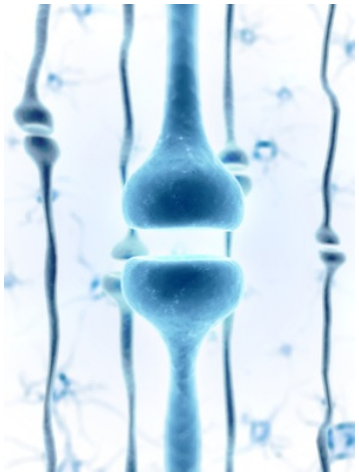
Neuronal circuits and single synapses- new imaging approaches with high resolution

Technical Journal Club
Josephin Wagner
30.06.2015

Introduction

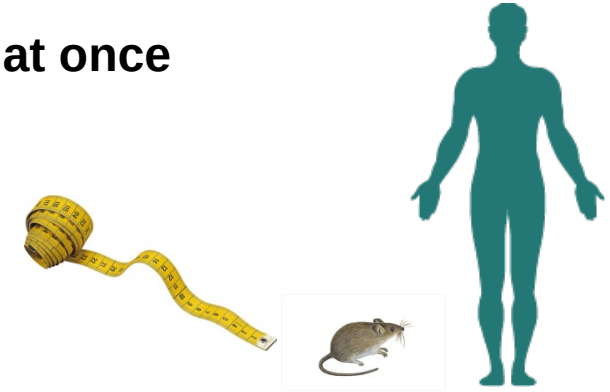
- Only electron microscopy provides resolution for complete neuronal circuits and synapses reconstruction
- Behavior is generated by interacting neurons on brain- wide circuits
- Reconstruction and computation of entire brain is highly desirable



- Preservation of ultrastructural details is needed

Issues to solve

- Neuronal synaptic connectivity is widely distributed over their dendrites and axons
- The length of all branches of one single cell exeeds 1 cm in mice and 1 m in human
- Whole brain mapping require **high resolution** and **large contiguous volumes**
- On day: Image a single brain to trace **all axons at once**



…How to proceed ?

Paper 1

Staining and embedding the whole mouse brain for electron microscopy

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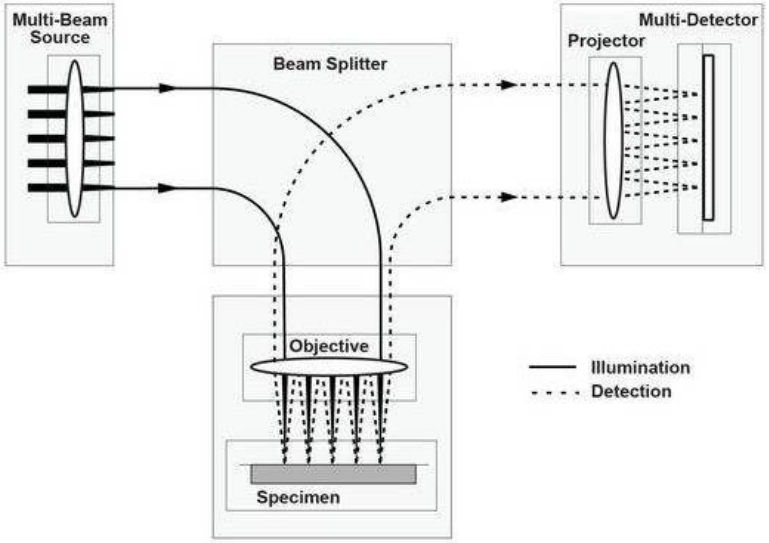
Methods- multibeam SEM imaging

Multibeam scanning electron microscopy

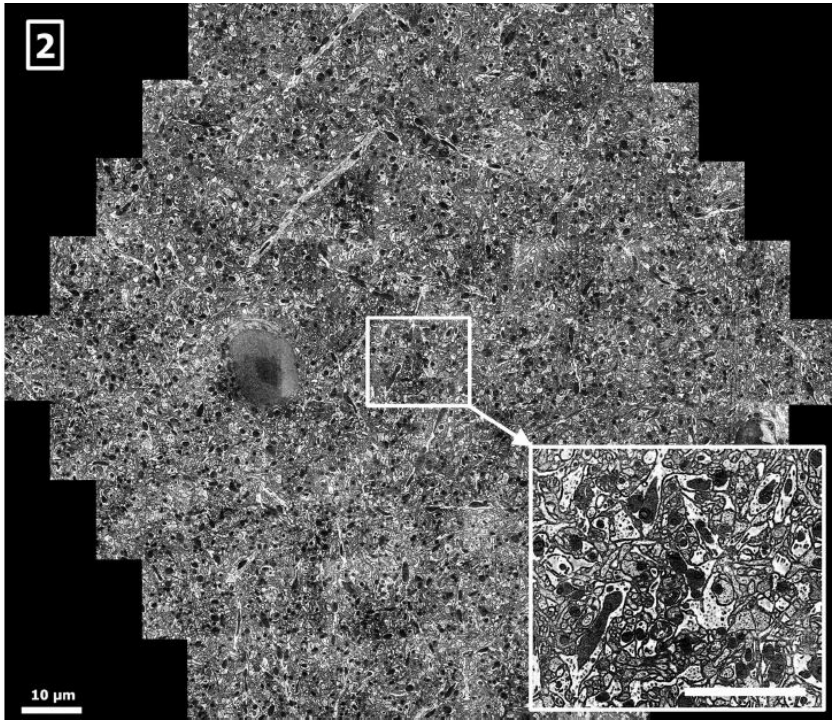


Schematic of the multi-beam microscope

- Multiple beams of 61 primary electrons in parallel (increases speed)
- Image of a hexagonal sample area 100-μm wide
- Primary electrons focus on specimen and separated by a beam splitter (magnetic field) from the secondary electrons
- All electron beams form 61 individual images which are then merged into a single, large area micrograph

Methods- multibeam SEM imaging

Multibeam scanning electron microscopy



- Cerebral cortex of mouse brain (block-face), showing unmyelinated neuronal and glial processes,3.8 nm pixel size, 270 electrons per pixel
- Capable of high throughput imaging (till 1GHz)

Methods- Sample preparation

- Staining and embedding procedure of the entire brain with long immersion time
- Provided uniform myelin staining and preserved ultrastructure
- Multibeam electron microscopy was applied to follow myelinated axons with low error rates

Methods- Sample preparation

wb PATCO (whole brain periodic-acid–thiocarbohydrazide-OsO_4):

	incubation steps					
staining protocol	primary		secondary		tertiary	
	solution	temp (°C)	solution	temp (°C)	solution	temp (°C)
Os	80 mM OsO_4	20	-	-	-	-
Os -> PbAsp	80 mM OsO_4	20	lead aspartate	20	-	-
Os -> UA	80 mM OsO_4	20	50 mM uranyl acetate	20	-	-
wbPATCO	90 mM periodic acid, 0.1 M cacodylate, pH 7.4	2	100 mM TCH, 0.1M cacodylate, pH 7.4	50	80 mM OsO_4	20
OTO	80 mM OsO_4	20	100 mM TCH	50	80 mM OsO_4	20
rOTO	80 mM OsO_4, 72 mM ferrocyanide, 0.1 M cacodylate, pH 7.4	20	100 mM TCH	50	80 mM OsO_4	20

- Best suitable staining with buffered periodic acid in combination with TCH (thiocarbohydrazide) immersion (48h)
- TCH = osmiophilic reagent, with periodic acid oxidation provides osmium black with good pigment qualities in tissue
- OsO4 = post fixative after perfusion
- Method originally designed to stain glycogen and polysaccharides
- Resulted in enhanced myelin contrast (high levels of glycolipids and cerebrosides in myelin)

Methods- Block face imaging

Assessing Staining quality and uniformity:

•Coronal block face mosaics at 80nm pixel

•Cross sectional surface cutted with 8 mm diamond knife (Diatome)

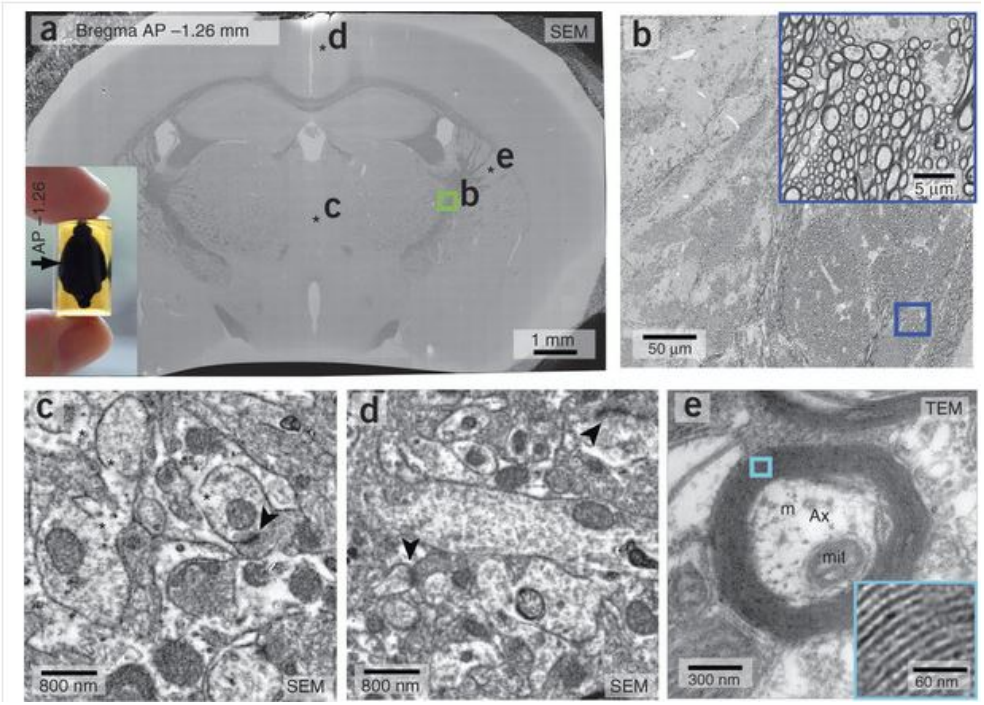
•Tissue coated with platinum carbon using electrobeam evaporator (allow charge dissipation during imaging)

•Mosaic imaging using a customer written software (Mathlab)

•975 parts where combined to a final mosaic, in total 330 *µ*m field of view

Results- Block face imaging

Whole mouse brain stained with wbPATCO and embedded with Quetol



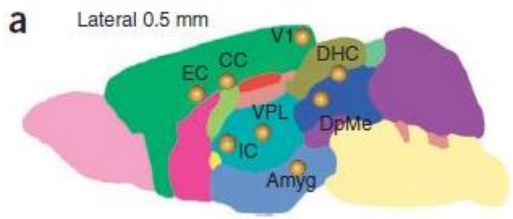
- staining and tissue preservation were uniform across the entire section
- Ultrastructural preservation was good and consistent in deep and superficial regions

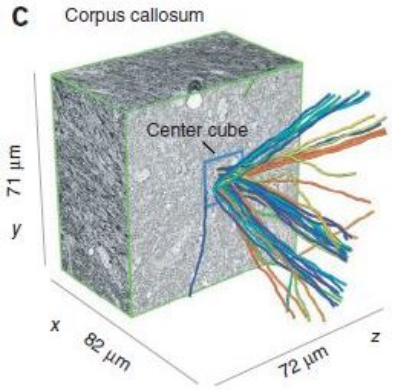
Methods- Serial block face imaging (SBEM)

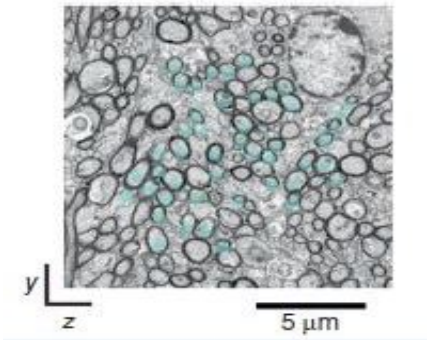
Determine myelinated axon traceability with wbPATCO stained brains:

- Stacks from several locations of the brain (8 regions of interests, ROI)
- Sample was trimmed till first ROI and marked
- Small volume sample of a ROI was cutted and imaged with SBEM

Results- serial block face imaging (SBEM)



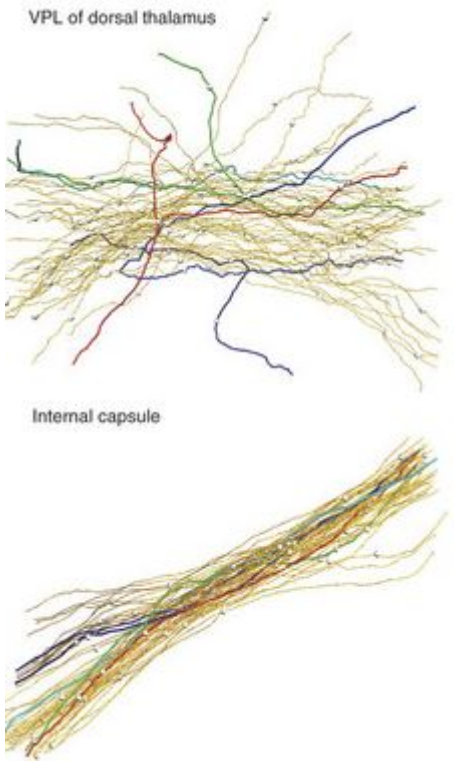




- SBEM shows axon traceabilty in 8 ROI`s
- Example: Corpus callosum with 50 traced axons
- Axons followed dominant direction in white matter
- Single images from the stack, showing axon tracing seeds

Results- serial block face imaging (SBEM)

3D reconstruction: Analysis of axon morphology



- Axons followed more tortuos path in gray matter (thalamus vs. internal capsule)
- Nodes of Ranvier was larger in gray matter (higher rate of branching)
- Myelinated axons where traced with low error rates

Evaluation- Axon traceability analysis

- 50 axons randomly choosen using Knossos software

- To quantify myelinated axons, Nodes of Ranvier where detected at 10 μm

- Unmyelinated axons had stretches with low contrast, hardly detectable

Conclusion- Paper 1

- Staining provides uniform and sufficient contrast in EM to trace myelinated axons with low error rates
- Methods also alow recognition of unmyelinated processes, but low membrane contrast prevent reliable tracing
- Wb PATCO staining unsuitable for tracing complete neuronal circuits

Paper 2

What`s new ?

High-resolution whole-brain staining for electron microscopic circuit reconstruction

Shawn Mikula[1,2] & Winfried Denk[1,2]

NATURE METHODS | VOL.12 NO.6 | JUNE 2015 | 541

Methods- BROPA sample preparation

- BROPA = **b**rain wide **r**educed **o**smium staining with **p**yrogallol mediated **a**mplification

- Optimization of whole brain staining due to long series of immersion steps, preserves ultrastrucural details like chemical synapses, thinnest neurites, spine necks

Methods- BROPA sample preparation

Obstacles:

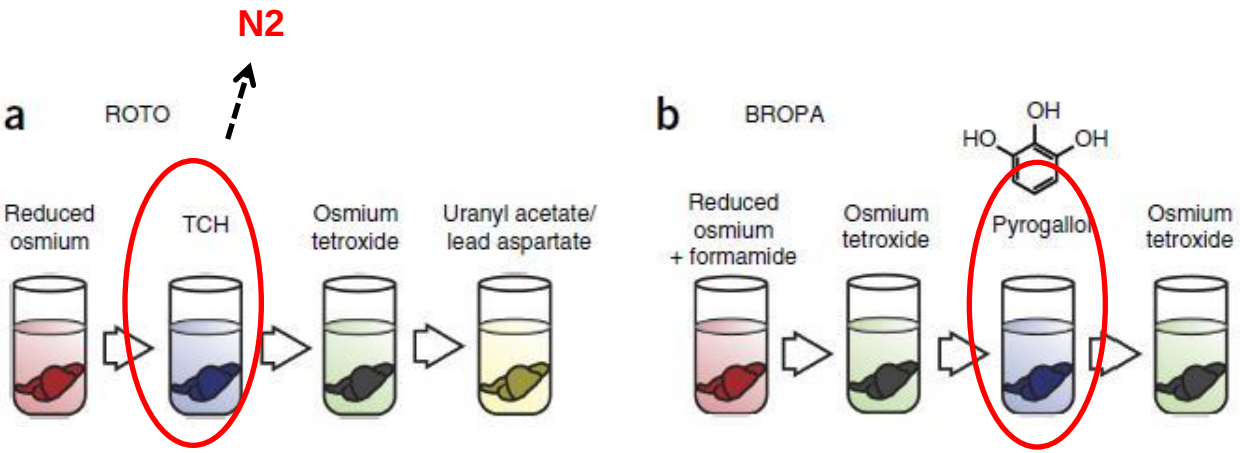
•no stainings beyond 200 *µ*m

•Larger samples where disrubted (bubble formation, N2)

•Highly charged molecules where unable to pass membranes

•Osmotic imbalance during aledhyde fixation occured

Methods- BROPA sample preparation

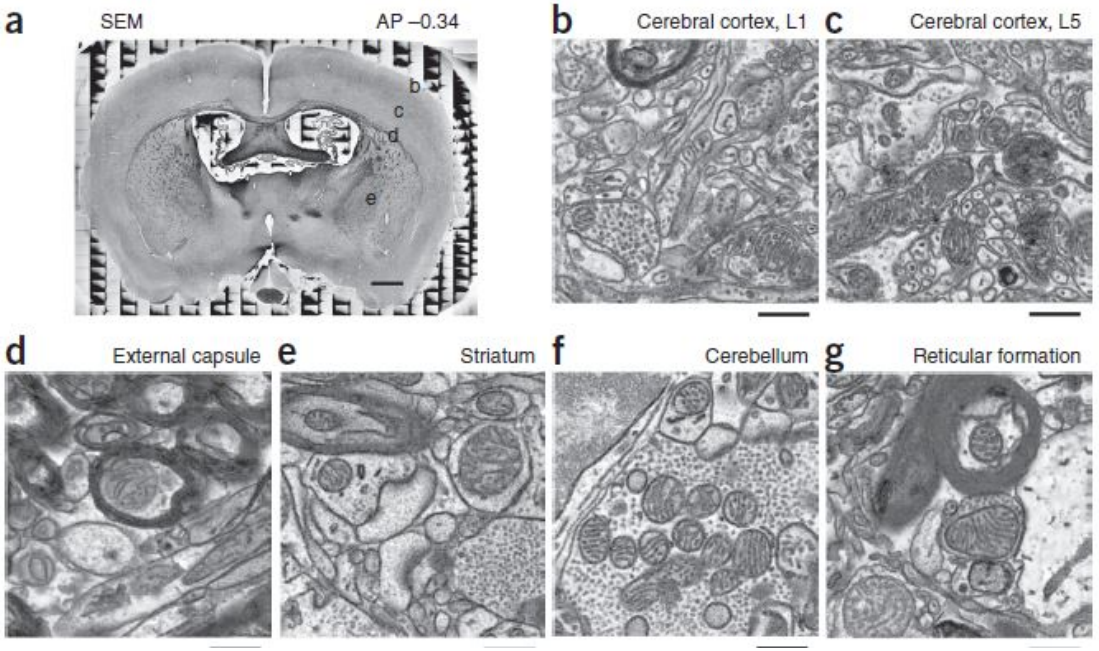


- Good ultrastructure
- No staining beyond 200 *µ*m
- Limited penetration
- Large sample disruption (N2 liberation from TCH)

- Pyrogallol (trihydroxybenzene) corrects osmotic imbalance and keeps extracellular space intact
- Improved stain penetration

Evaluation of BROPA

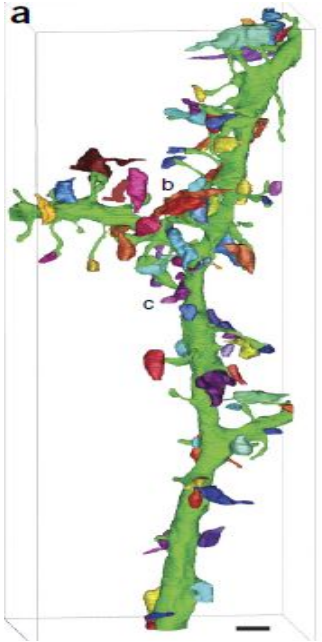
SEM imaging: Evaluation of BROPA-prepared brains



Evaluation of BROPA

Synapse identification

- Selection of SBEM stack (striatum)
- 25 neurites where chosen, among these most higly branched where analysed
- Human annotator performed volume 3D reconstruction using ITK-SNAP software and labeled corresponding presynaptic boutons



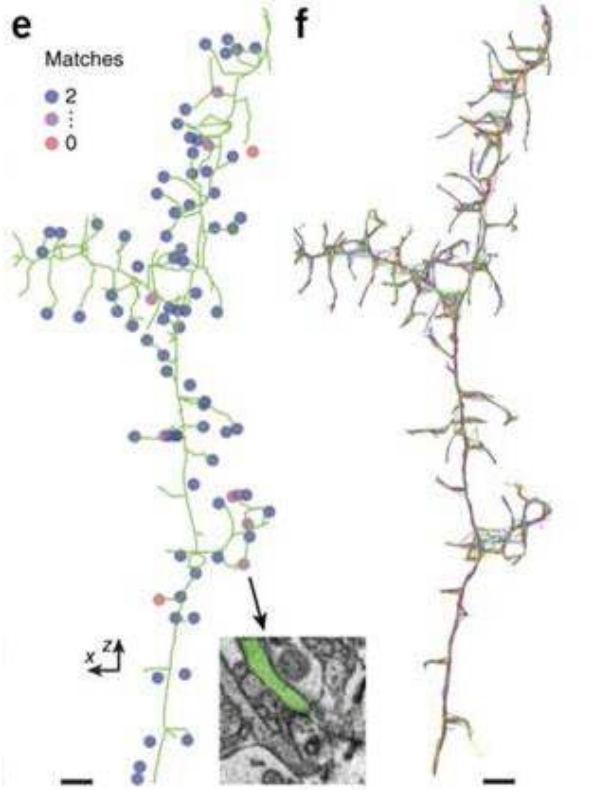
dendrite in the striatum and 74 synaptic boutons (randomly colored) from SBEM data set

http://www.nature.com/nmeth/journal/v12/n6/extref/nmeth.3361-sv1.mov

Evaluation of BROPA

Synapse identification

KNOSSOS
3D IMAGE VISUALIZATION AND ANNOTATION



- Further annotators identified all synapses in common in skeleton tracing (Knossos- visualization-annotation software)
- Tracing of chemical synapses, small neurites, spine necks and small-caliber axons possible

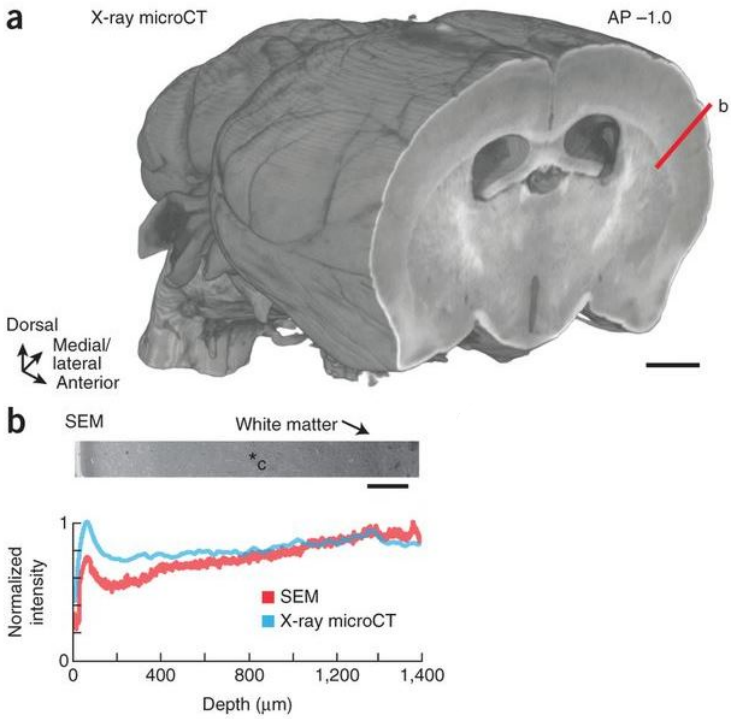
Evaluation of BROPA

Traceability analysis

- Errors are eliminated by RESCOP (**r**edundant **s**keleton **c**onsensus **p**rocedure **a**nalysis)
- Statistical software model: Analyses neurite connectivity
- Disagreement rate less than 2- 5%

Evaluation of BROPA

X-ray micro CT imaging

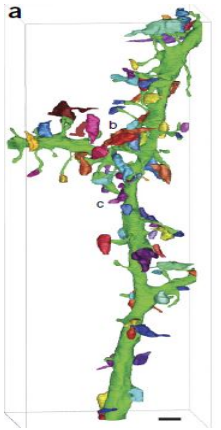


- Assessing staining uniformity, internal distortions or structural damage
- Image contrast similar to SEM
- Quickly test an embedded brain for defects and distortion
- Provide a complete data set without hidden defects

http://www.nature.com/nmeth/journal/v12/n6/extref/nmeth.3361-sv4.mov

Conclusion- paper 2

- BROPA appear to meet all requirements for complete neural circuit reconstruction
- Ultrastructure identifies chemical synapses, small neurites, spine necks, small-caliber axons
- Limited number of cells should provide insights into computational questions
- Combination of automated segmentation and manual proofreading might succeed in reconstructed a whole mouse brain one day



Thank you for your attention!