Lecture 8:
Extending Unbiased Stereology to 3DEM

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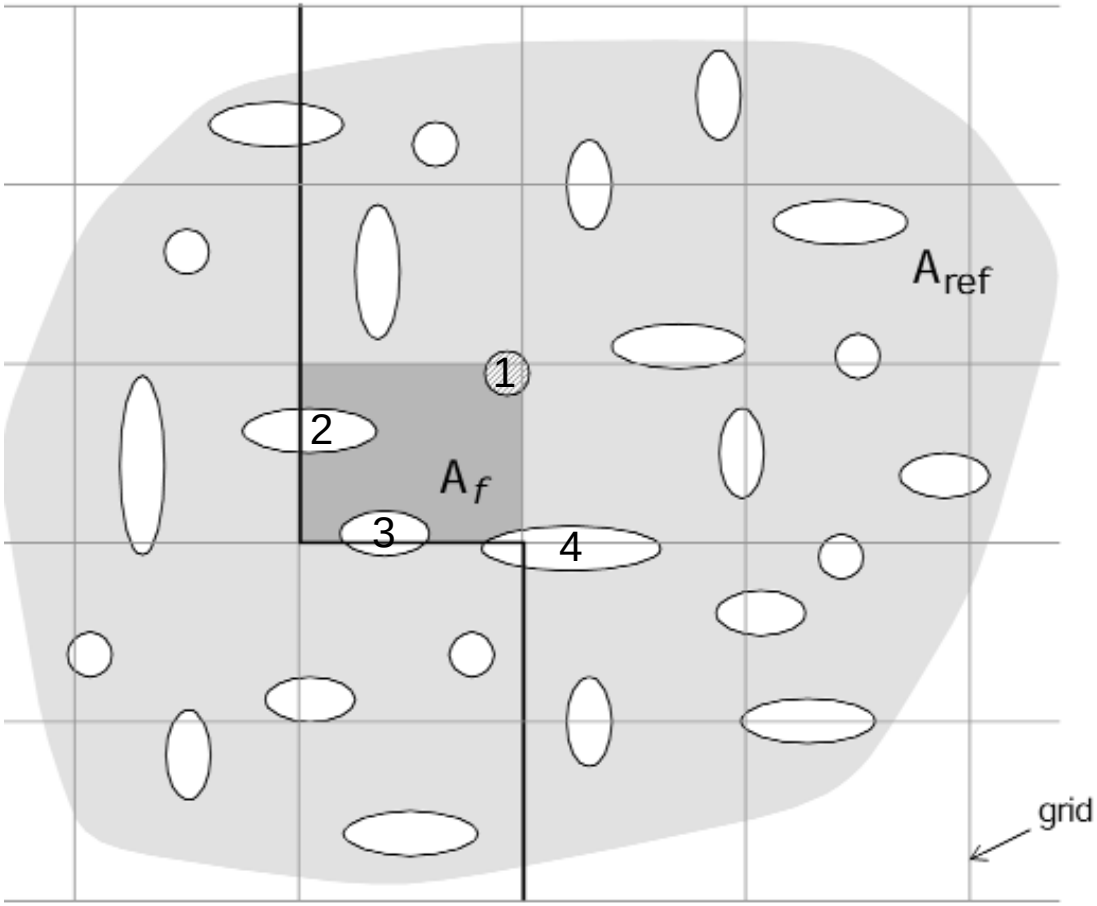
Required Reading:

Fiala and Harris, J. Am. Med. Inform. Assoc. 2001, 8:1-16.

What is Unbiased Stereology?

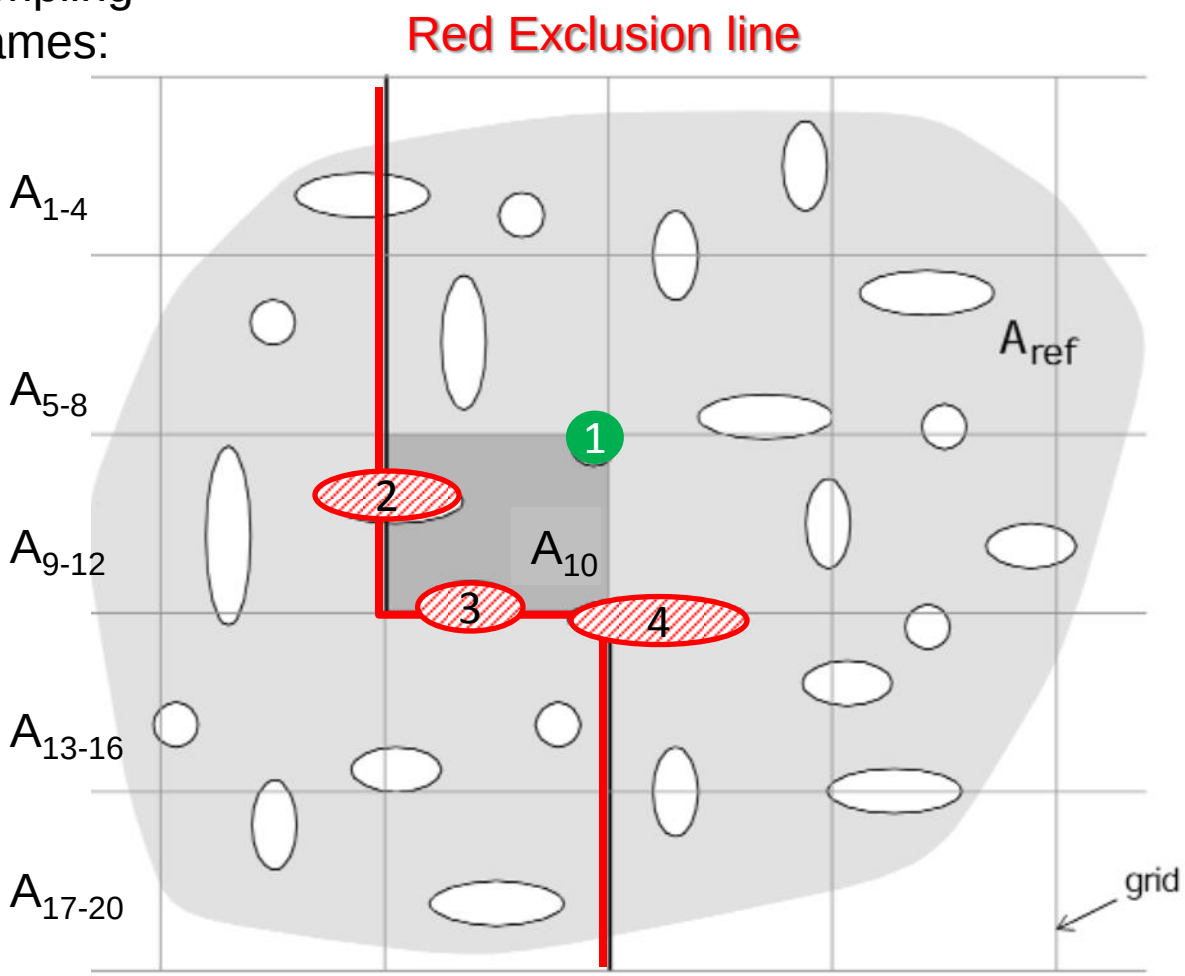
- Stereology is the science of quantifying 3D objects from their 2D profiles.
  - As seen in sections from light or electron microscopy, or single frames from tomography
- What does ‘unbiased’ mean in this context?
  - The sampling strategy ensures that the 3D object is not counted more than once based on profiles in the sections.

How would you avoid over-counting objects whose profiles appear in more than one sampling frame (A_f)?



Use exclusion lines

Sampling Frames:



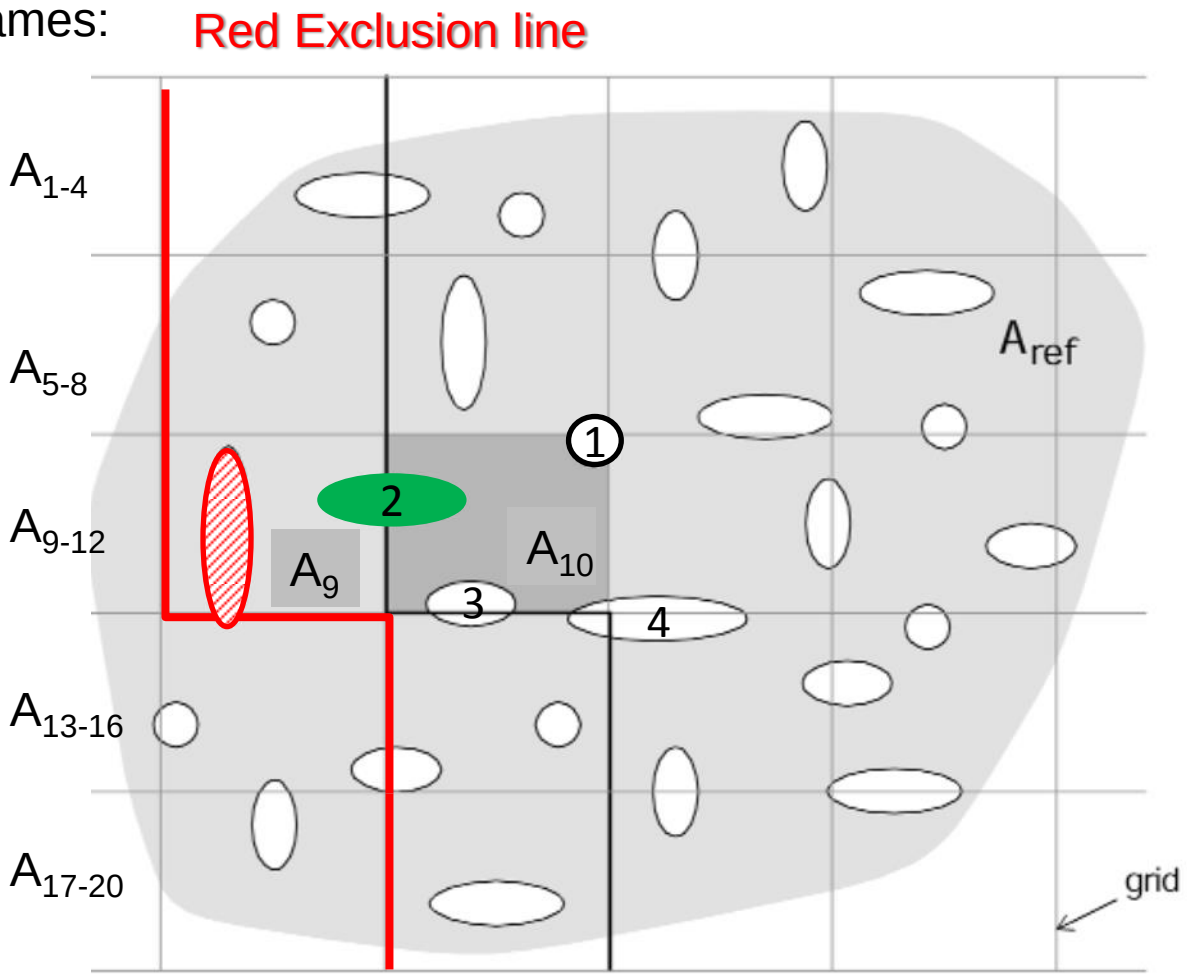
Consider Sampling frame A_{10}.

Only object 1 enters sampling frame A_{10} without touching the exclusion line.

The other 3 objects encounter the exclusion line and are not counted in this sampling frame.

Consider adjacent sample A_9

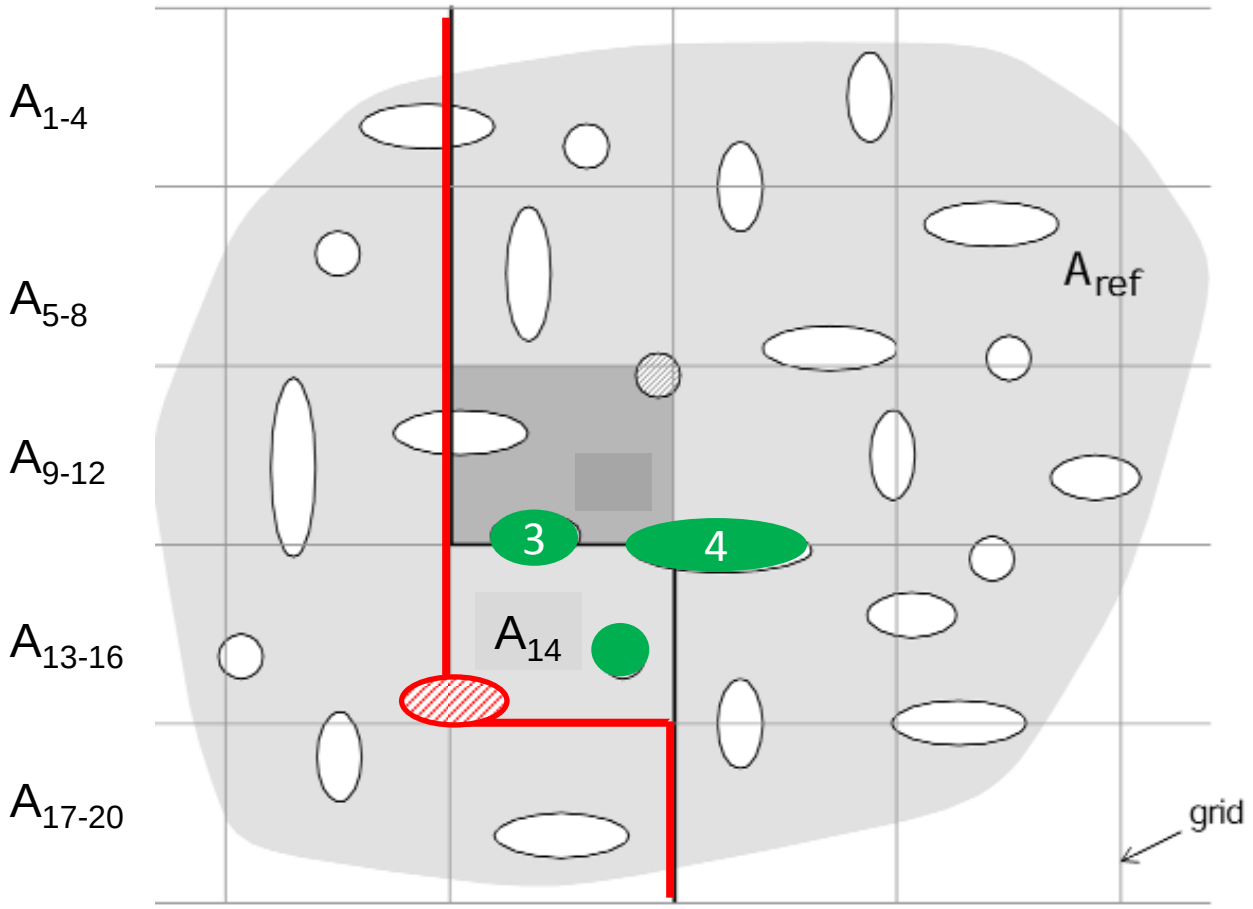
Sampling Frames:



Object 2 will only be counted in sampling frame A_9, not A_{10}

Consider adjacent sample A_{14}

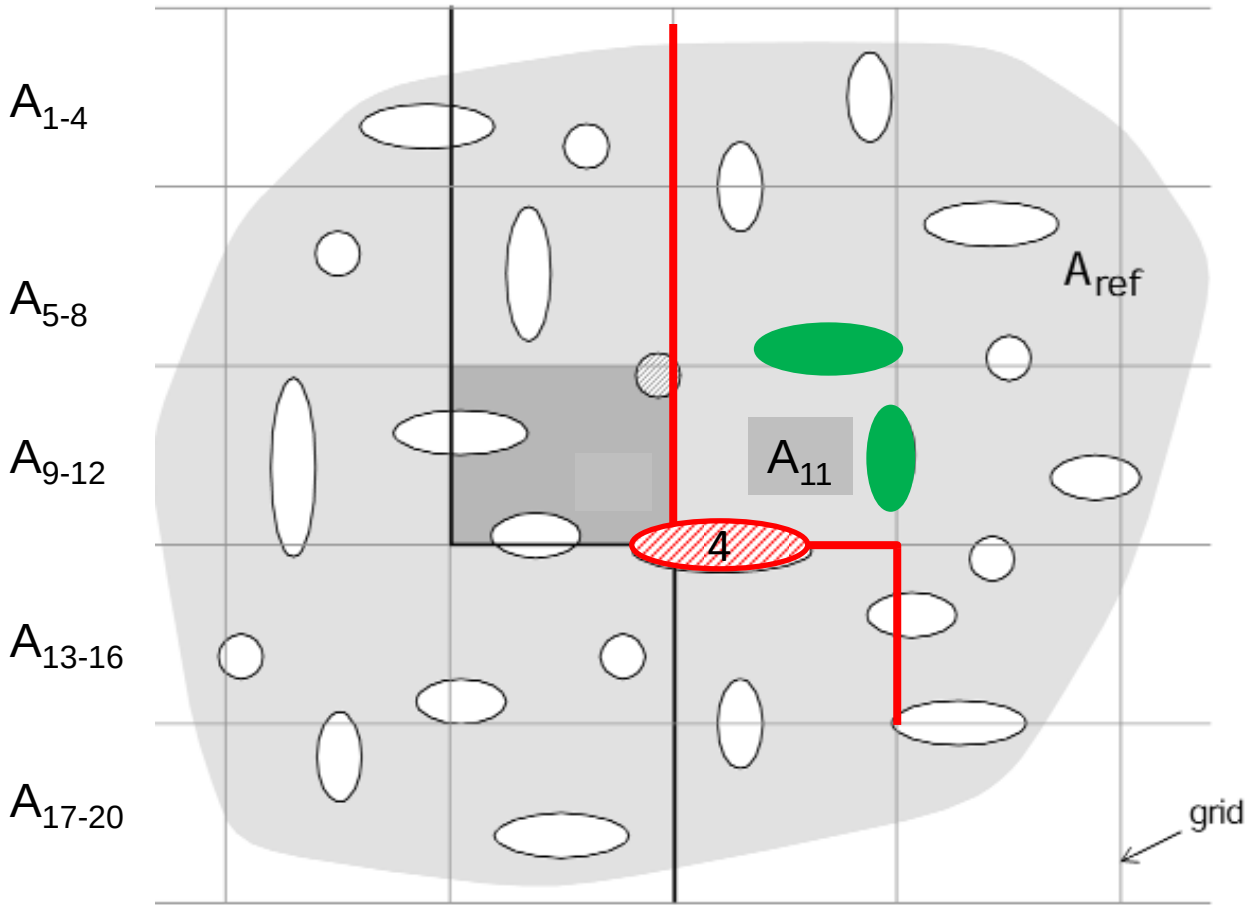
Sampling Frames:



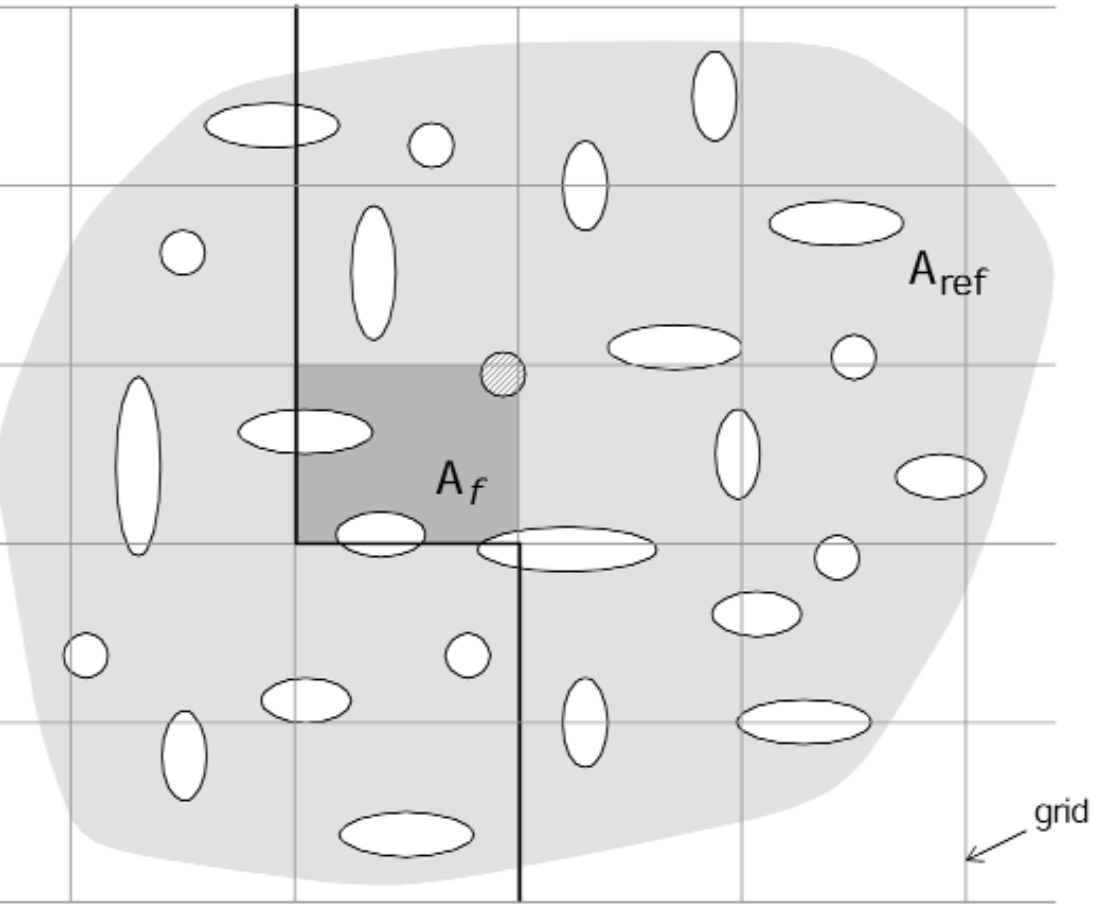
Objects 3 and 4 will only be counted in sampling frame A_{14}

But not in sampling frame A_{11}

Sampling Frames:



Now we can quantify the density of profiles (N_A) in A_{ref}



$$N_A = \frac{\sum_f Q_f^-}{\sum_f \beta_f A_f}$$

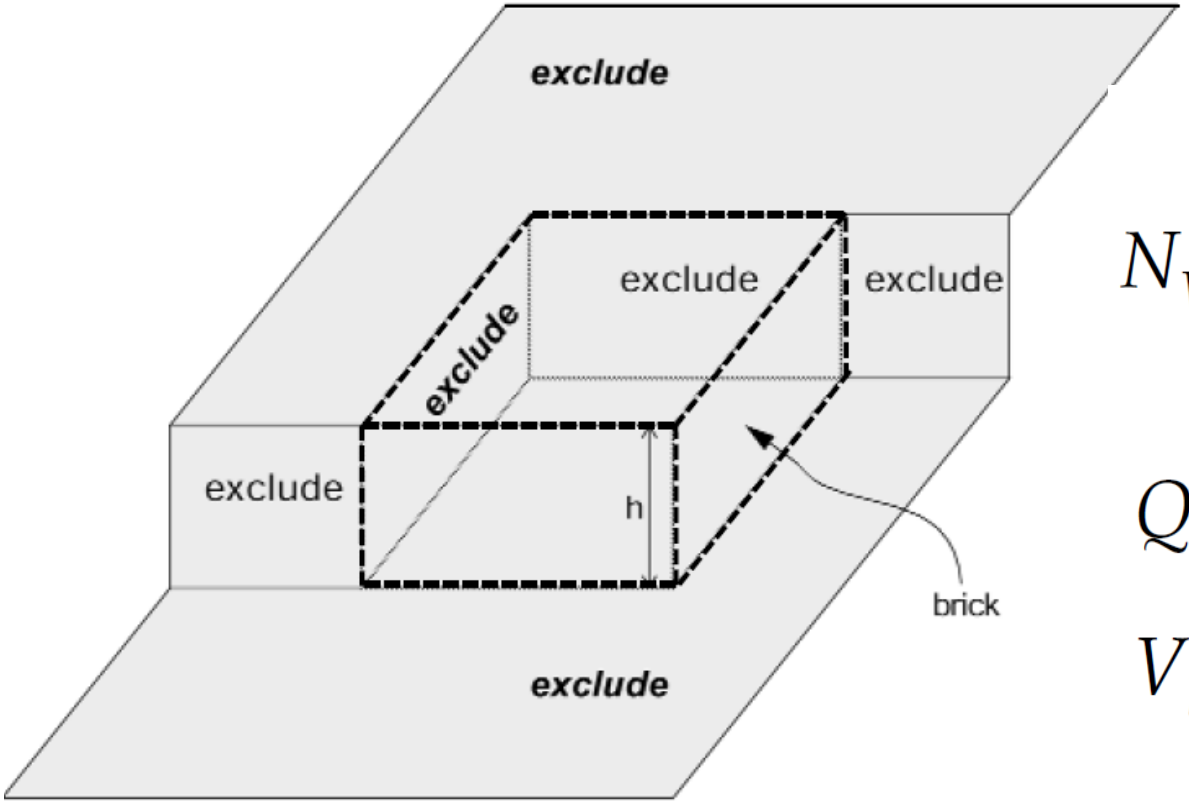
Q_f^- = count per frame

A_f = frame area (should be >3x larger than the largest profile)

β_f = fraction of frame within A_{ref}

Fiala and Harris, J. Am. Med. Inform. Assoc. 2001, 8:1-16.

Extending unbiased frame to unbiased Brick with exclusion planes



$$N_V = \frac{\sum_b Q_b^-}{\sum_b V_b}$$

Q_b^- = count per brick

V_b = brick volume (should be larger than the largest object)

Section thickness determination Needed for accurate volumes.

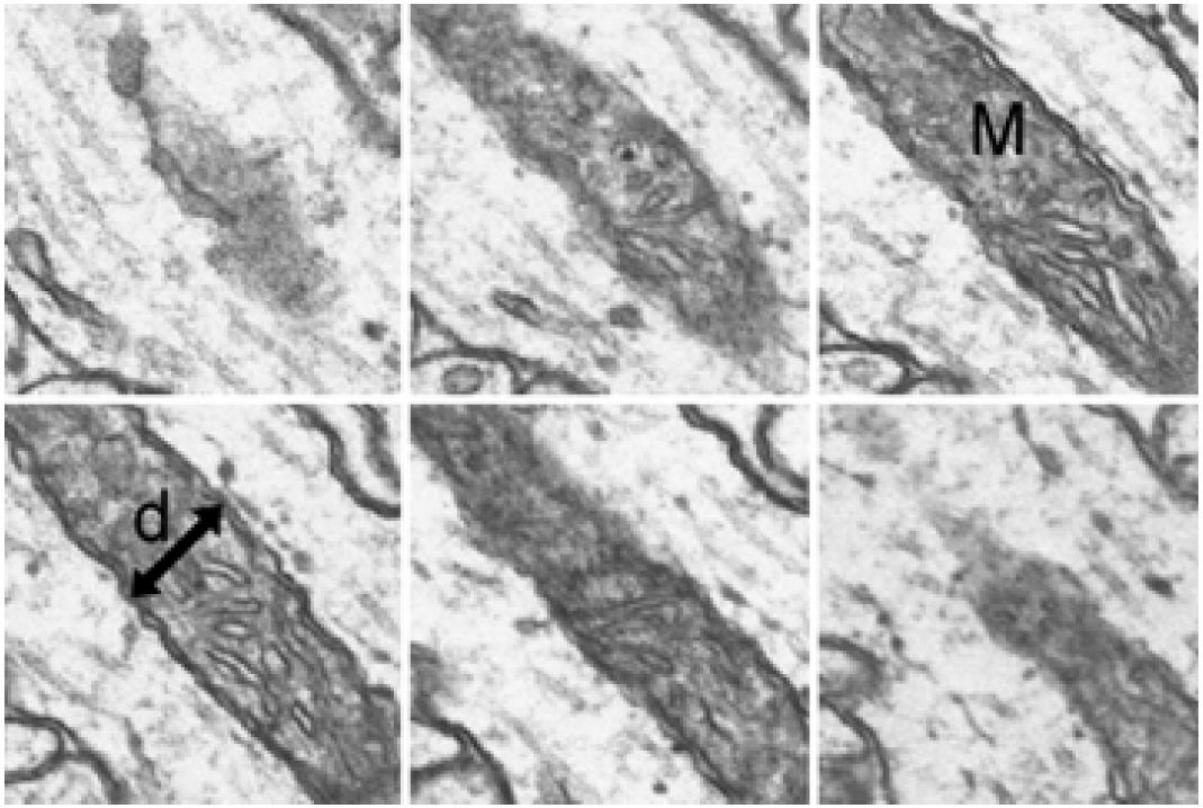


Figure 4 A sequence (*from upper left to lower right*) of six serial sections that pass longitudinally through a mitochondrion (*M*). At the central section, the diameter (*d*) is measured. Since the mitochondrion is cylindric, the ratio of the diameter to the number of sections spanned by the mitochondrion is an estimate of section thickness. On the first and last section, the mitochondrion may appear as a gray wall at the point where the diameter was measured. In such cases, depending on the darkness of the gray wall, a fractional value of either 0.25, 0.5, or 0.75 is used instead of the full section count. For the case shown, the mitochondrion spans four sections fully and about 0.25 sections at either end, for a thickness estimate of $d/4.5$.

For these projects, we have already determined section thickness and it has been entered into the series – you can find it in the section list.

Fiala JC, Harris KM (2001) Cylindrical diameters method for calibrating section thickness in serial electron microscopy. J Microscopy 202(3):468-472.

All brick should be same size otherwise can not take means of bricks:

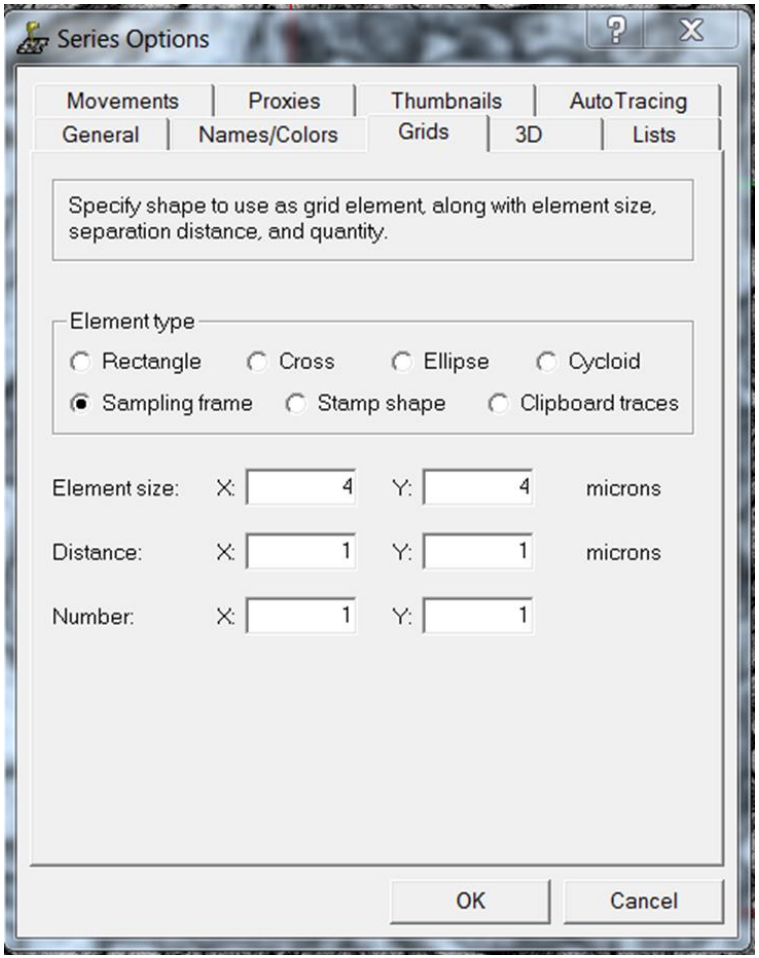
Mean of the samples

$$N_V = \frac{\sum_b Q_b^-}{\sum_b V_b} = \frac{1}{B} \sum_{b=1}^{B} \frac{Q_b^-}{V_b}$$

ONLY when ALL V_b in the data sets are the same.

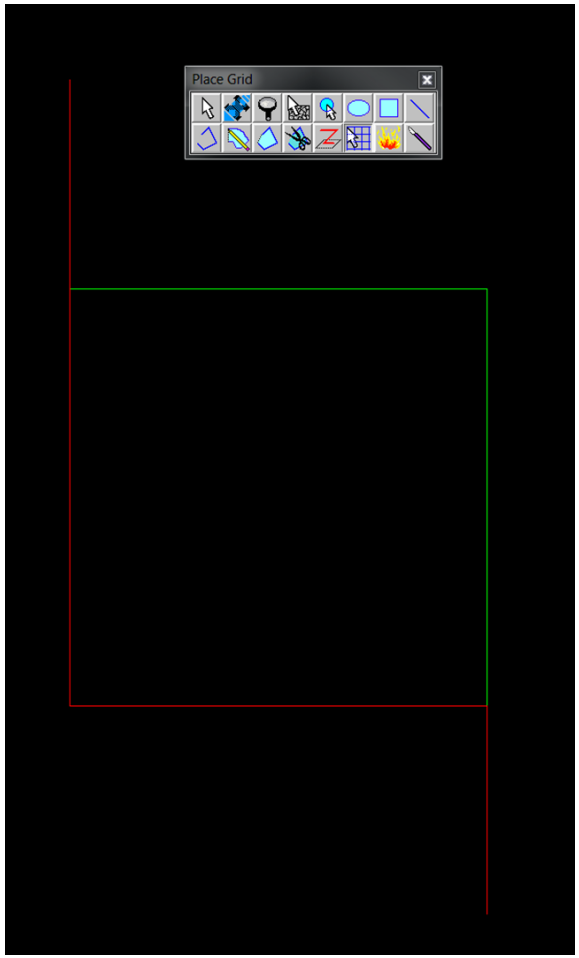
- For axonal boutons – determine maximum volumes and multiply by 2-3 (depending on number of sections available) for V_b
- Same for Spines – determine max volumes x2-3
- Make Bricks span enough sections to contain those volumes and draw your brick exclusion lines to be at least 2x the largest bouton or spine.

Creating a Sampling Grid in RECONSTRUCT™

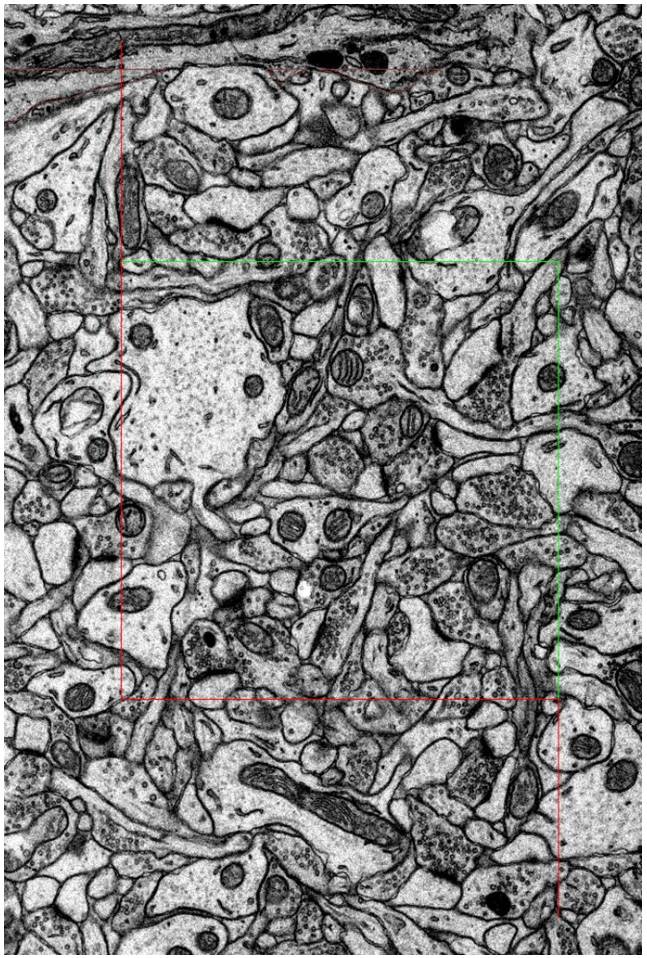


- Set up your Grid Tool in Series Option → Grids.
- Element size – use what is needed for your object – e.g. 4x4 microns is set here.

Use Grid Tool to place the Grid on your central section of interest:



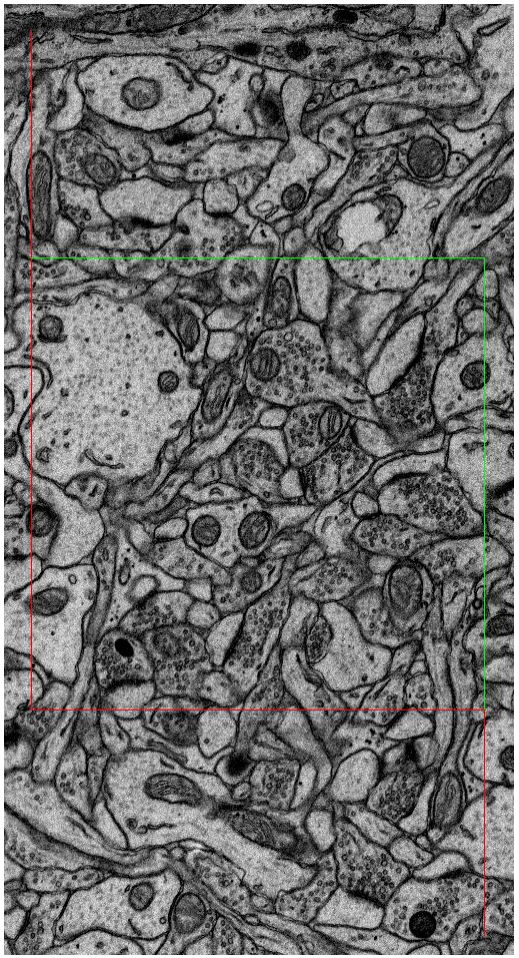
Sect 96



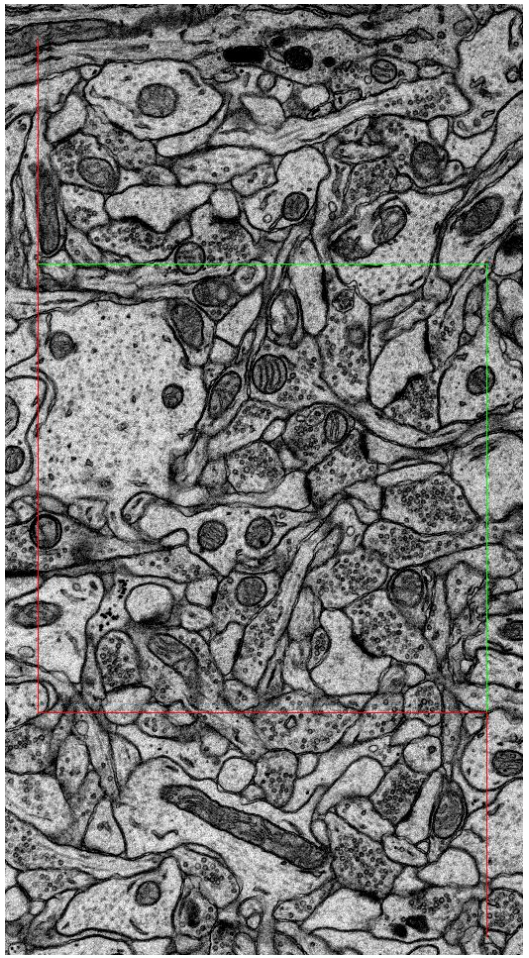
Red will make the exclusion planes, Green will make inclusion planes of the brick

Copy and paste to adjacent sections in both directions to span enough (~50).

Sect 95



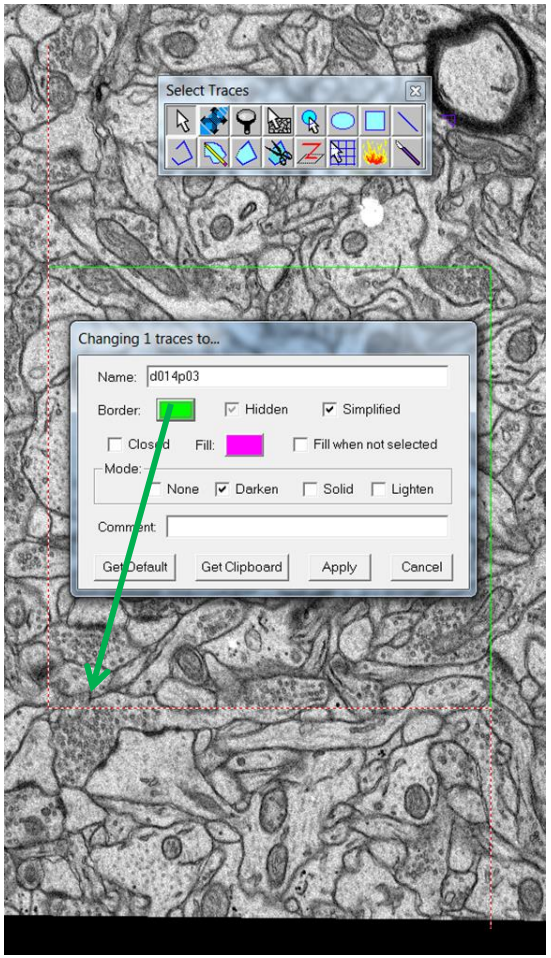
Sect 97



Do not try to place the bricking frame on every section, it is impossible to place it in the same place that way.

Top Inclusion plane = Last section of Brick: Count/include objects that cross this plane

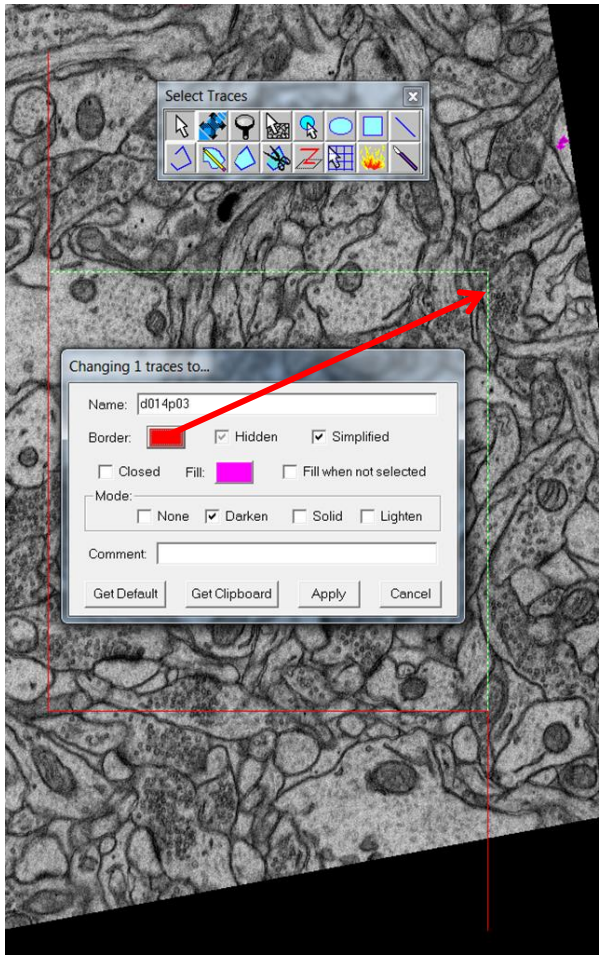
- Select the two red lines, do ctrl A and change the border attributes to green.
- Include an object that enters the brick from this side, as evidenced by its touching any edge or the middle of the frame (however, not the extended former exclusion lines alone).



Section 121

Bottom exclusion plane – First section of the Brick: Change all borders to Red

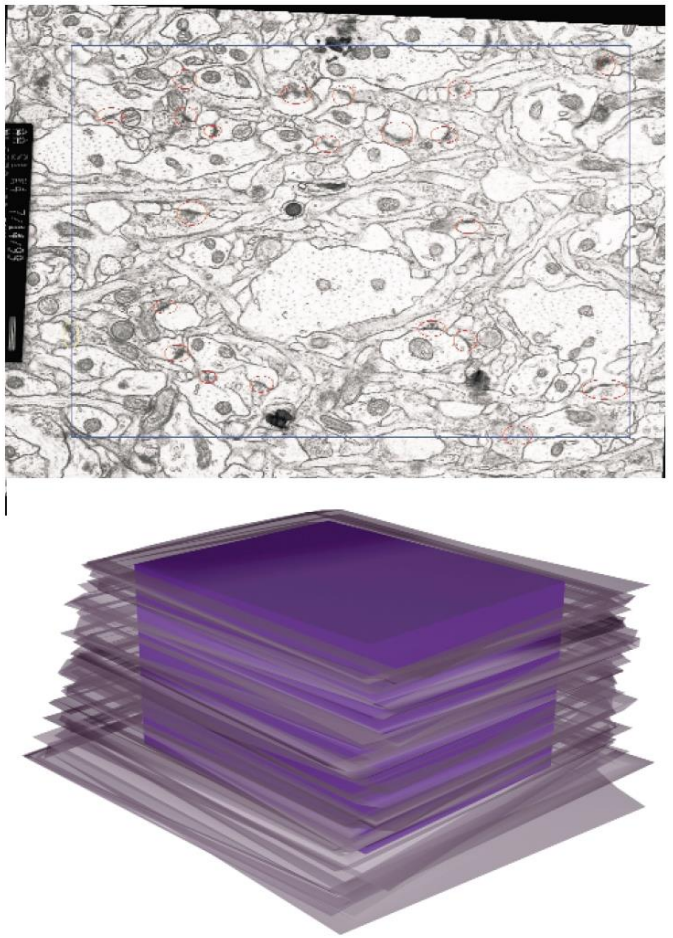
- Select the two green lines, do ctrl A and change the border attributes to RED.
- Exclude an object that enters the brick from this side, if it touches any edge, the extended exclusion lines, or the middle of the frame.



Section 71

Reference Volume (Fig. 10)

- The aligned sections are not perfectly overlapping.
- Hence, the overall reference brick (purple) is a subset of the image volume (gray).
- Multiple sample bricks within the reference brick should have non-overlapping exclusion planes.



Caveats regarding unbiased stereology

- For sample sizes less than objects of interest
  - Risk not being able to identify the objects accurately.
  - Introduce huge count variability
- Popular paired-section dissectors
  - Recommended to count at 100 synapses / set for accuracy – in this example that would be 33 pairs.
  - do not give correct counts (See figure 11)

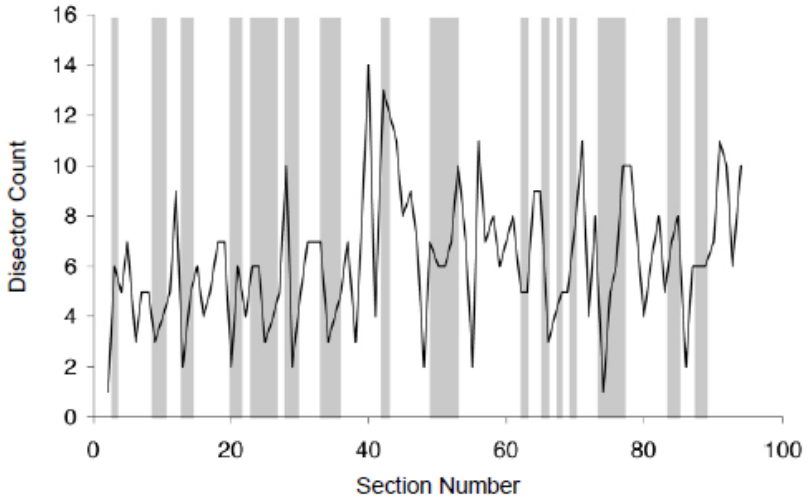


Fig. 11: Each pair of sections in the series represents a disector of one section thickness. The solid line shows the count (Q^-) obtained for each such disector. The gray bars show the random selection of 33 of the disectors for estimation of synaptic density.

Real synapse count in the reference brick:

- ***262 synapses / 100 µm³***

Random sets of 33 dissector pairs

- ***153 / µm³ - 242/ 100 µm³***

Four non-overlapping Sample bricks

- ***265 synapses / 100 µm³***

Issues of volume changes during tissue processing are corrected by ratios:

- We do not know exactly how tissue is distorted by the fixation, processing and dehydration steps.
- However, ratios of subsets of objects are not affected by this distortion.

Note typo in paper the S and O are reversed in this side of the equation

$$\frac{N_{V_S}}{N_{V_O}} = \frac{\frac{\sum Q_S^-}{V_T}}{\frac{\sum Q_O^-}{V_T}} \times \frac{\sum Q_S^-}{\sum Q_O^-}$$

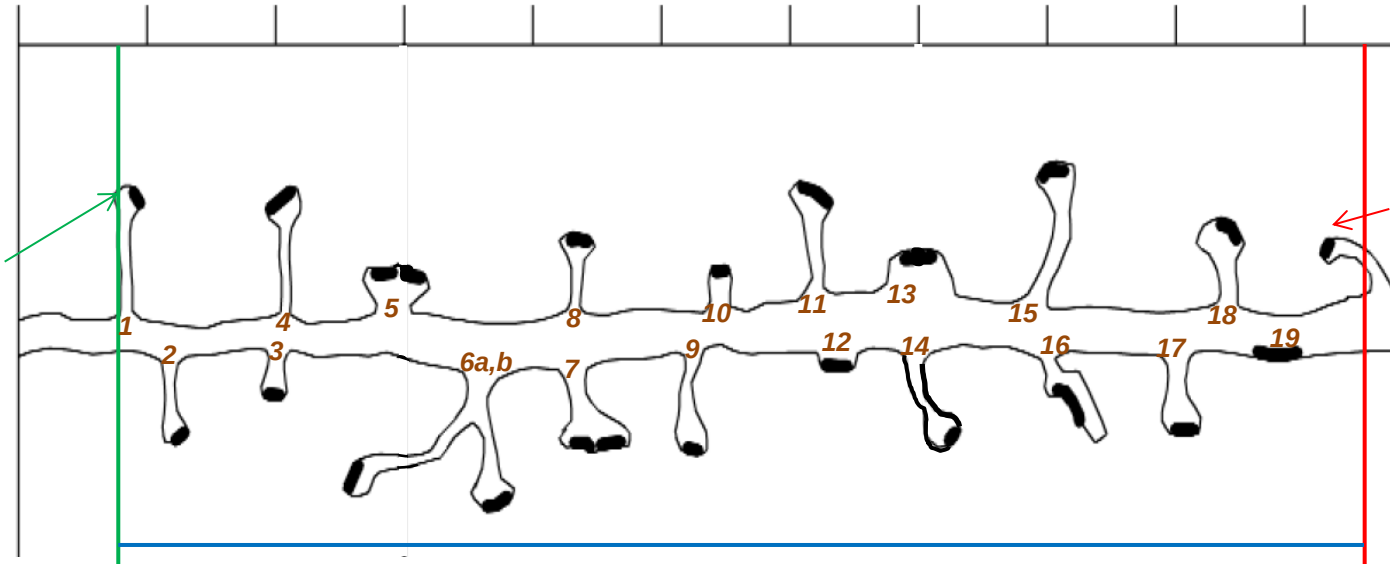
Q^- = count per brick
V = brick volume
O = all objects
S = subclass of the object
T = total volume

To compute the volume of your brick:

$$Brick\ Volume = X \times Y \times ST \times \#Sections\ Spanned$$

- X= Element x value; Y= Element y value;
- ST = section thickness.
- Note, the green and red lines are two open traces; hence, you can not compute the volume directly from them in RECONSTRUCT.
- Reconstruct could compute this value for you; but then you must trace the perfect closed square at the corners of your sampling frame and copy across sections – the math is simple enough.

Unbiased per-Length Analysis
Longest available:



Whole spine is reconstructed, cut-off based on origin

No part of the spine is included because last origin is excluded.

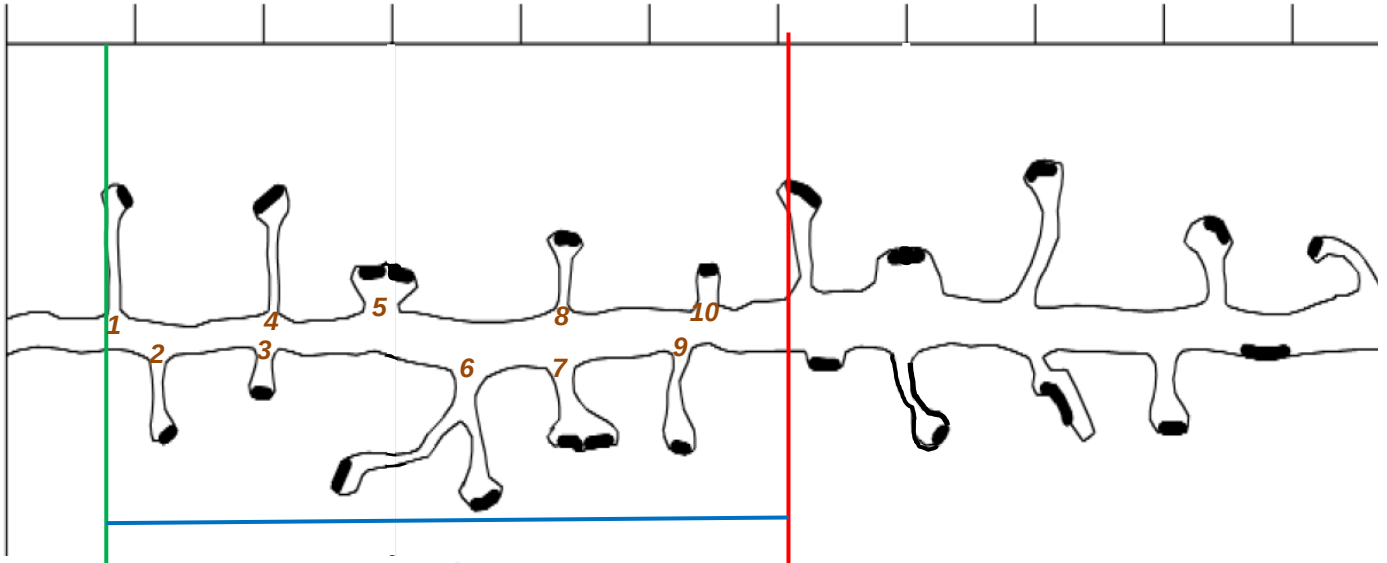
l

Begin left side of the origin of complete spine #1

End left side of the origin of last spine whether complete or not.

$$density = \#/l$$

Unbiased Length l_i should be matched across sample dendrites



Begin left side of origin of complete spine #1

End left side of origin of spine to give ~equal segment lengths across data sets.

Most reliable estimates for density $(\#/l)$ are achieved when the sample l_i dimensions are the same across conditions and series.