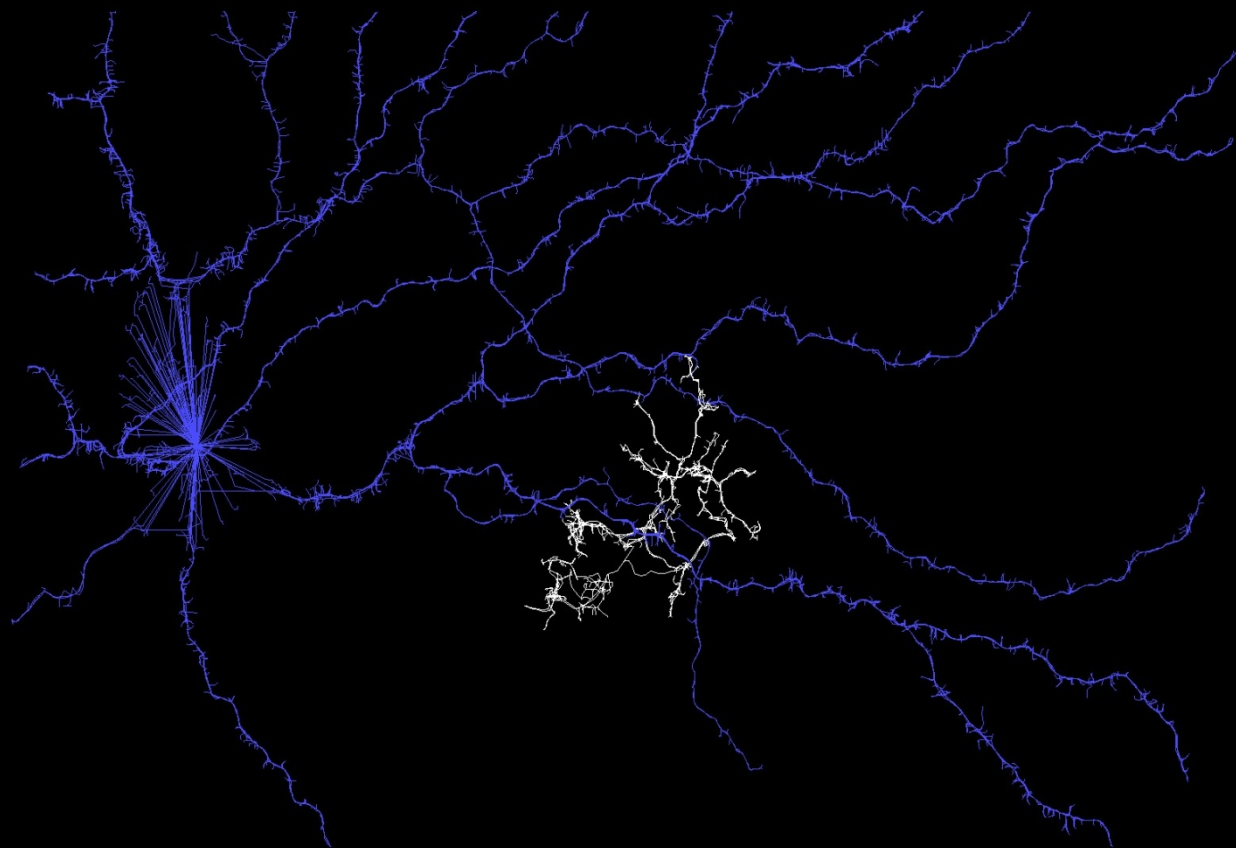




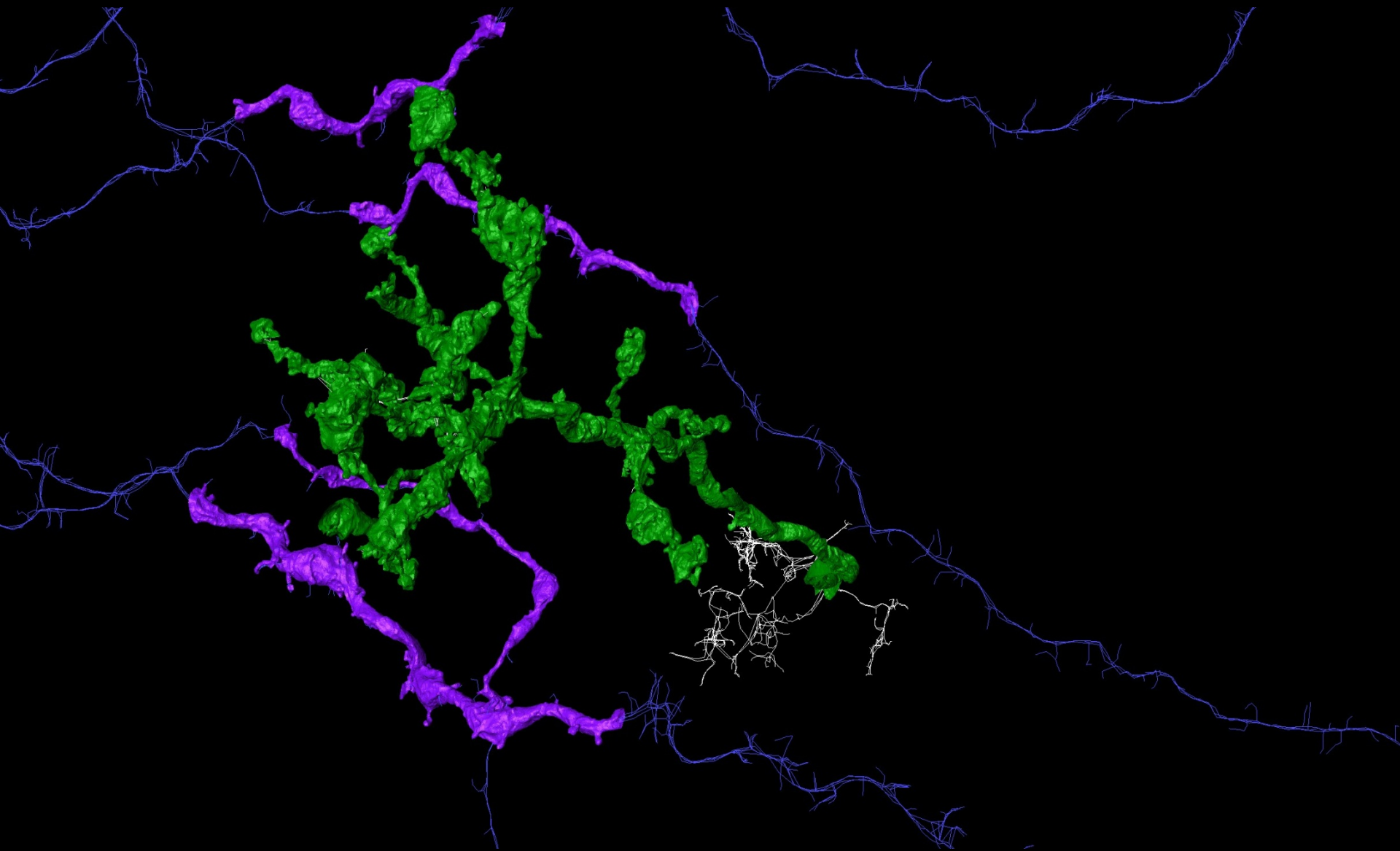


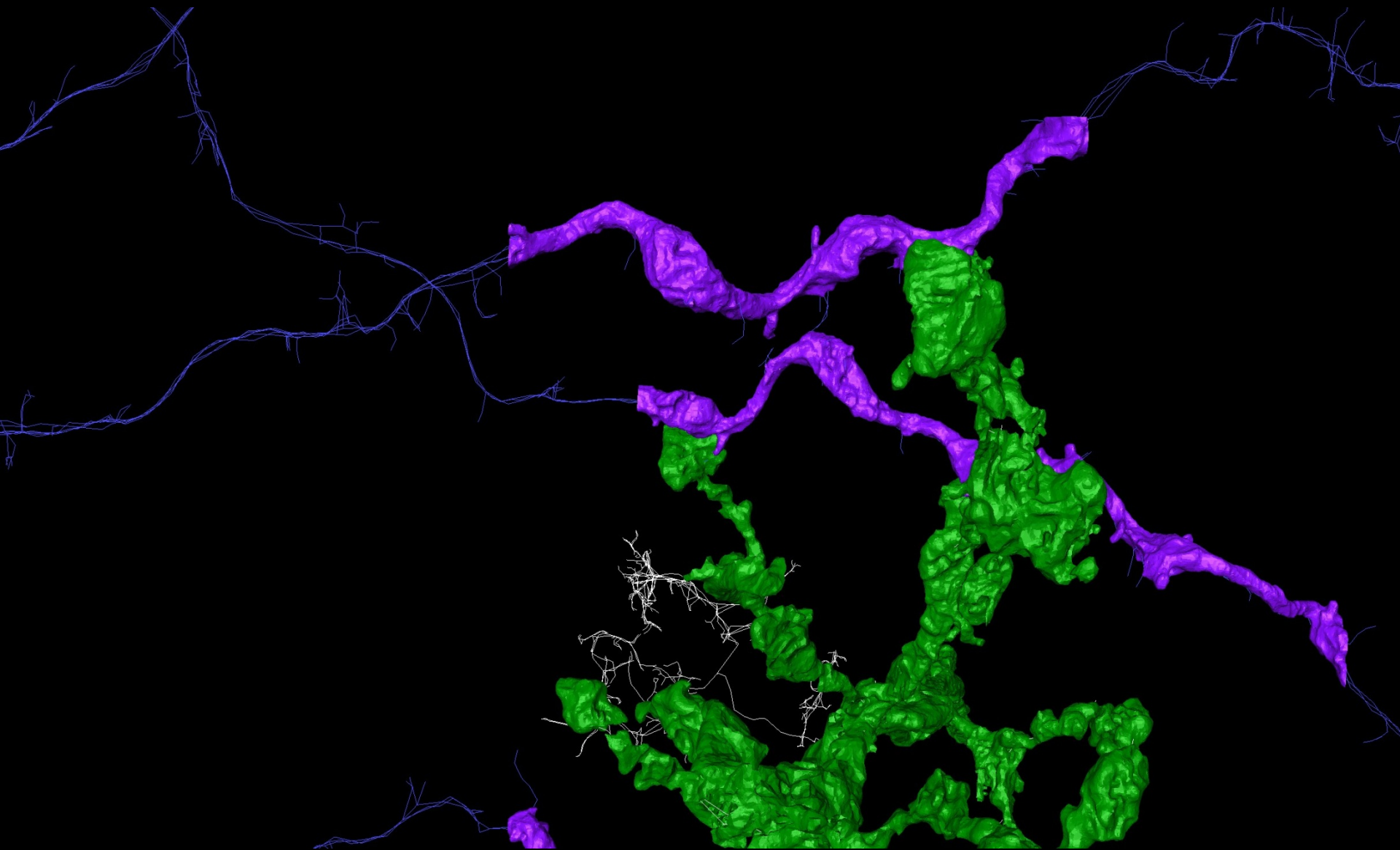


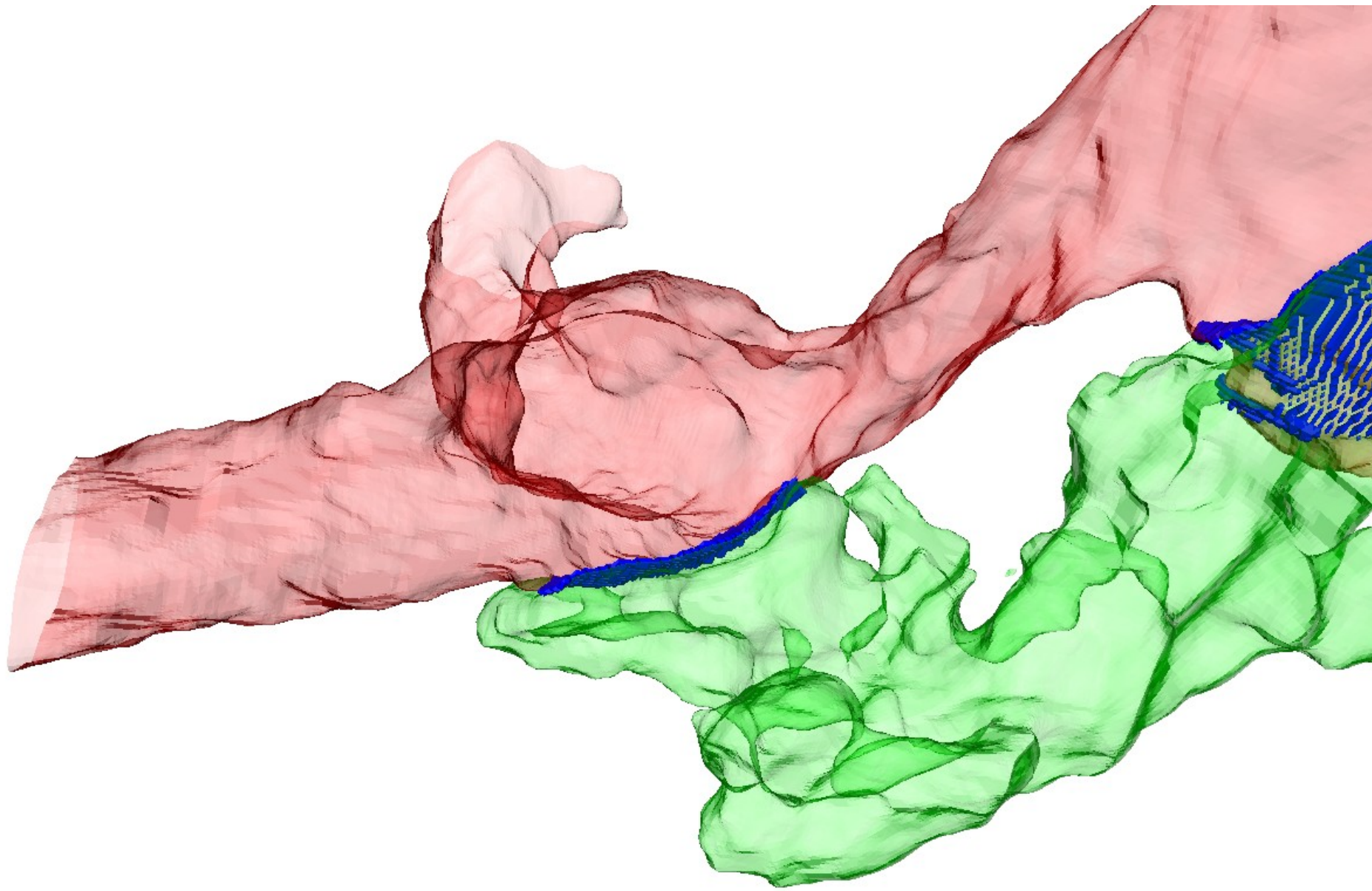


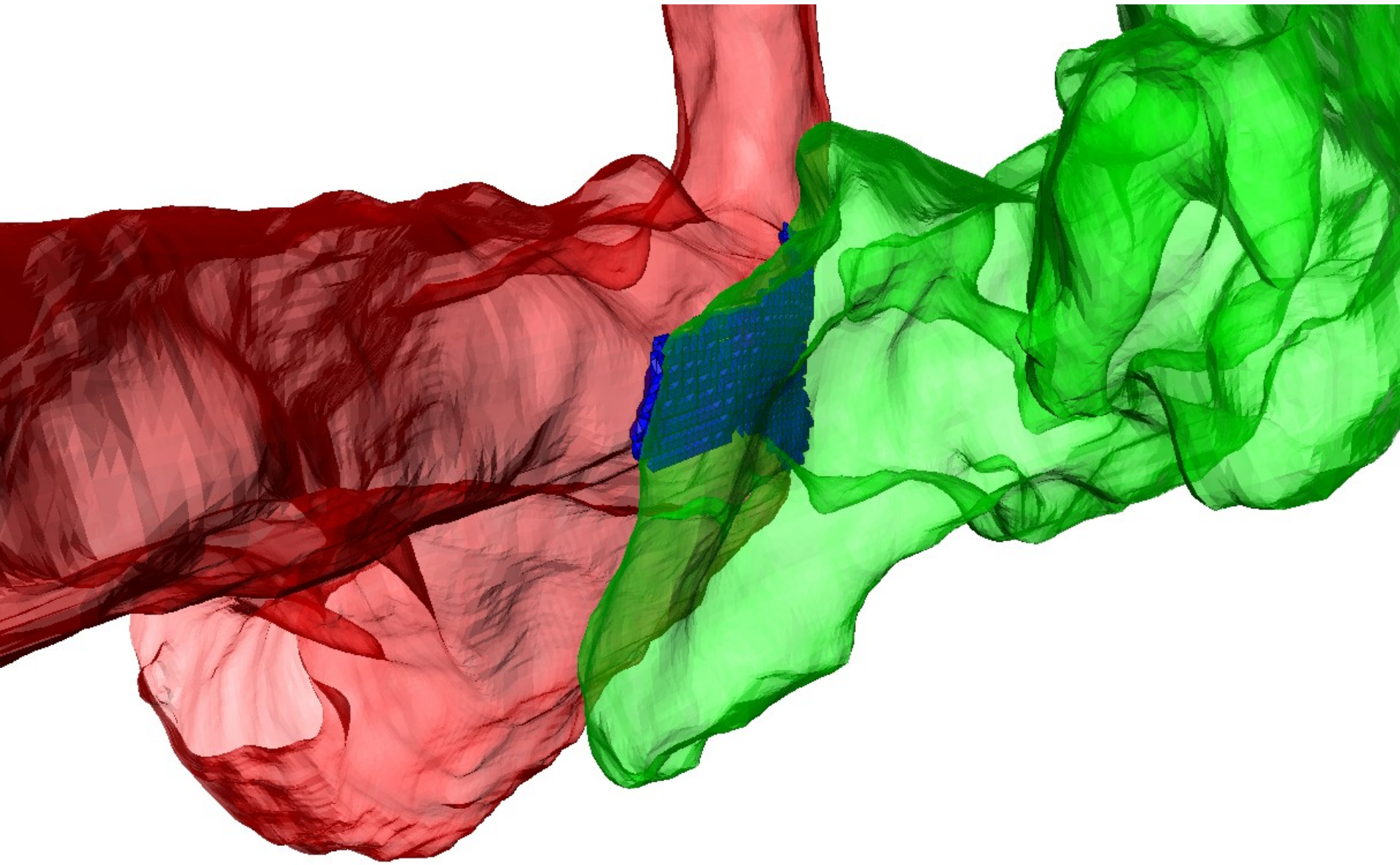




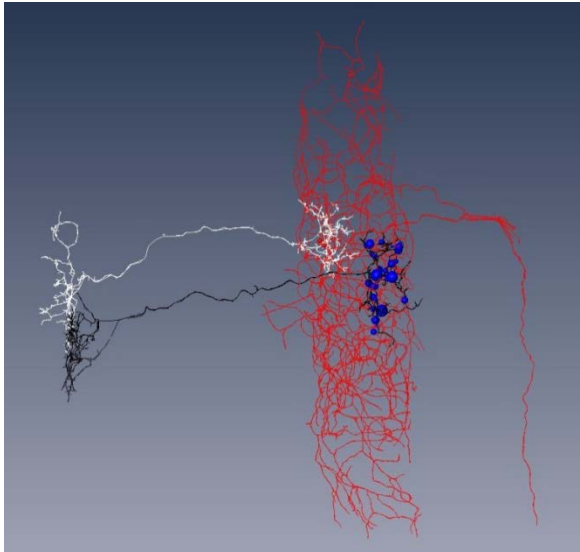




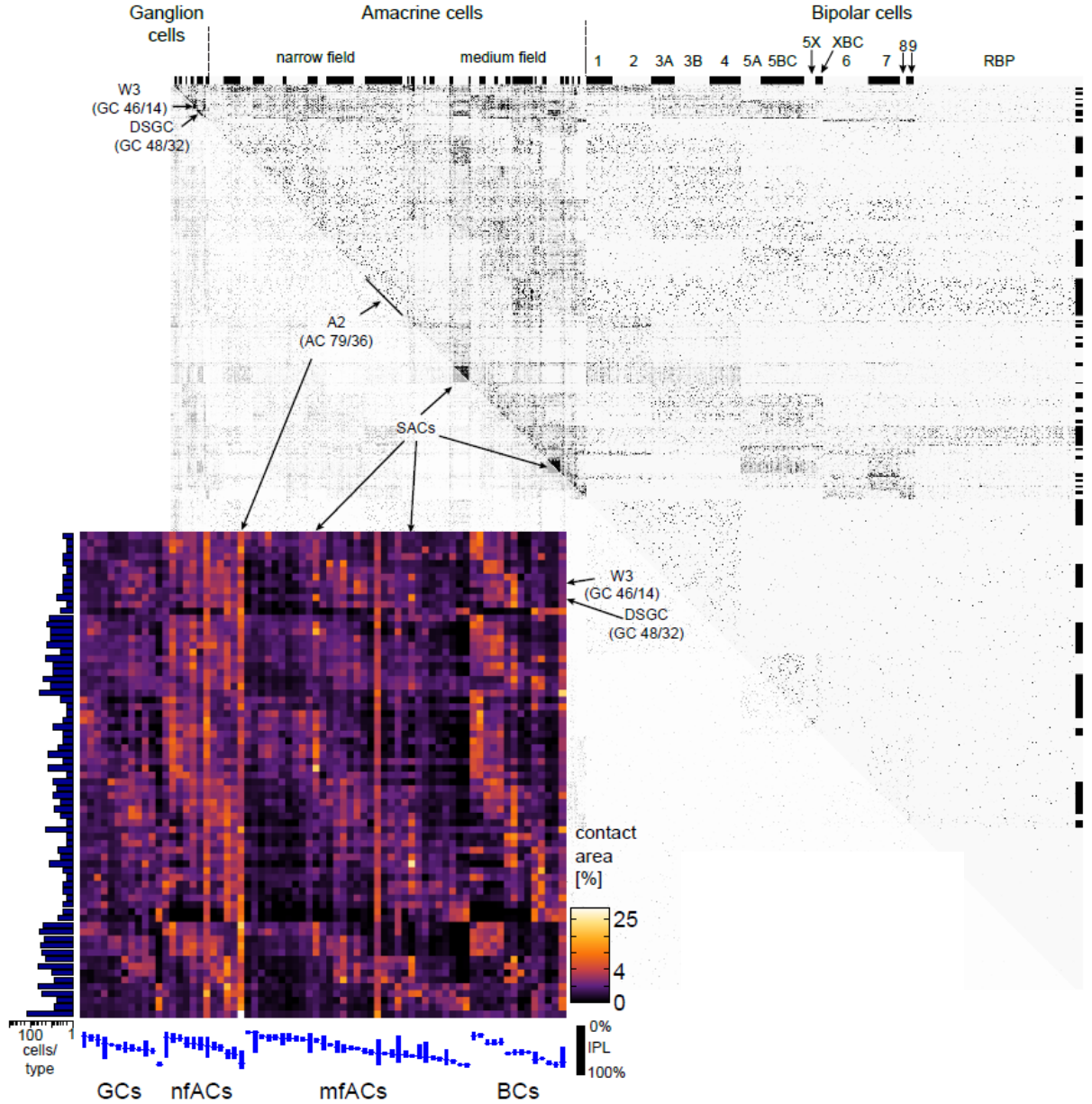




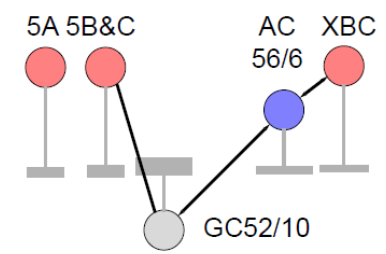
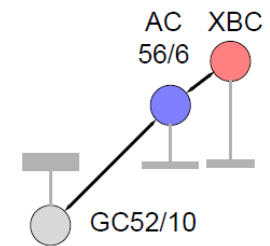
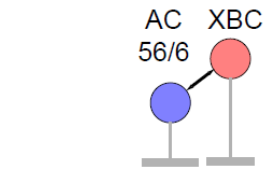
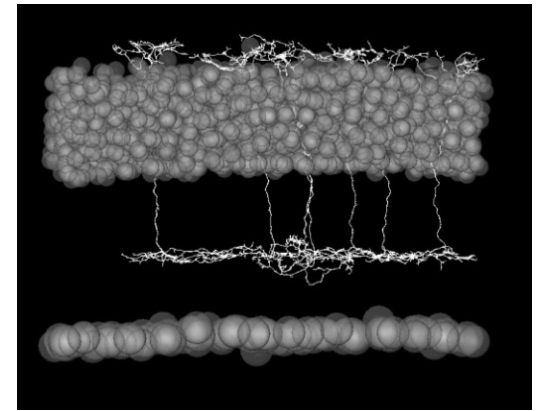
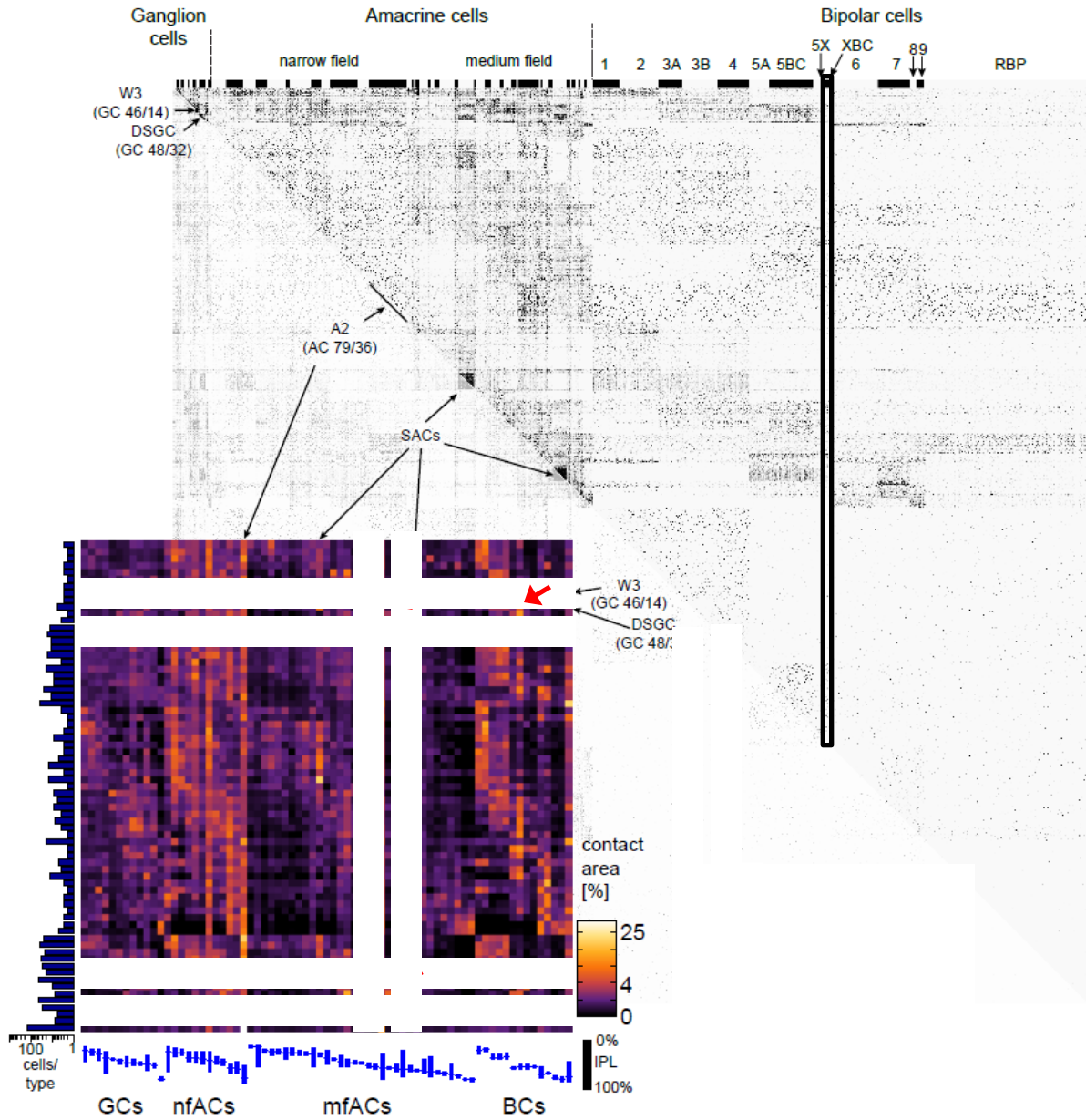
Dense reconstruction of mouse retina



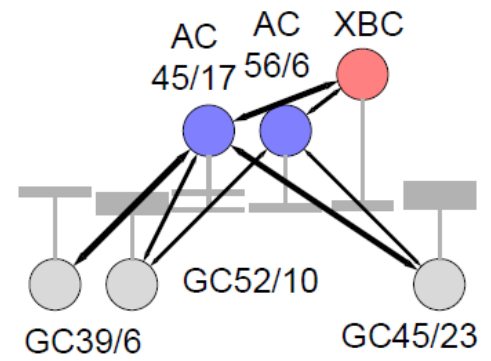
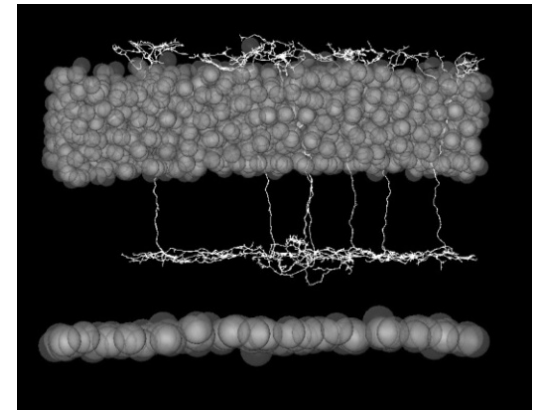
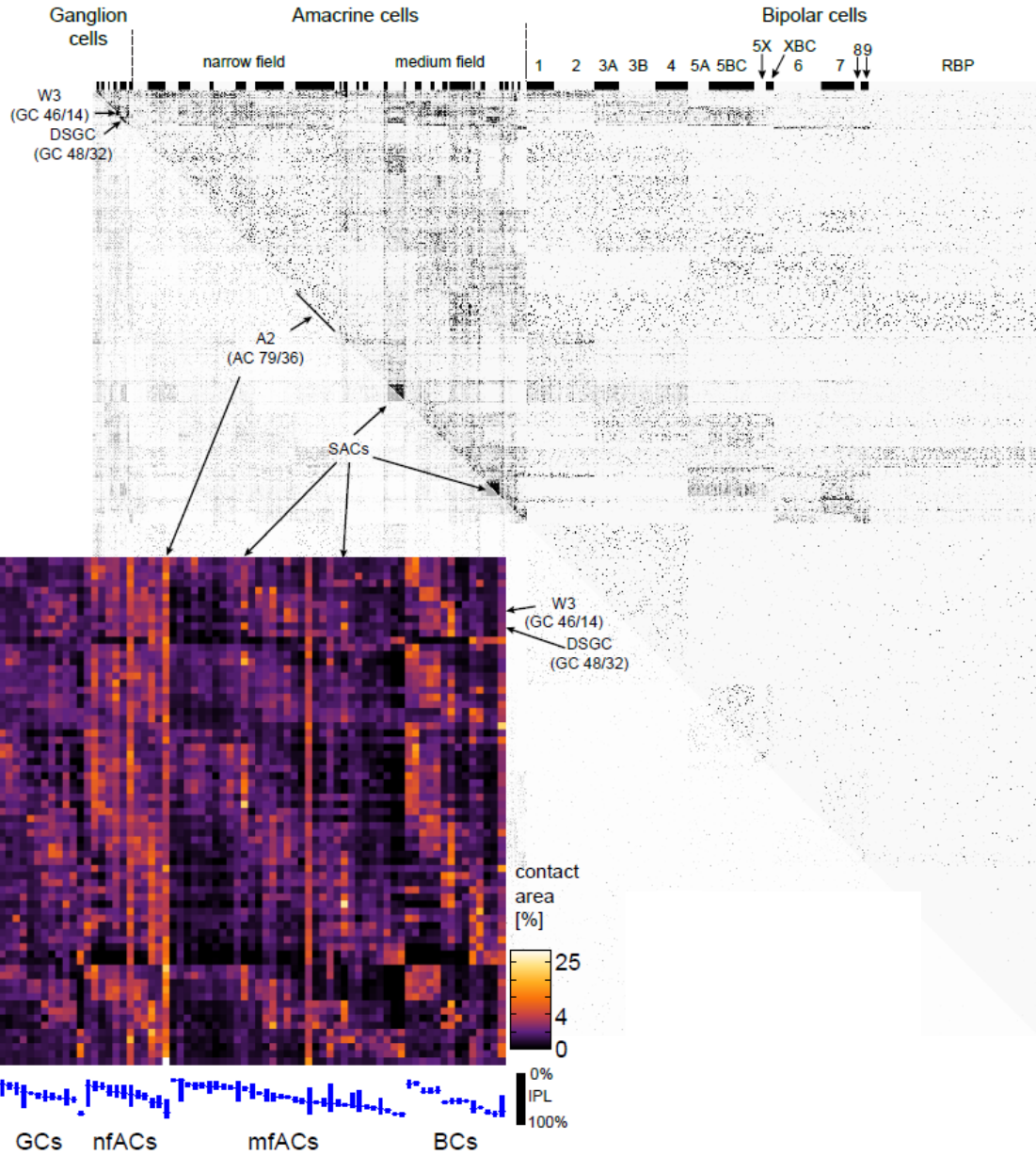
- Zeroes rule out possible circuits
- Defined cell-types by connectivity
- Identified novel cell types
- Proposed novel circuits



Proposed XBC circuits

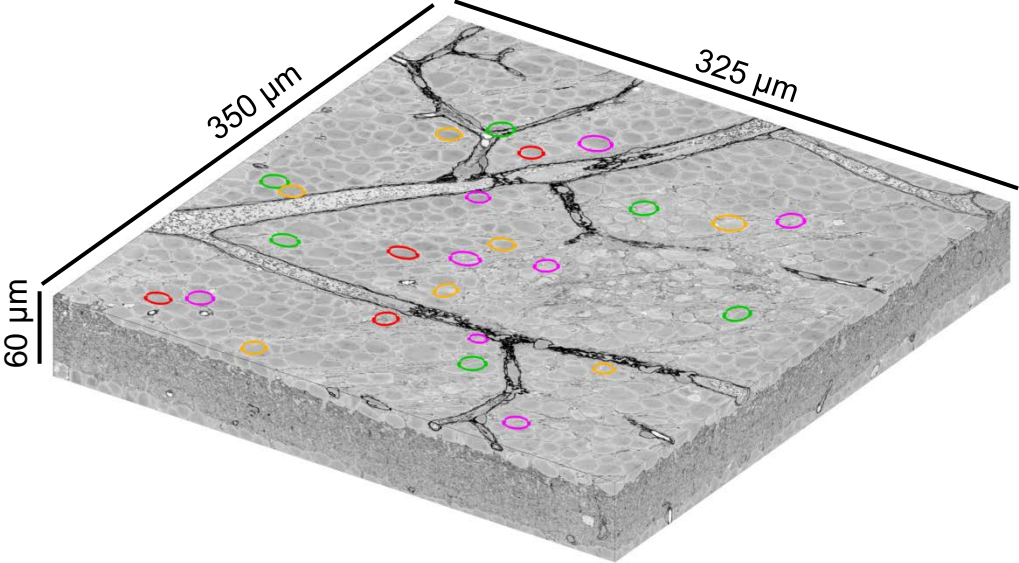


Proposed XBC circuits

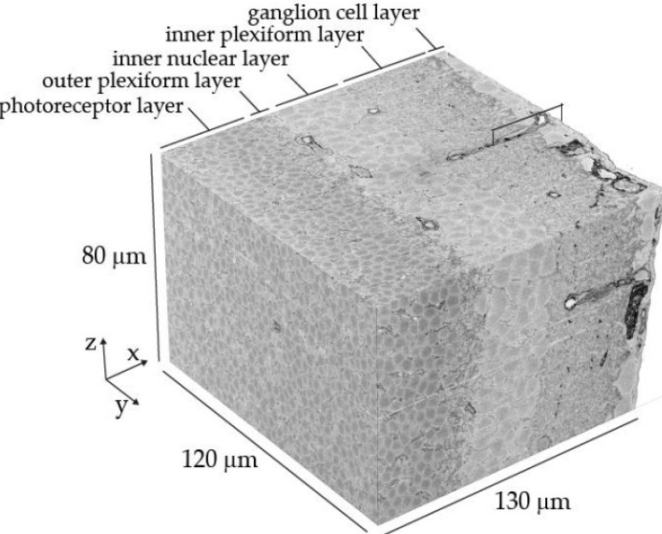


Neuronal circuit analysis of SBEM mouse retina data / are connectomes useful?

Sparse, targeted reconstruction following 2-photon calcium imaging

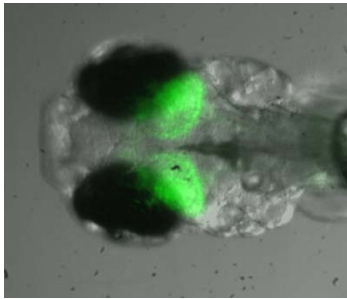
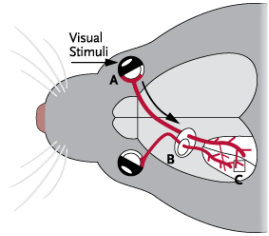
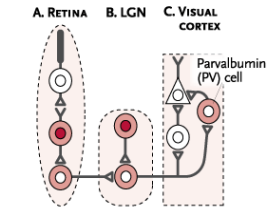


Dense, comprehensive reconstruction



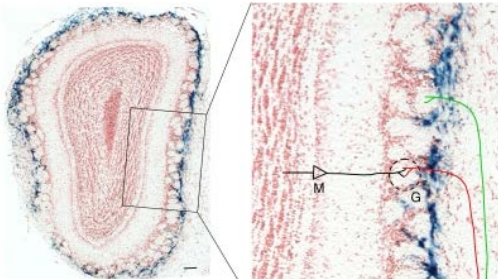
Dense, comprehensive reconstruction following volume 2-photon calcium imaging

What we are working on now



1. Connectivity of retinal ganglion cell axons in the LGN and SC
 - combine long range axon tracing with locally dense EM reconstruction

2. Functional imaging in the optic tectum with subsequent dense EM reconstruction
 - possible to acquire entire visual pathway



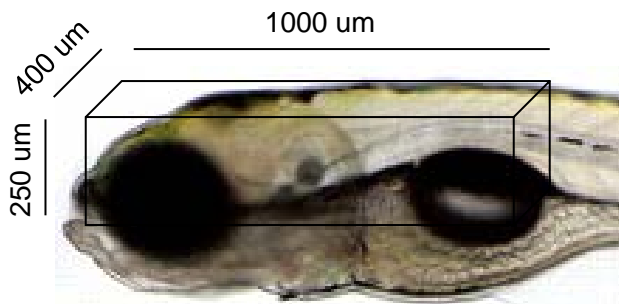
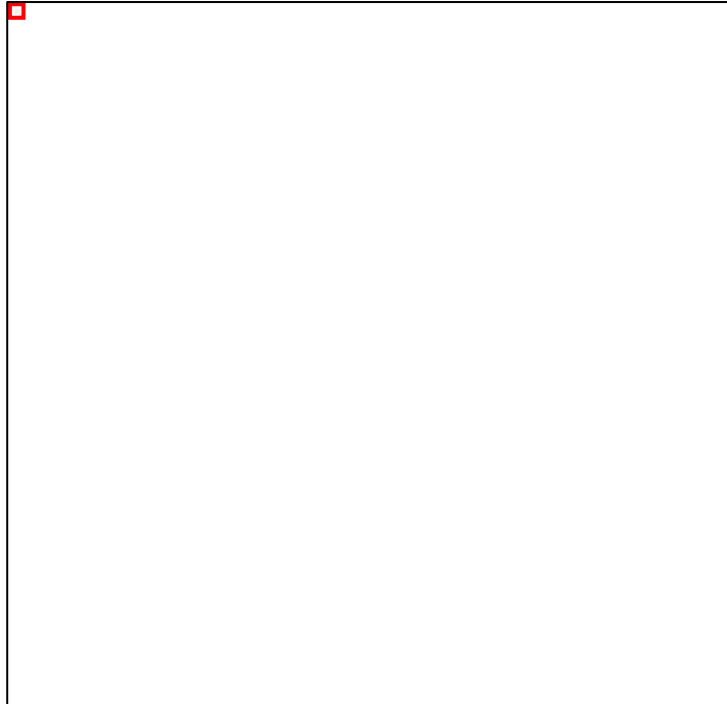
3. Olfactory bulb glomerulus dense EM reconstruction
 - possible to acquire olfactory 'column'

Is a connectome of the larval zebrafish feasible?

Yes, but how long will it take?

10^5 postsynaptic neurons

10^5 presynaptic neurons



Acquisition: 4-6 months, $60 \times 10^6 \mu\text{m}^3$

Analysis: 2 hrs / μm^3 (manual annotation)
= 57700 work years

Let's assume a 5 year goal:

11540 human annotators for 5 work years

115 human annotators for 5 work years
with a 100 speed-up using ML algorithms

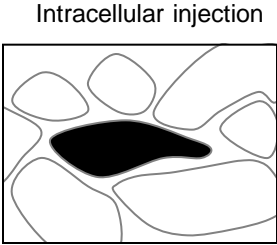
11540 times speed-up ($\sim 2.6 \text{ s} / \mu\text{m}^3$) using machine learning algorithms

CHAMPION NO. 110
CLASS. N° 110

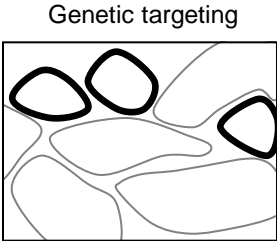
How can we speed up analysis?

1. Make the data easier to analyze

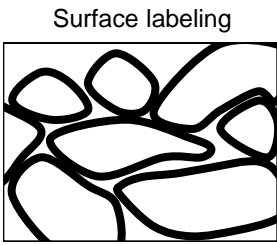
Staining strategies



individual cells

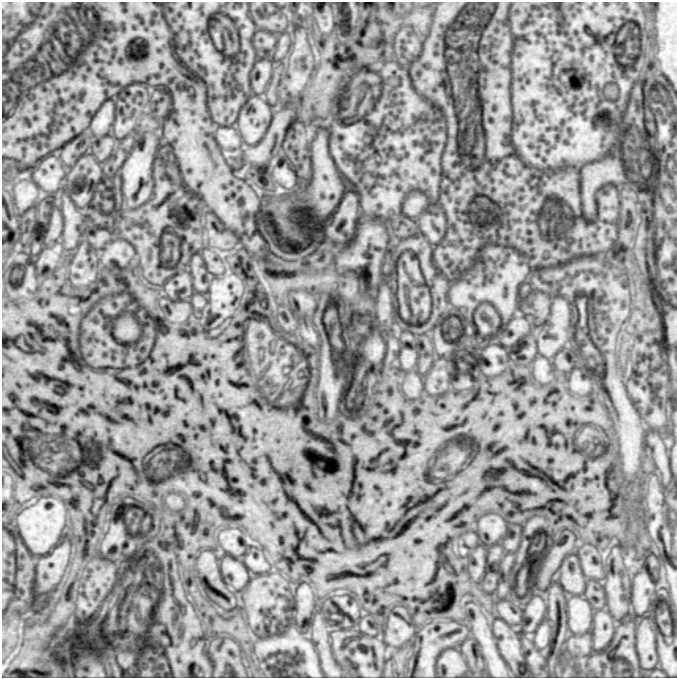


subpopulations



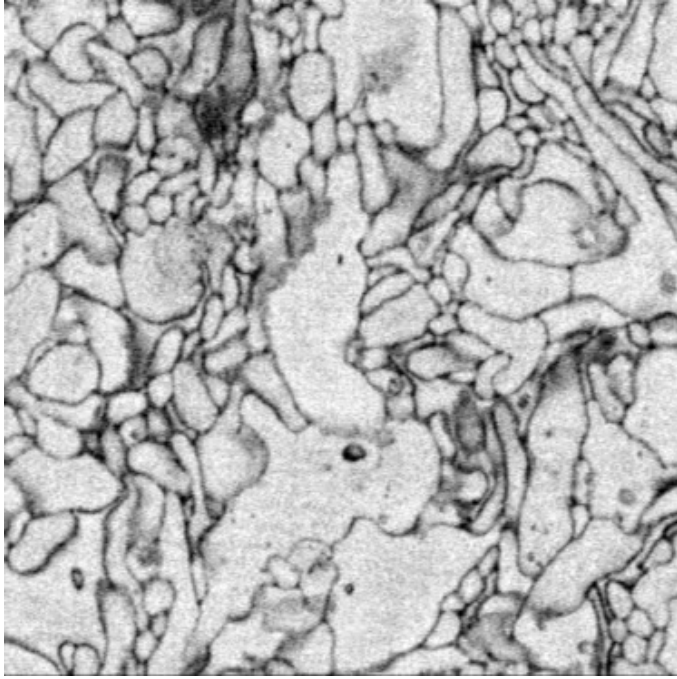
full volumes

Conventional *en bloc* stains
OsO4, uranyl acetate, lead citrate



12 x 12 x 25 nm³

1 μm

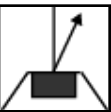
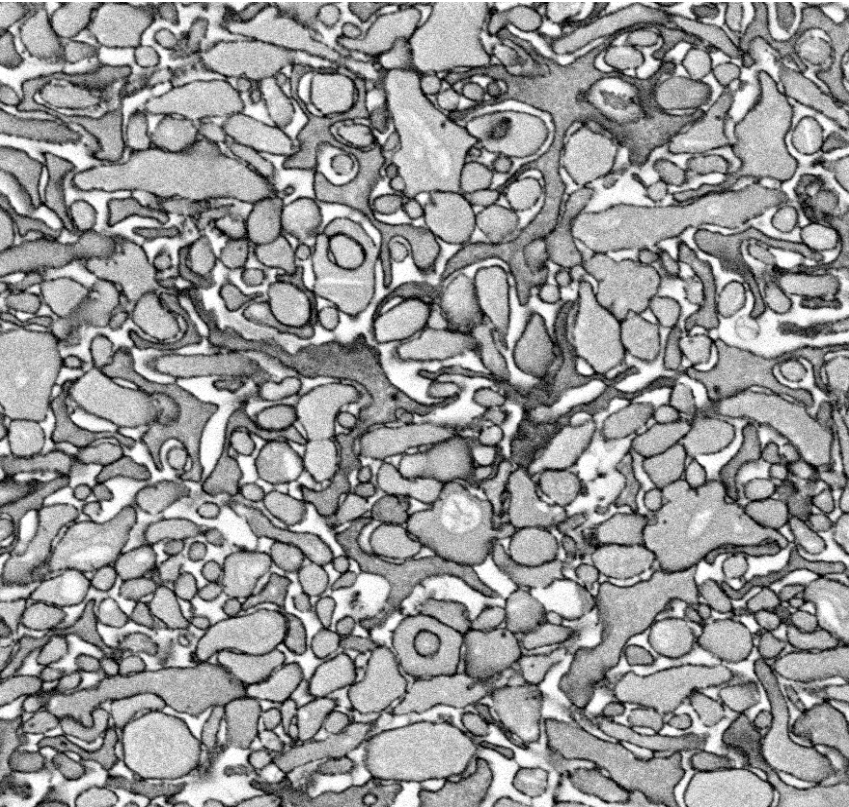


16.5 x 16.5 x 25 nm³

1 μm

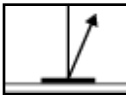
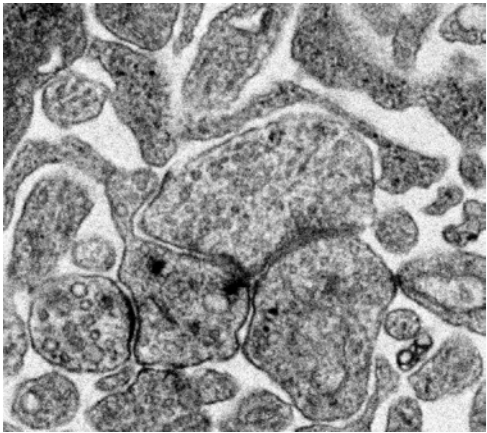
Mouse retina, inner plexiform layer

Extracellular space preservation



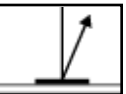
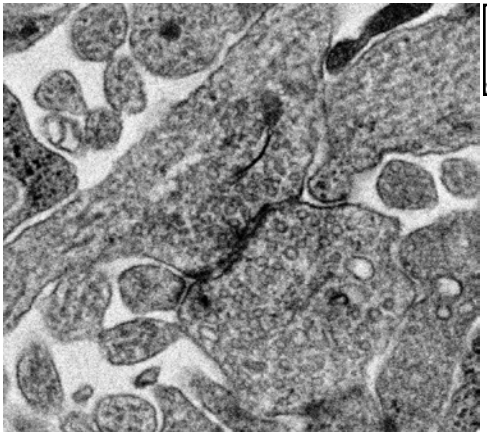
+ DAB

1 μm



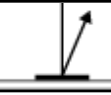
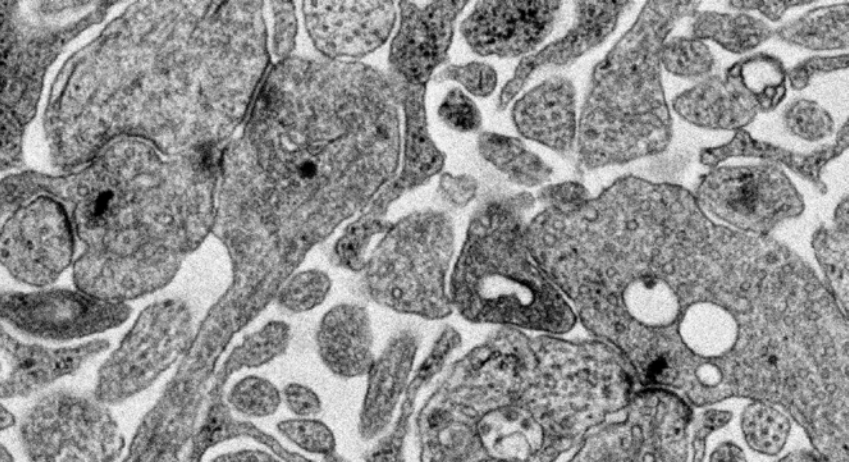
- DAB

100nm



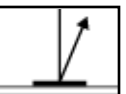
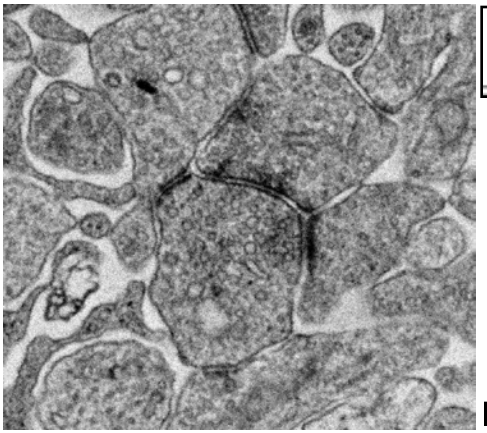
- DAB

100nm



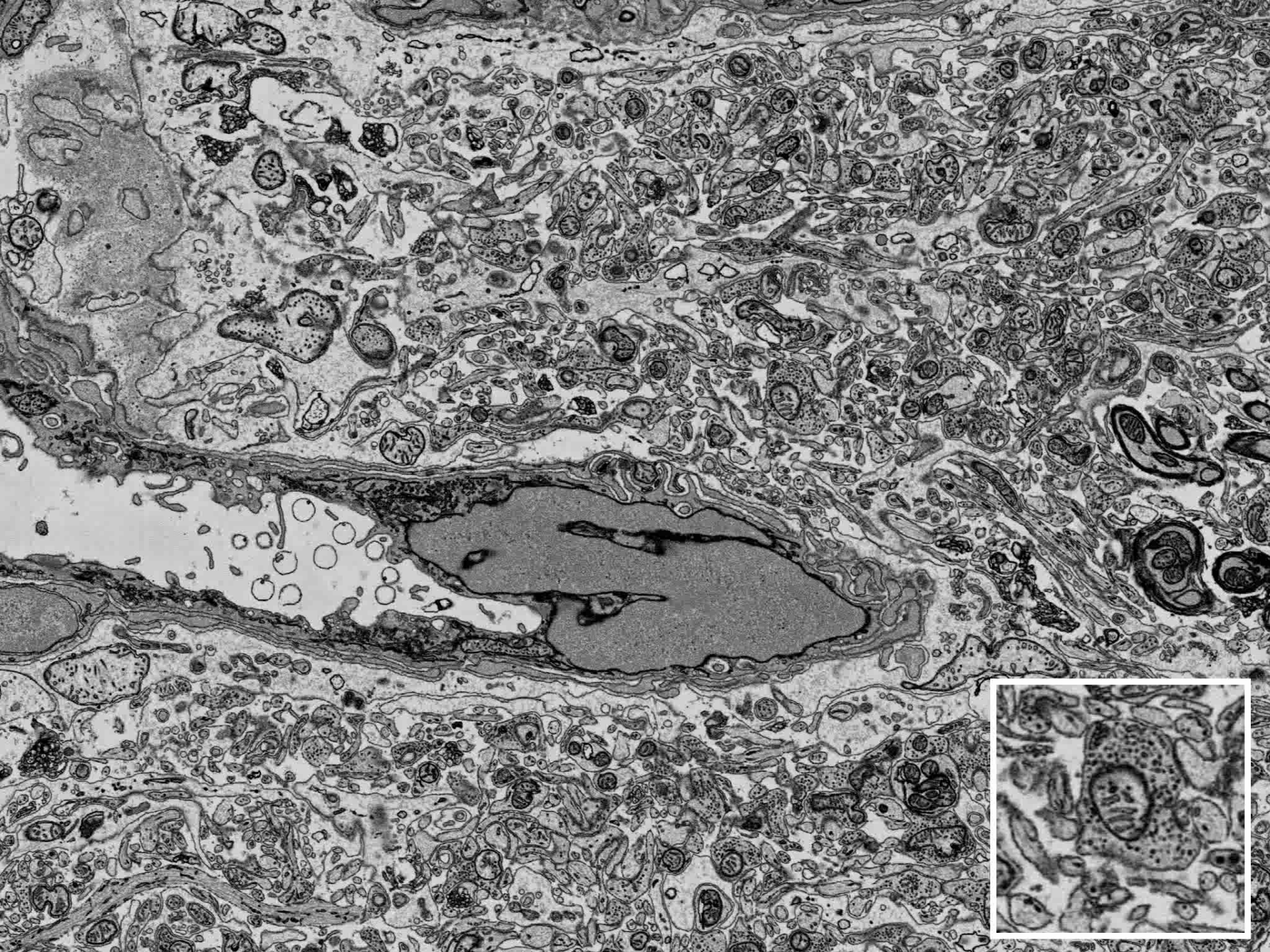
- DAB

100nm



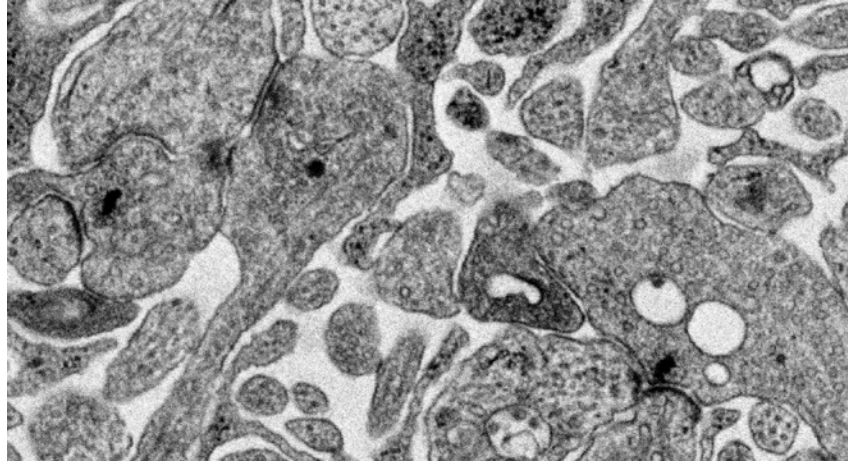
- DAB

100nm



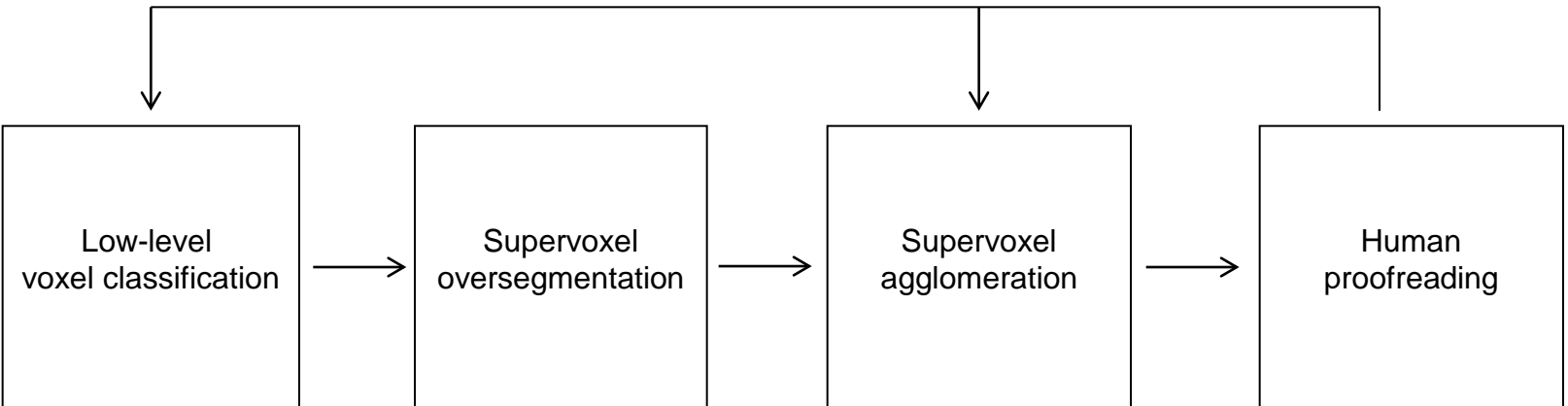
How can we speed up analysis?

1. Make the data easier to analyze



2. Position ourselves to take advantage of latest ML algorithms

NIH analysis pipeline (in progress)



e.g. Alex Krizhevsky's deep conv. NN code

e.g. conn. comp

e.g. feature-based learning

similar to eyewire.org but fun, and mobile

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