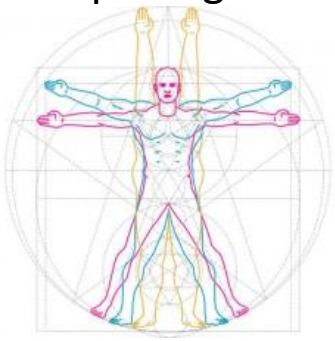
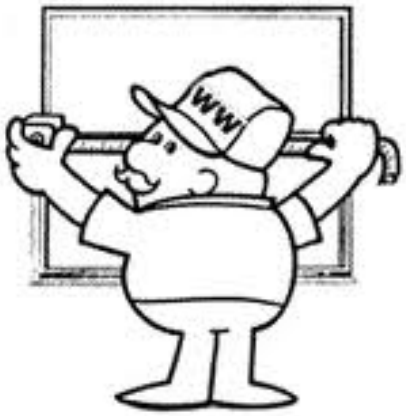


# Quantitation



- Sampling
- Morphometry
- Stereology
- Pattern Analysis
- Image Analysis

**ACA4 (2022) - Mike Mahon**
School of Medicine, Faculty of Medicine & Health Sciences
Keele University

# Questions

- Why measure ?
- What do you want to measure ?
- How do we measure ?
- Are the results unbiased, precise, accurate, valid, meaningful ?

# Questions

- Why measure ?
- What do you want to measure ?
- How do we measure ?
- Are the results unbiased, precise, accurate, valid, meaningful ?

# Why measure ?

" …. When you can **measure** what you are speaking about and express it in numbers, you know something about it; but when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of ***Science***, whatever the matter may be."

***Lord Kelvin (1883)***

# Why measure ?

- Obtain Absolute Data
- Variability / Constancy
- Relative / Comparative Data
- Experimental v Control Data
- Disease v Healthy Data
- Treatment v Control Data
- Data on Changes / Growth / Ageing / Differences
- Data on Structure / Function Relationships
- Data to Predict / Mathematical Models

**"The plural of anecdote is <u>not</u> data !"**

- Why measure ?
- What do you want to measure ?
- How do we measure ?
- Are the results unbiased, precise, accurate, valid, meaningful ?

# What? Things which may be measured

- Structures (3D)
  - Number, size, surface area, length, volume, shape, …
- Mass
  - Weights of organelles, cells, tissues, organs, people, ...
- Shapes & Arrangement
  - Macromolecules, organelles, cells, tissues, organs, people, …
- Chemical Constituents
  - Storage products, DNA
- Activity – Time (4D)
  - Enzyme activities, intracellular events, cell turnover, movement

See also 3D shapes – Geometric Morphometrics (Frigot) .. Next week!

What ?

# What to Measure

- Whole Body
  - Height, Weight, Girths, Composition, Anthropometry, Somatotype, Auxometry
- Whole organ
  - Volume: Water displacement, Specific Gravity, Hydrostatic weighing
- Size
  - Lengths, Widths
- Amount
  - Lengths, Lv; Surface areas, Sv; Volumes, Vv
- Numbers
  - $N_A$, Nv, N
- Shapes
  - Roundedness, Indentedness, S:V ratios, Form Factors, Tortuosity
- Orientations
  - Angles, Isotropic, Anisotropic, Branching
- Locations & Patterns
  - Random, Clumped, Dispersed, Related

What ?

# Examples

- Proportion of muscle, fat, bone, skin in limb
- Size of muscle fibres, Haversion systems, glomeruli …
- Number of neurons in the brain/ spinal cord/ DRG
- Number of fibrocytes per unit of tendon
- Length of capillary network in ligament
- Amount of lipid in heart
- Surface area of villi in gut, alveoli in lung
- Orientation/branching of Purkinje fibres in cerebellum
- Relationship of necrotic cells to capillaries
- Angle of pennation in muscles

What ?

# Applications

- Anatomy, Neuroanatomy, Embryology
- Histology, Histochemistry, Autoradiography, …
- Pathology
- Radiology
- Food Science
- Metallurgy
- Materials Science
- Geology
- Ecology
- Geography
- Social Sciences
- Astronomy

- Why measure ?
- What do you want to measure ?
- How do we measure ?
- Are the results unbiased, precise, accurate, valid, meaningful ?

How ?

" One ounce of thought is worth one ton of equipment."

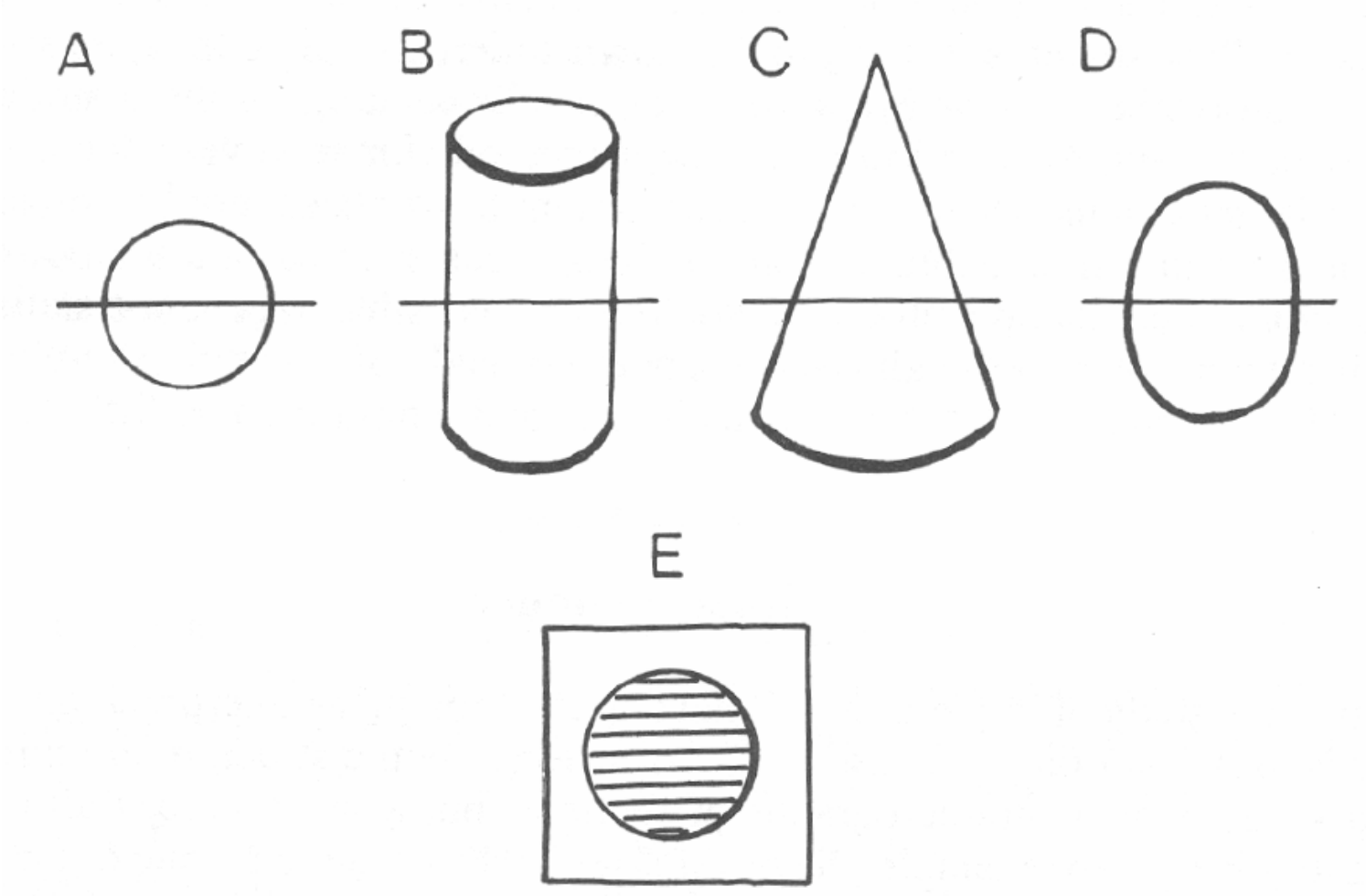
***Lord Rutherford***

How ?

# Pre-Quantitation


- Qualitative Analysis
  - Observation / Visualisation / Form / Organisation
  - Recording
  - 2D/3D Interpretation / Serial:Thick Sections / Reconstruction
  - Functional Interpretation
- Subjective Quantification
  - Variability
  - Amount, many, more, larger, ++++
  - Activity
- Relate to other levels of organisation, up, down
- Relate to other methodologies – Physiol, Biochem, Living
- Artefacts / Misunderstandings

# 2D < > 3D



# 2D < > 3D

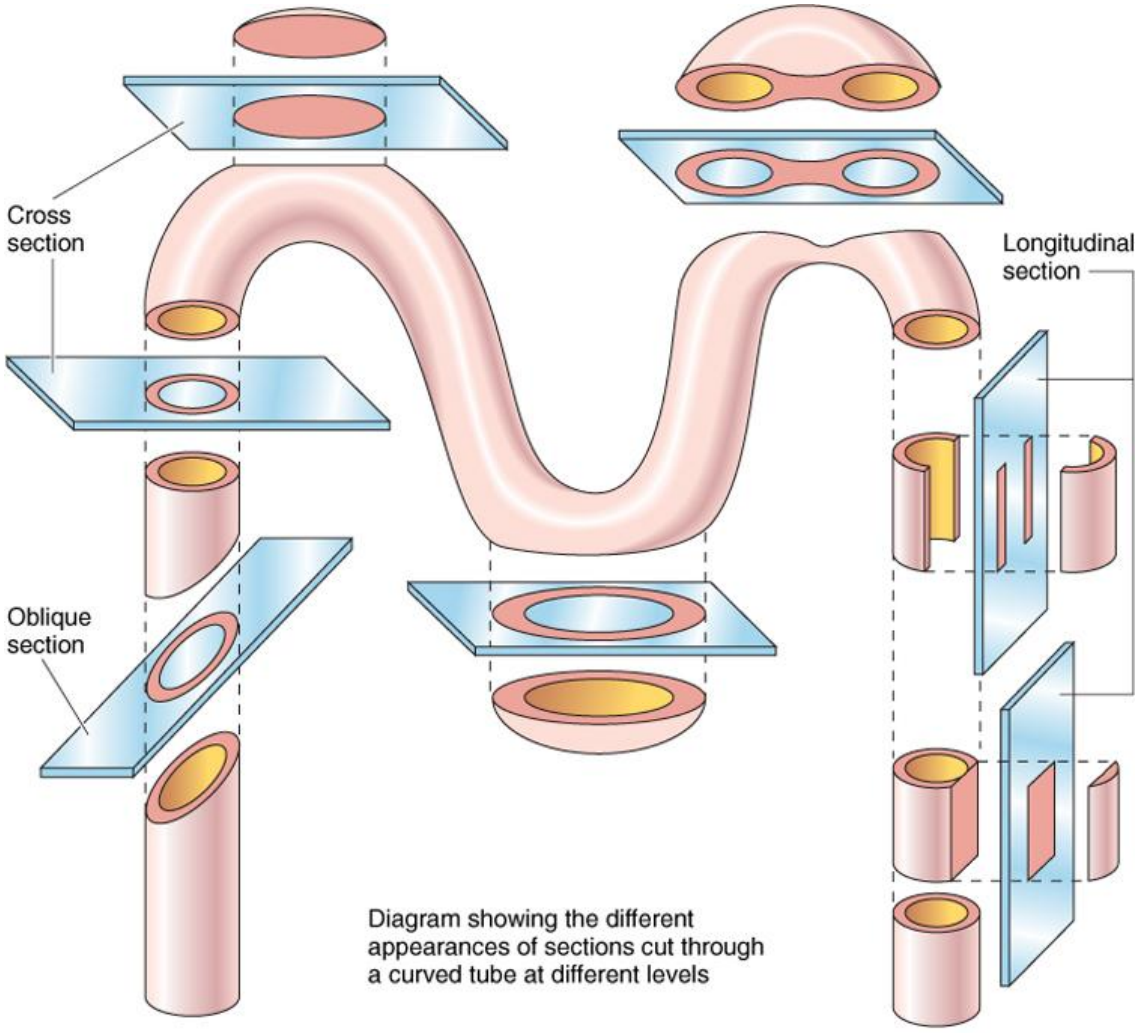
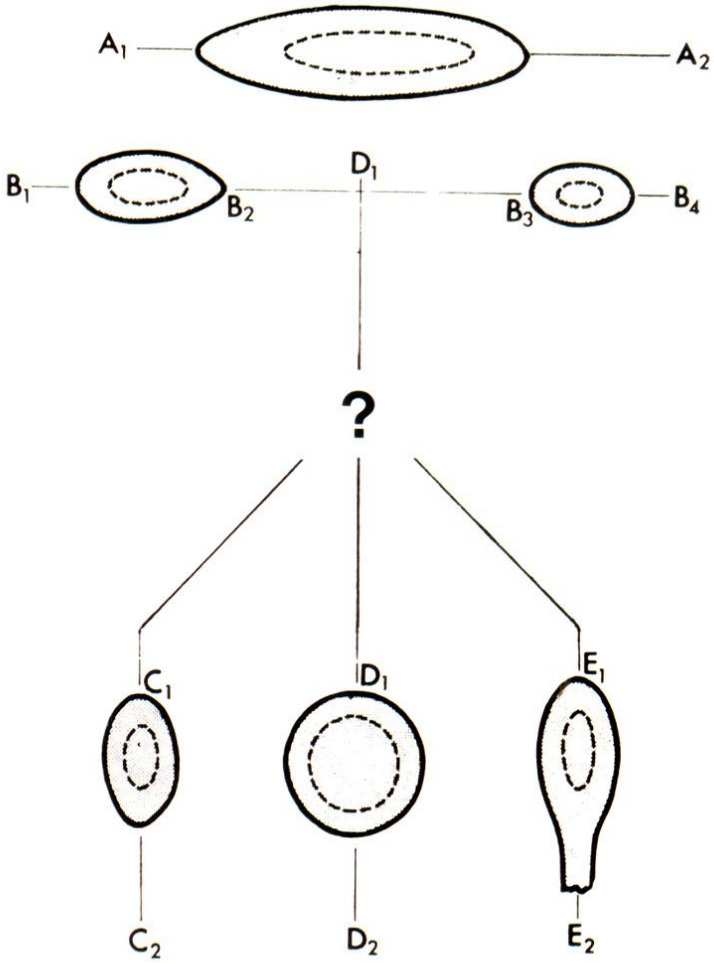
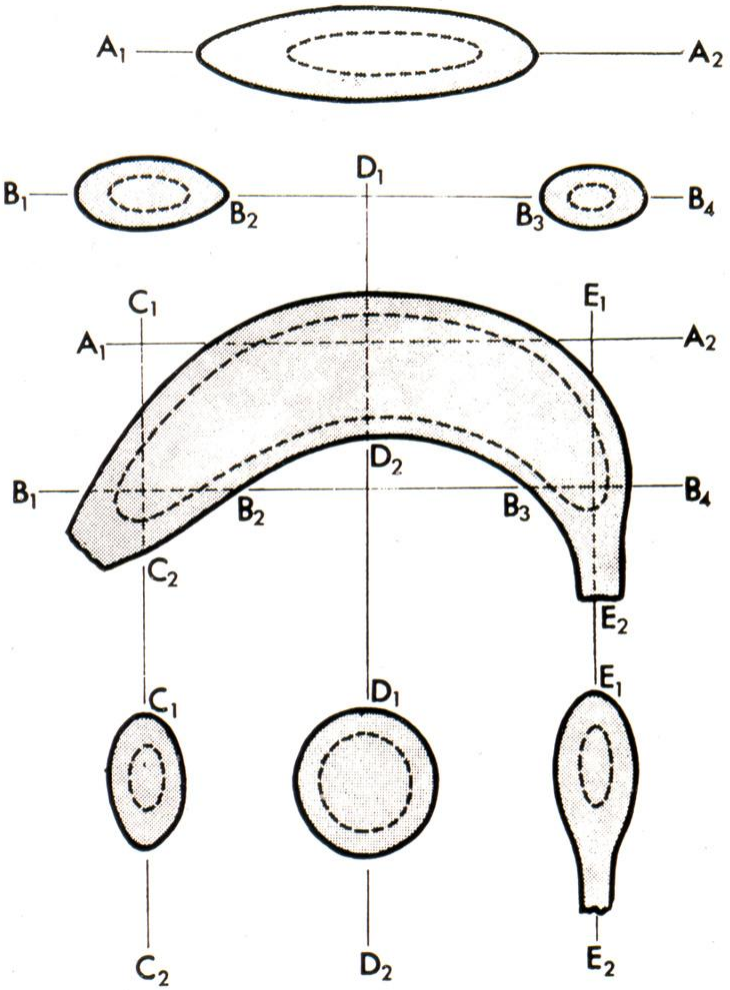



Diagram showing the different appearances of sections cut through a curved tube at different levels

© Elsevier. Gartner & Hiatt: Color Textbook of Histology 3E - www.studentconsult.com

# 2D < > 3D


A1
A2
B1
B2
D1
B3
B4
?
C1
D1
E1
C2
D2
E2


A1
A2
B1
B2
D1
B3
B4
C1
E1
A1
A2
D2
B1
B2
B3
B4
C2
E2
C1
D1
E1
C2
D2
E2

How ?

# Quantitation

## Optical Properties

- Analytical Microscopy
  - Reflectometry, Phase Contrast/Refractometry, Polarising, Interference, 'Weigh cells', Microdensitometry
- Semi-Quantitative
  - Rating scales, ++++

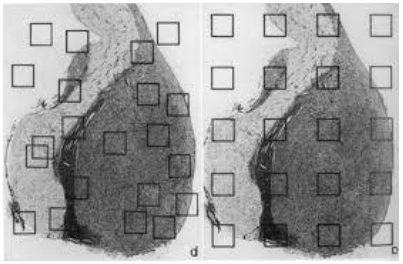
## Geometrical Properties

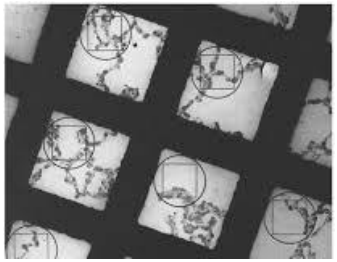
- Sampling
- Morphometry (… directly)
- Stereology (… indirectly 2D/3D)
- Pattern Analysis
- Image Analysis

How ?

# Sampling

- Identify Object
  - Staining
  - Magnification
- Representative
  - Is tissue Homogeneous (Isotropic) / Irregular (Anisotropic)
    Heterogeneous (Anisotropic)
    Gradiential (Anisotropic)
- Random
  - Completely random
  - Systematic stratified random sampling
  - Zonal oblique sector analysis

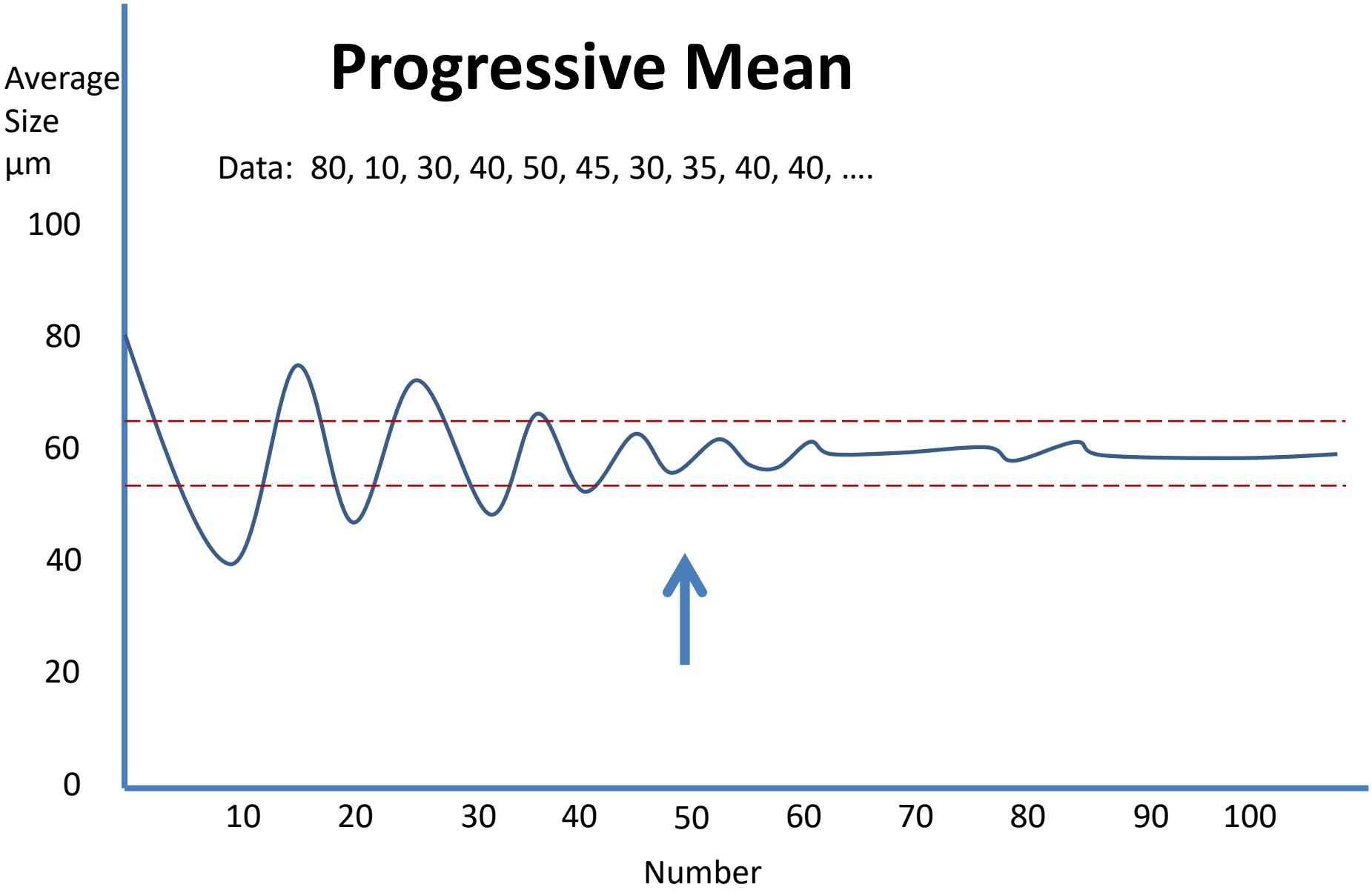




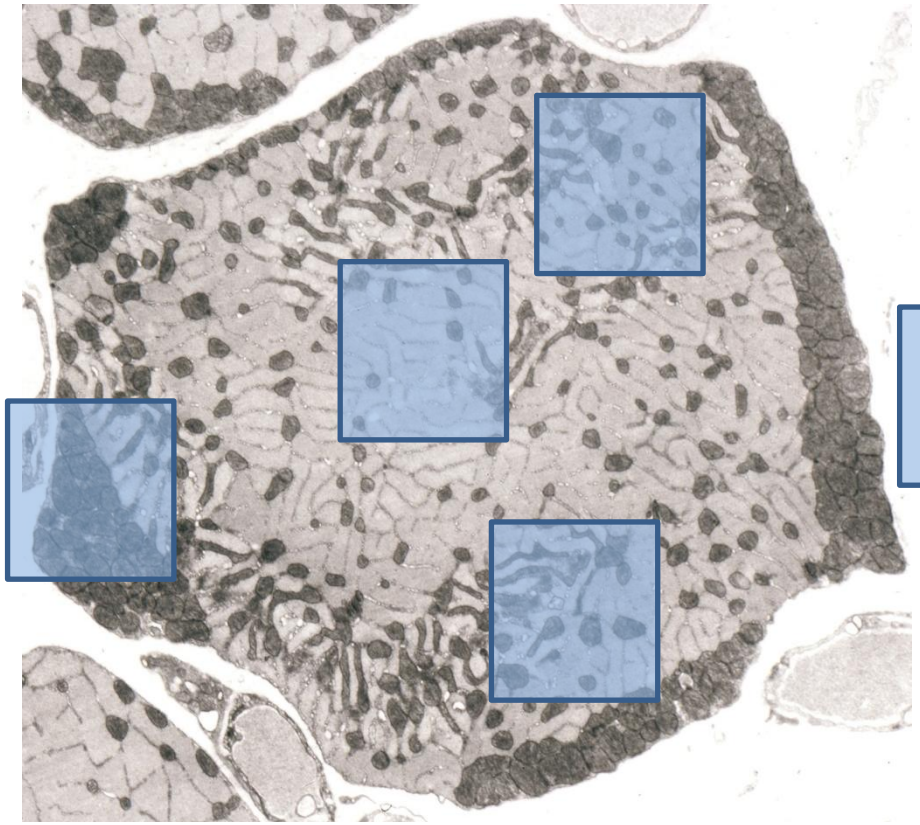
- Manual / Automated
- How many samples? (Experimental Design)
  - Individuals / Organs / Blocks / Sections / Micrographs / Items / Measures
  - Hally Formula RSE= SQRT (1-Vv)/SQRT n
  - Progressive mean, Log Plots
  - Do More, Less Well !

# Progressive Mean

Data: 80, 10, 30, 40, 50, 45, 30, 35, 40, 40, ….



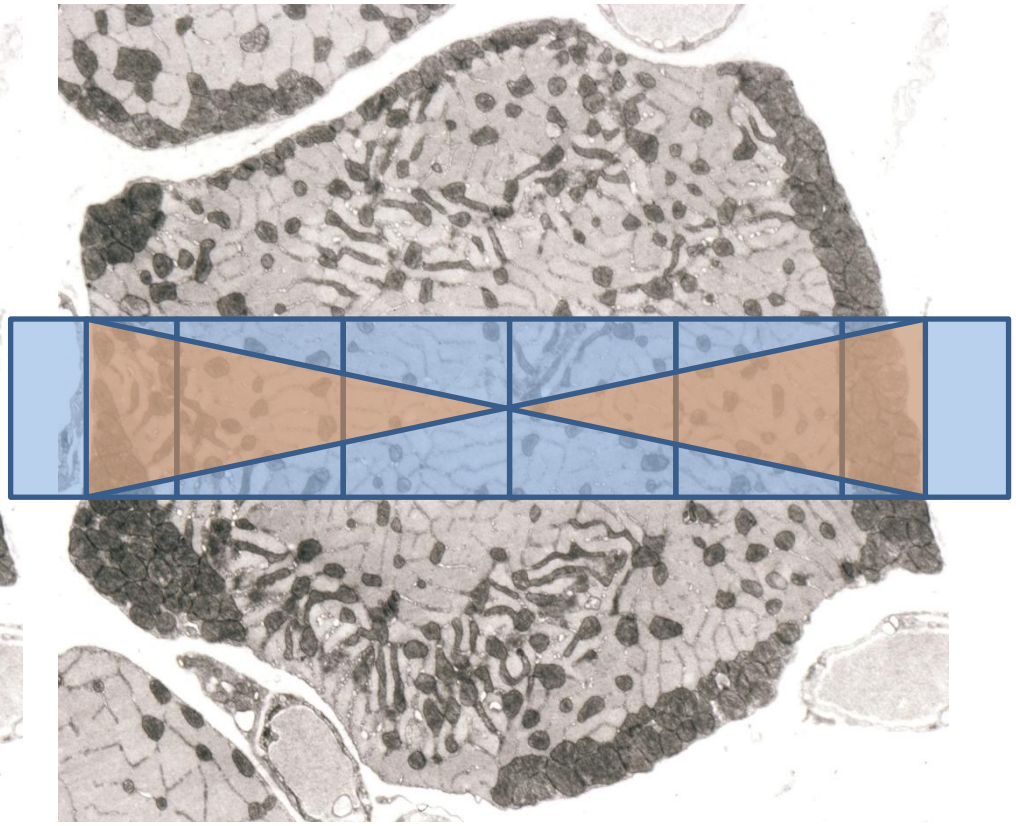
## Skeletal Muscle – Random Sampling Micrographs at x18,000



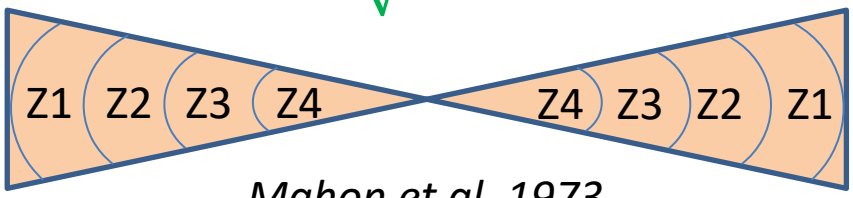
X

Sampling: $\leq 1, RSE, Prog.Mean$

## Skeletal Muscle – Zonal Oblique Sector Analysis Method (ZOSAM)



√



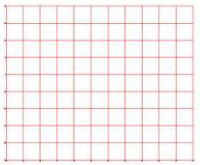

*Mahon et al, 1973*

How ?

# Which Method ?

- Morphometry
  - direct measurement of structures

- Stereology
  - extrapolation from 2D to 3D using simple counting methods



- Image Analysis
  - combination of above using digital imaging and computers in manual or automatic modes
  - plus data presentation and analysis


See Russ, 2019 Robust Measurements

How ?

# Morphometry 1

Measurement of Form

- Magnification calibration

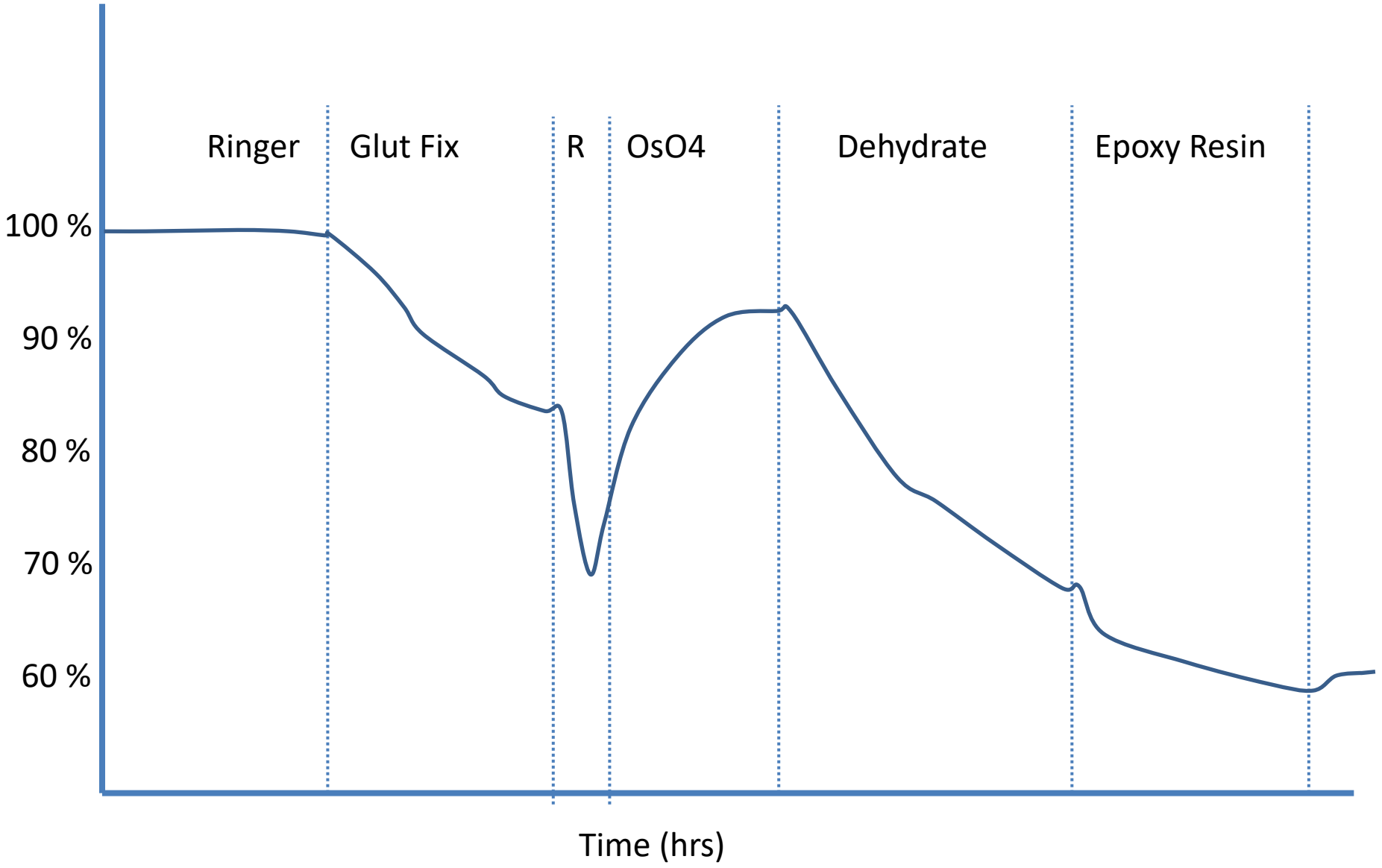
  - Known Objects, Graticules, Beads, Crystals, Hysteresis, Axes (printing, distortion)
- Section Thickness
  - Weigh, Calipers, Inteferometry; Holmes effect
- Preparation effects
  - Fixation, dehydration, embedding, sectioning, staining
- Equipment & Time available / Costs

# Tissue Preparation Effects

- Fixation Effects (Sissons, Goldspink, Strickland 1960s, 1970s)
  - Flemmings - 3-15%
  - Carnoys -19-36%
  - Bouins -23%
  - Formalin -23-30%
  - Zenker -30-40%

- Length change 10%
- Areal change 21%
- Volume change 33%

- Need a Standard ….. Ringers, Frozen, …


Ringer
Glut Fix
R
OsO4
Dehydrate
Epoxy Resin
100 %
90 %
80 %
70 %
60 %
Time (hrs)

# Practical

**Part**

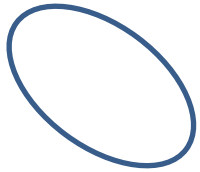
1. Magnification Calibration
2. Morphometry
3. Stereology
4. (Pattern/Shape analysis)

How ?

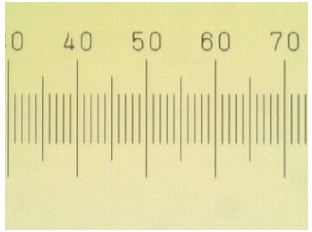
# Morphometry 2

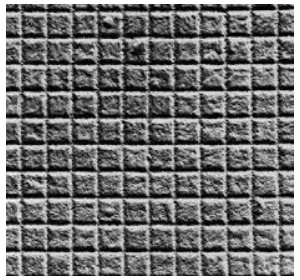
## Measurement of Form

- Volumes, Lengths ⚠ , Surfaces ⚠, Numbers ⚠ ⚠ ⚠, Thickness
- Diameters & Areas
  - Longest, shortest/narrow, Orthogonal, Feret, Average, Random chord
  - From area ($\pi r^2$), Circle of best fit



- Shape
  - Axial ratio, S:V ratios or Form factors($A/P^2$, $1=4\ \pi A/P^2$), Angularity (Gulfs & Peaks)
  - Shape of best fit (Identikit), Reconstruction
  - Fourier Analysis, Fractals (Mandelbrot)
- Equipment
  - Rulers, Calipers, Graticules, Stage Micrometer, Cut & Weigh
  - EM: Diffraction Grating, Latex Spheres, Crystals
  - Filar micrometer, Image Shearing micrometer
  - Photographs, Drawings, Projection (Camera Lucida/Drawing Tube)
  - Thread, Map Measurer (Opisometer), Planimeter
  - Stereological lattices
  - Image Analyser



# Length

- Dependent on Magnification
- How long is the coastline of Britain? (Mandelbrot (1967) Science)

U 200 km
L 2400 km

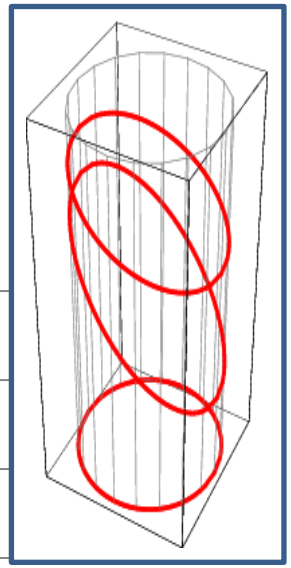
U 50 km
L 3400 km

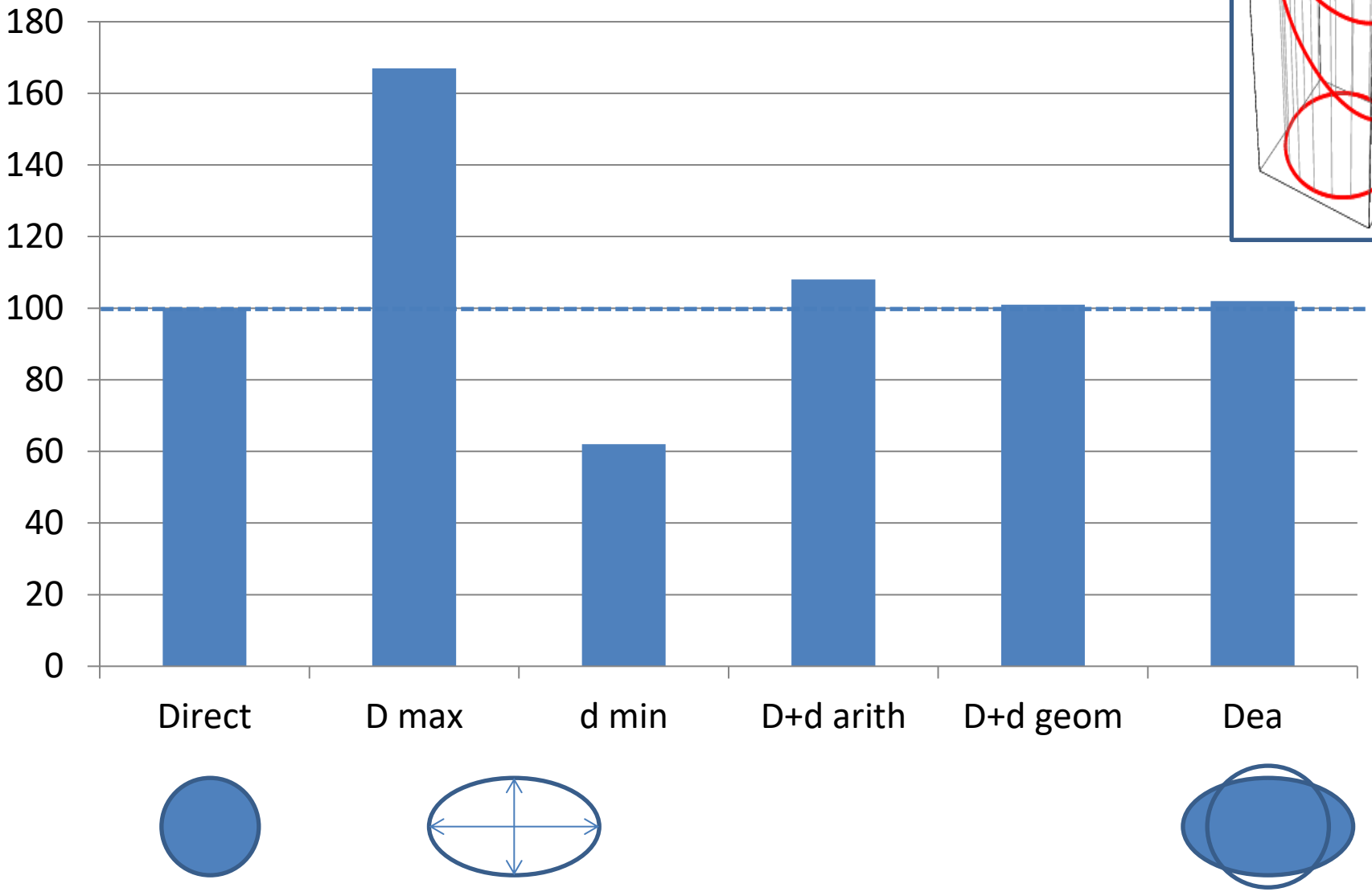
U ?? km
L 12450 km

[See also Russ et al 2018
The Problem of Perimeter]

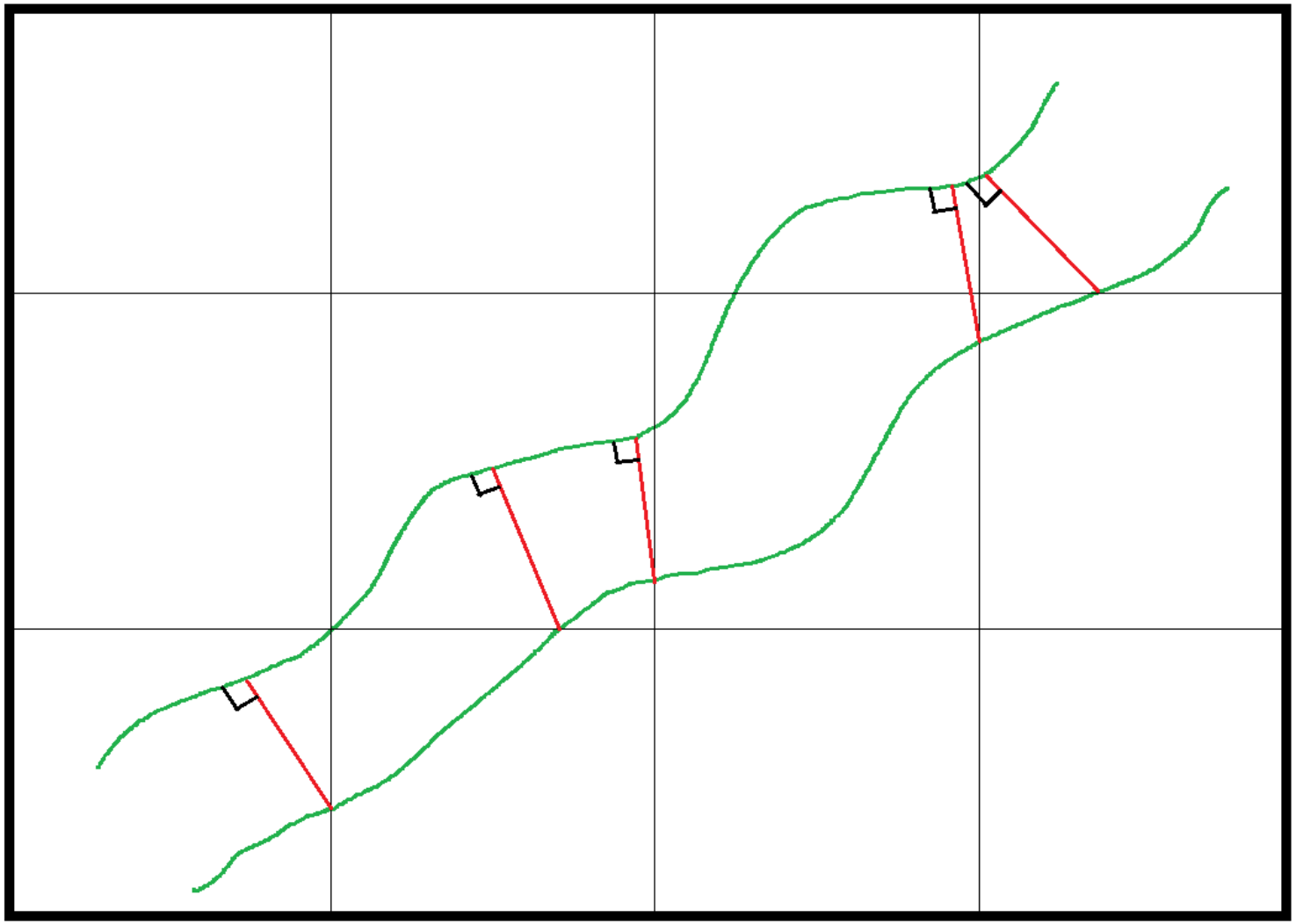
Fractal Dimension of Wigglinesss 1.25 !

# Diameter (Muscle Fibres)

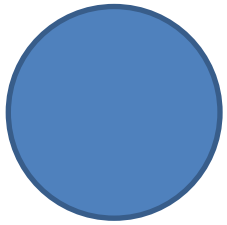


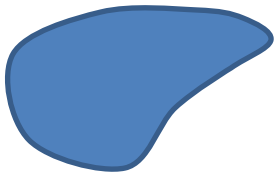


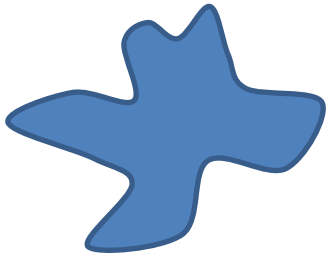
Thickness Measurements



# Shapes

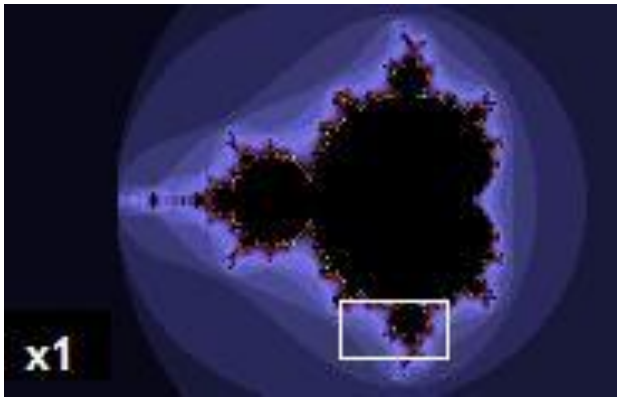


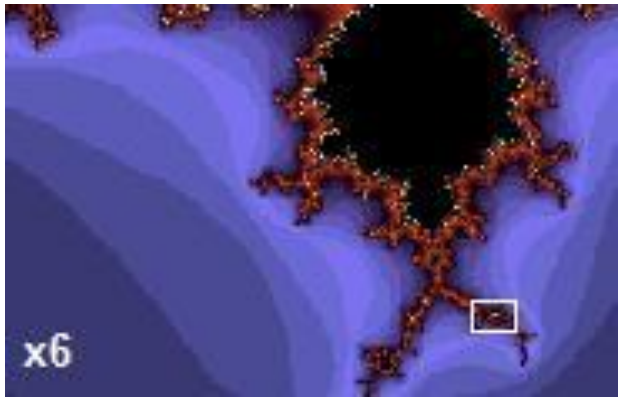


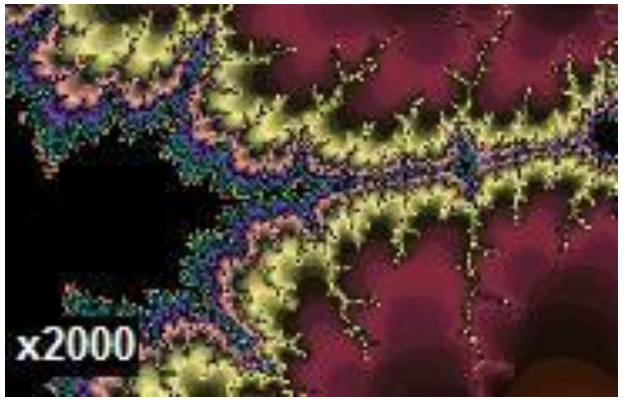


| | | | |
|---|---|---|---|
| Form Factor | 1.0 | 0.8 | 0.4 |
| Gulfs | 0 | 1 | 5 |

$F=(4 \times \pi \times \text{Area})/\text{Perimeter}^2$



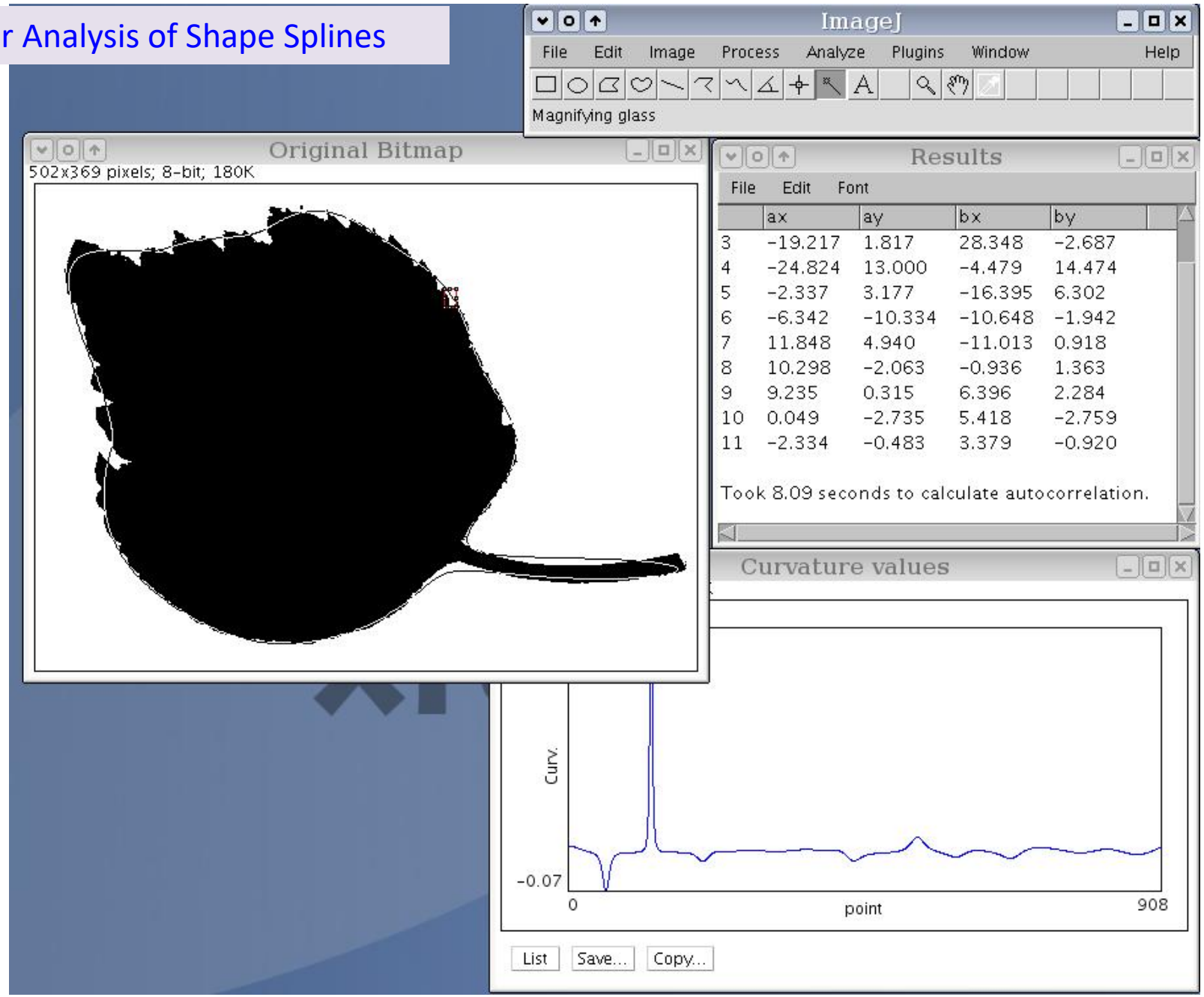



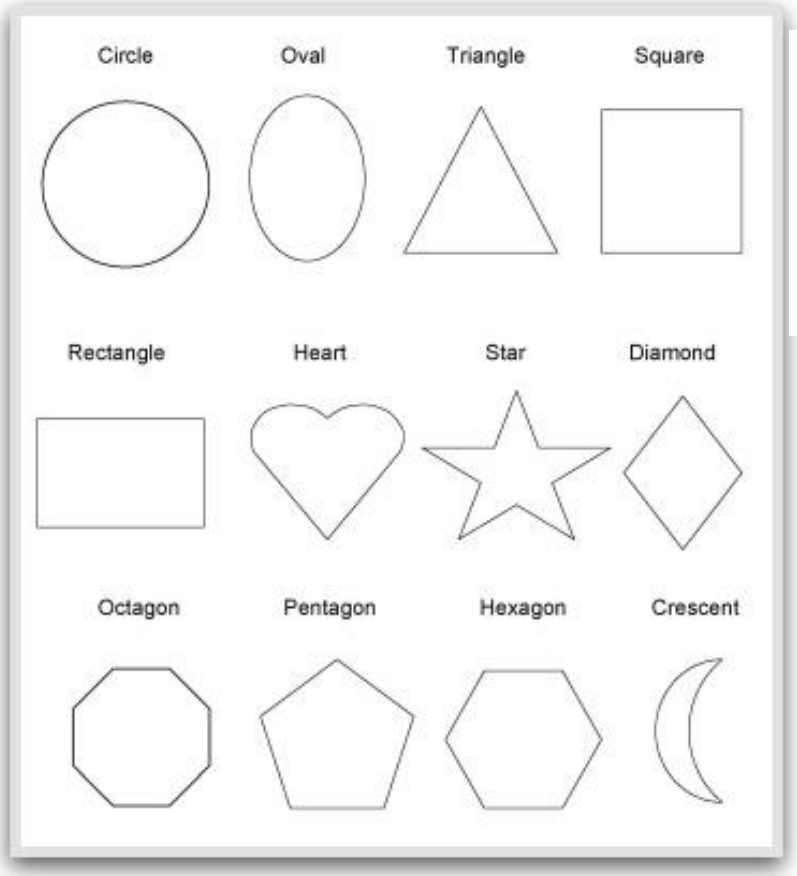



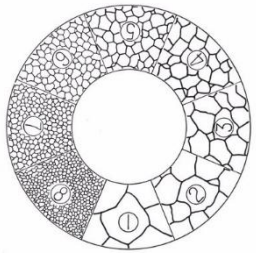

Fractals – Mandelbrot Set

See Mandelbrot, 1982 The Fractal Geometry of Nature

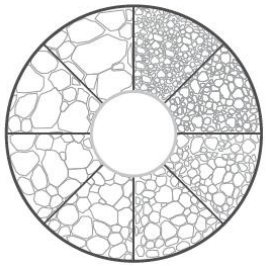
# Fourier Analysis of Shape Splines

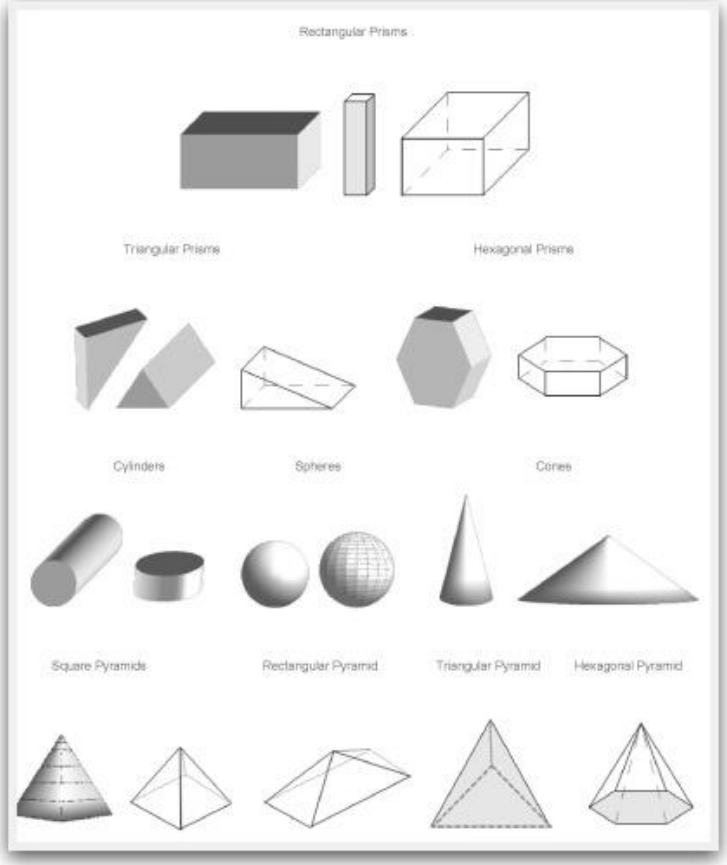




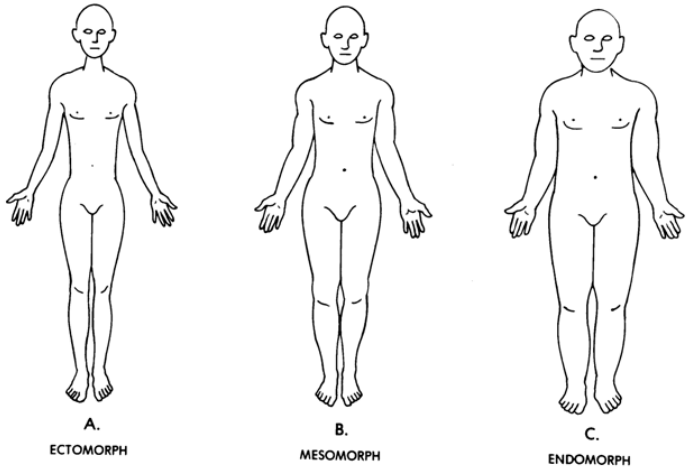



Identikit Match





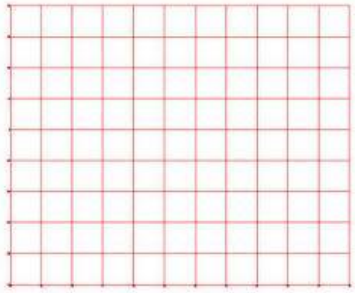

Somatotypes



How ?

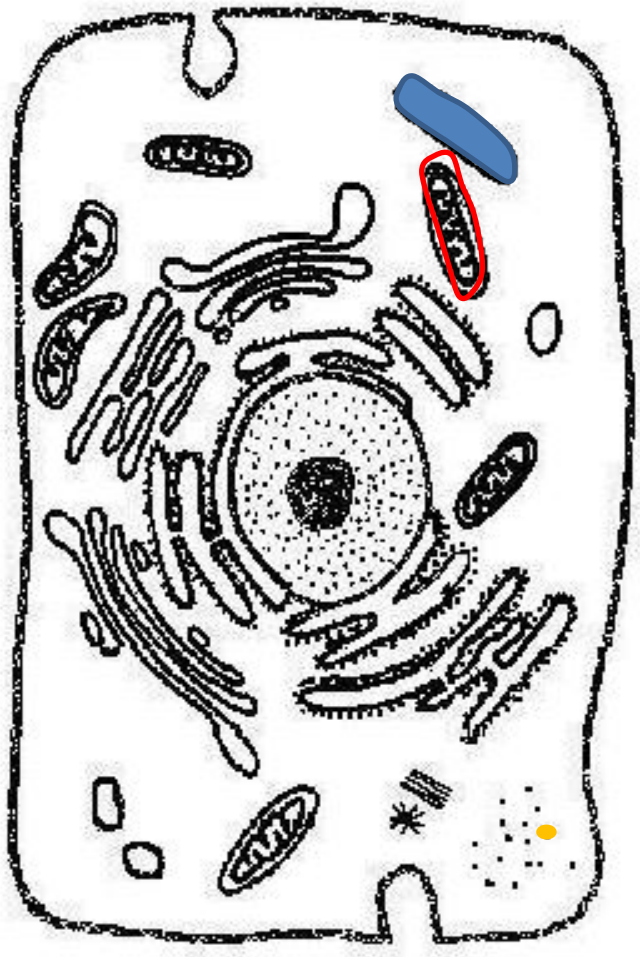
# Stereology 1

Extrapolation from 2D to 3D



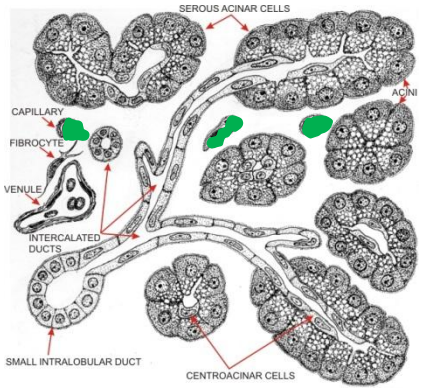
Geology, Metallurgy, Biology, Engineering, Astronomy

- History
  - 1777 Buffon
  - 1850s Delesse, Sorby
  - 1961 ISS, Models; Elias, Weibel, Williams, Mayhew, Cruz-Orive
  - 1983 Unbiased/Designer: Gunderson (Cavalieri, 1635)
- Dimensional Reduction
  - Volumes, Surface Areas, Lengths, Numbers
  - Volume=3D, Area=2D, Length=1D, Point=0D
  - Vv, Sv, Lv, Pp  Point Counting, Line Cuts, Counting
- Equipment
  - Probes (Sections, Lattices, Tally Counters, Image Analysers)
  - Isotropic Probes, Merz Lattices, Cycloids

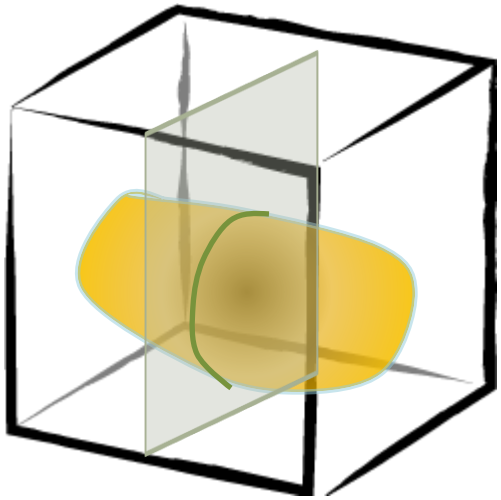


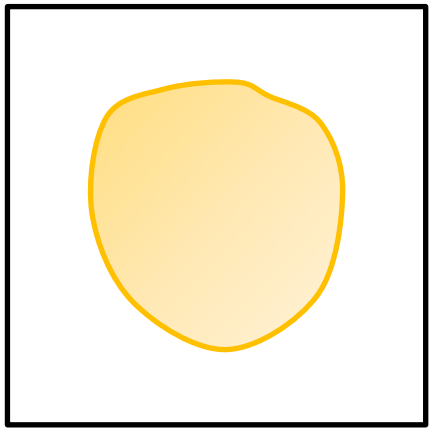
# Dimensional Reduction

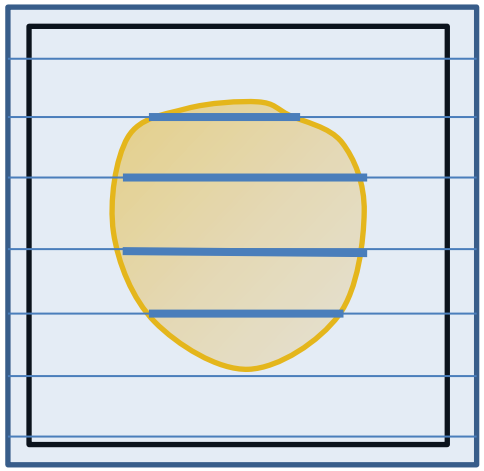
| OBJECT | | SECTION | |
|---|---|---|---|
| 3D VOLUMES | → | AREAS | 2D |
| 2D AREAS | → | LENGTHS | 1D |
| 1D LENGTHS | → | POINTS | 0D |
| 0D POINTS | → | Hit | 0D |
| | | Miss | X |

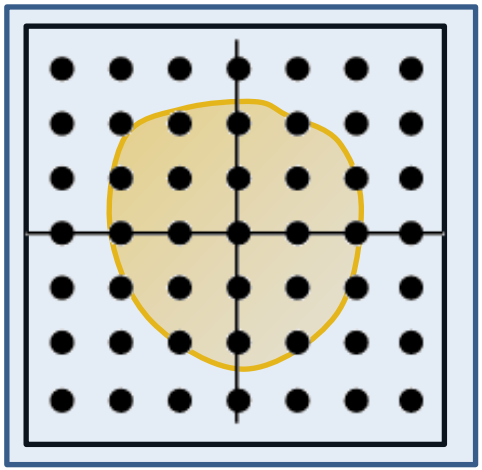


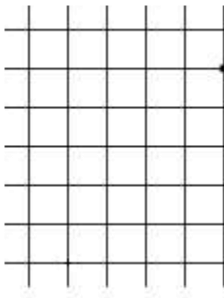

# Volume Fraction







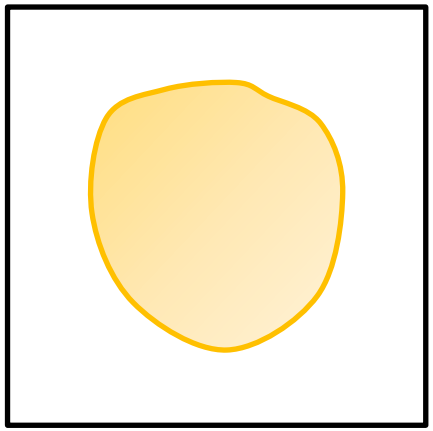




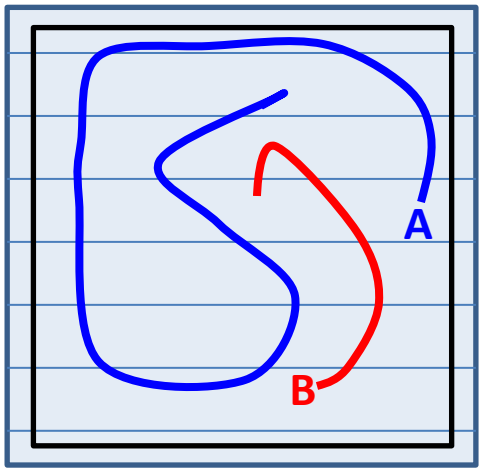
$V_V = A_A = L_L = P_P$

| Reconstruct | Planimetry<br>Cut & Weigh<br>Count Squares | Measure<br>Opisometer<br>Computer | Count |
|---|---|---|---|

# Surface Density



$$S_V = L \times 4/\pi$$



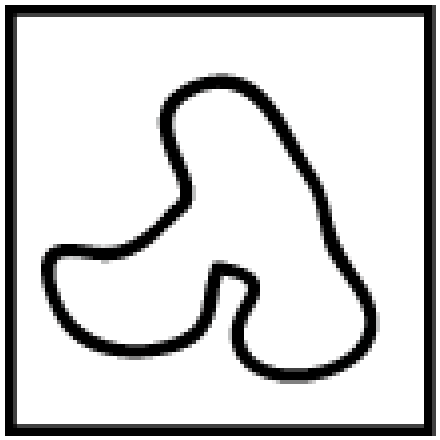
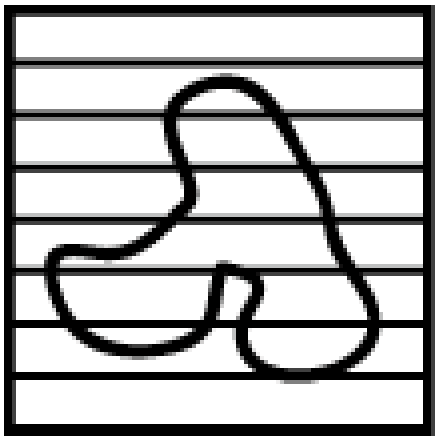
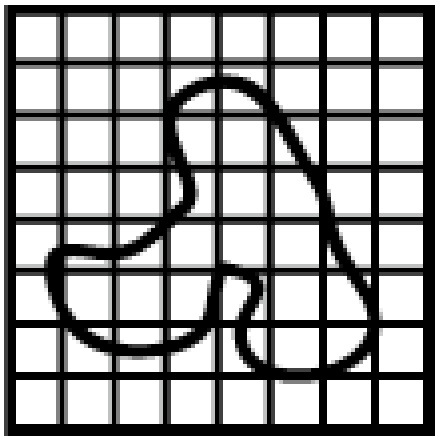
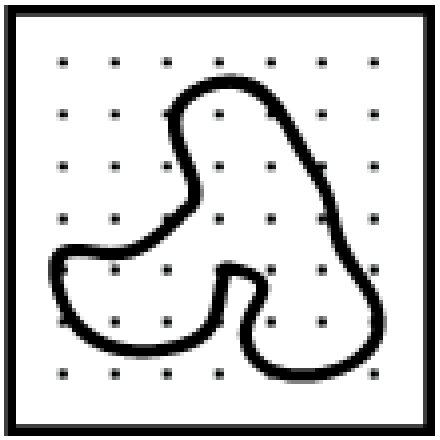
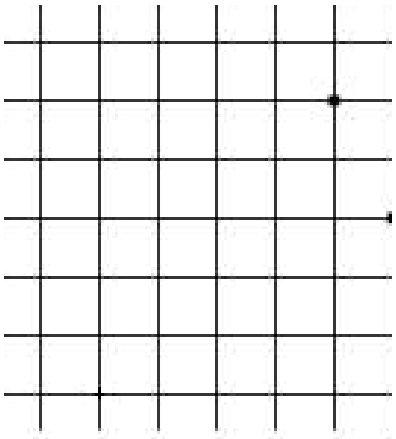
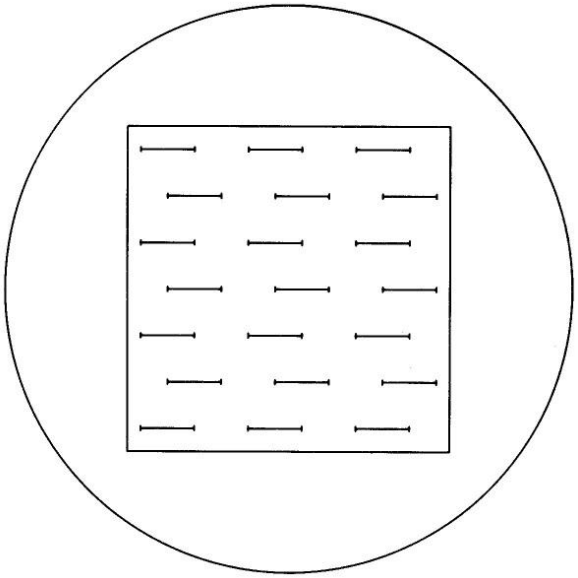
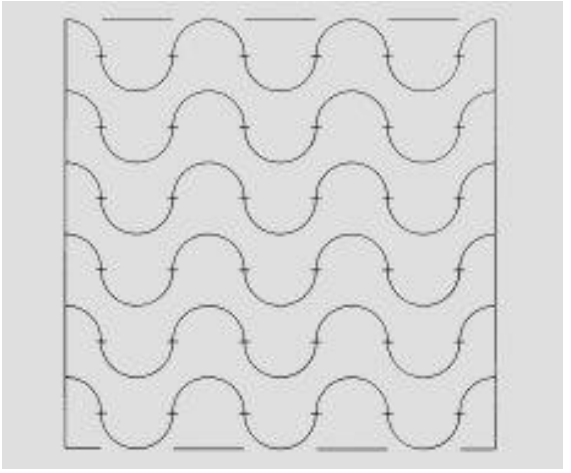
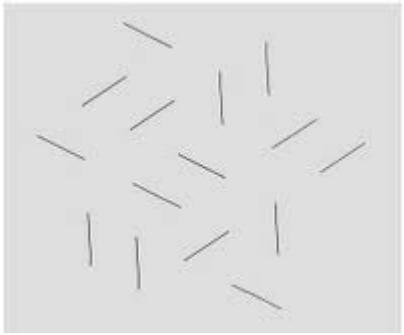

A= 14 cuts

B= 5 cuts

$S_V = 2 \times I_L$

$L = \pi / 2 \times I_L$

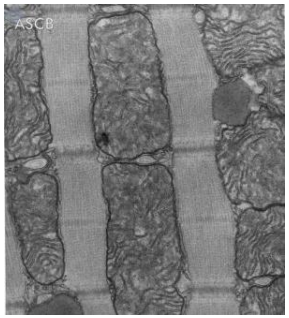
Buffon's Needle (1777)

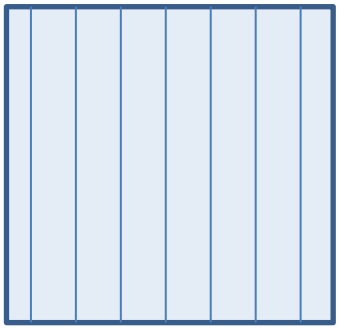
# Stereology Lattices (Probes)

Use Hally formula for number of hits

# Anisotropic Tissues







- ISO $Sv = 2I_L$ 2.00
- Aniso $0^0$ $Sv = \pi / 2\ IL$ 1.57

  $90^0$ $Sv = \pi / \sqrt{2}\ IL$ 2.22

*Weibel, 1980*

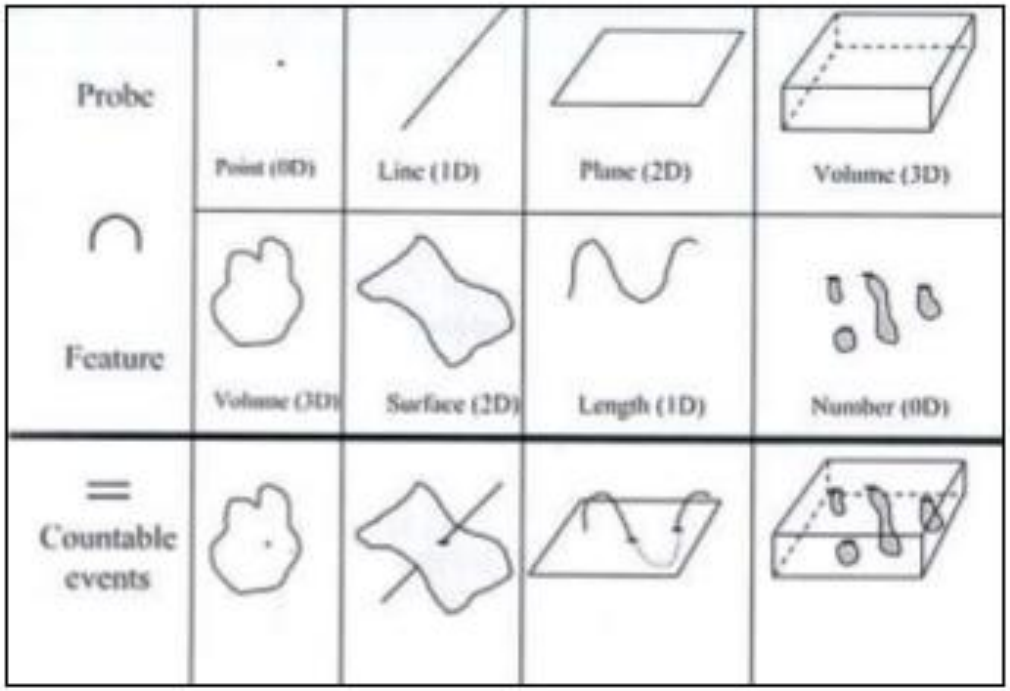
or use line lattice at $19^0$ to L.S. *Eisenberg, 1974*

or use Merz Isotropic lattice

or use Linear lattice x2 & ‘measure’ orientation

# Summary

- Geometric probes used for the sampling
  - Points for volume
  - Lines for surface area
  - Planes for lengths
  - Volume for numbers
- Geometric probes are required to report 3D data



# Summary



## *Basic relationships in stereology*

**TABLE 2.1**

*Relationship of measured (○) to calculated (□) quantities*

| Microstructural feature | Dimensions of symbols (arbitrarily expressed in terms of millimeters) | | | |
|---|---|---|---|---|
| | $mm^0$ | $mm^{-1}$ | $mm^{-2}$ | $mm^{-3}$ |
| Points | $P_P$ | $P_L$ | $P_A$ | $P_V$ |
| Lines | $L_L$ | $L_A$ | $L_V$ | — |
| Surfaces | $A_A$ | $S_V$ | — | — |
| Volumes | $V_V$ | — | — | — |

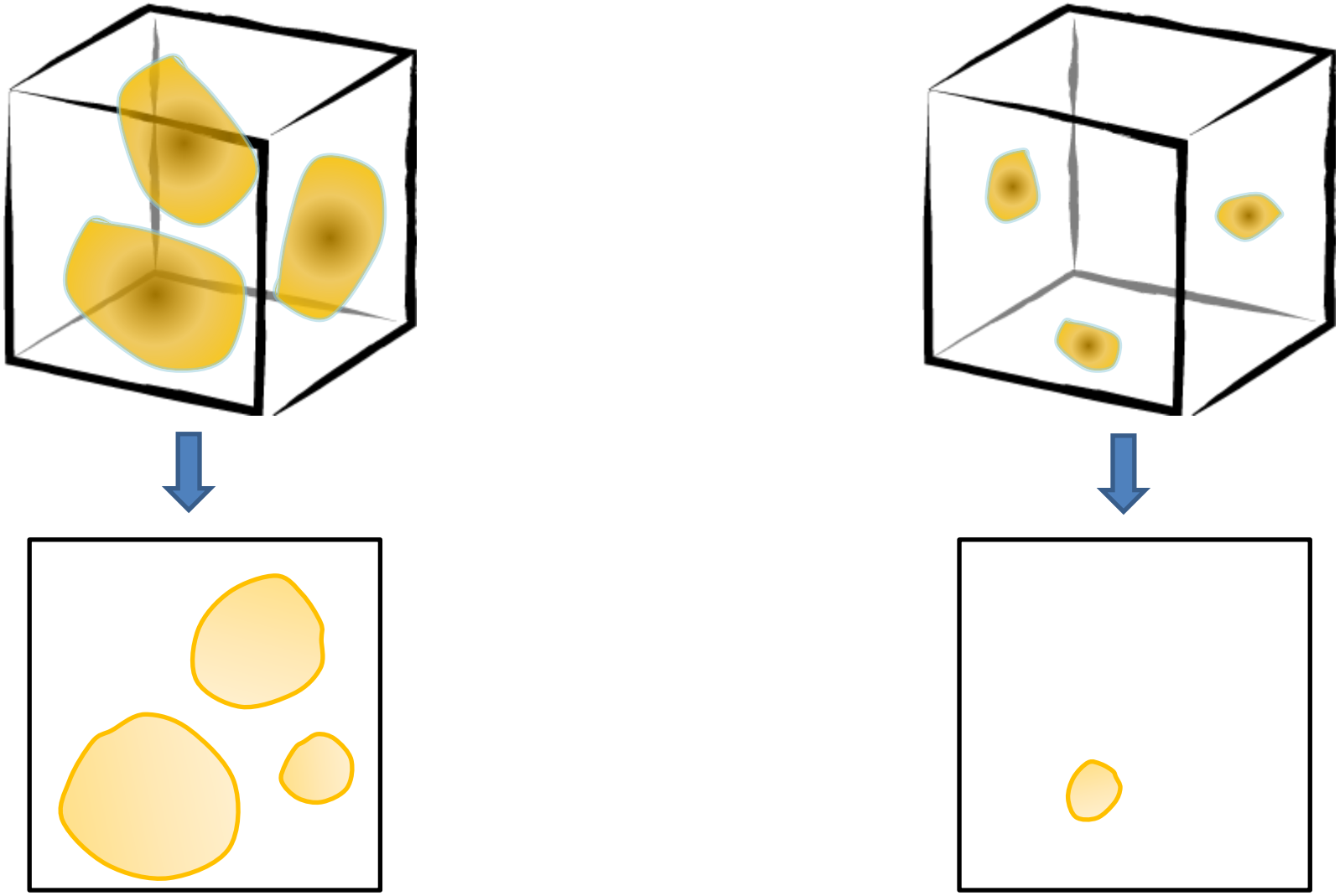
Underwood, *Quantitative Stereology*, 1971, Addison-Wesley

Please acknowledge Carnegie Mellon if you make public use of these slides


# Numbers

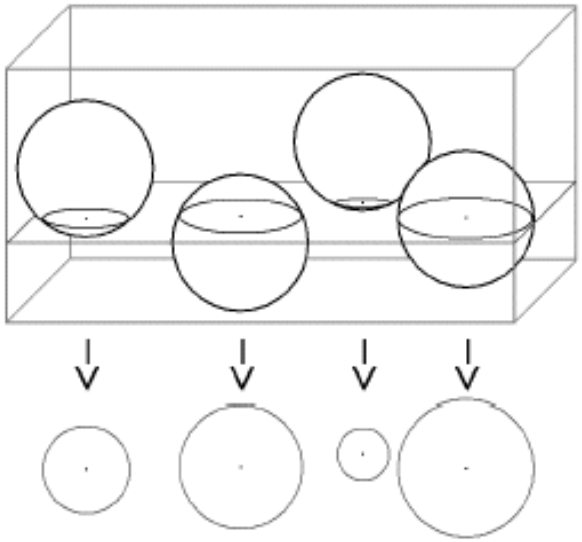
- N, Nv, NA
- Counting Objects or Profiles ?
  - Loose cells/smears or sections?
- Depends on size and shape, section thickness
- Reconstruct
- Correction procedures (for Size, Shape, Populations)
  - Abercrombie (1946) D=dx4/ π
  - Schwartz-Saltykov (1958) Unfolding
  - Avoid: use Design Based Stereology

# Numerical Density

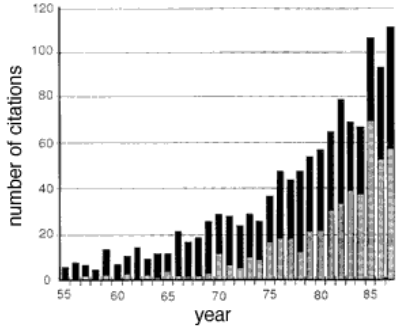


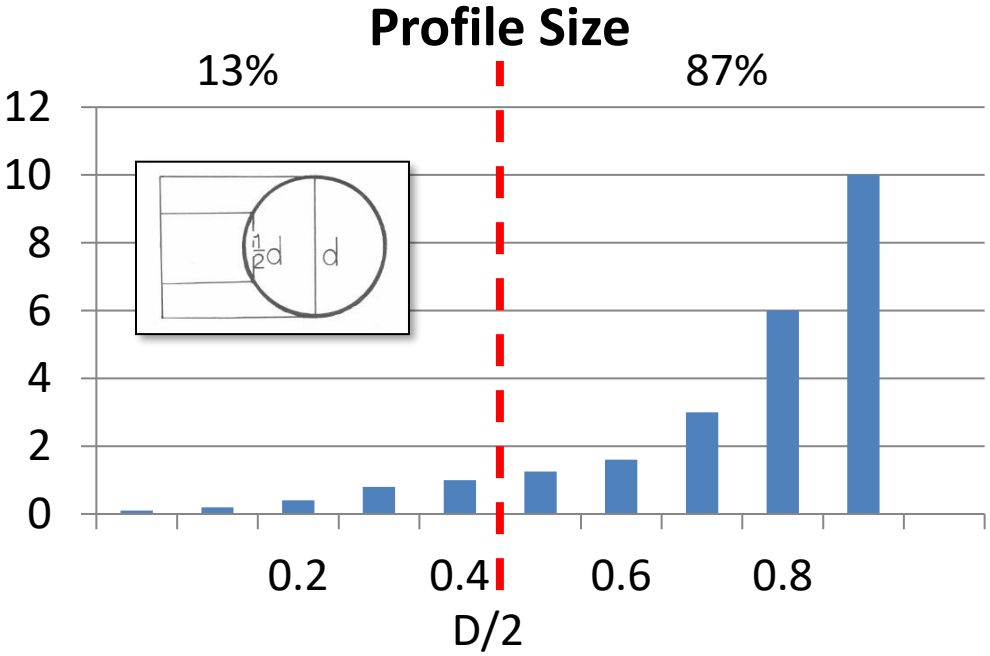
$$N_V = N_A / \bar{D}$$

# **Size** corrections



Monodispersed (Same Size)




Abercrombie (1946) Correction Factor …

$D = d \times 4/\pi$ (ie x1.273)

Polydispersed (Different Sizes)

Schwartz / Saltykov Unfolding
….. Shape (Ellipsoids, Cylinders, …) !!

# **Size** corrections

**Holmes Effect** – if objects small relative to section thickness

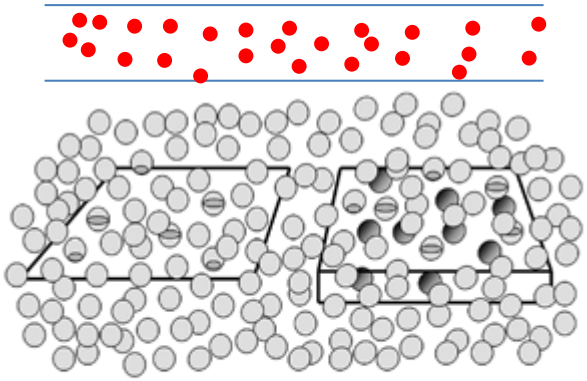
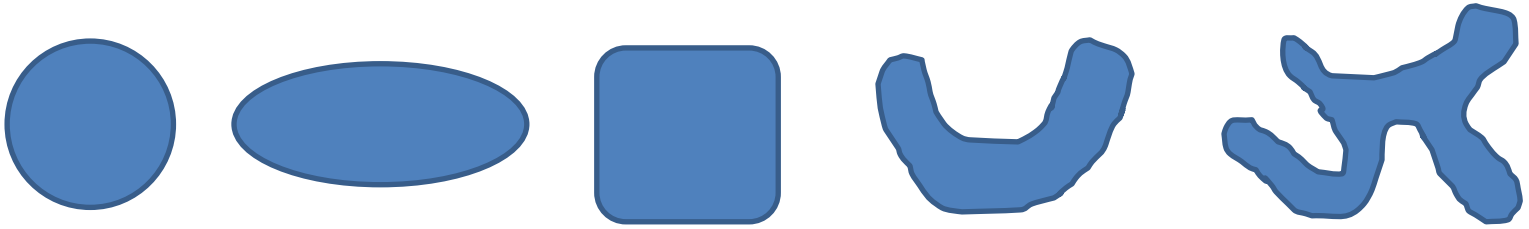


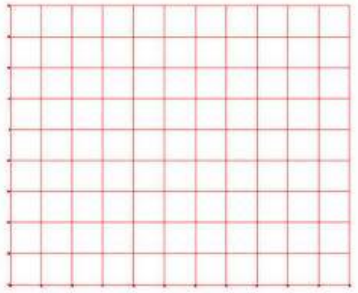
Fig. 6 The Holmes effect not only interferes with size estimation, but also with the determination of volume ratio since particles overlap (right side). In sufficiently thin sections (left side) this problem doesn t exist.

**Shape Effect** – mean ‘diameter’ for spheres, ellipsoids, cubes etc based on axial ratios or volume/surface ratios (complex, biased)

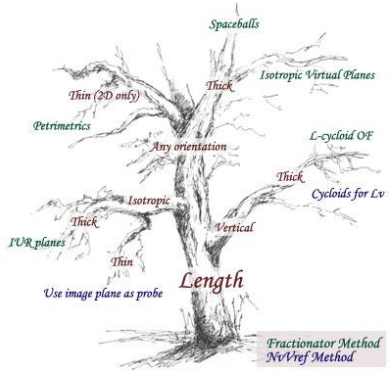


How ?

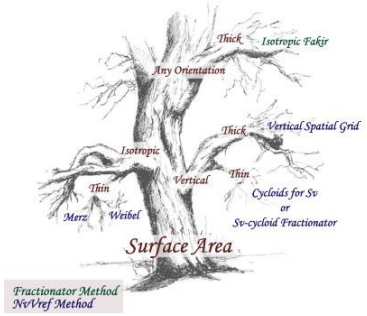
# Stereology 2



- New Design Based Stereology
  - Fractionator, Disector, Nucleator, Surfactor, Proportinator, Selector, Rotator, Cycloids
  - Surface Weighted Star Volume
  - Unbiased Brick, Isotropic Fakir
  - Spaceballs, Petrimetrics

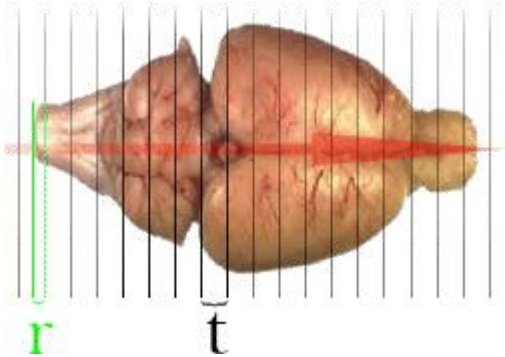


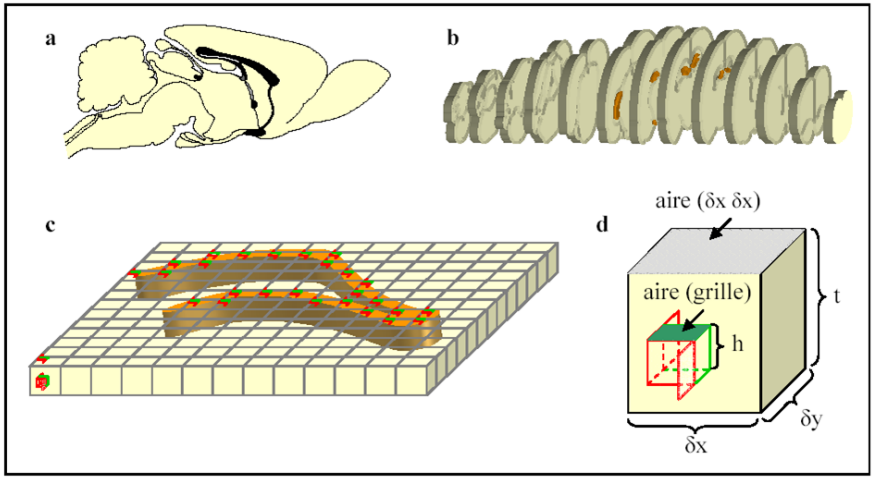

- Equipment / Design
  - Specialised Sampling; IUR, VUR, SRS sections
  - Thick sections, Optical sections
  - Unbiased Counting Frames

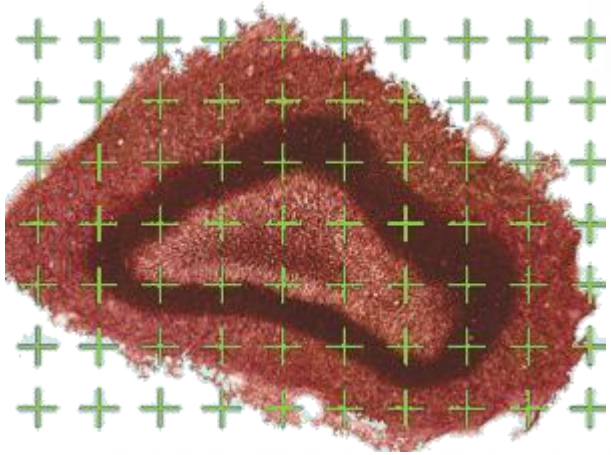


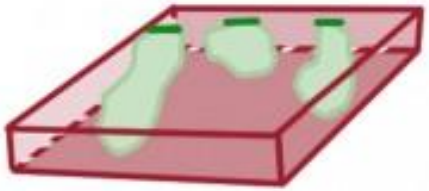

*Gunderson, Sterio 1980s* *See Howard & Reed, 1998, 2010* www.stereology.info

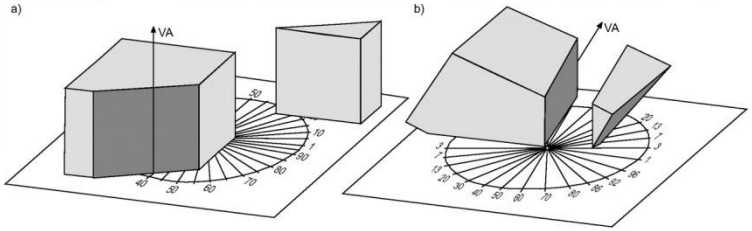
# Cavalieri Method

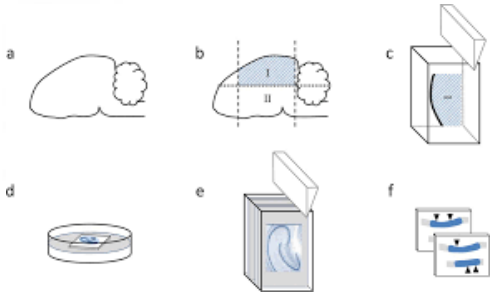


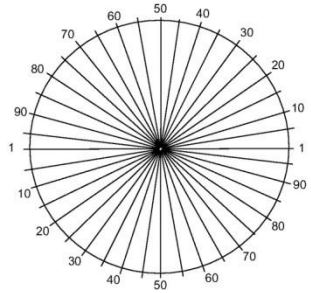



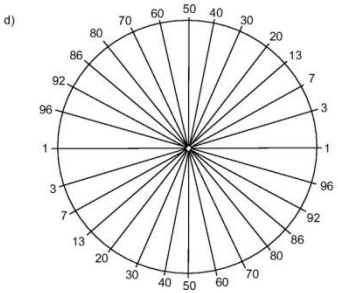







J Microsc. 2022 Sep 12. doi: 10.1111/jmi.13141. Online ahead of print.

# Improving cavalieri volume estimation based on non-equidistant planar sections: The trapezoidal estimator

Disector for counting
Total Number in a Volume

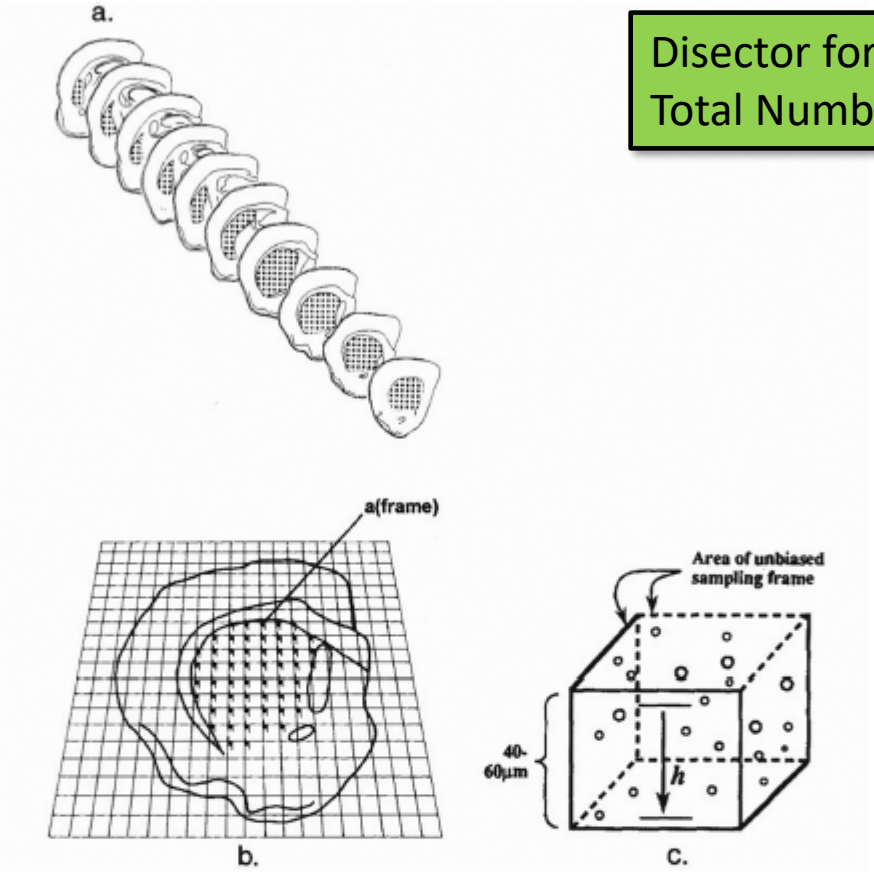
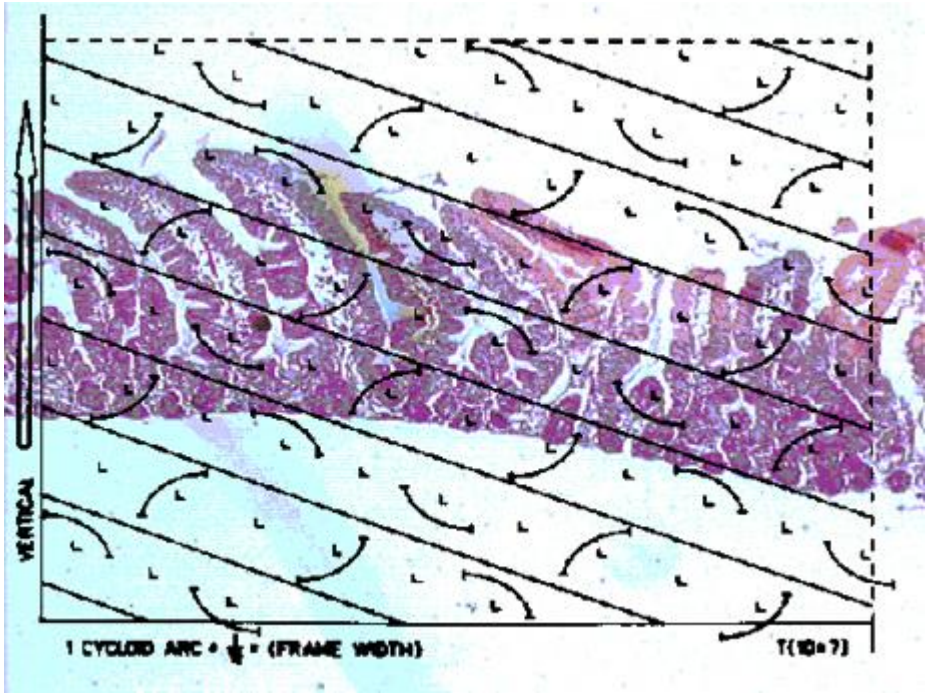
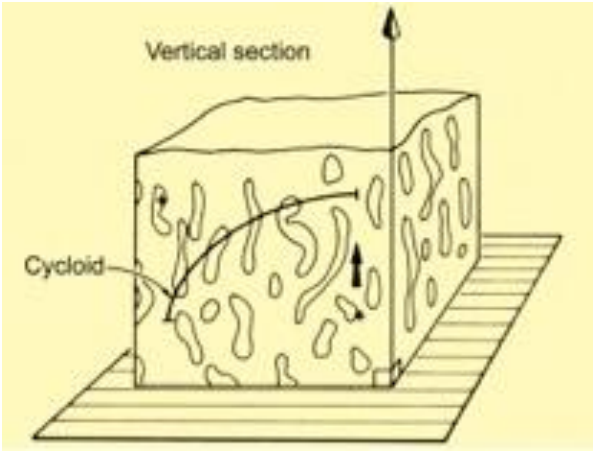


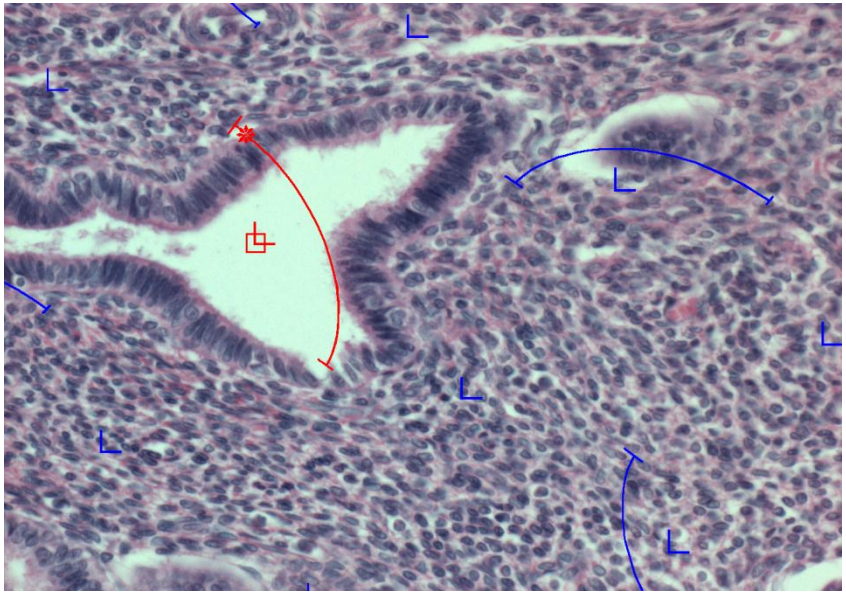

Fig. 1. *Schematic illustrations of a worked example through the rat striatum of Cavalieri's method (a, b) and the optical disector method (b, c). In (a) a systematic series of sections through the striatum, after a random start, is illustrated. A systematic array of points (i.e. dots) is overlayed on the striatum and the number of points (P) falling on the striatum in each section are counted. These points are then summed to obtain the* $\sum P$ *for the entire striatum in a specific cerebral hemisphere. The value for* $\sum P$ *is substituted into Cavalieri's formula to calculate the absolute volume (see text for further details). In (b) systematic sampling throughout the striatum, after a random start, is illustrated. This strategy is used to measure the Nv in a specific section. The a(frame) refers to the area of one unbiased sampling frame, among a number of these frames, within the striatal boundaries of one section. The Nv of neurons was measured within each a(frame) and its disector height. This analysis to estimate the Nv was completed for all the sampled sections in (a). The counting of disector neurons through the height (h) of a sampled disector volume is illustrated in (c). (a, b) Modified from West et al. (1996), with permission. (c) From Oorschot (1994), with permission.*

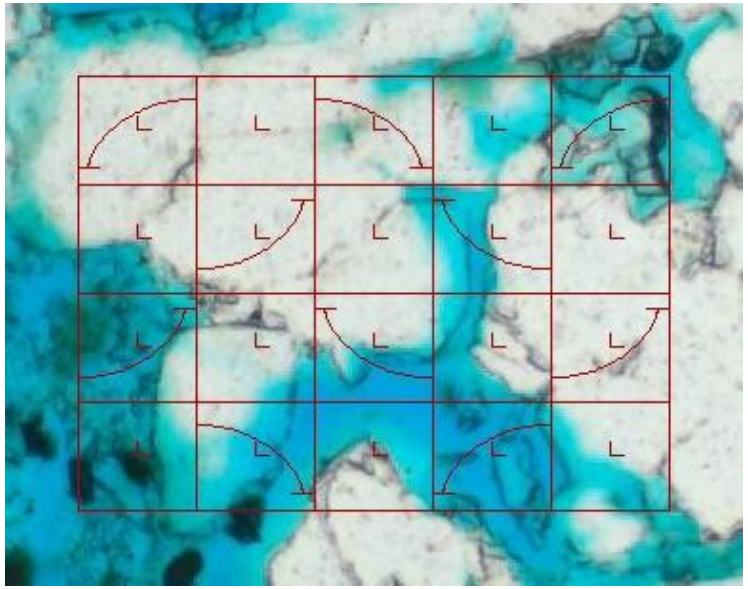
Oorschot, 2011

# Cycloids for estimating Surface Area in a Volume



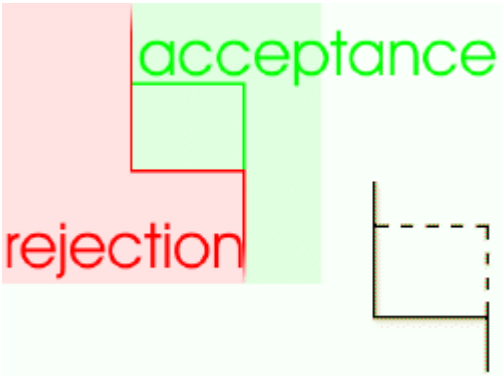



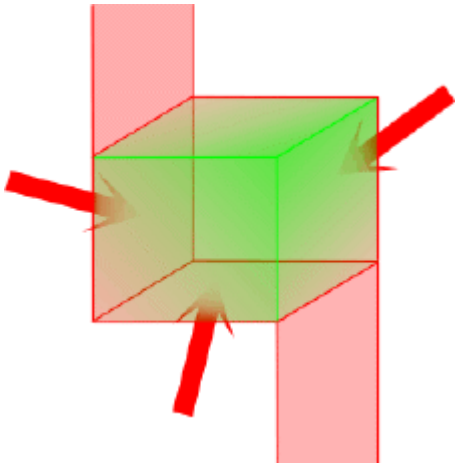


# Unbiased Stereology

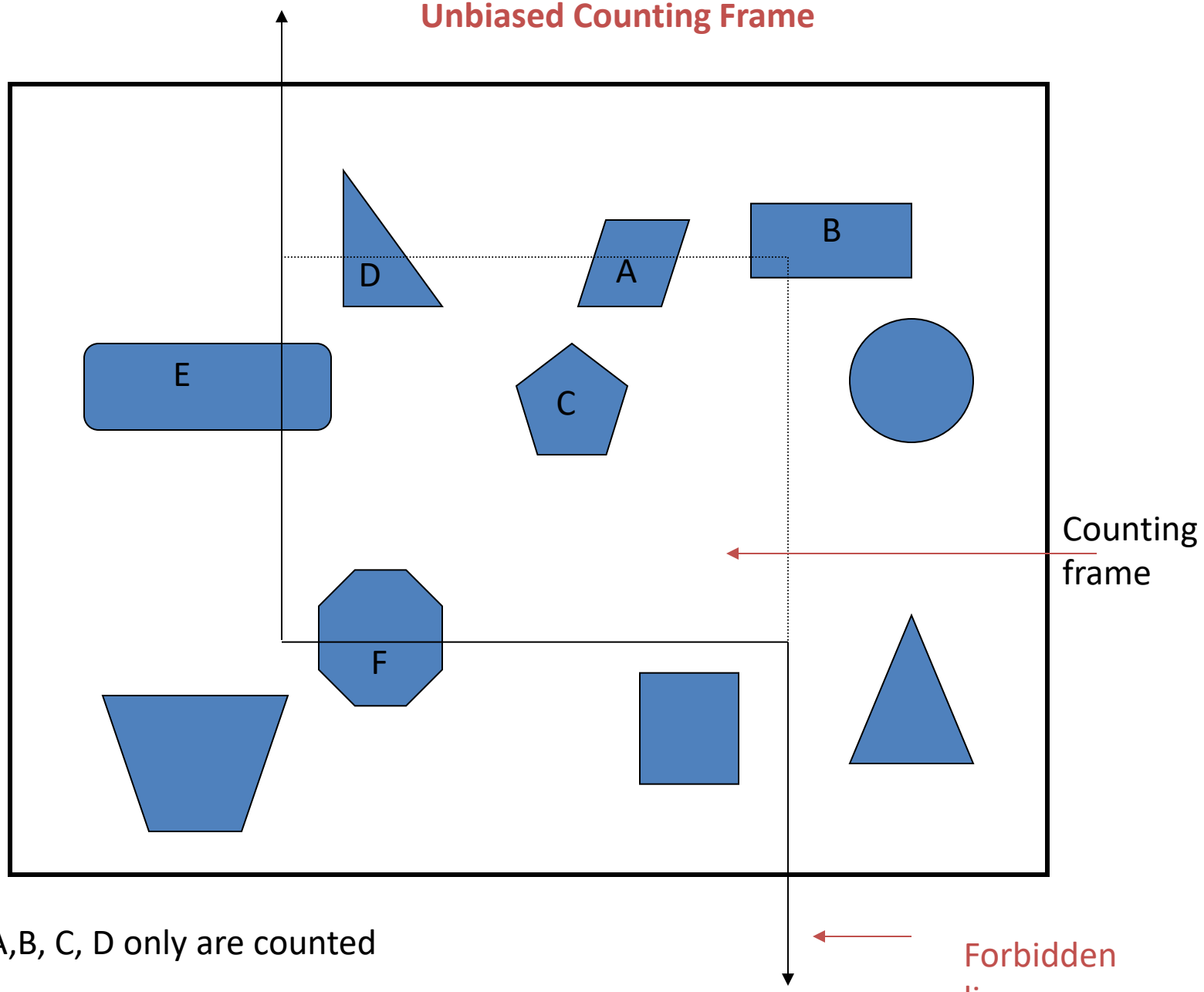
Gunderson




- Measuring (see Picture 1)
  Since small objects are more likely to fit as complete objects within a measuring field it is best to remove this bias by measuring ALL objects within the frame and ALL objects hitting the dashed (allowed) lines but NOT those hitting the solid (forbidden) lines.
- Counting (see Pictures 1 & 2)
  Objects allowed in the reference section (Picture 1 - A,B,C,D) are then checked in the next (look-up section (Picture 2)). If they DO NOT APPEAR in the look-up section these are the objects which are counted - ie object A ONLY.
  Therefore one object occupies a volume equal to the frame area times the section spacing.
  This is called the Disector Method.
- See: Howard CV, Reed MG (1998) Unbiased Stereology. RMs Handbook 41; Bios Scientific.



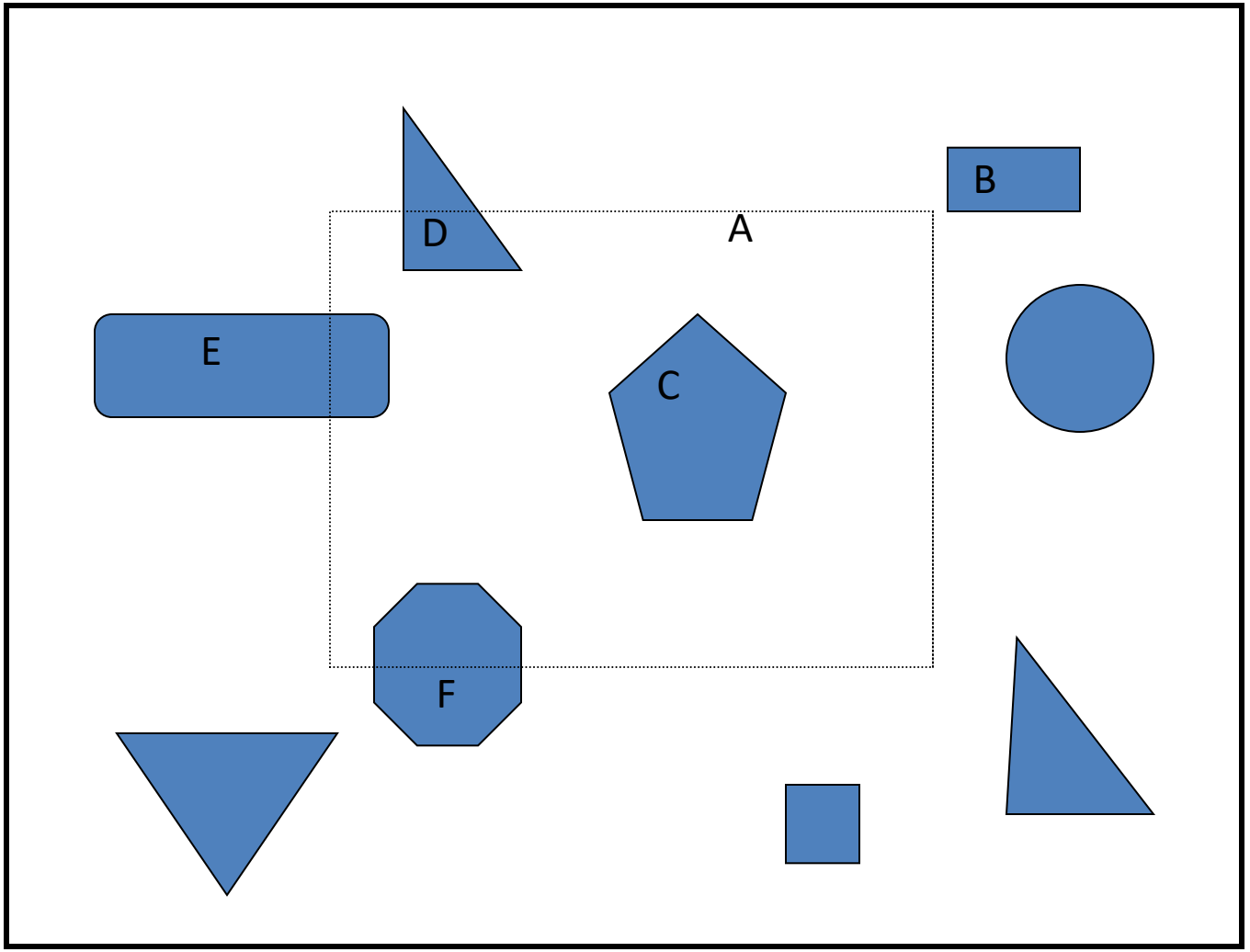
## Unbiased Counting Frame




Objects A,B, C, D only are counted

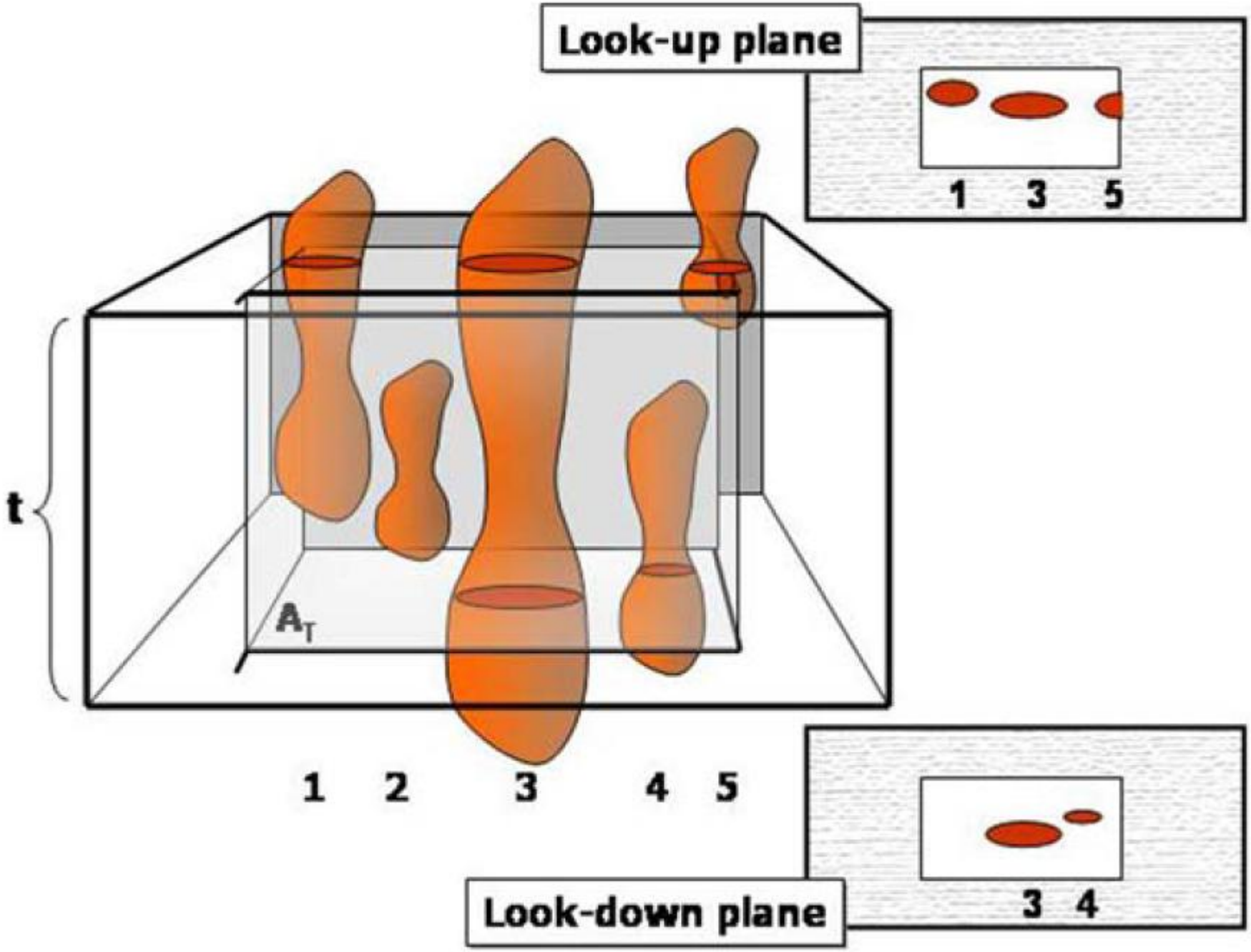
1) Reference Section

# Disector method

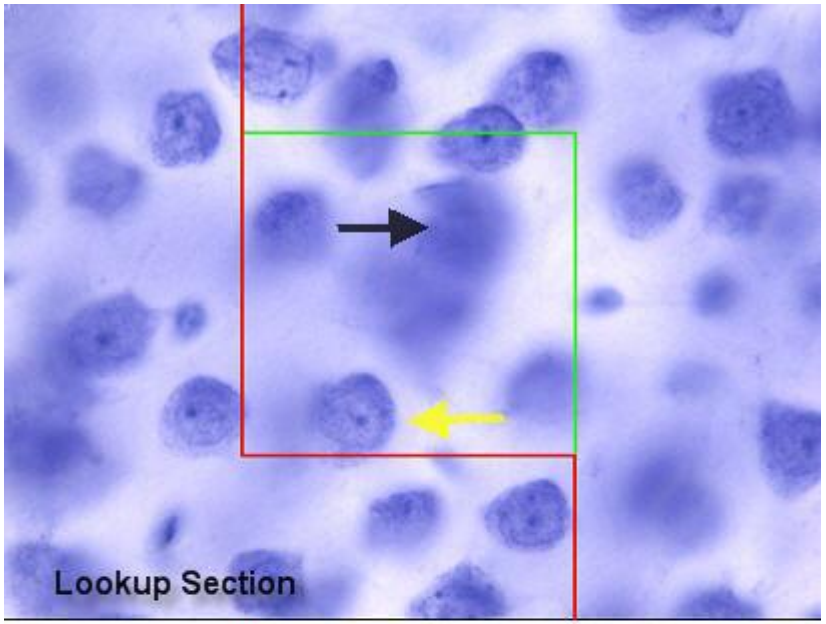


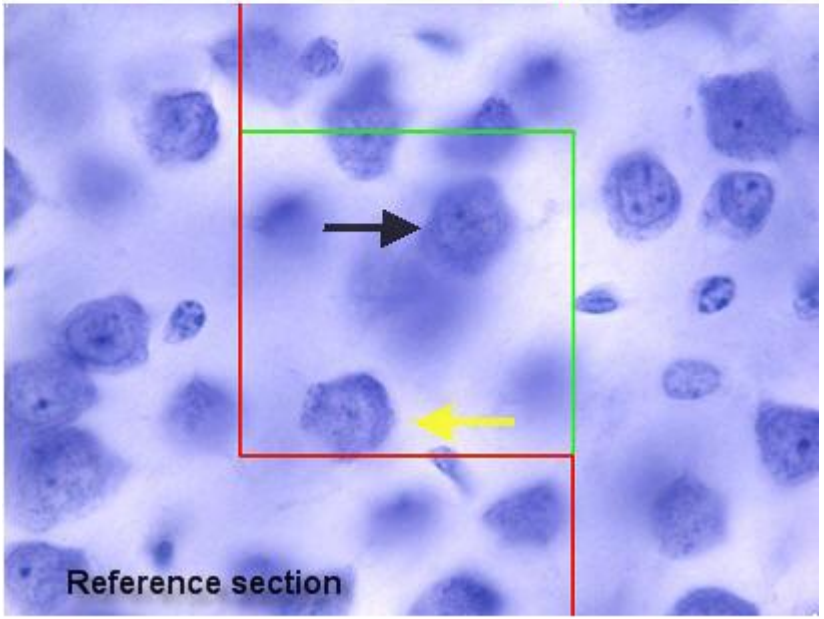

## 2) Look-Up Section

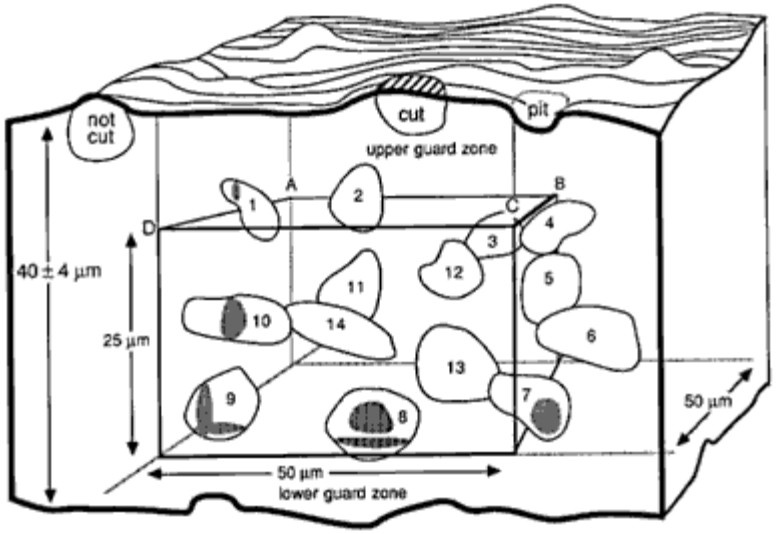
Of the objects counted in the reference section (A,B,C,D) only the objects NOT present in the look-up section are counted, ie only object A is counted.



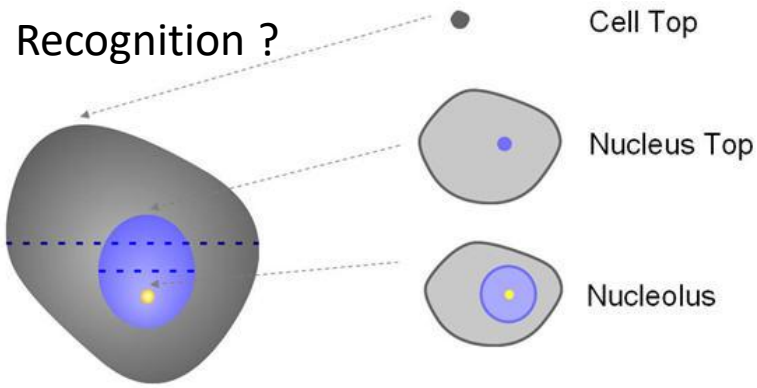

*Mandarim-De-LaCerda (2003)*

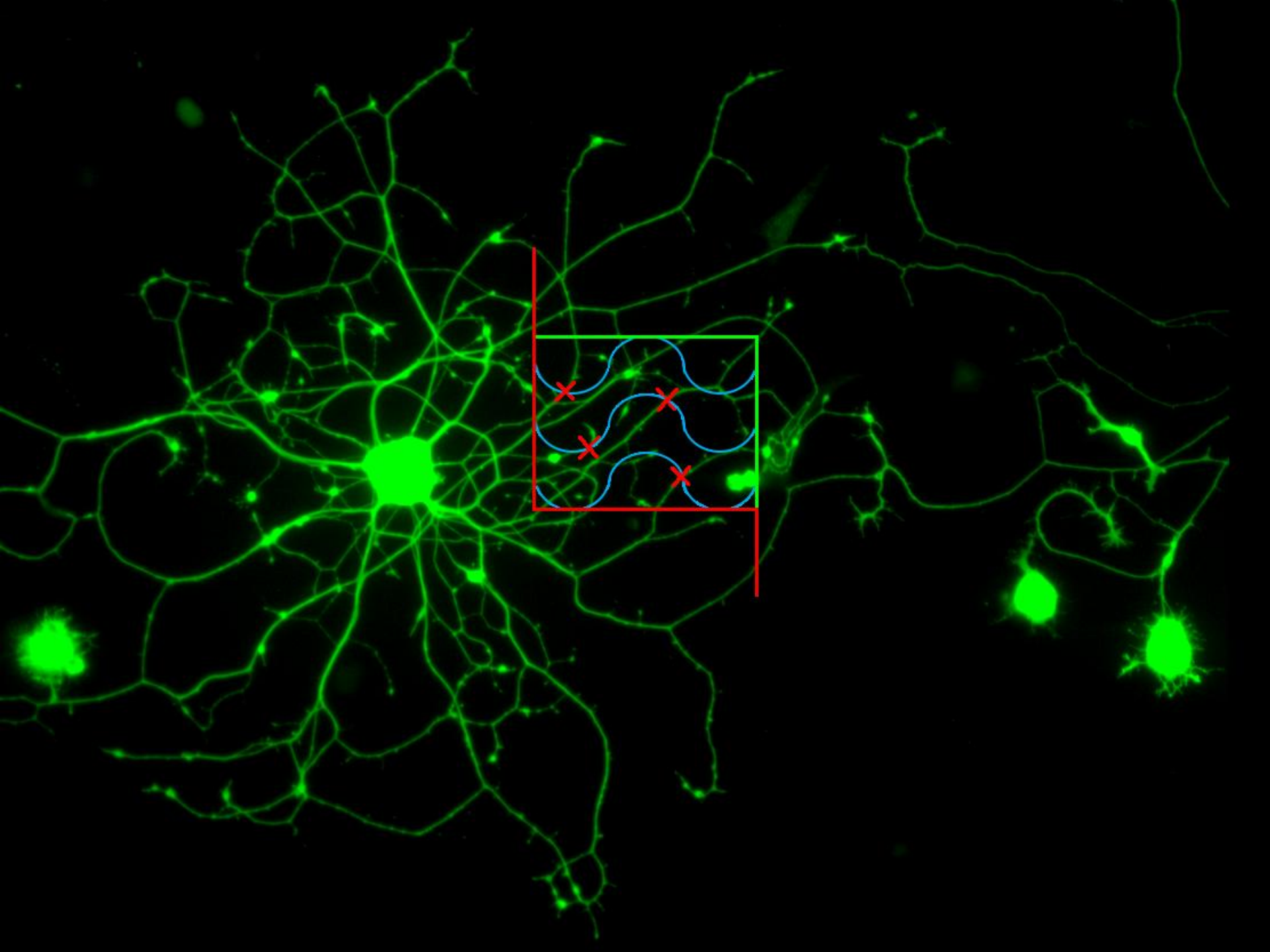



Recognition ?



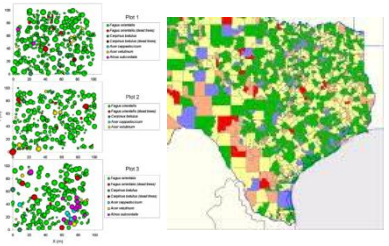


# Commercial Stereology Software Packages

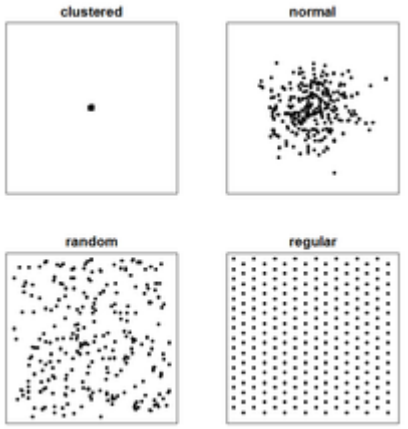
How?

# Pattern Analysis

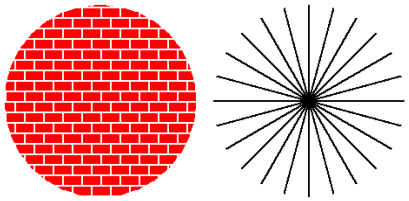
## Measurements of 'Organisation'

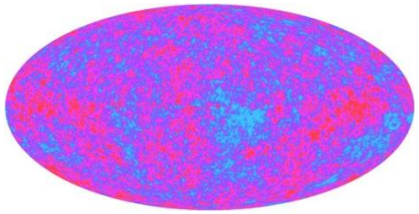


- Location / Distribution / Spatial Arrangement / Association / Connectivity / Interaction
  - ???: Random, Regular, Clumped, Dispersed, Associated/Related
  - Distance:  Nearest Neighbour, Mean free path
  - Grouping: Enclosed, Contiguity, Runs Test, SPAM
  - Autocorrelation
  - Tesselation / Joins / Overlay methods
  - Regional Density, Point Swarms

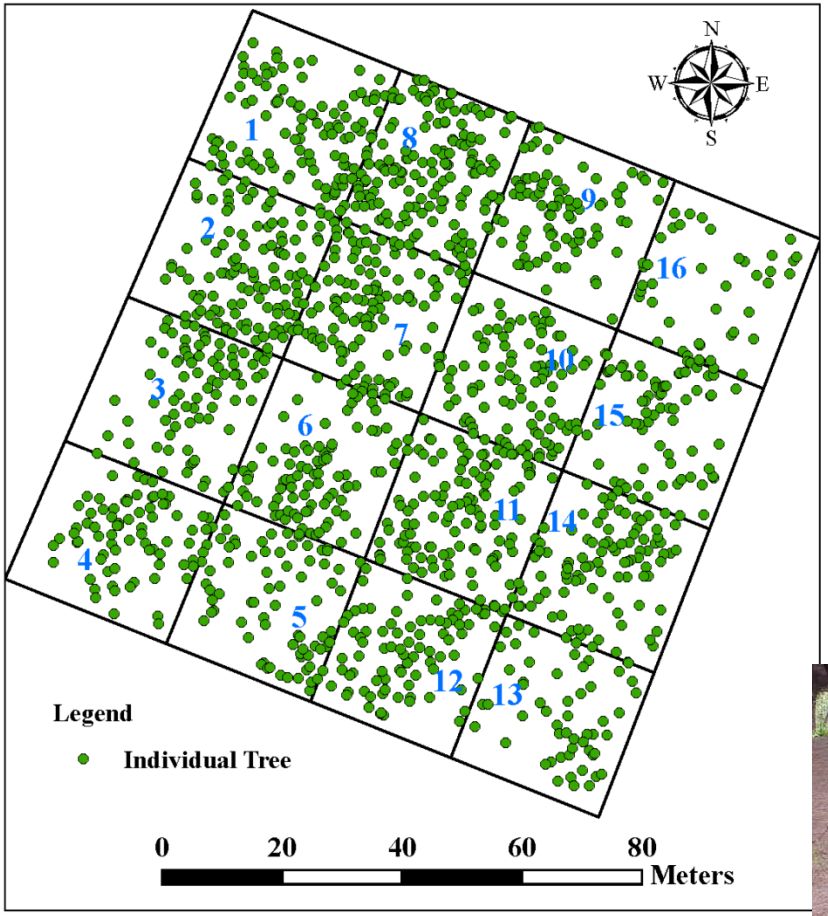



- Orientation / Branching
  - Dendritic methods (fields, segments, nodes)
  - Isotropic, Anisotropic

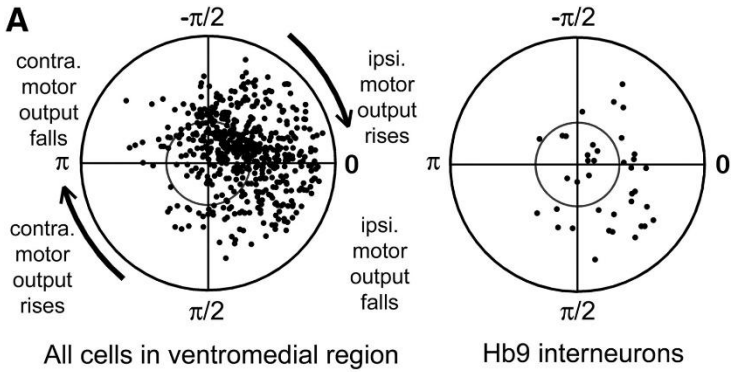


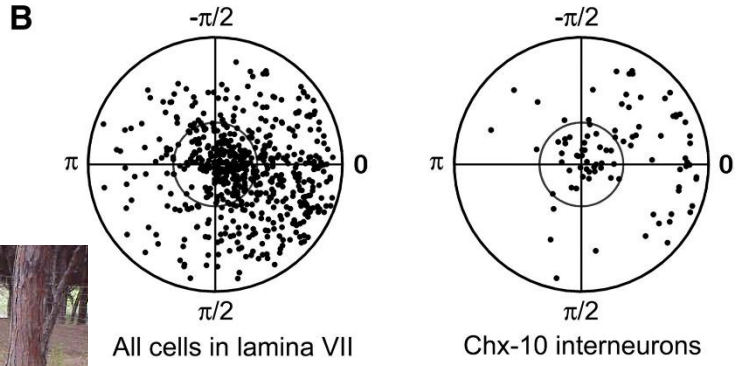


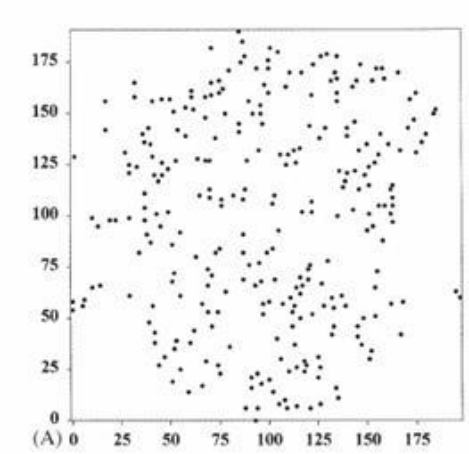
***See:*** *Uylings, Berry, Aherne, Underwood, Johnson, Sokal & Rohlf, James, Mahon, Cruz-Orive, Diggle, Unwin*

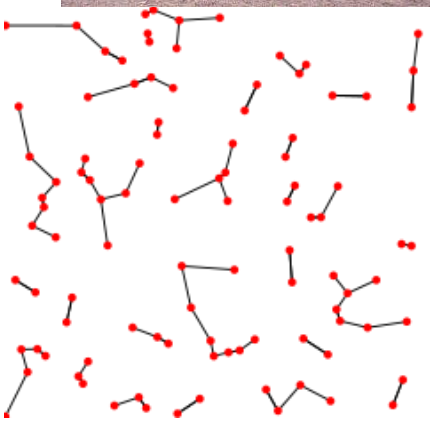

N
W
E
S
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Legend
Individual Tree
0
20
40
60
80
Meters

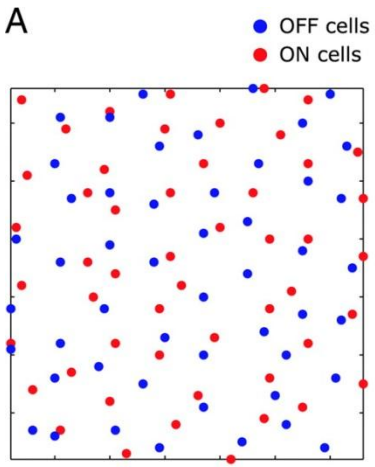
Examples


A
-π/2
contra. motor output falls
ipsi. motor output rises
π
0
contra. motor output rises
ipsi. motor output falls
π/2
All cells in ventromedial region
-π/2
π
0
π/2
Hb9 interneurons


B
-π/2
π
0
π/2
All cells in lamina VII
-π/2
π
0
π/2
Chx-10 interneurons


175
150
125
100
75
50
25
0
(A) 0
25
50
75
100
125
150
175

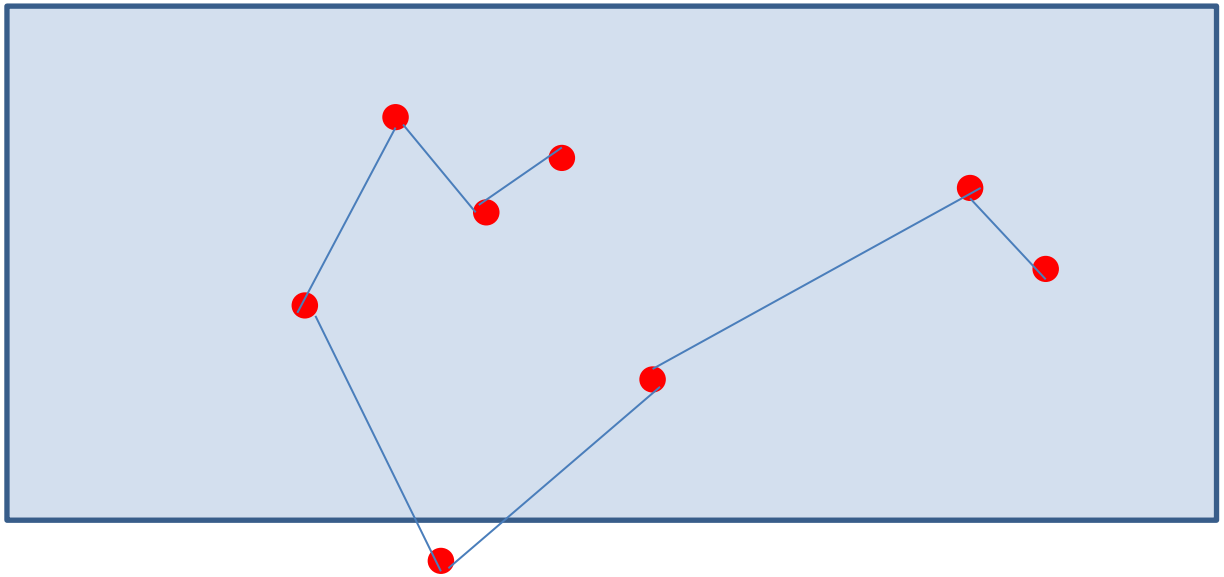



A
OFF cells
ON cells

# Nearest Neighbour Analysis

- Developed from Ecology studies (Poisson distribution) *Clark & Evans, 1954, Kendal & Moran, 1963*

- Average distance between objects or Mean area / cell



- Begin at random cell / point *Problems:* reflexive pairs / high densities

Predict expected result for random population and compare

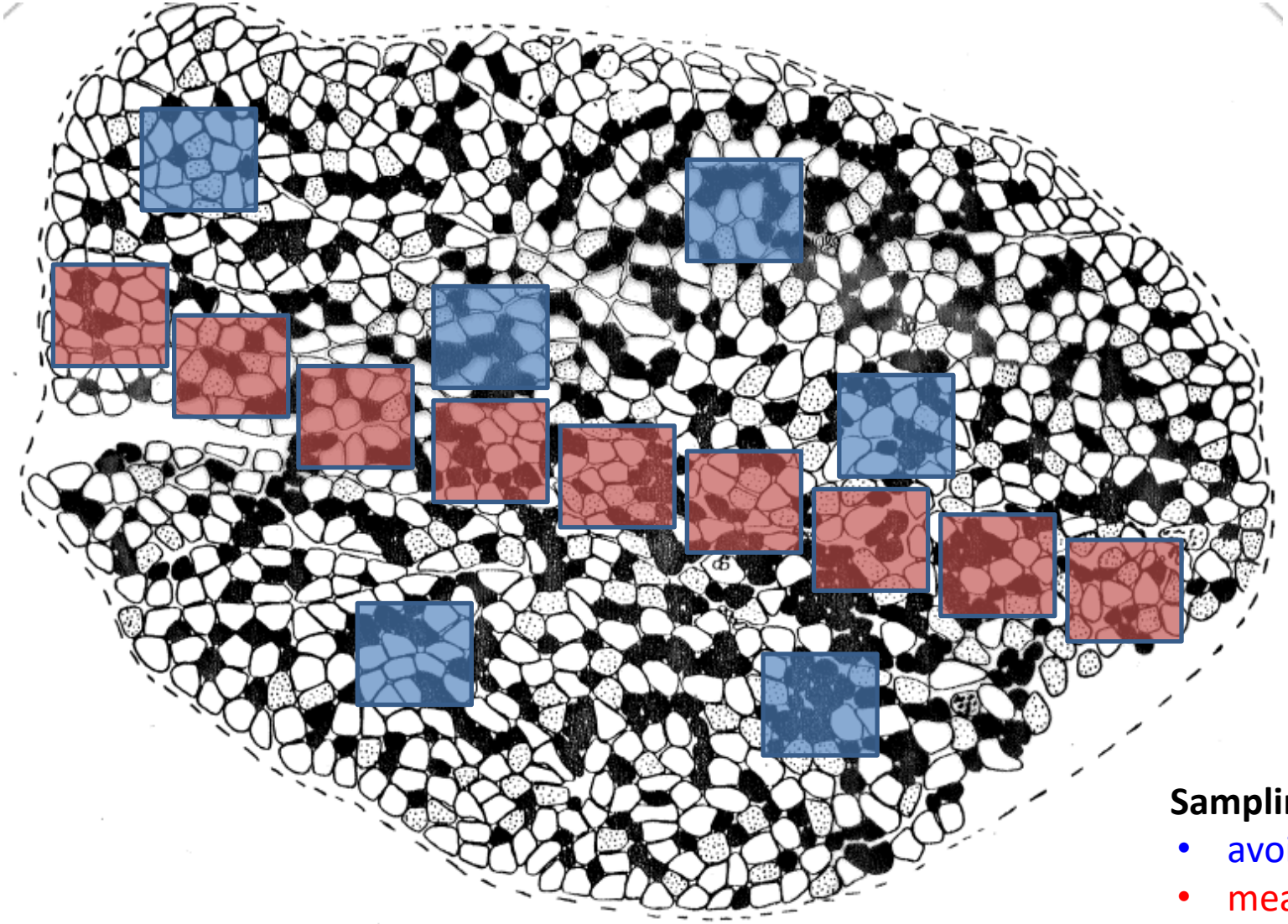
2D (eg muscle) $\bar{d} = 1/ (2 \times \sqrt{N_A})$

3D (eg brain) $\bar{d} = 0.554/(\sqrt[3]{N_V})$

Index of Dispersion $I_d = d_{obs}/d_{exp}$

Also, **Mean Free Path** (edge-edge) *James, 1977* $\lambda = (1-V_V)/N_L$ in µm

# Homogeneity / Heterogeneity

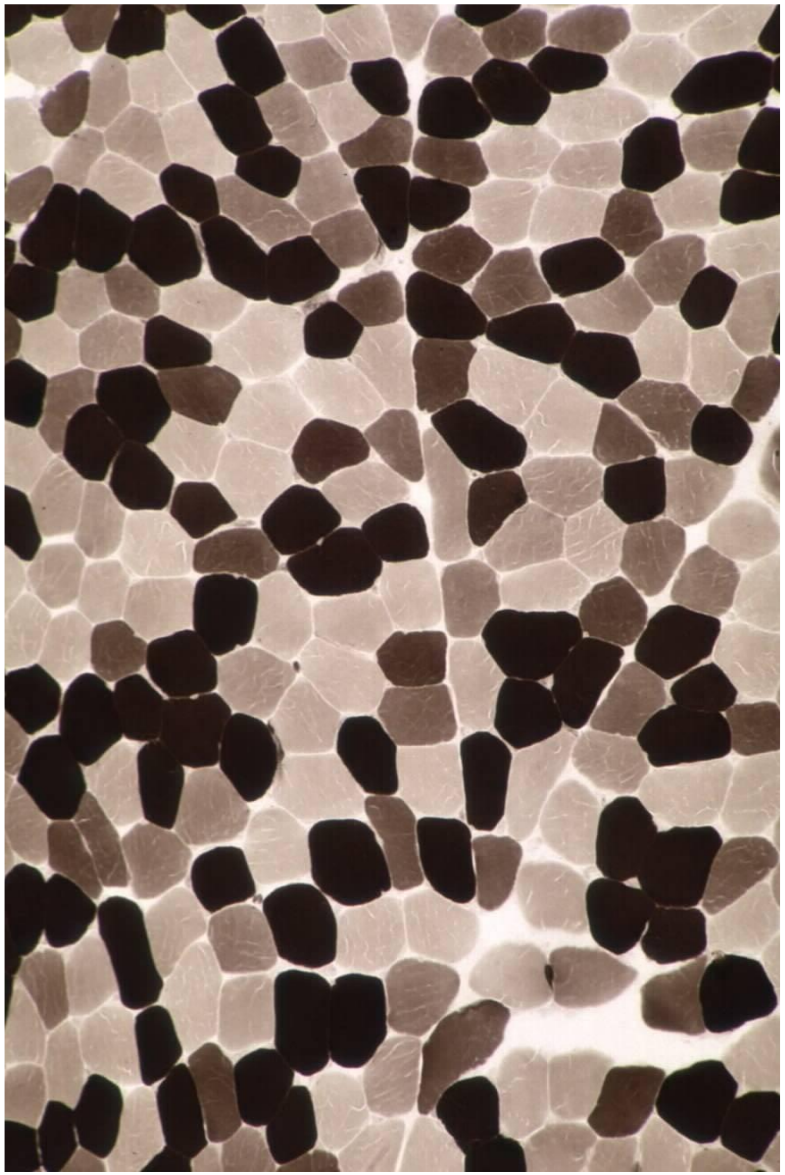


**Sampling :**

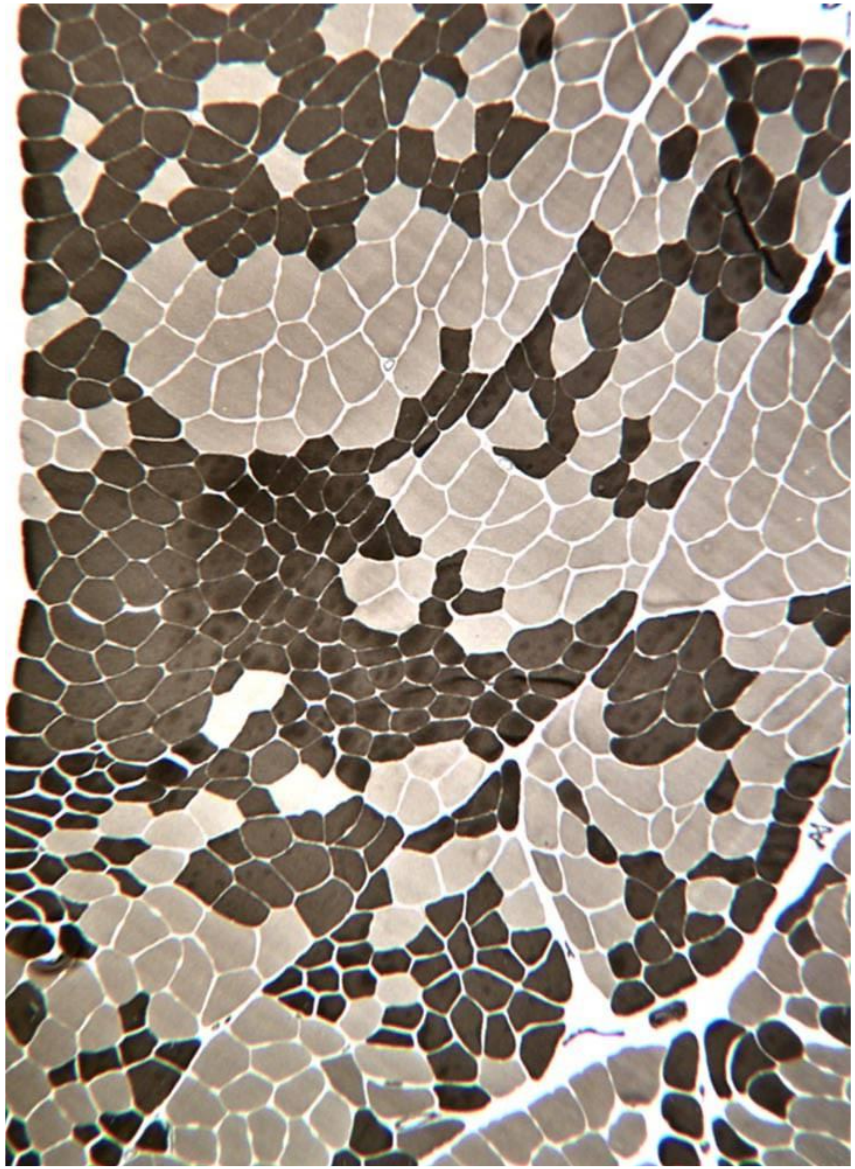
- avoid it
- measure it

# Randomness ?

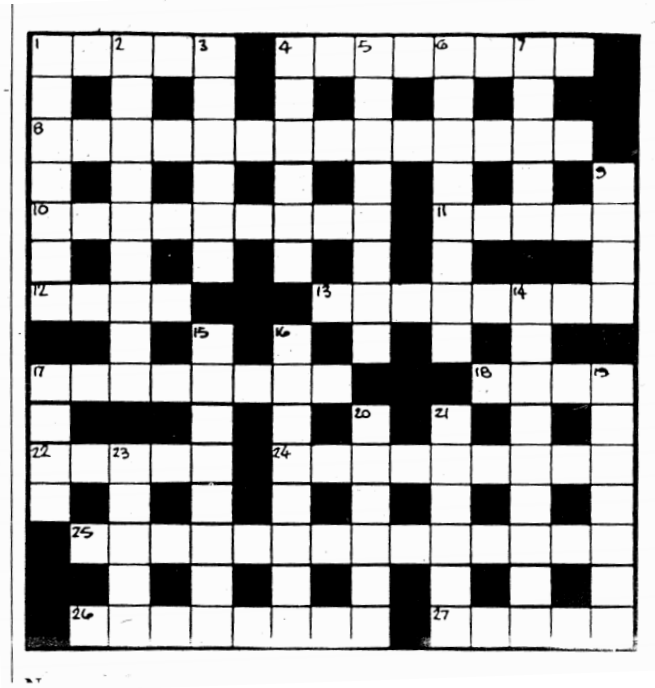
Healthy Muscle



Diseased Muscle



# Randomness ?



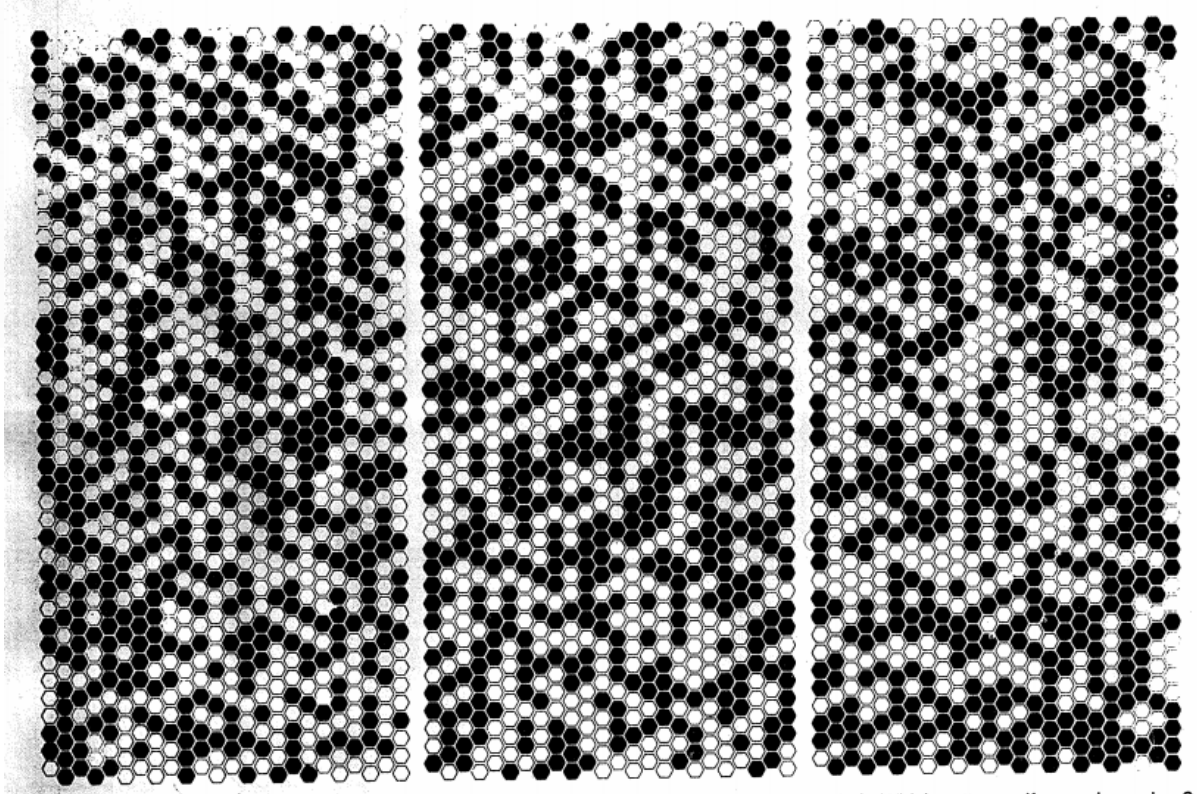


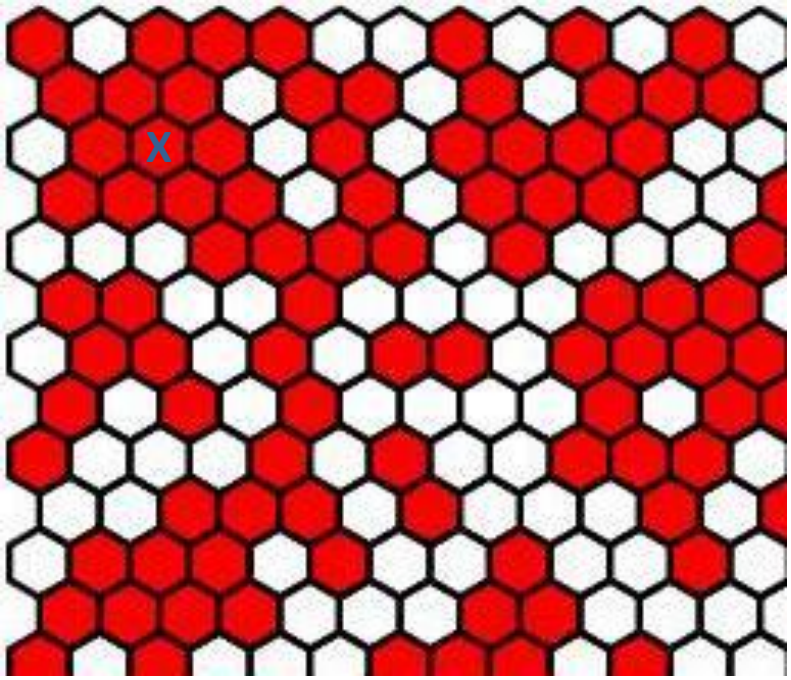
**FIGURE 6.** This figure shows three stochastic patterns with 1008 cells each; half of them are dark. Which pattern—if any—is random? The answer is given in the Appendix.

# DISTRIBUTION

## Enclosed “cell” method

- Observed versus Expected
- Predicted E= $Np^7$ +/_ SD
- Depends on percentage occurrence
  - 30% R = 0 enclosed
  - 50% R = 1
  - 70% R = 8
  - 90% R = 50

*Johnson, 1973*



# DISTRIBUTION

## Runs Test

- RRRR W RRR WWWW R WW  r = 6
- R W R W R W R W R W R W R W R  r = 15

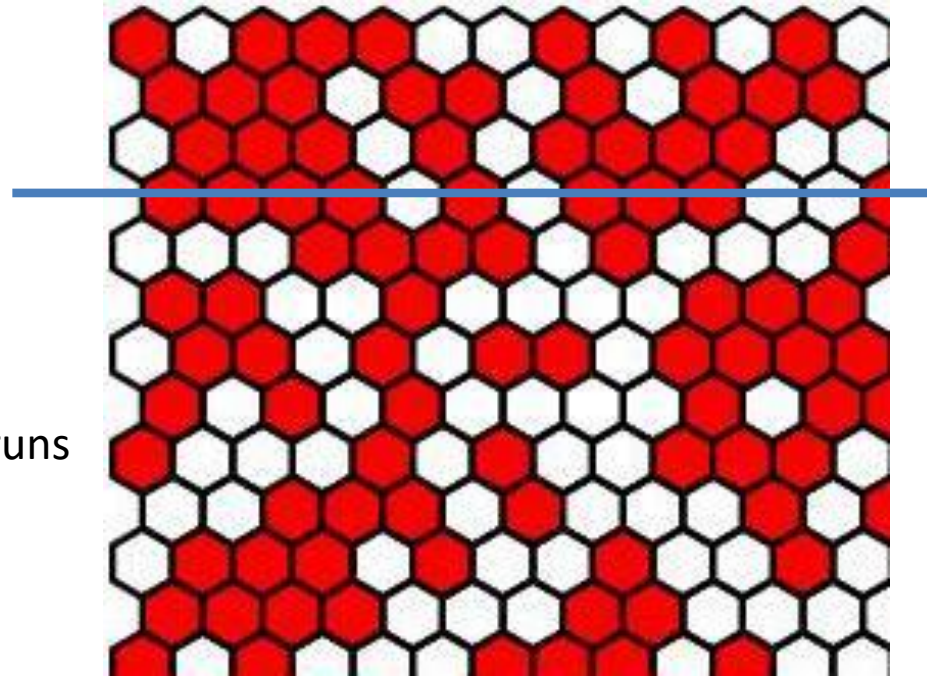
- N = 15,  n1 R = 8,  n2 W = 7

- Exp F = [2 x (n1 x n2 / n1 + n2)] -1  = 6.5

- T = (F - Exp F) / SD

- Distribution IS Random
- Distribution IS NOT Random

- Predict Runs  eg 100 cells 60% R = 47 +/-5 runs



*Sokal & Rohlf, 1973*

# DISTRIBUTION

## Run Lengths (Clumps)

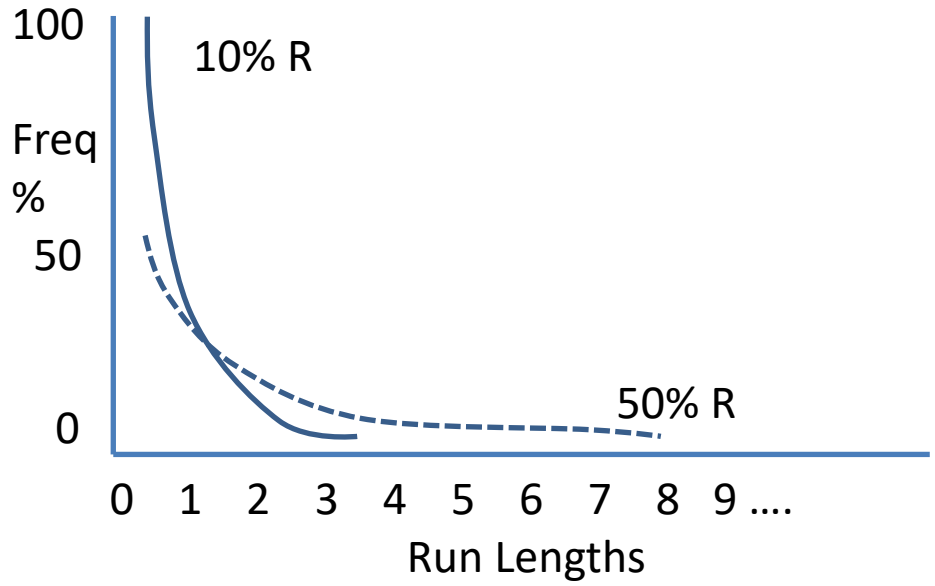
*Roach, 1968*

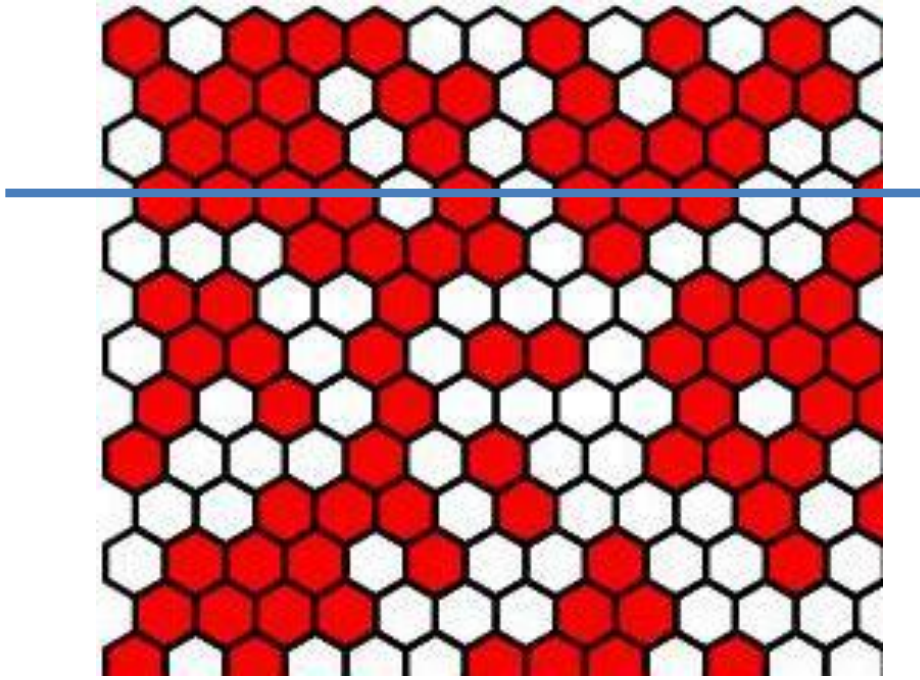
- RRRR W RRR WWWW R WW — Avlength R = 2.66
- R W R W R W R W R W R W R W R — Avlength R = 1.00

- Predict expected run lengths for a random distribution

$Cl_R = N_R + P_{RL^{R-1}} \times (1-P_R)^2$

$Cl_W = N_R \times P_R \times (1=P_R)^{LW}$



# DISTRIBUTION

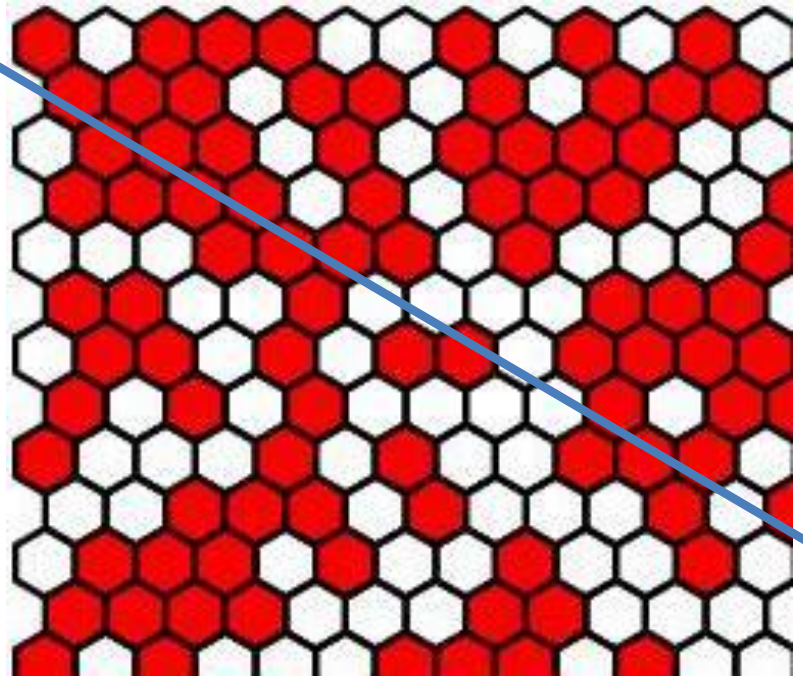
## Contiguity

*Underwood, 1970* *Gurland, 1975* *James, 1980*

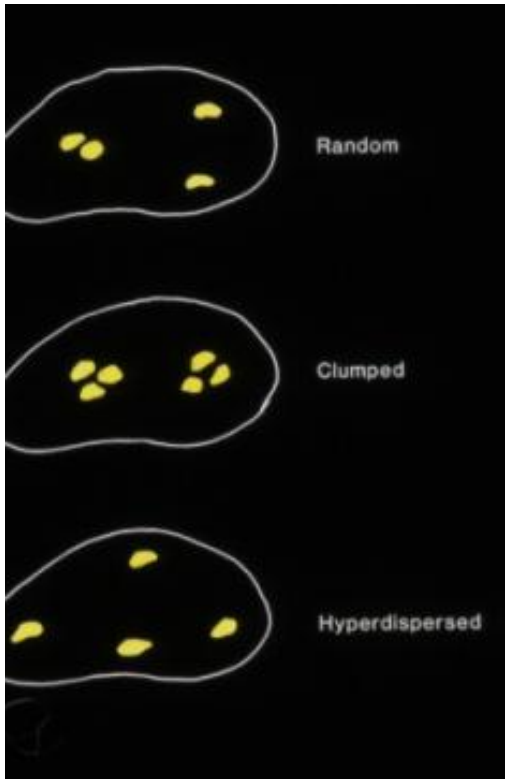
- Apply test line and look at intersections with boundaries
- Need to know length of test line and

$N_{RR}$ = R_R
$N_{WW}$ = W_W
$N_{WR}$ = W_R_W

- Use stereological SV formulae modified for 2D
- $L_A = \pi/2 \times P_L$
- Estimate Interface lengths for $LA_{RR}$, $LA_{WW}$, $LA_{WR}$
- **Index of Contiguity** $C_{RR} = LA_{RR}/(LA_{RR} + LA_{WR})$

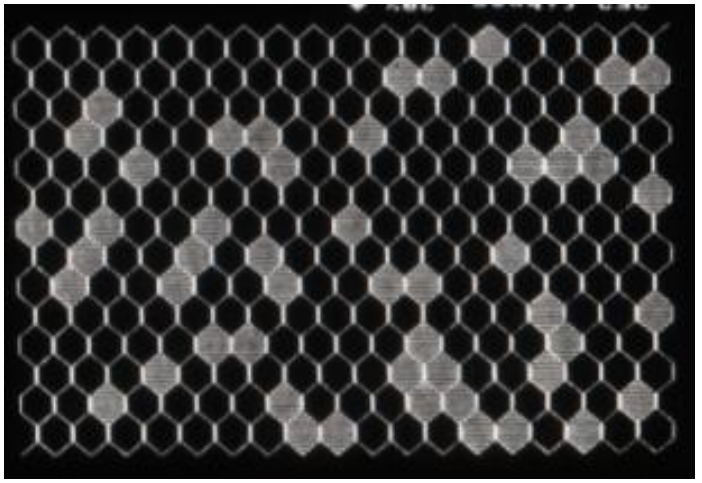


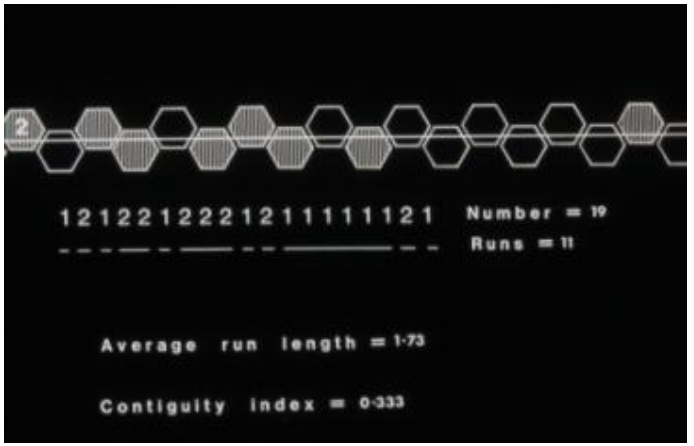
# DISTRIBUTION

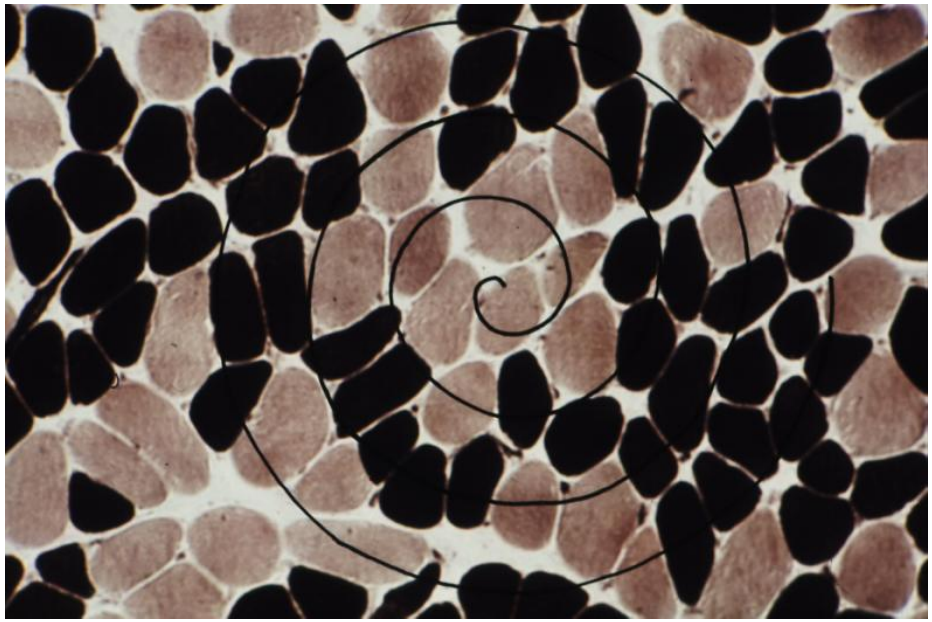


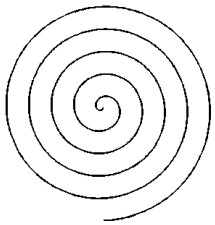


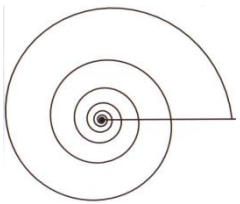
Spatial/Spiral Pattern Analysis of Muscle



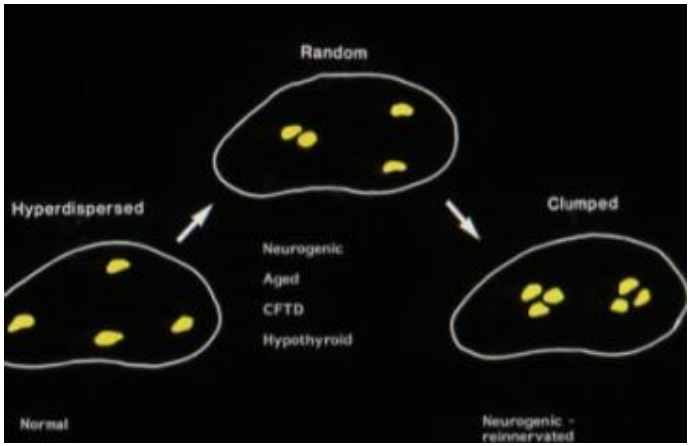


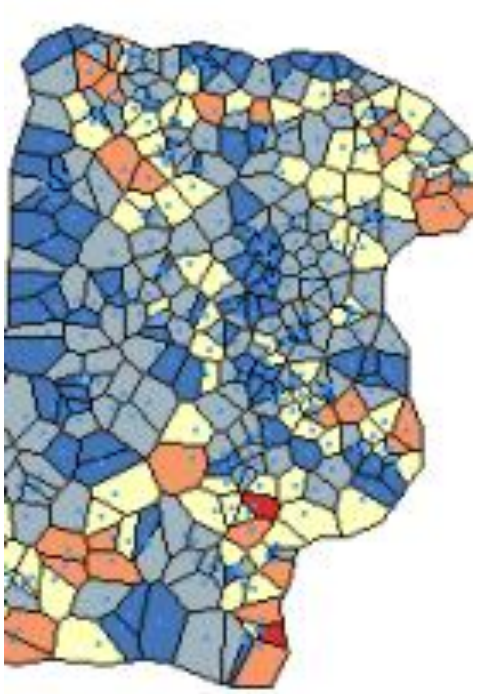


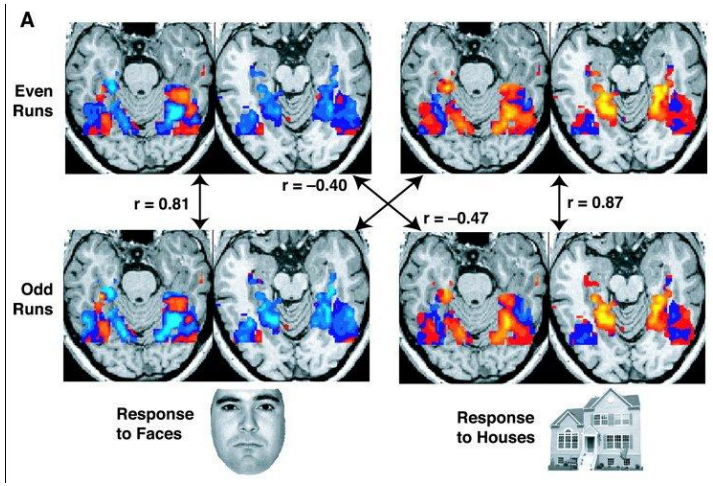


Mahon, 1985



# ASSOCIATION

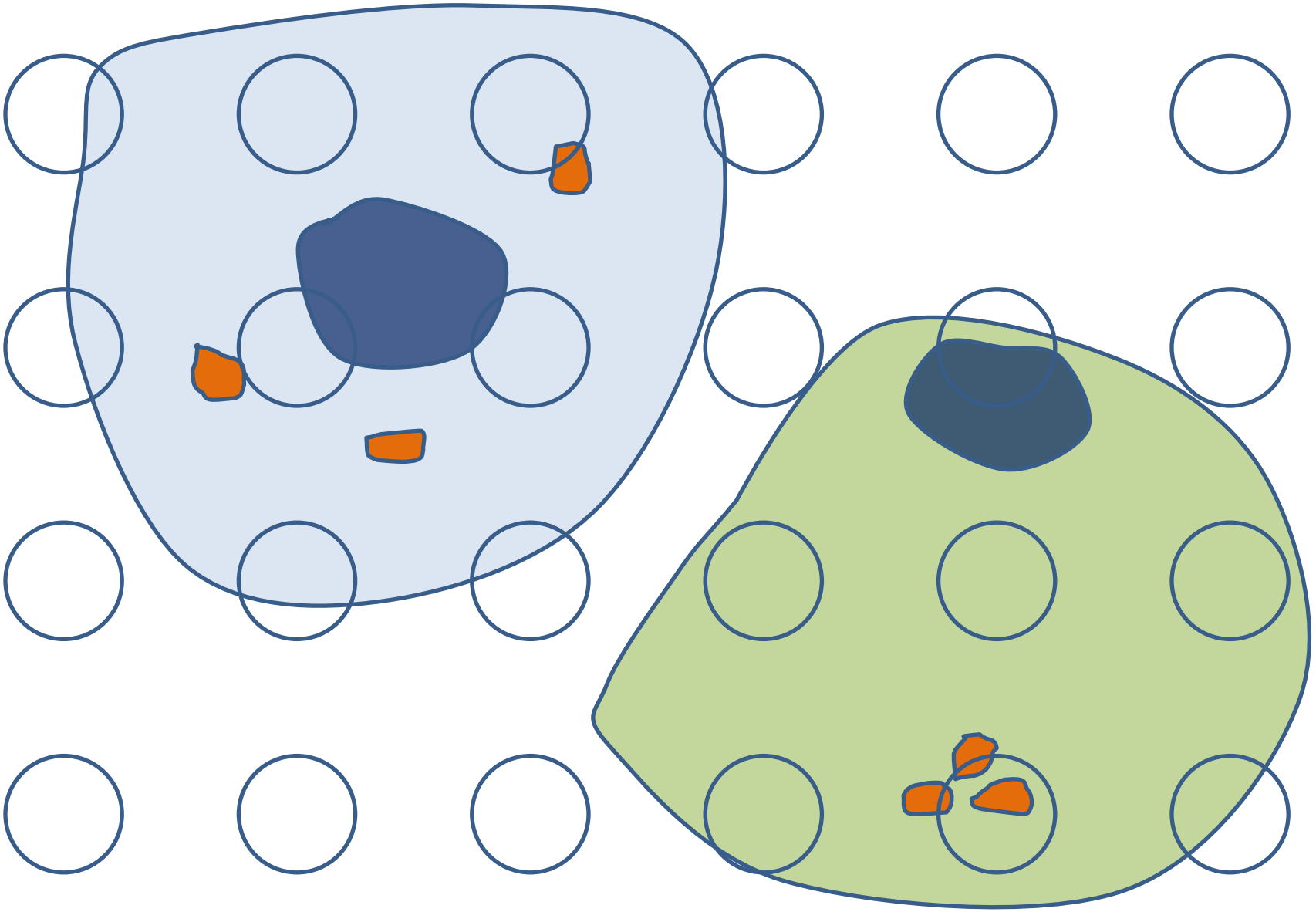




# Measure of Association – Circle Overlay Method

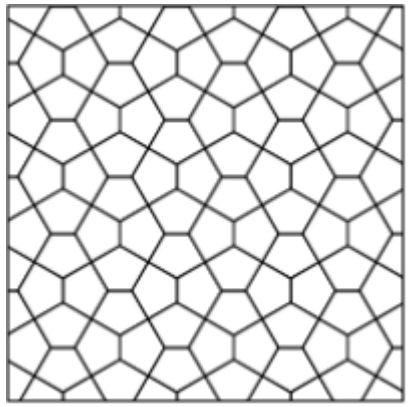
*Cruz-Orive, 1976*

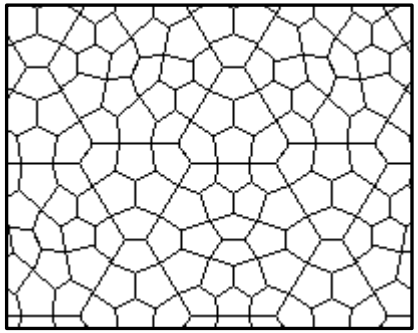
eg: Eccentric nuclei, organelle clumping or autoradiography *(Williams, 1977)*

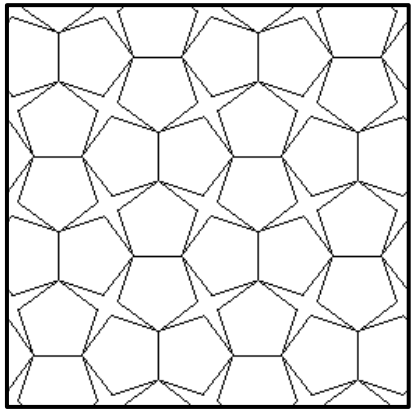


Problems: lattice size & spacing

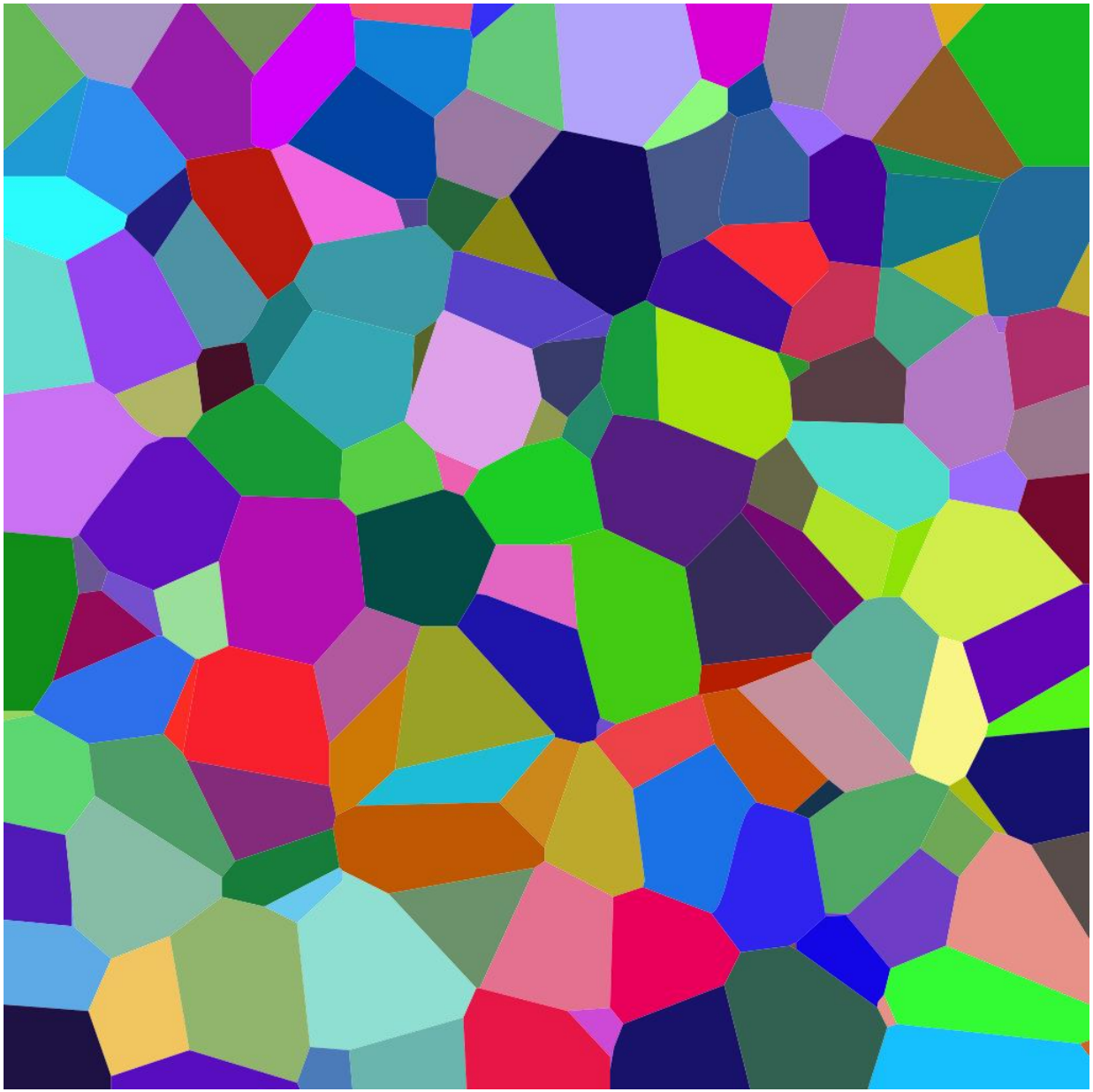
# Tesselation



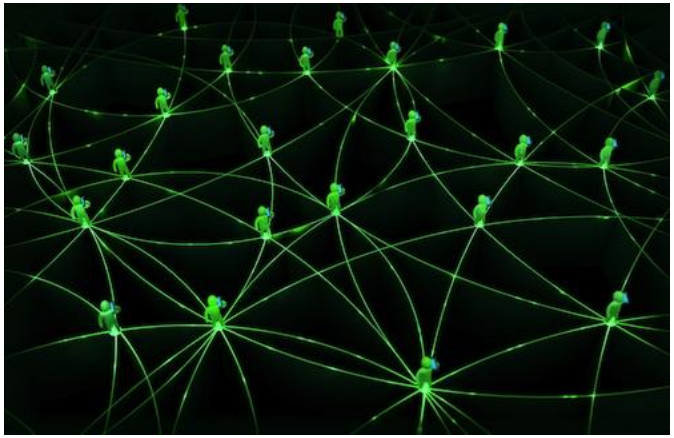


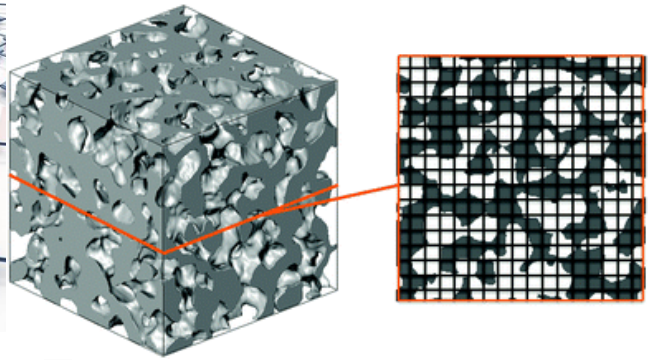


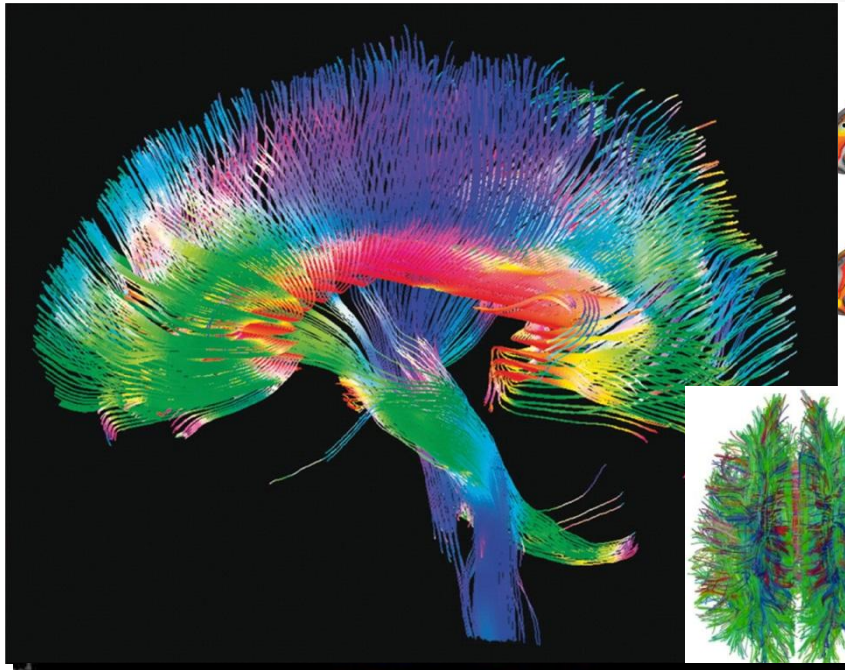
Apply lattices and use mathematical concepts of
“Lattice tesellation of congruent domains”

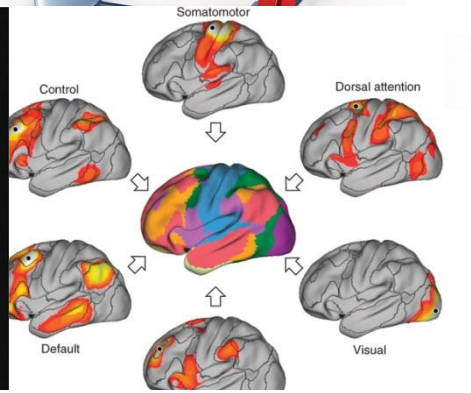


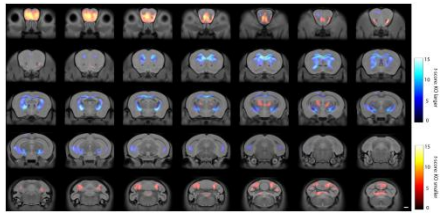
# CONNECTIVITY

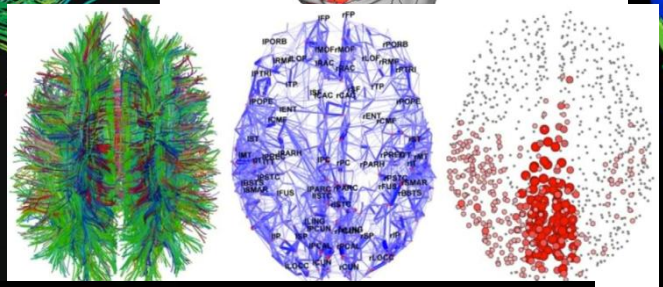


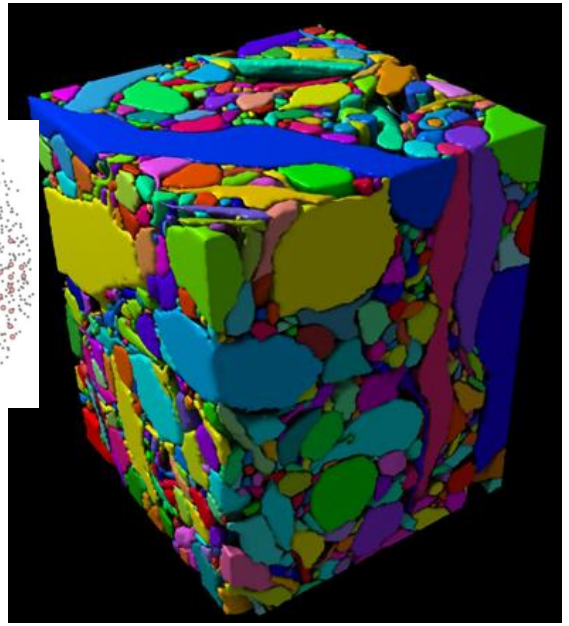


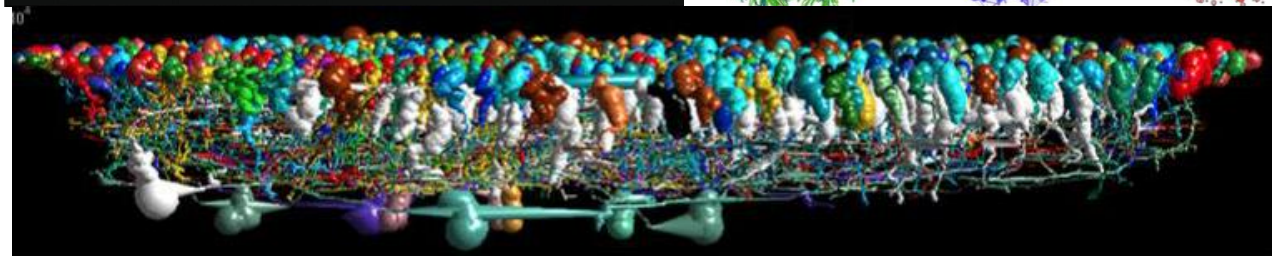




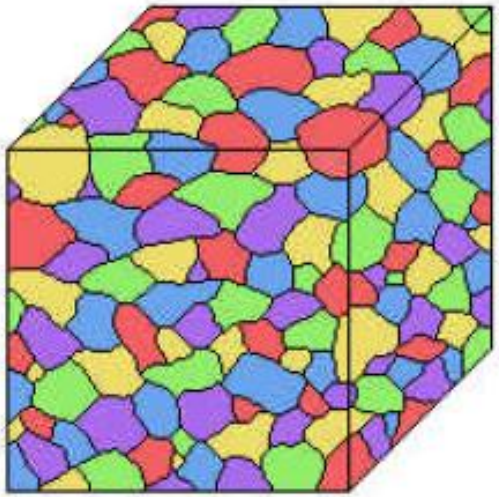




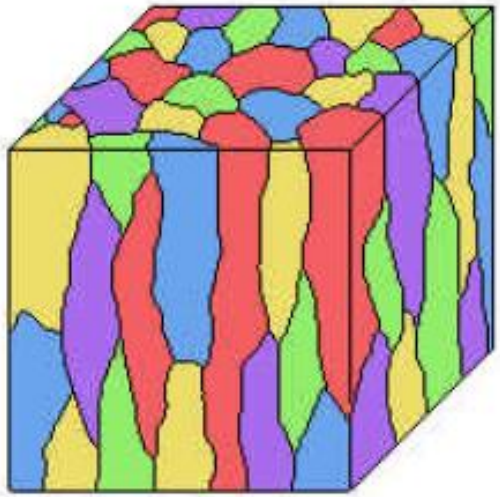


Brain Forest !

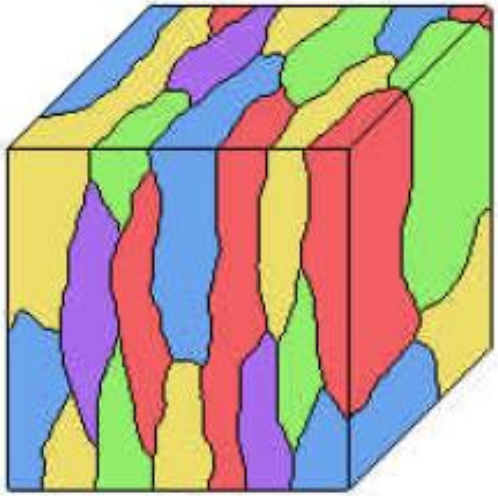
# ORIENTATION



a



b



c

Apply line lattice

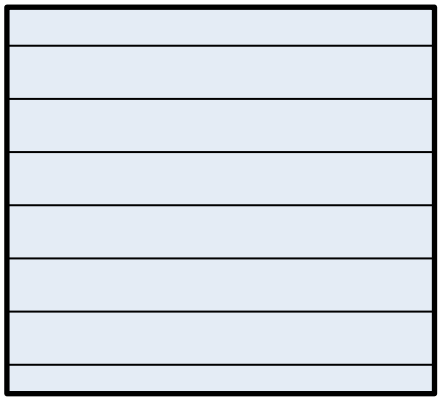
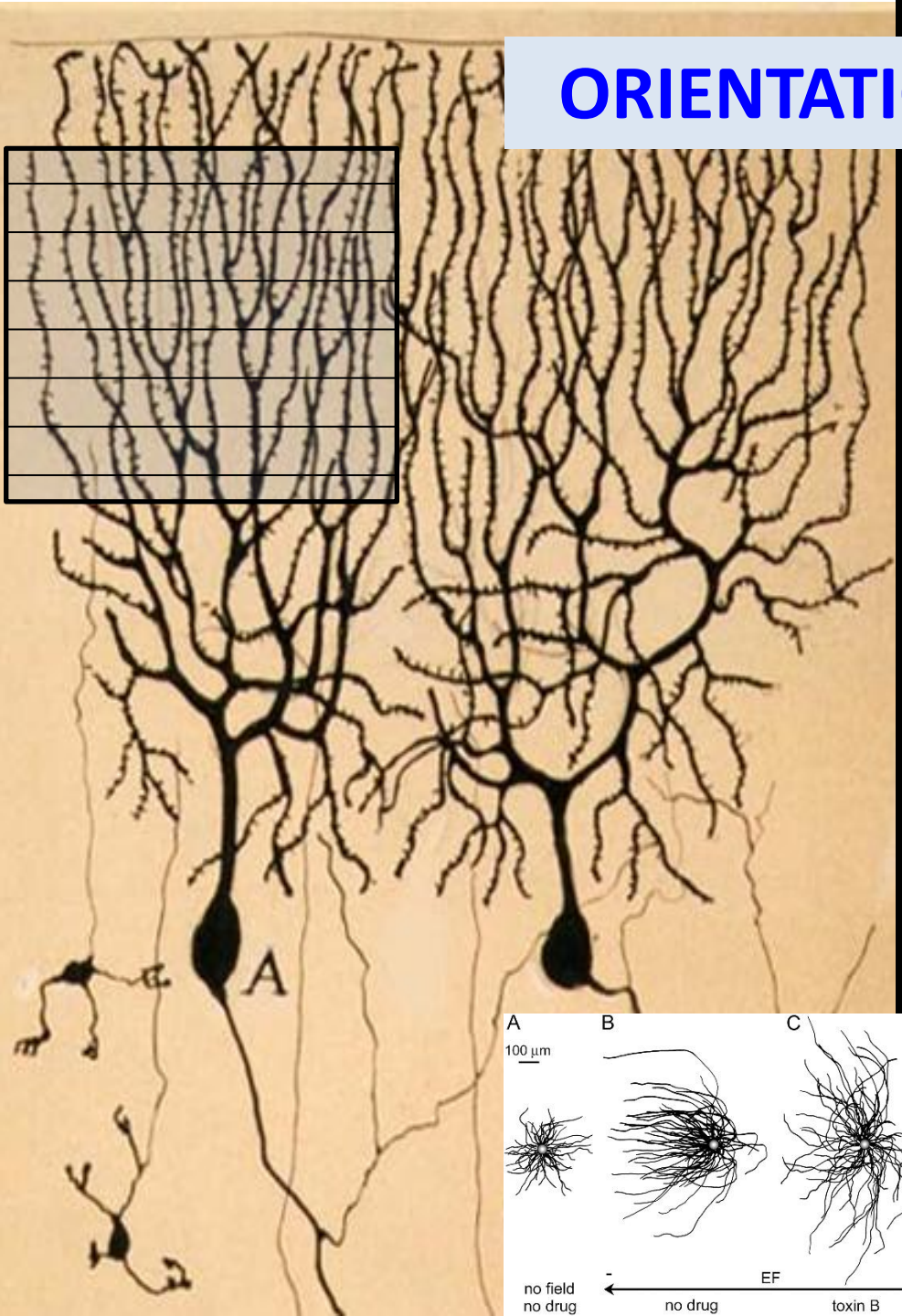


Figure 5. Examples of some simple kinds of preferred orientation: a) equiaxed; b) needles; c) plates.

# ORIENTATION





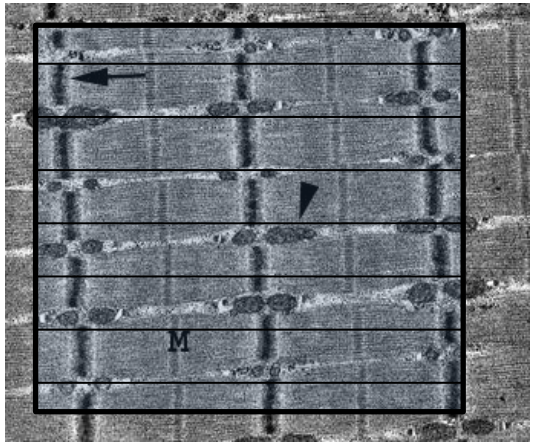


# ORIENTATION

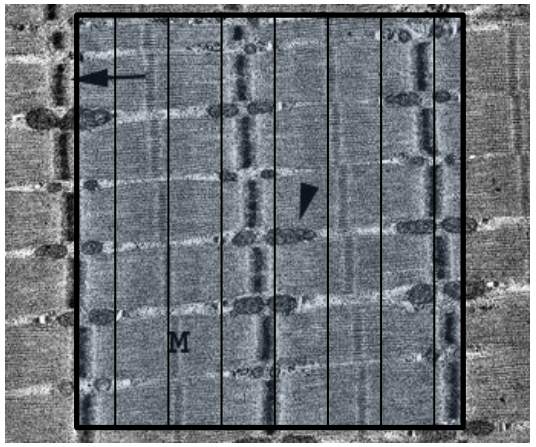
Sample/Probe it to – avoid it or measure it

- Cut section in ONE plane
- Apply line lattice in TWO orientations

II




⊥

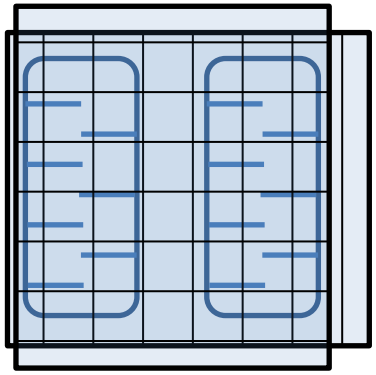



Remember for Isotropic structures $S_V = 2 \times I_L$

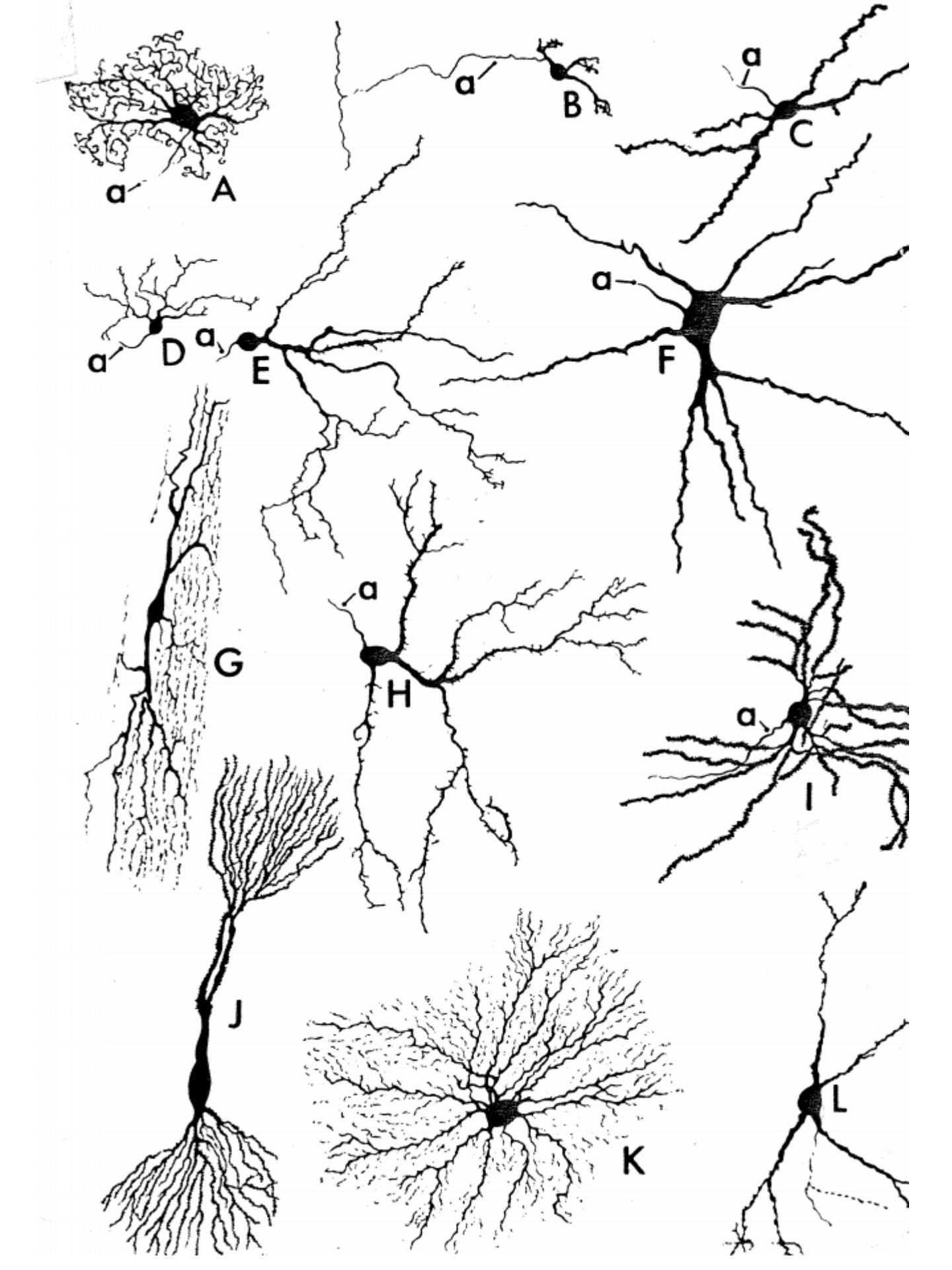
$$\mathbf{Sv} = \pi/2\ I_{L\perp} + 2I_{L\parallel} - \pi/2\ I_{L\parallel}$$

Degree of orientation of surfaces

$$\mathbf{\Omega} = \frac{I_{L\perp} + I_{L\parallel}}{I_{L\perp} + 4/\pi\ I_{L\parallel} - I_{L\parallel}}$$



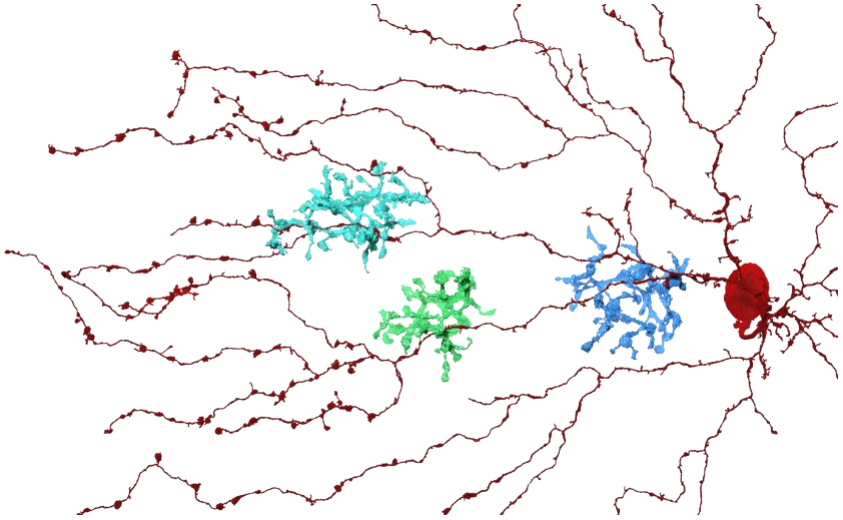
NT James, 1980s

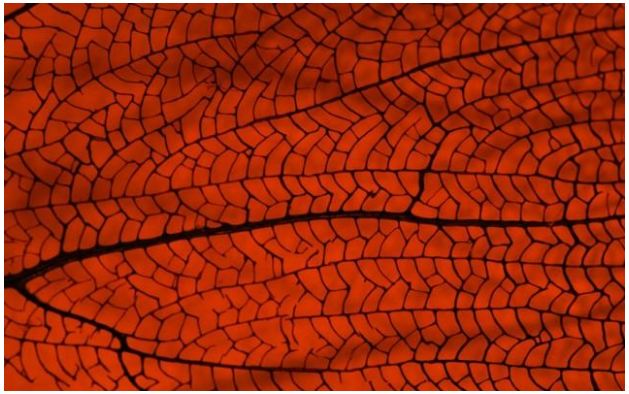

a
A
a
B
a
C
a
D
a
E
a
F
G
a
H
a
I
J
K
L

# BRANCHING


Sparse growth of dendrites
in an aging, inactive brain

Typical dendritic growth in an active brain



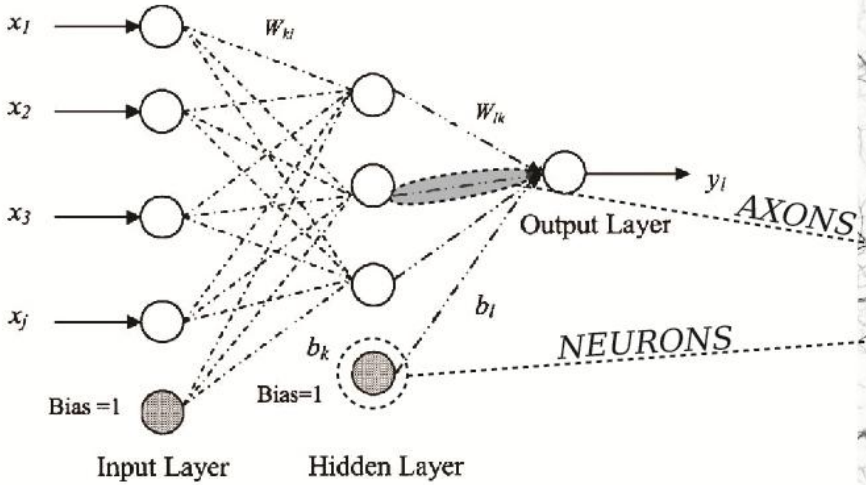


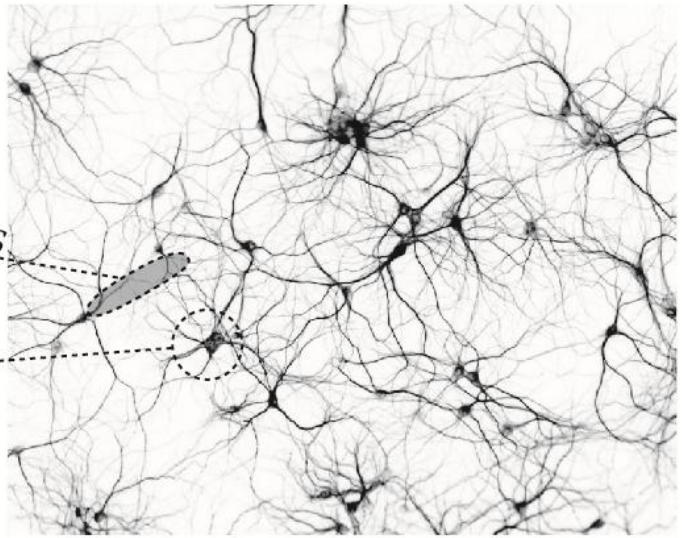
**Topology**

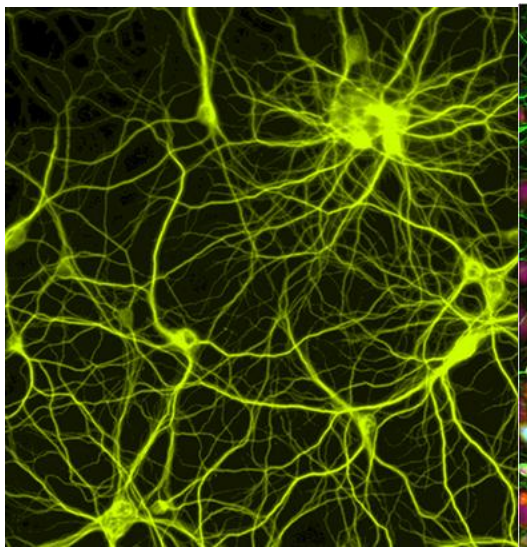
- Arbor or Tree analysis
- Vertex / Branch / Segment
- Bifurcations / tri .. / multi ..
- Angles

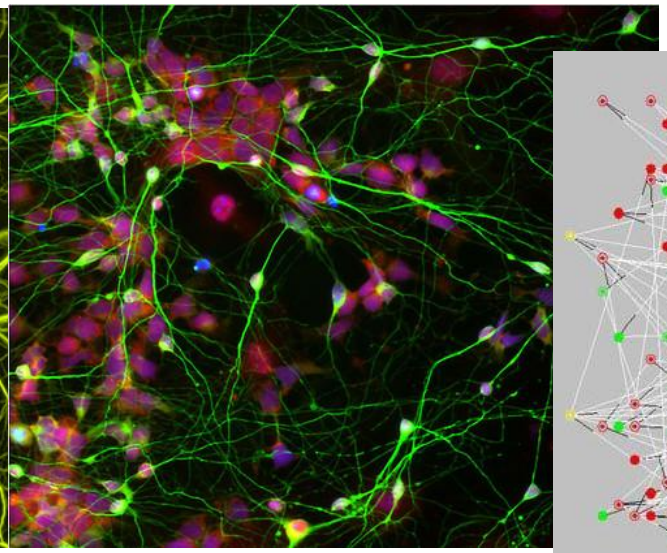
*Berry, 1980s*

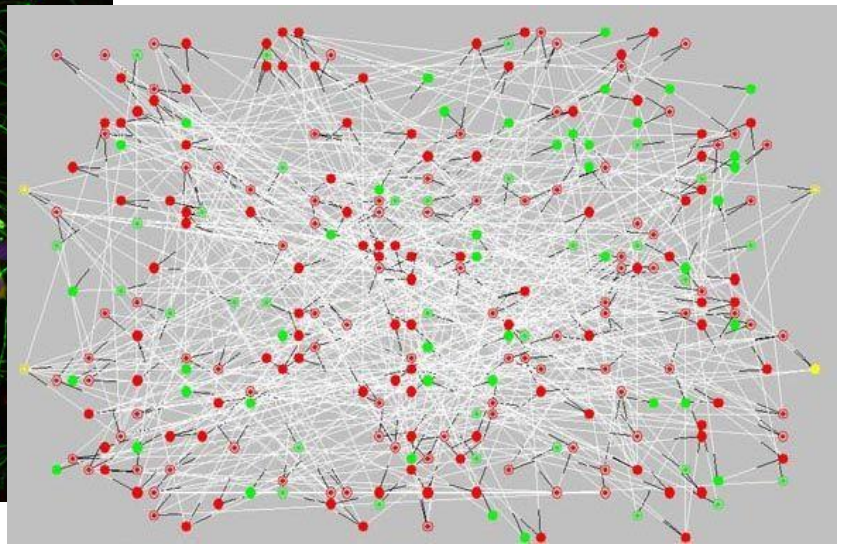
# NEURAL NETWORK MAPPING



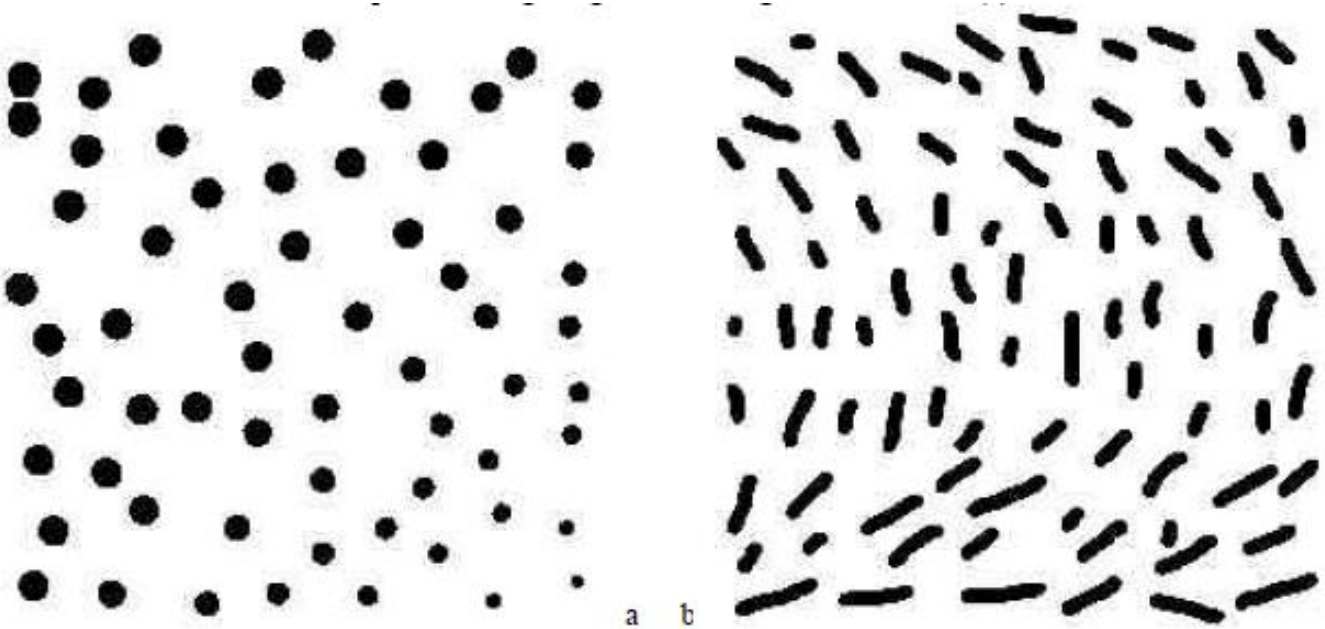







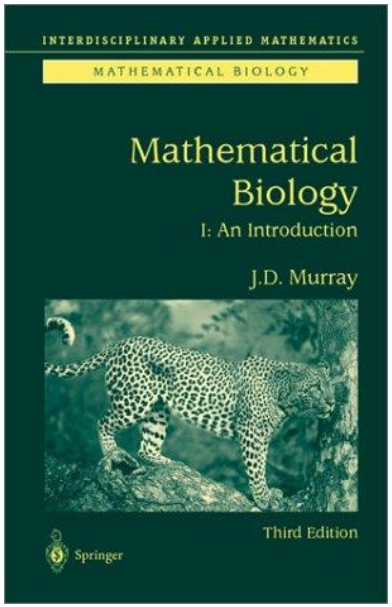
Artificial Intelligence (9th June 2014) Passed the Turing Test !

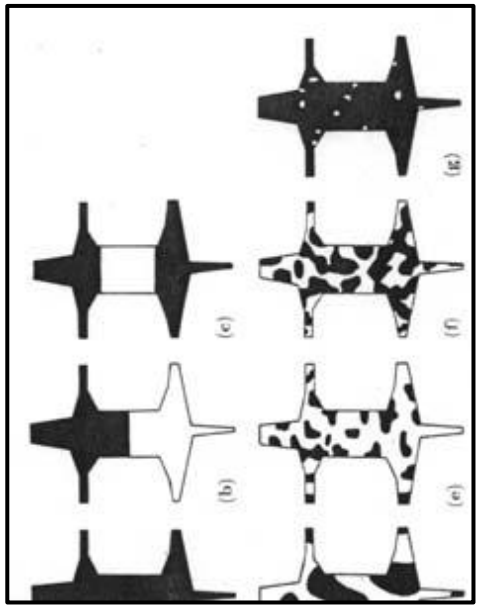


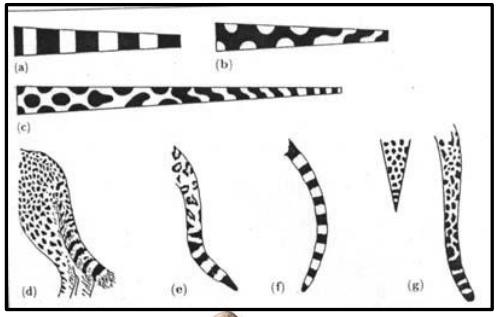
a b

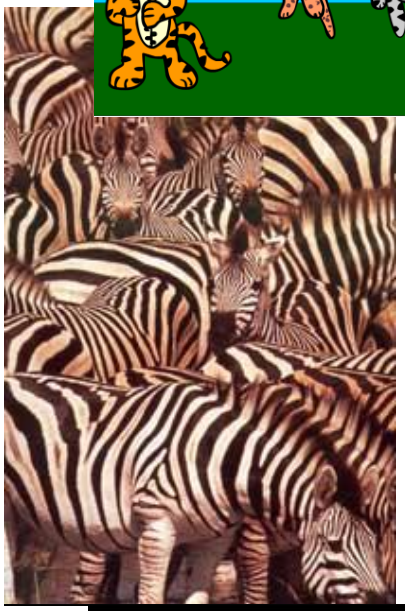
Figure 10. Examples of gradients: a) size; b) orientation.

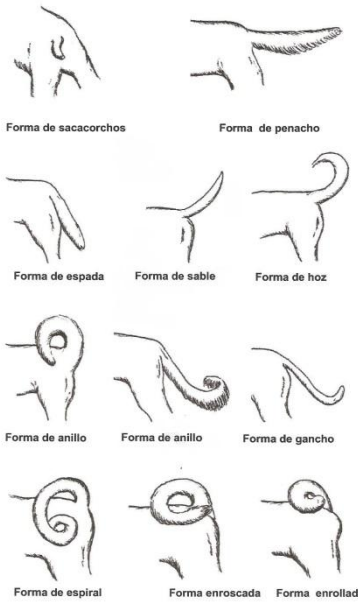
# Mathematical Modelling of Topography & Development



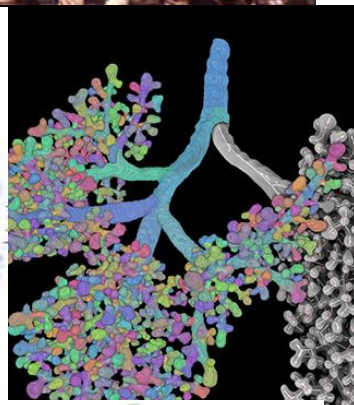



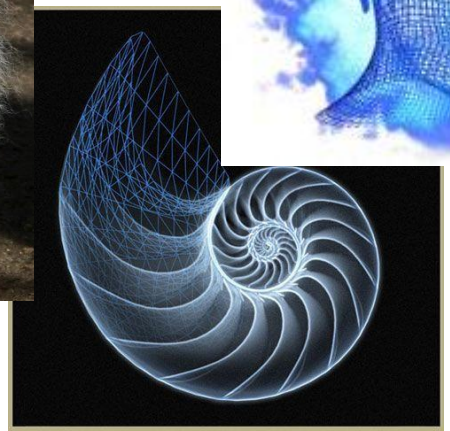


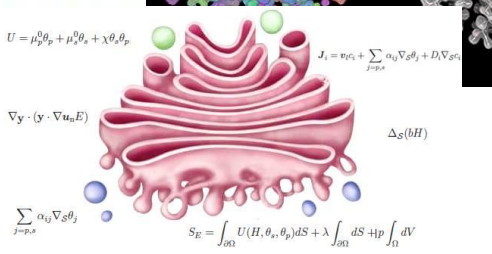



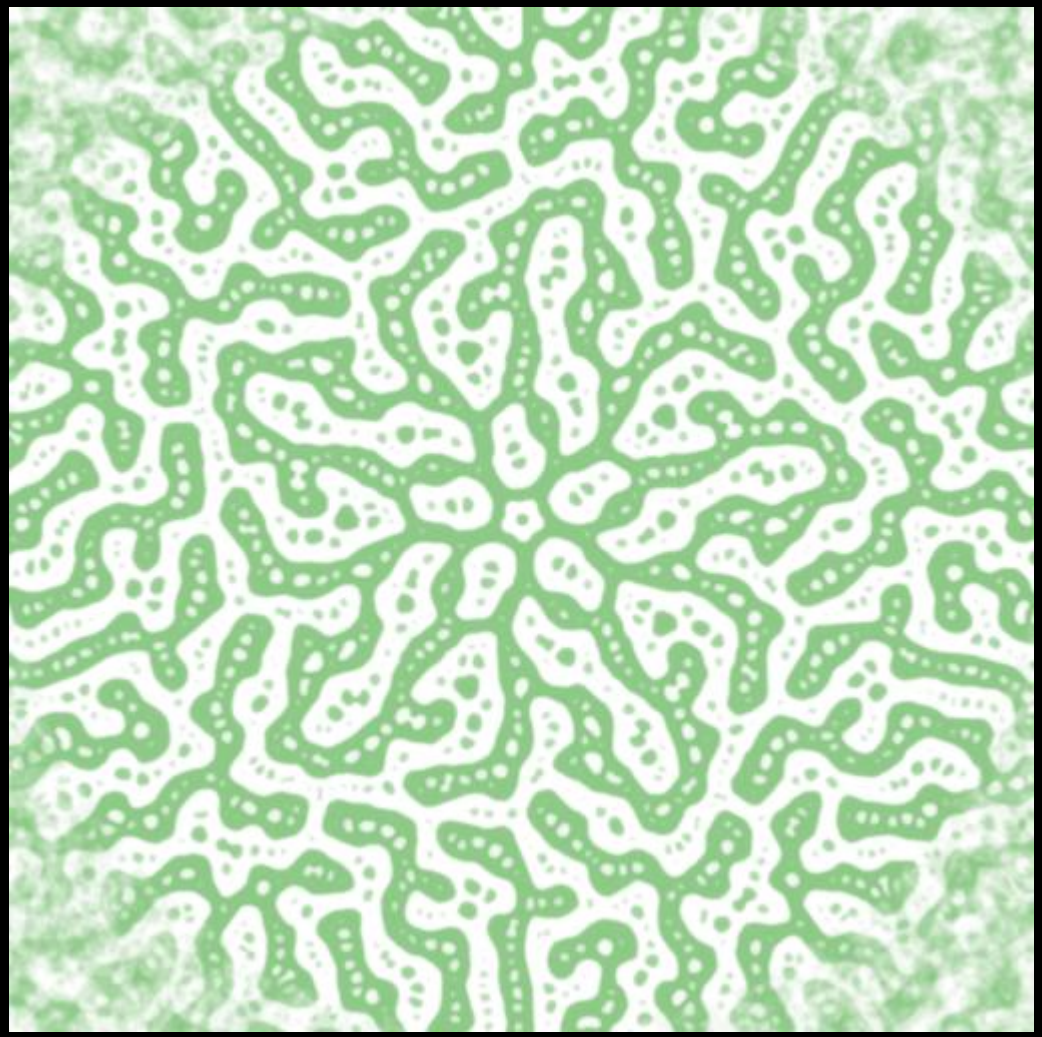






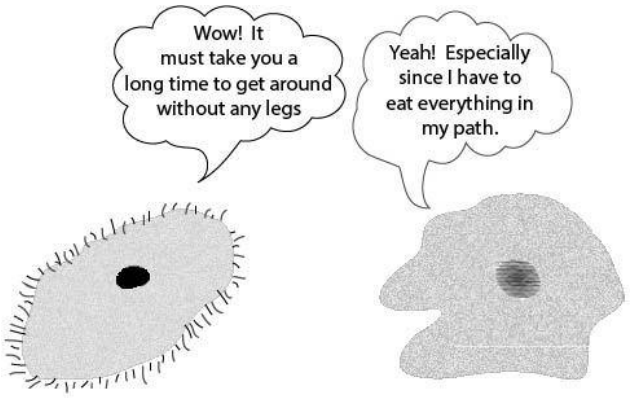






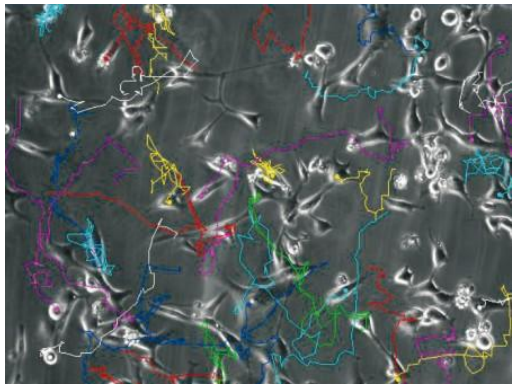
# Movement

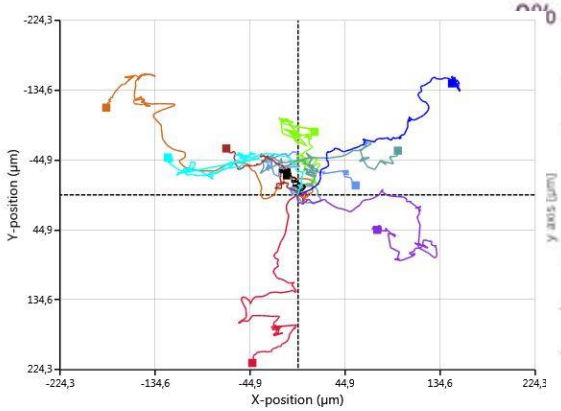
How ?

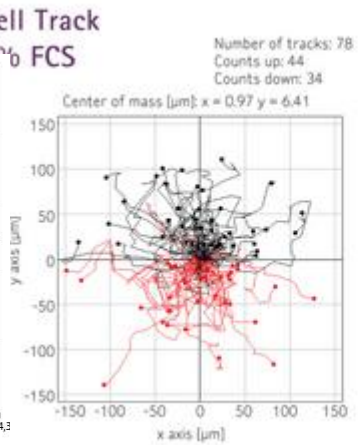


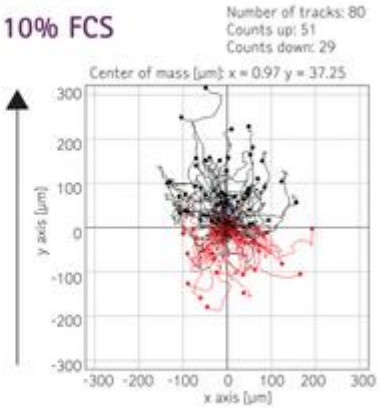

- Organisms, cells, organelles
- Subjective recording

- Tracing
- Direction
- Velocity
- Diffusivity
- Association





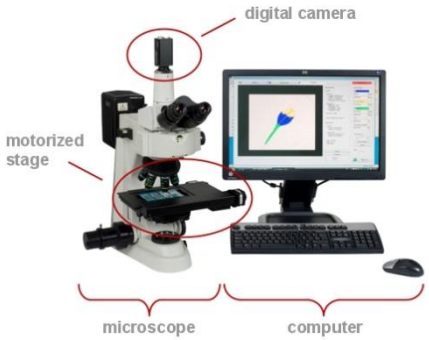



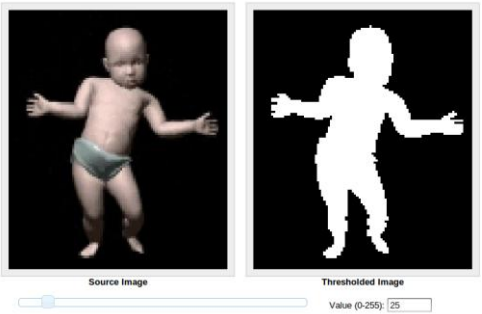
How ?

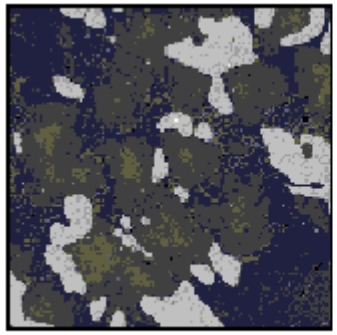
# Image Analysis

- Automated/Computerised Measurement vs Eye/Brain
- Early: 1969-1990 … then software based
  - MOP, Quantimet, Magiscan, Imagan, …. NIH Image-J, Matlab, LAS, Image Pro, i-Solution, QuPath
- Procedure
  - Sampling
  - Calibration
  - Image capture
  - Segmentation
    - Thresholding, Edge Detection, Erosion/Dilation
    - Object detection
  - Measurement
    - Size, shape, number, density (IOD?), arrangement, …
  - Data analysis and display
- + / -
  - Speed & Measurement / Identification, User, Cost, GIGO

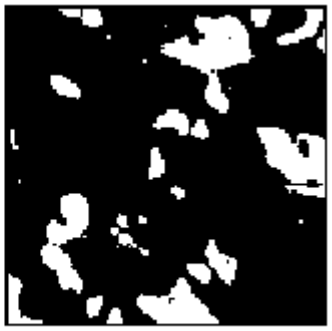


# Thresholding

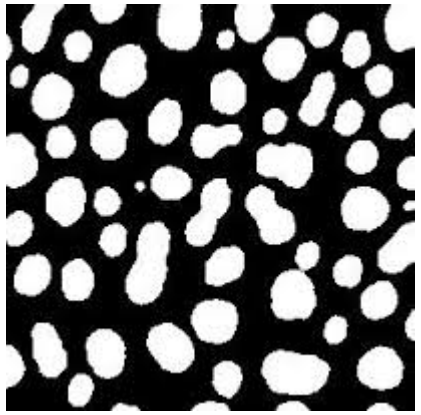


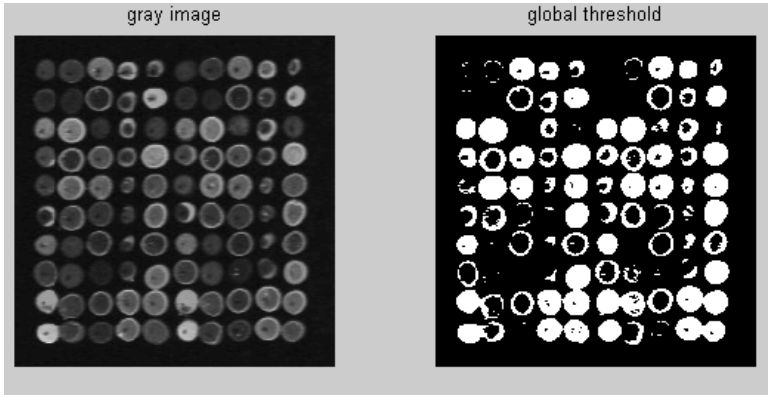



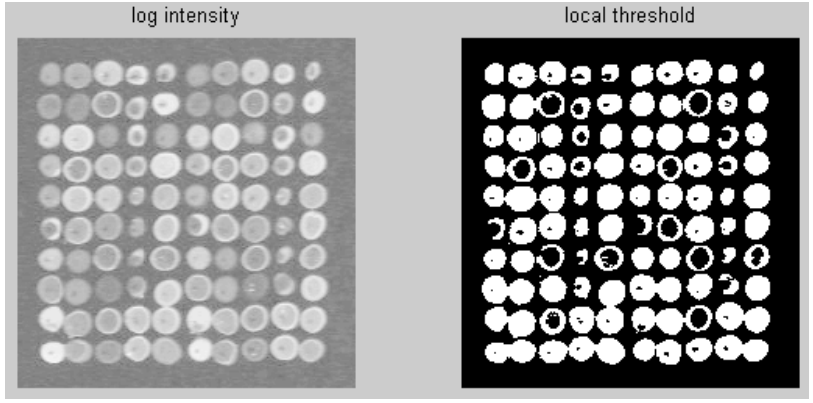
Original image



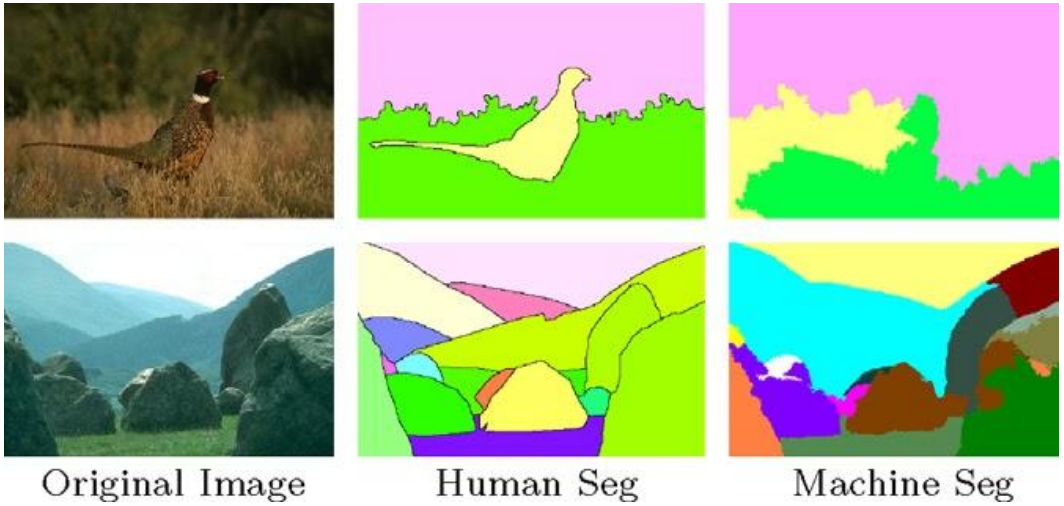
Thresholded binary image



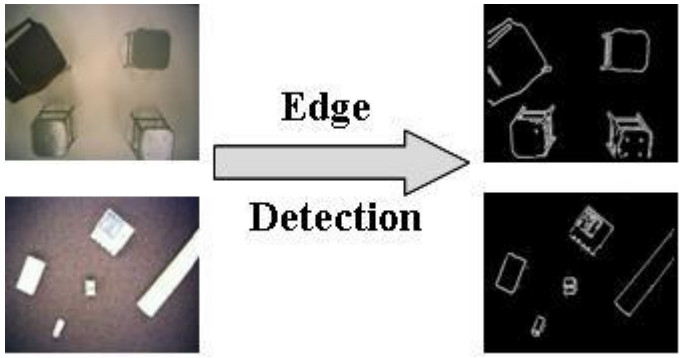


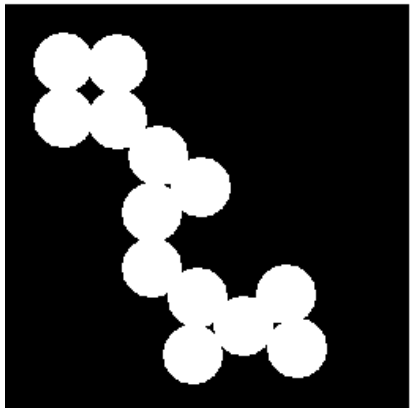

# Segmentation

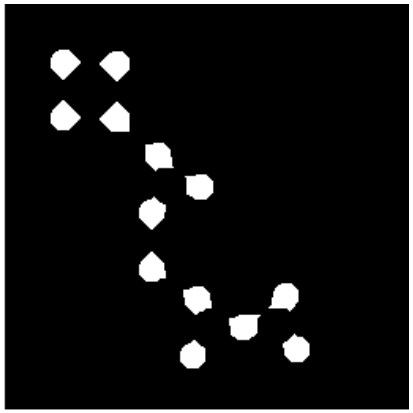


Edge Detection

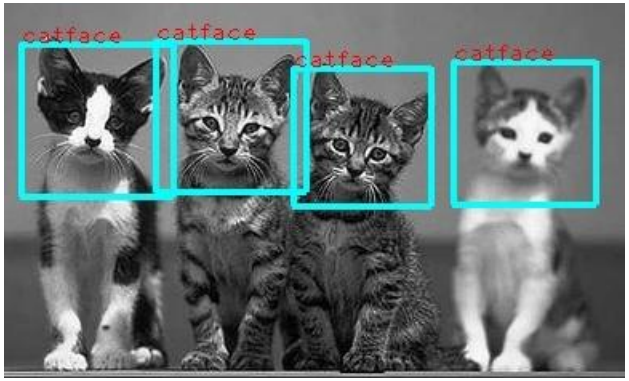



Erosion / Dilation

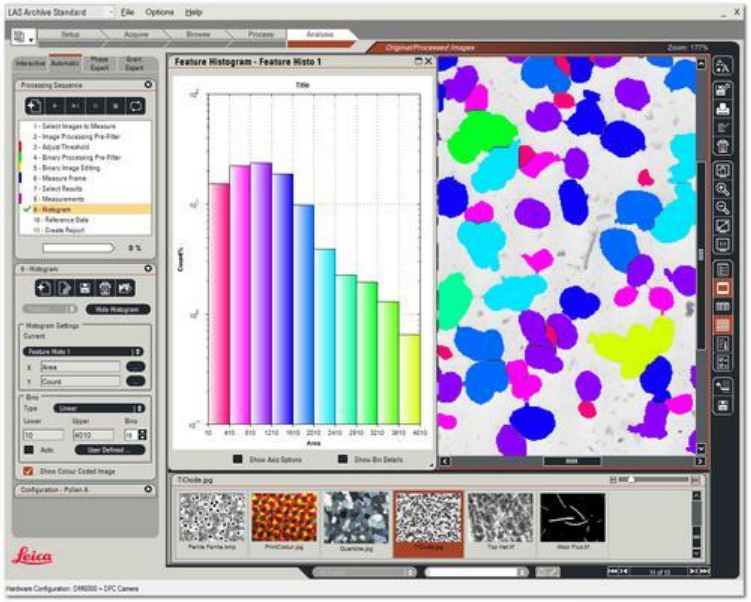


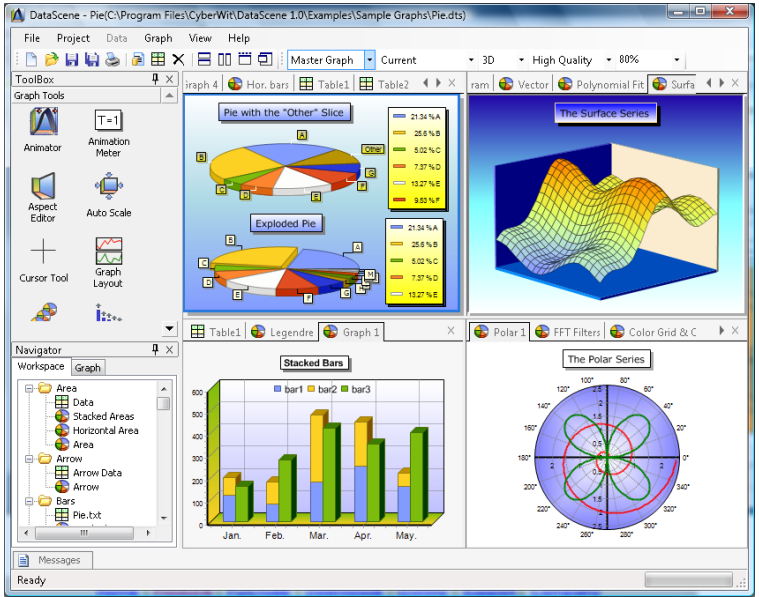


Object Detection



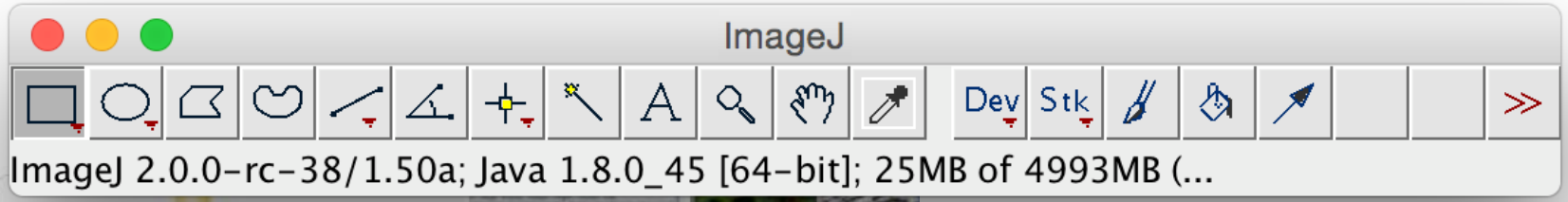
**Problems** – brilliant results output **But** does the user know what the machine is doing and have they considered bias, caveats etc. ?? !!



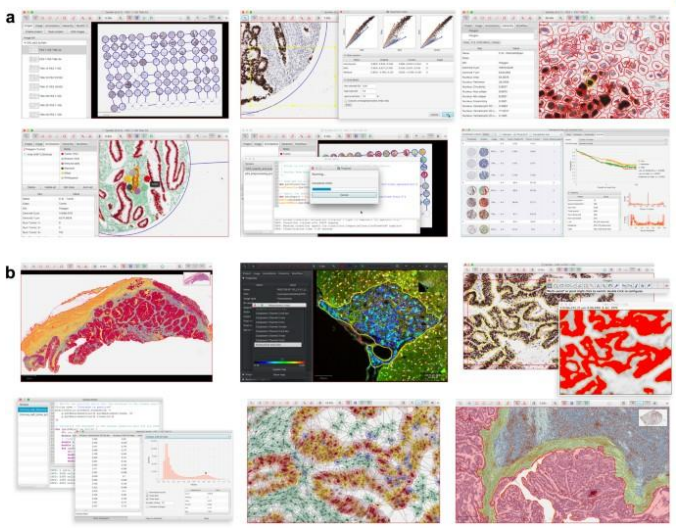


**THANKS TO MAGISCAN – IMAGE ANALYSIS IS NO LONGER THE PRESERVE OF THE SPECIALIST**





ImageJ




**QuPath**

Quantitative Pathology & Bioimage Analysis

Results ?

# GIGO

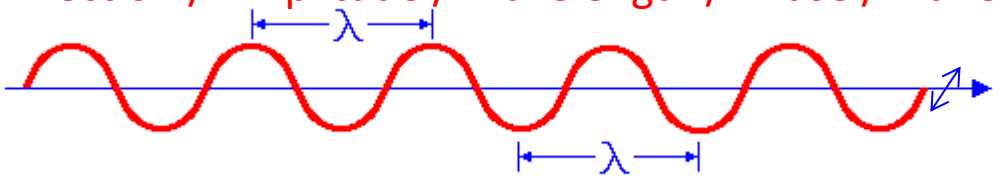
**Garbage In – Garbage Out !**

"Garbage to ten decimal places is still garbage !"

How ?

# Analytical Microscopy

Direction / Amplitude / Wavelength / Phase / Plane

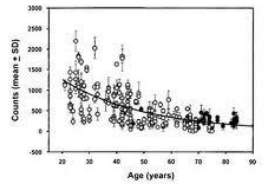



- Reflectance (Number)
- Microdensitometry (Amount)
- Fluorometry (Fluorescence)
- Phase Contrast / Refractometry (Density)
- Interferometry (Density, Thickness, Mass)
- Polarisation / Polariometry (Orientation)

How ?

# Microdensitometry 1

How much substance present?



The decline in macular pigment optical density with age, as measured by resonance Raman spectroscopy.

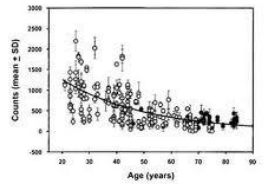
- Measure Areas, Volumes and Densities
- Histochemistry (Specificity/Stoichiometry/Localisation/Section thickness/ …)
- Bouger-Beer-Lambert Laws
- Absorbance & Transmittance
- Homogeneous Parallel Monochromatic Spot Light
- Optical Density
- Distribution Error
- Integrated optical Density
- Equipment: Flying-Spot microscope, M86, Magiscan

1930s, 1970s, now

How ?

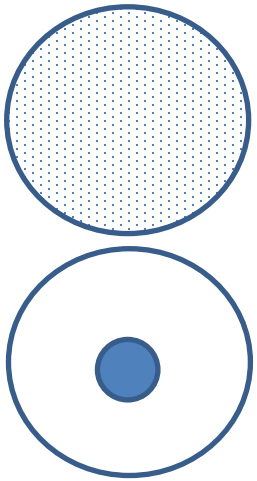
# Microdensitometry 2

How much substance present?



The decline in macular pigment optical density with age, as measured by resonance Raman spectroscopy.

- **Beer:** Concentration $\alpha$ Absorbance
- **Lambert:** Absorbance = $\log_{10}$ Incidence/Transmit
- **Concentration** = g/cm$^3$ or g/ area & thickness
- **Distribution Error:** Uniform v Non-Uniform

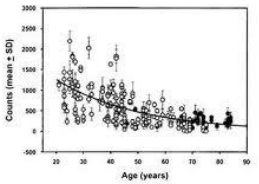


| T | OD | IOD (ArxOD) |
|---|---|---|
| 50% | 0.3 | 0.3 |
| ??% | 30 & 0 | 0.3 |

Application

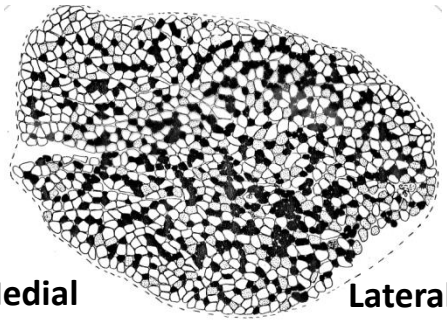
# Microdensitometry 3

How much substance present?



The decline in macular pigment optical density with age, as measured by resonance Raman spectroscopy.

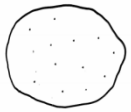
- **Oxidative Enzyme distribution in a limb muscle ?**

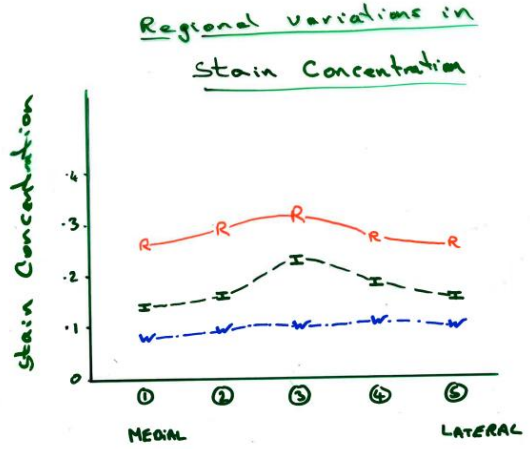


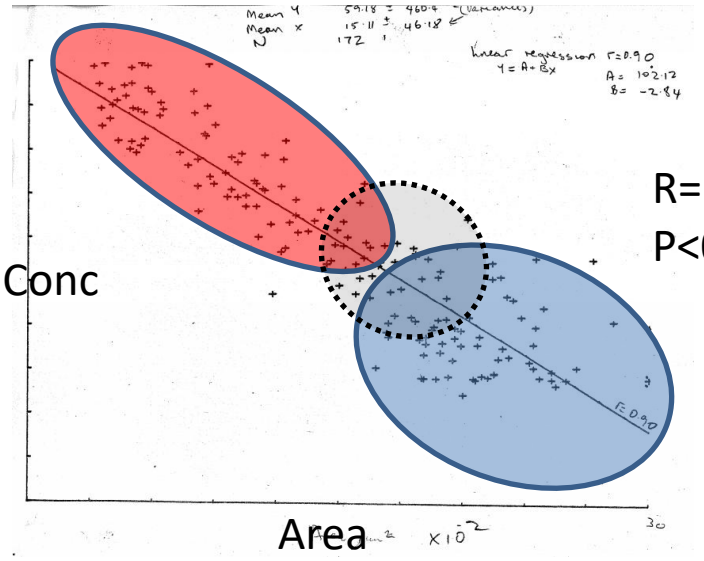


**Red**


**Intermediate**

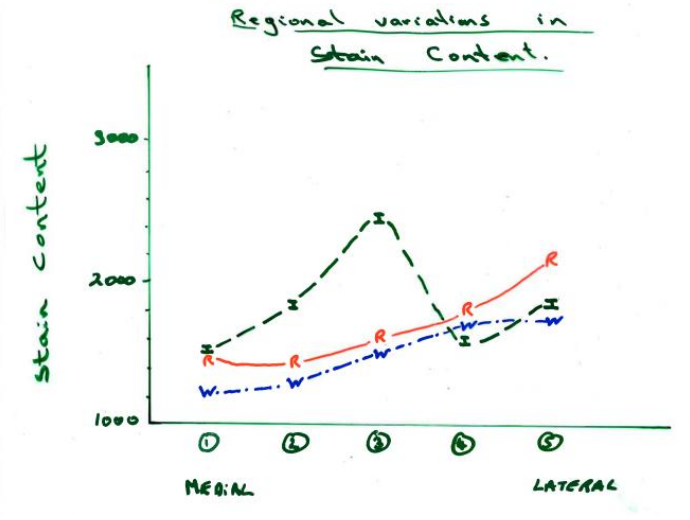

**White**




R= -0.90

P<0.001



- Why measure ?
- What do you want to measure ?
- How do we measure ?
- Are the results unbiased, precise, accurate, valid, meaningful ?

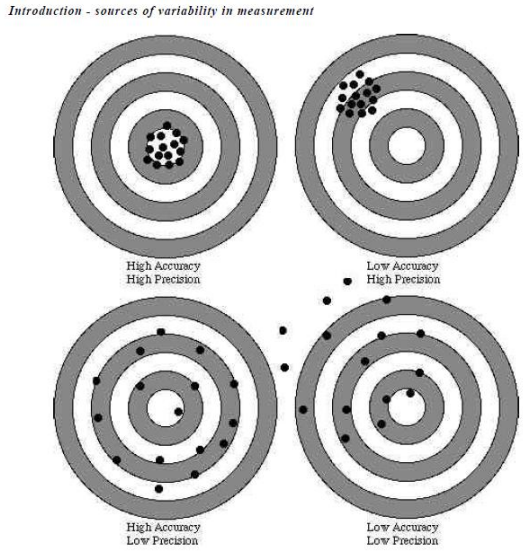
Interpretation ?

# Statistics !!!

- Means
  - Arithmetic, Harmonic, Angular
- Non-Parametric, Parametric
  - Gaussian
  - Poisson
  - Monte Carlo, Bose-Einstein, Autocorrelation
  - MANOVA, Hotelling test, Bonferroni
- Data presentation
  - Binning, Linear, Areal, Percentages, …

# Statistics !!!

- Accuracy
  - Degree of closeness to true value
- Precision
  - Related to reproducibility and repeatability
  - Improve by increasing sample size
- Bias
  - Random or Systematic error
- Valid
  - Measurement system which is ACCURATE and PRECISE and UNBIASED



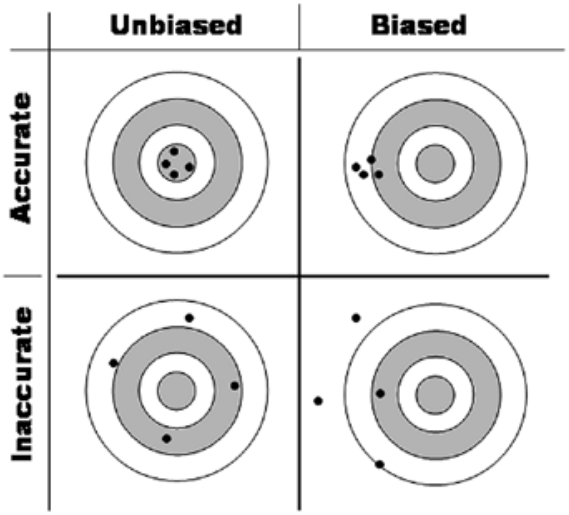
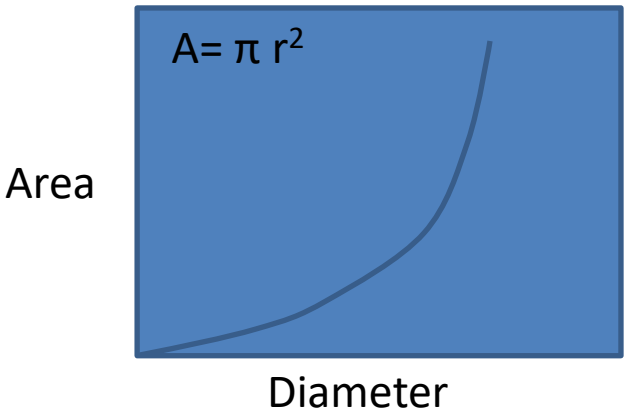
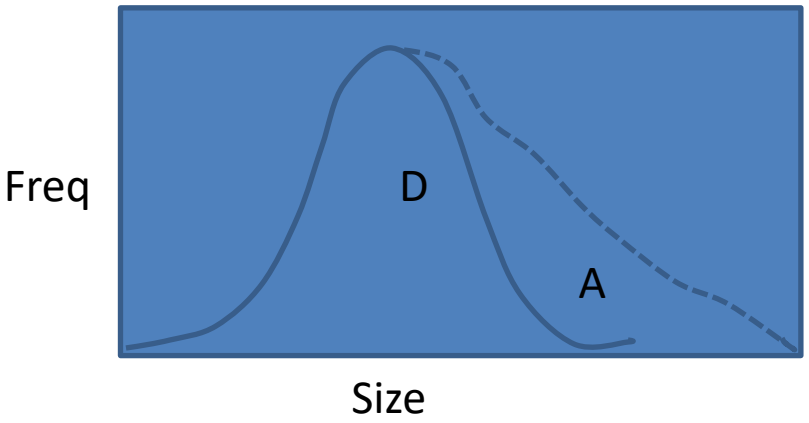




Fig. 2 Schematic illustration of the difference between accuracy and bias. The top row of targets shows accuracy that is the hits are closely clustered together. The bottom row shows inaccuracy and there is a marked scatter of hits. In the left hand column the average of the cluster of hits tends towards the bull s-eye, which means that they are unbiased. The right hand column shows the converse case, these hits are biased (based on Howard and Reed 1998).

# Problems in Data Analysis

- Means
  - Vv mito = meanPm/meanPt  or  mean of Pm/Pt
- Circles
  - meanDia and calculate area or meanArea and calculate dia



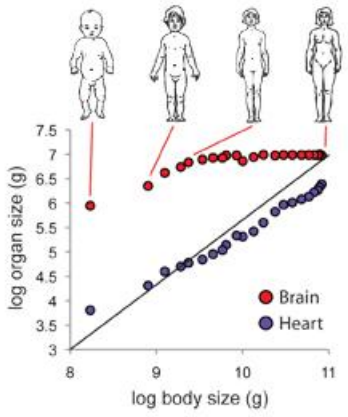



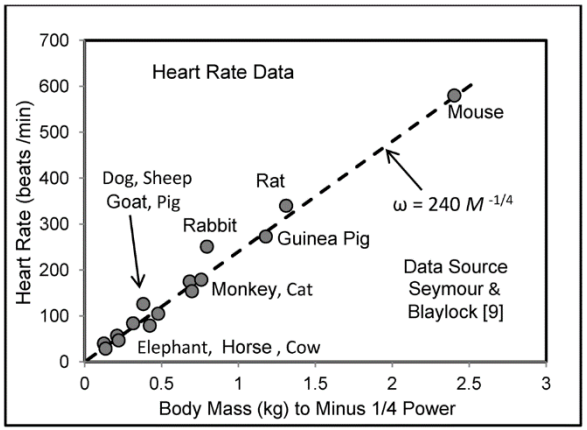
The Bigger Picture ?

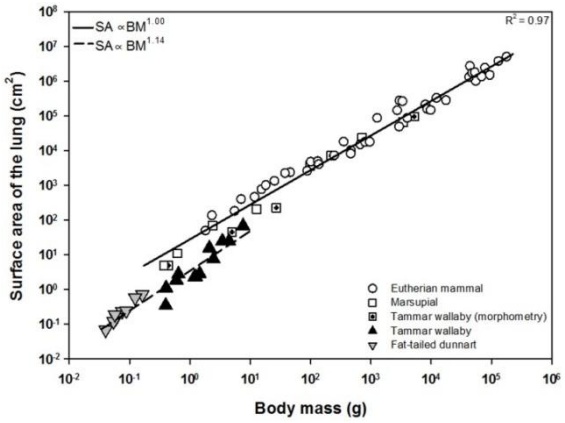
# Allometry

- Relationship of size, shape, anatomy, physiology, behaviour
- Snell (1892), D'Arcy Thompson (1917), Huxley (1932), Alexander (1970s), Weibel (1990s)
- Scaling
  - Organelles, cells, organs, organisms, populations
  - Within species
  - Between species

See also Lecture …
Geometric Morphometrics (Frigot)



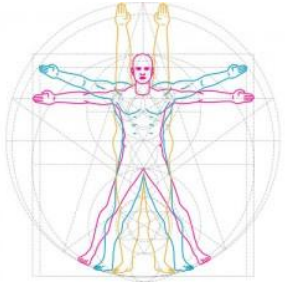



# How to conduct a measurement project

# How to analyse a measurement project

- What questions did they ask ?
- How reliable is the visualisation of objects?
- Did they sample correctly ?
- Did they use a valid measurement tool ?
- Did they measure precisely, accurately ?
- Did they make any assumptions about shape, size, orientation etc ? Should they ?
- Did they analyse/present the data correctly ?
- Is their structural/functional/clinical reasoning valid?

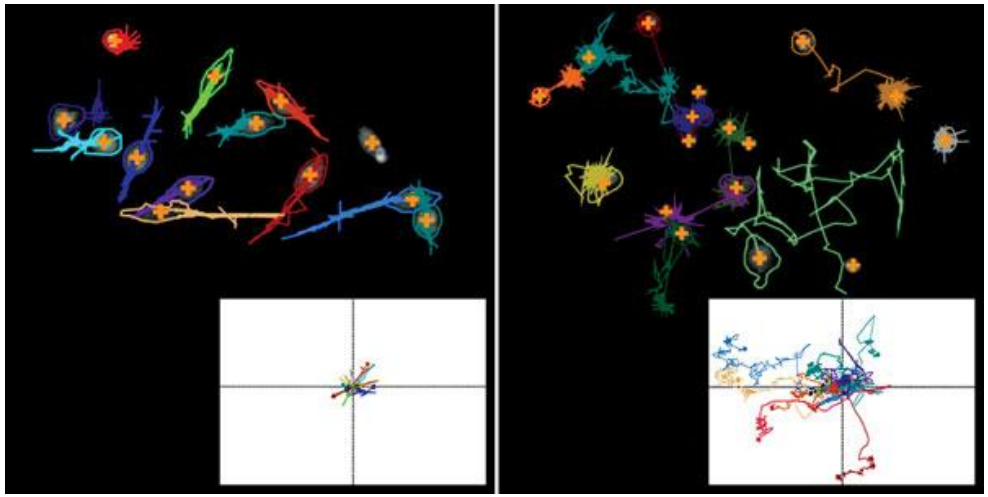


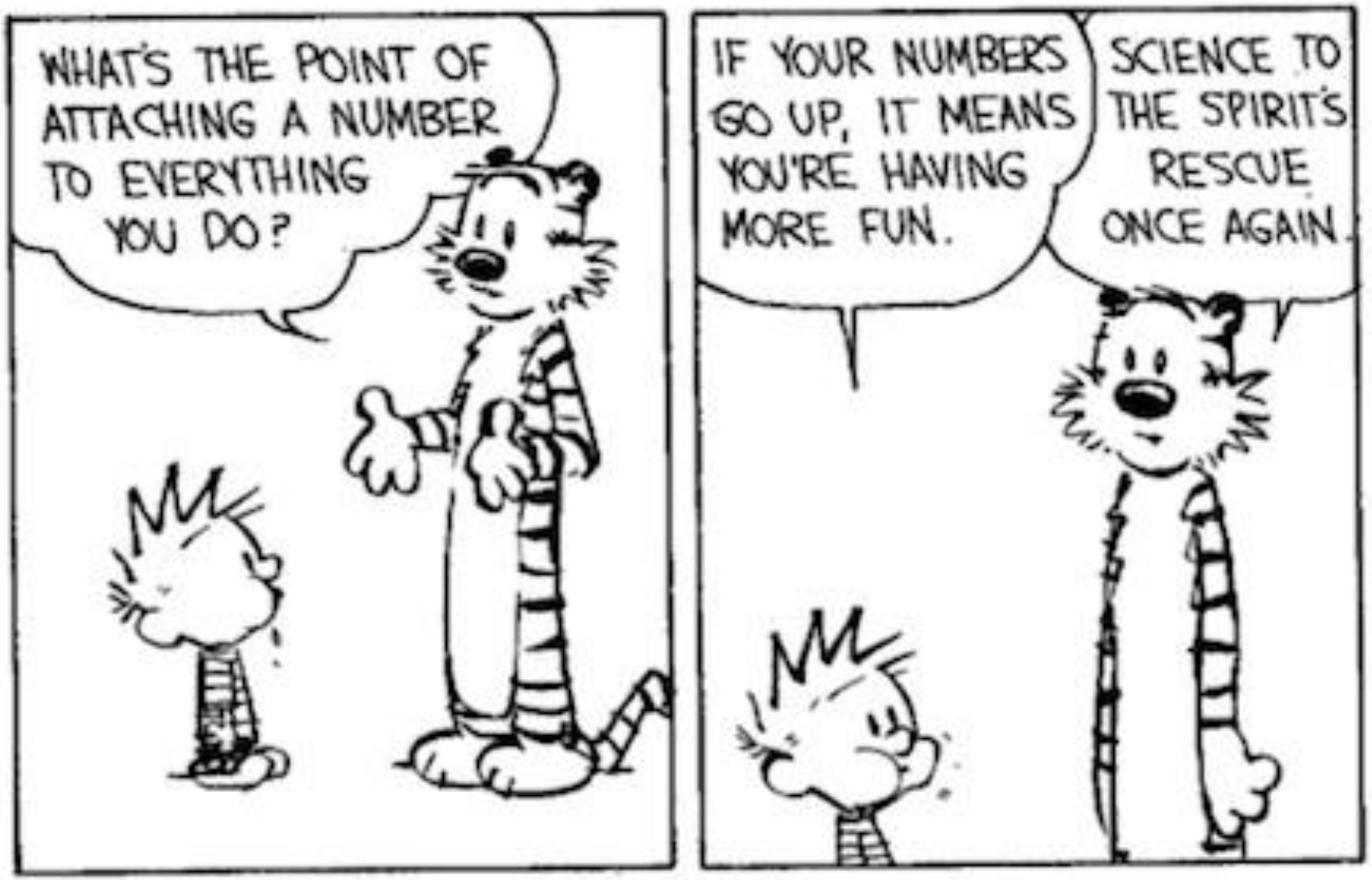
*… Caveat Emptor !*

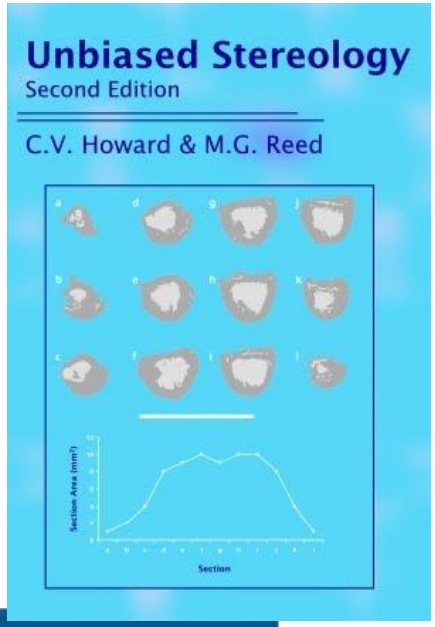
# Mike's motto ……

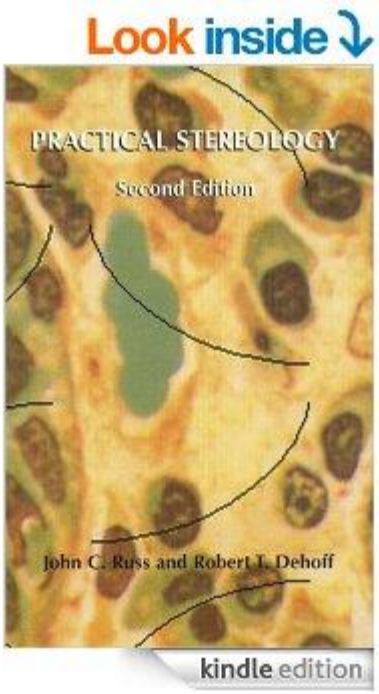
## If it doesn't move ……. Measure it !

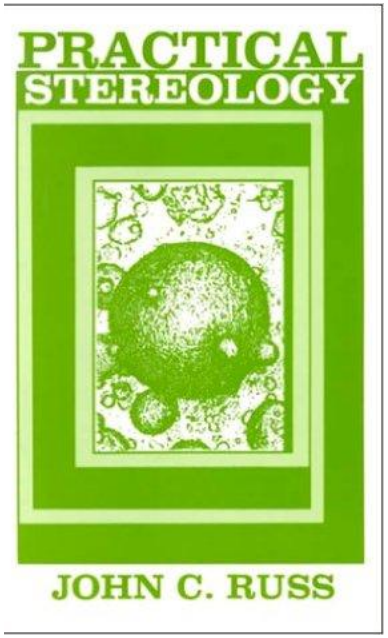
## If it moves ….. Measure its movement as well !!

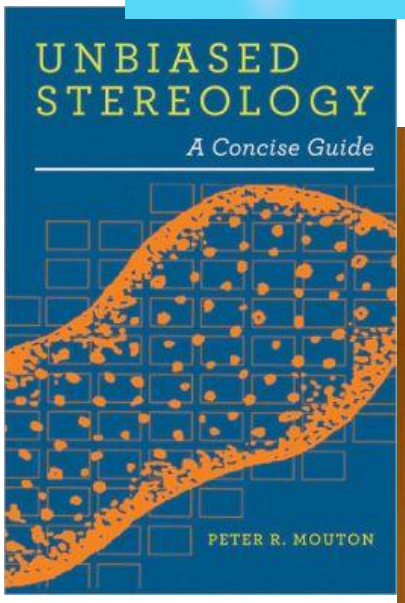


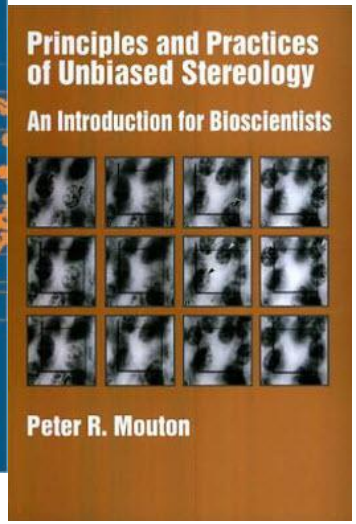

WHAT'S THE POINT OF ATTACHING A NUMBER TO EVERYTHING YOU DO?
IF YOUR NUMBERS GO UP, IT MEANS YOU'RE HAVING MORE FUN.
SCIENCE TO THE SPIRIT'S RESCUE ONCE AGAIN.

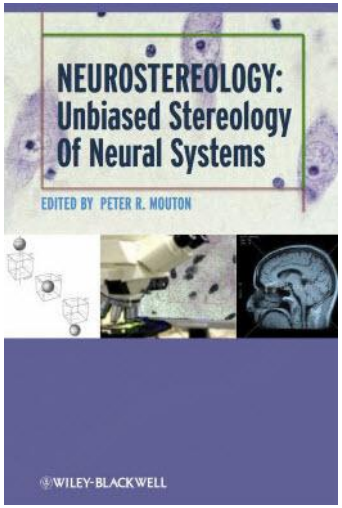


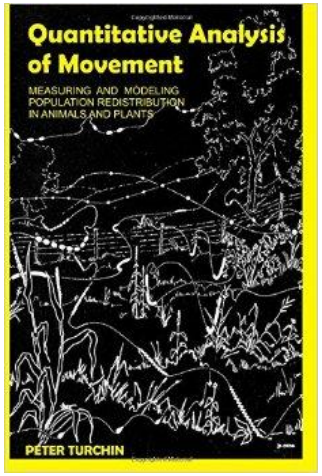




http://www.lab.anhb.uwa.edu.au/mb140/scope/stereology/stereology.htm

http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0001-37652003000400006

# Question

**What is the Answer to the Ultimate Question of Life, the Universe & Everything ?**

= ??

**Key Reference:** Douglas Adams “Hitchhikers Guide to the Galaxy”

Example Course

# PROGRAM

Venue: Medical Faculty, room B432 of the Dept. Anatomy & Neurosciences/Pathology, VU Uninversity Medical Center (VUmc), Van der Boechorststraat 7, Amsterdam

**Monday April 7, 2014**

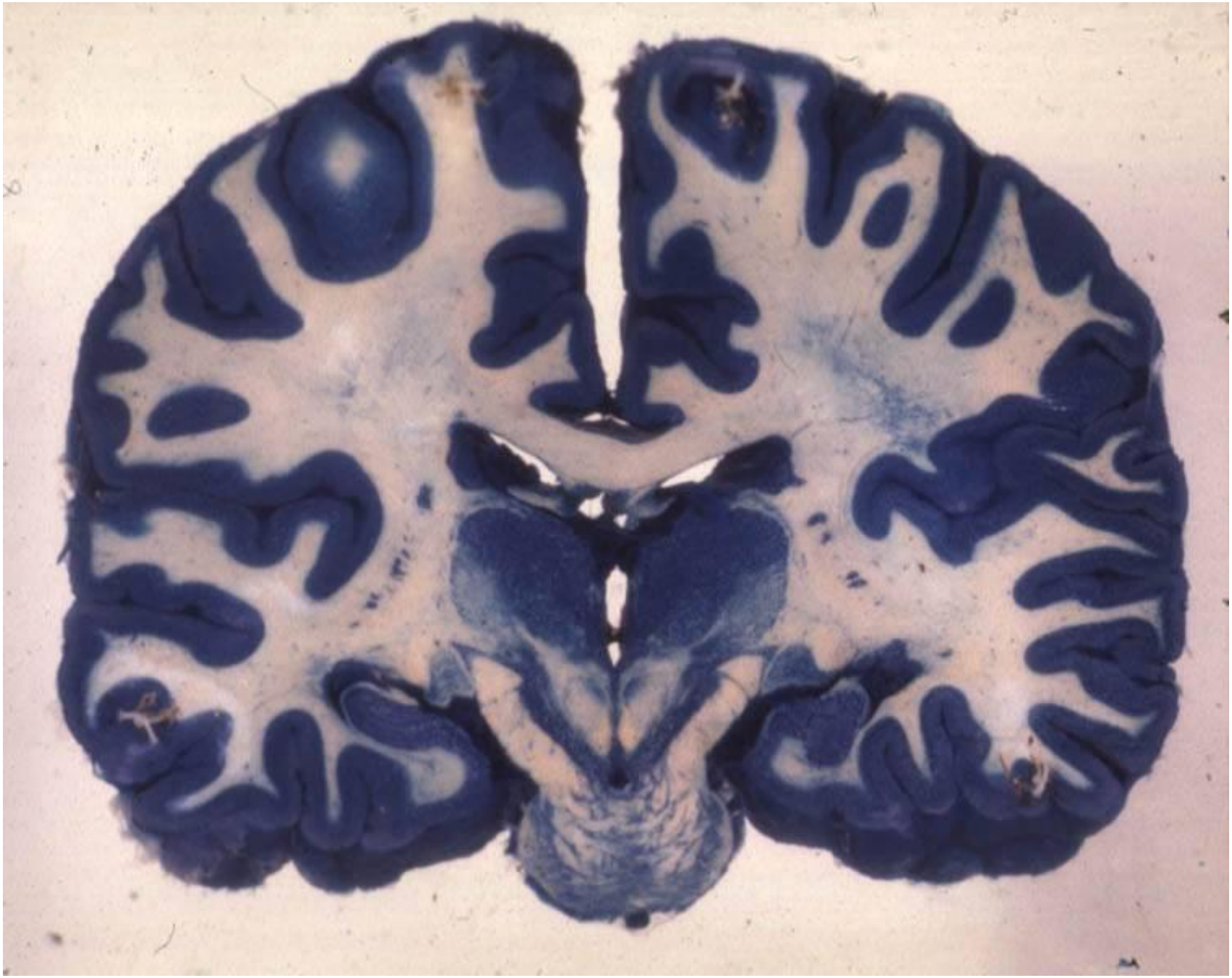
| | |
|---|---|
| 09:00 – 09:30 | **Introduction to the course (Wilma)** |
| 09:30 – 10:15 | **Principles of Stereology: 3D thinking, Sampling design** (Wilma) |
| 10:30 – 11:15 | **Unbiased estimation of volume in human and rat tissue (Harry)** Theory: Cavalieri's principle; systematic random sampling; thickness measurement; |
| 11:15 – 12:00 | **Practical exercise: estimation of the volume in rat neocortex (Harry en Wilma)**; systematic random sampling, cavalieri's principle |
| 12:00 – 12:45 | *Lunch break* |
| 12:45 – 13:30 | **Unbiased estimation of the total number of cells in human and rat tissue** (Wilma): over- and underprojection; dissector and fractionator method |
| **13:30 – 14:15** | **Confocal Microscopy, 3D object recognition and counting contacts in 3D (Floris)** |
| **14:15 – 15:00** | **3D reconstruction from multichannel confocal laser scanning image series** (Floris) |
| 15:30 – 17:30 | **Demonstration Part I: A) Neurolucida and Stereoinvestigator (Harry en Evelien); B) Workstations (Wilma); C) Confocal laser scanning microscopy (Floris); D) Densitometry (Pieter)** |

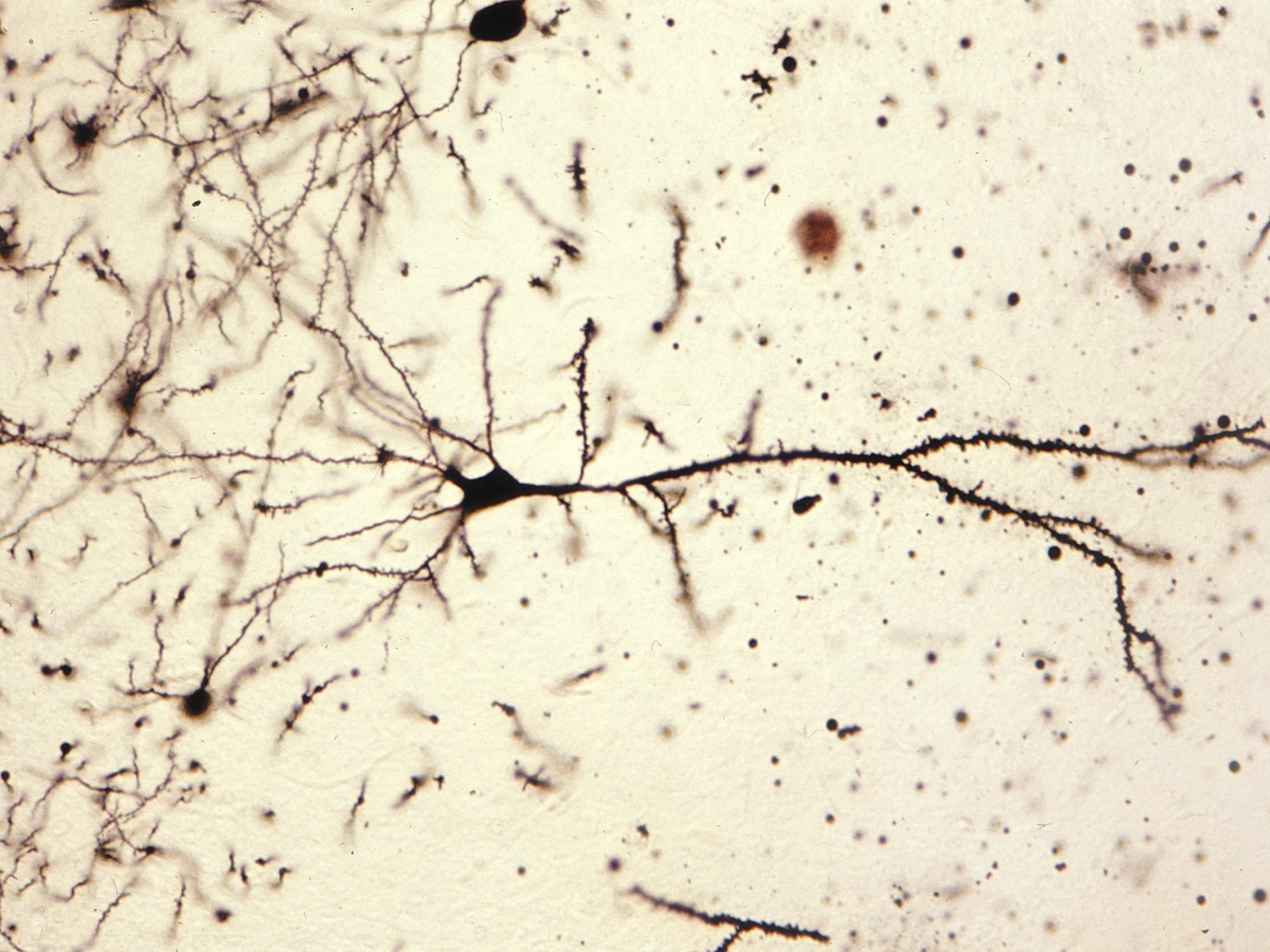
**Tuesday April 8, 2014**

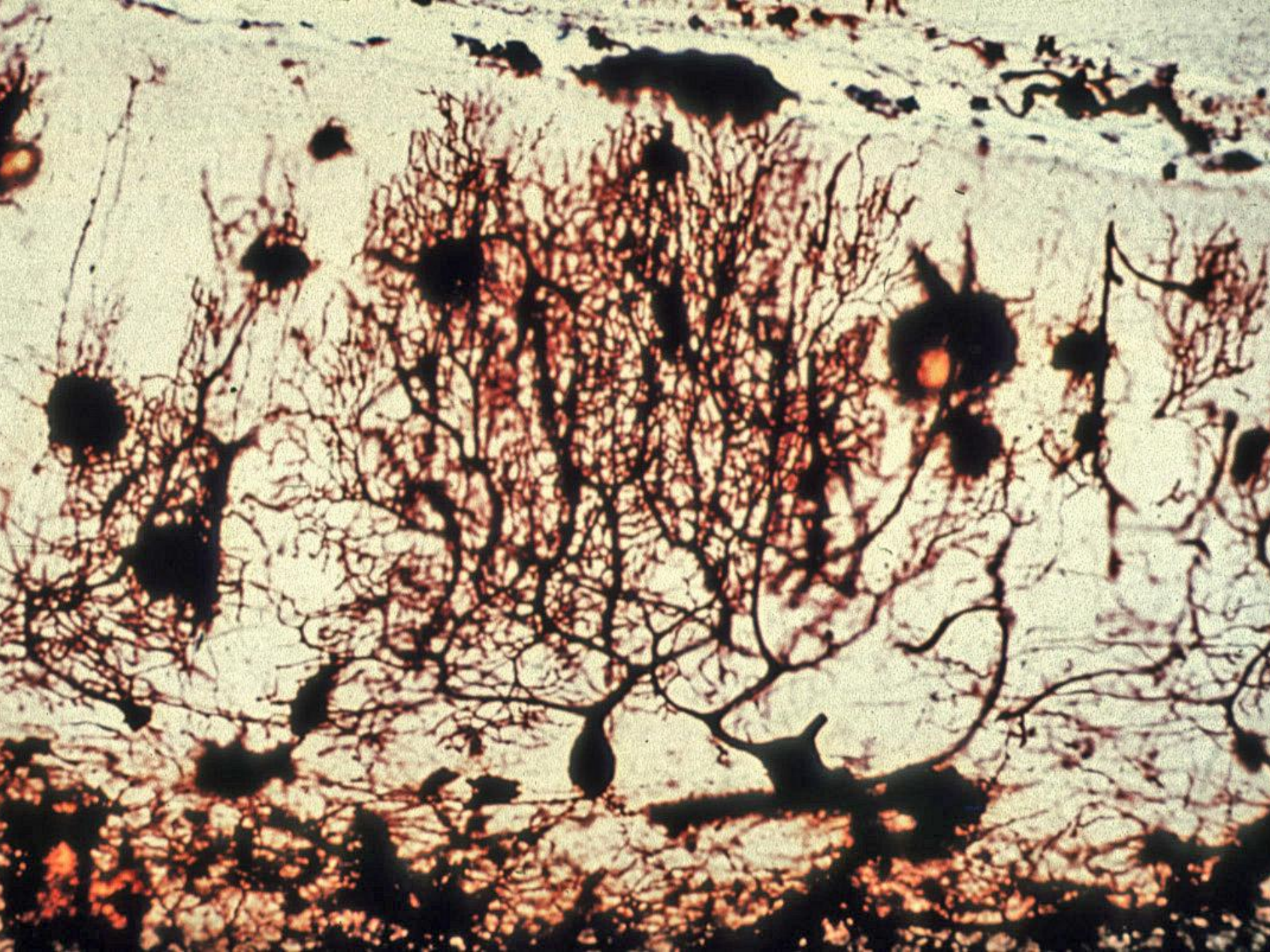
| | |
|---|---|
| 09:00 – 09:45 | **Neuroanatomical tracing and Morphometry (Floris)** |
| 09:45 – 10:30 | **Methods for 3D reconstruction and quantification of dendrites, synapses and spines (Wilma)** |
| **10:45-11:30** | **Brain Mapping using microscopic imaging techniques (Wilma)** |
| **11:30 – 12:00** | **What a single cell can tell us about the cerebral cortex (Cathrin Canto)** |
| *12:00 – 13:00* | *Lunch break* |
| 13.00 - 13.45 | **Anisotropy-Isotropy: how to overcome anisotropy? (Harry)** |
| 13:45 – 14.30 | **The nucleator principle: size estimation, spatial distribution (Harry)** |
| 14:30- 15:15 | **Vascular Morphometry (Wilma)** |
| 15:30 – 17:30 | **Demonstration Part I: A) Neurolucida and Stereoinvestigator (Harry en Evelien); B) Workstations (Wilma); C) Confocal laser scanning microscopy (Floris); D) Densitometry (Pieter)** |

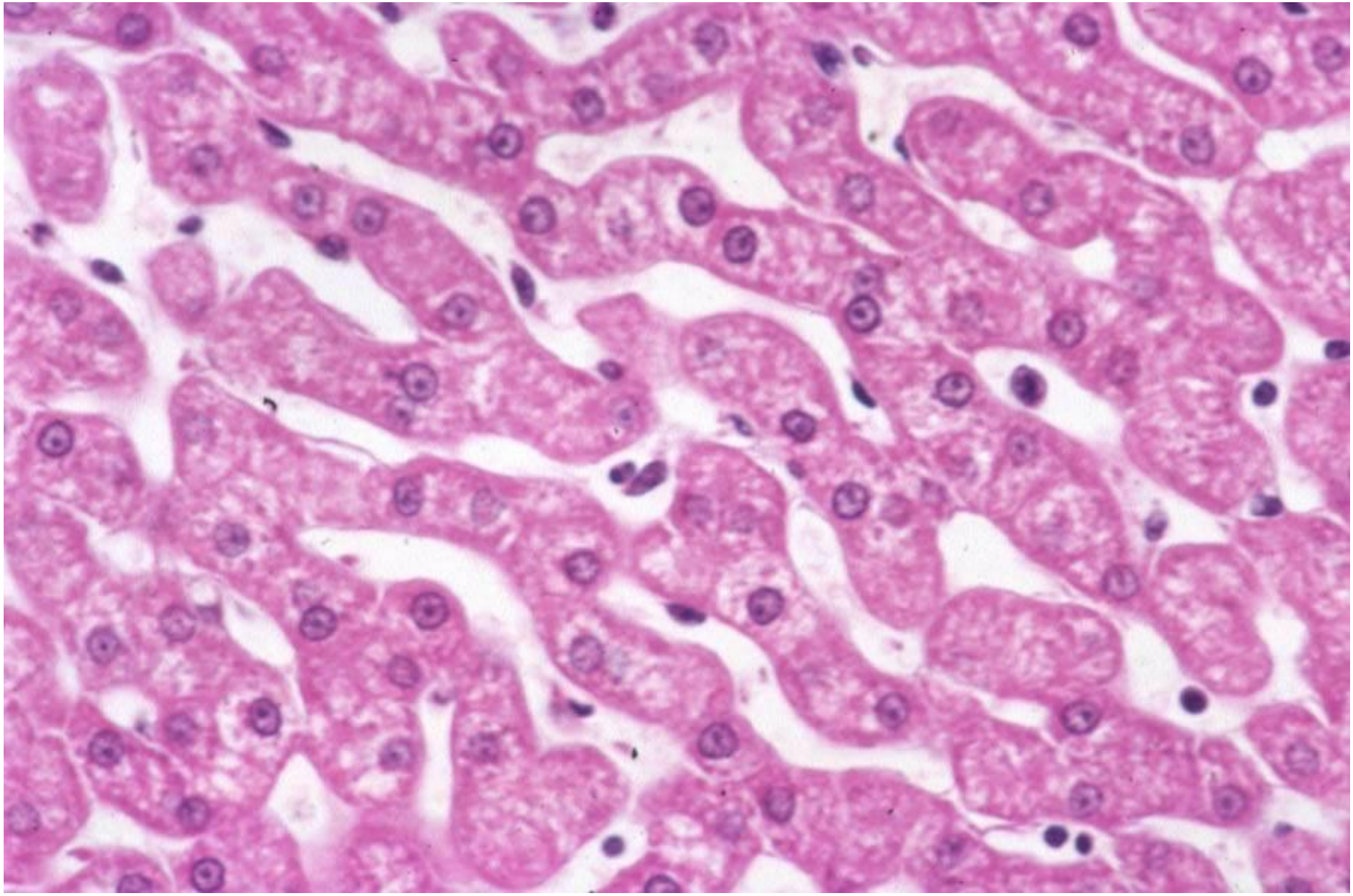
**Wednesday April 9, 2014**

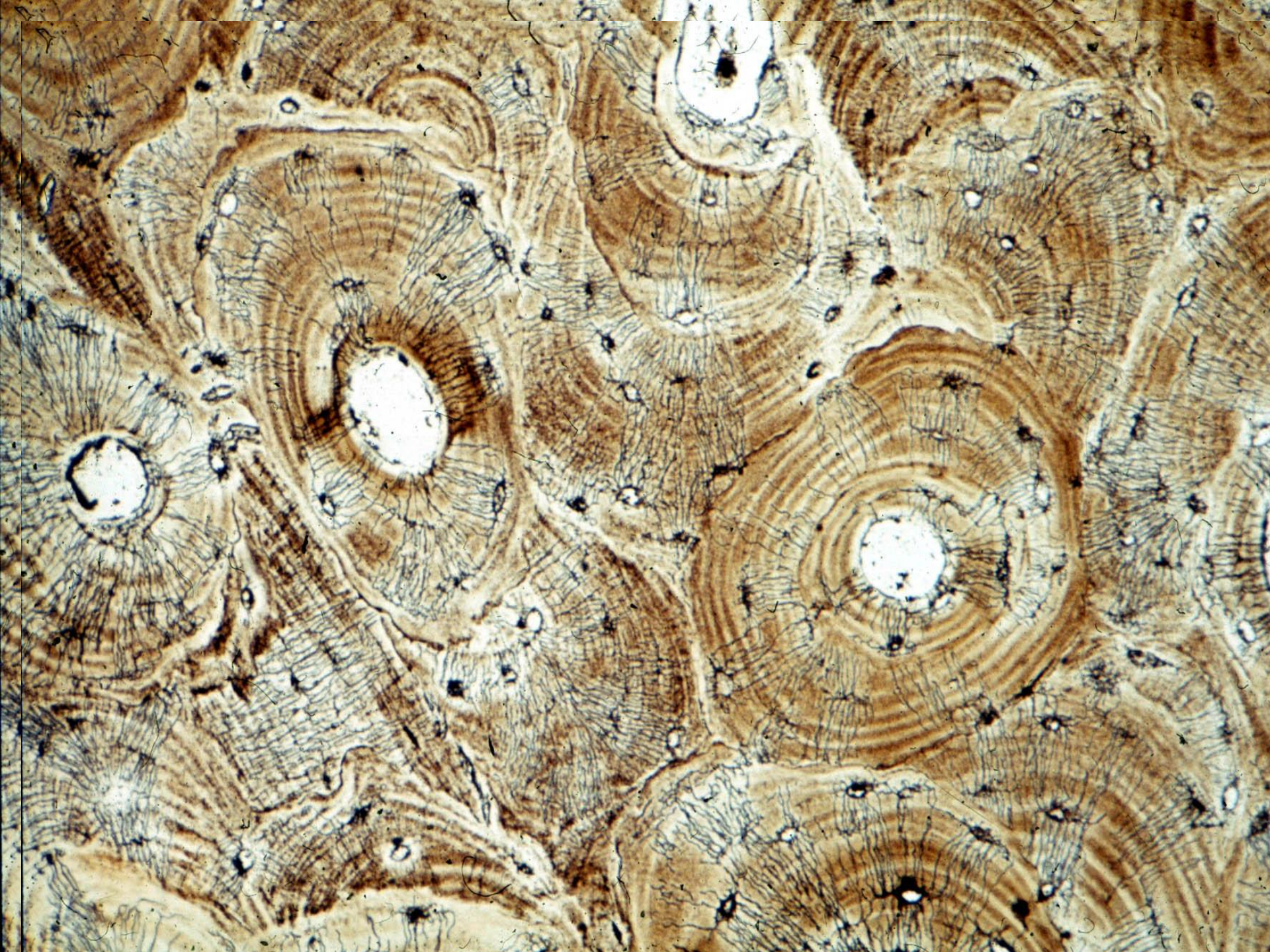
| | |
|---|---|
| 09:00 –09:45 | **Microscopy (J-185)** |
| 10.00-10.45 | **Microscopic imaging and densitometry (Pieter)** |
| 11.00-12:00 | **Structure and ultrastructure of biological samples (Wilma)** |
| *12:00 – 13:00* | *Lunch break* |
| 13:00 – 13:45 | **Histological pitfalls: delineation, tissue embedding and deformation, shrinkage, Immunohistochemistry (Harry)** |
| 13:45 -14:30 | **Estimation of precision (Harry)** |
| **14:30- 15:00** | **How to deal with biological variability? (Harry)** |
| 15:00 -17:00 | **Demonstration Part I: A) Neurolucida and Stereoinvestigator (Harry en Evelien); B) Workstations (Wilma); C) Confocal laser scanning microscopy (Anne-Marie en John Bol); D) Densitometry (Pieter)** |
| 17:00 – 18:00 | **Course evaluation and Farewell drink.** |

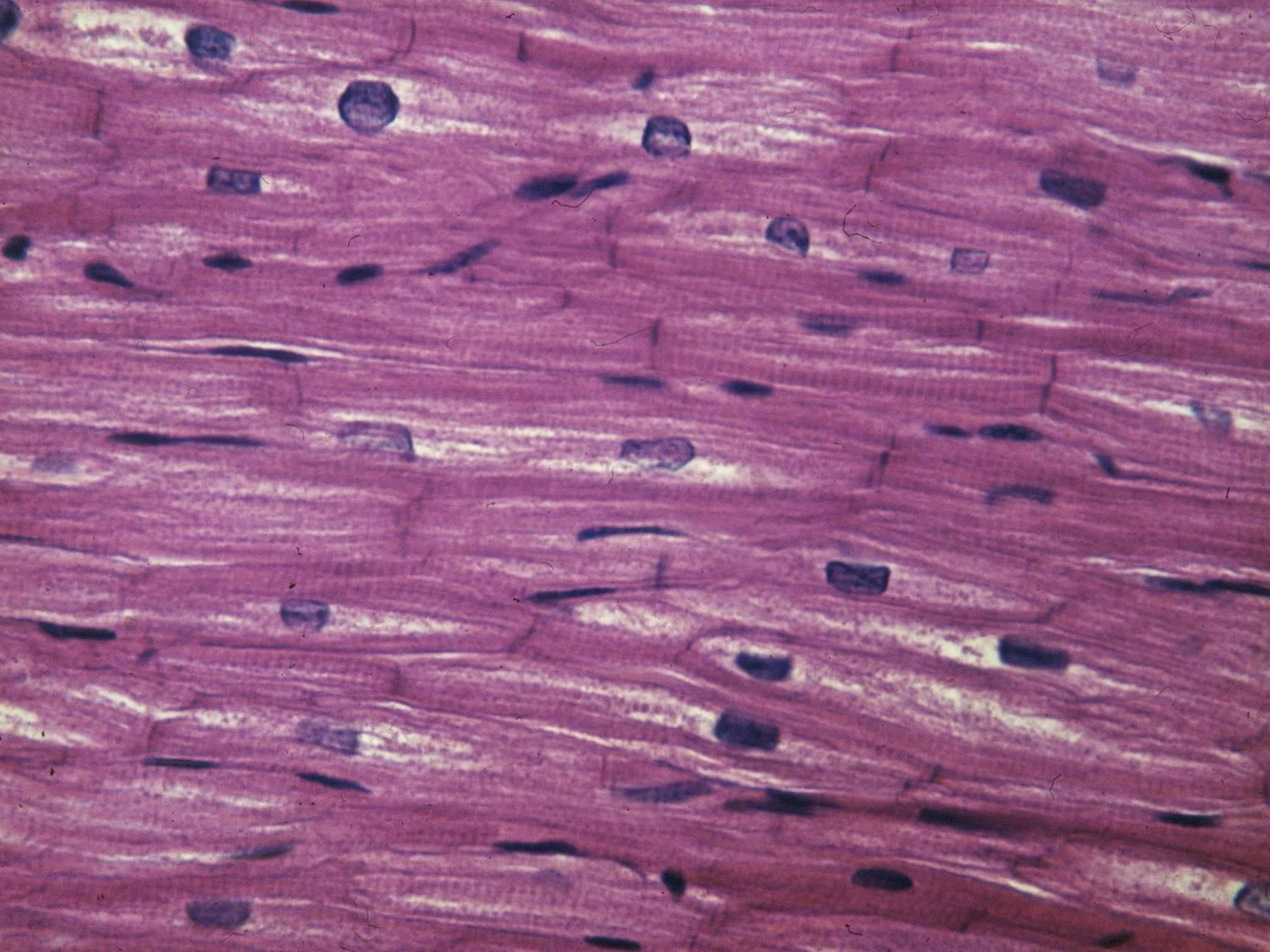


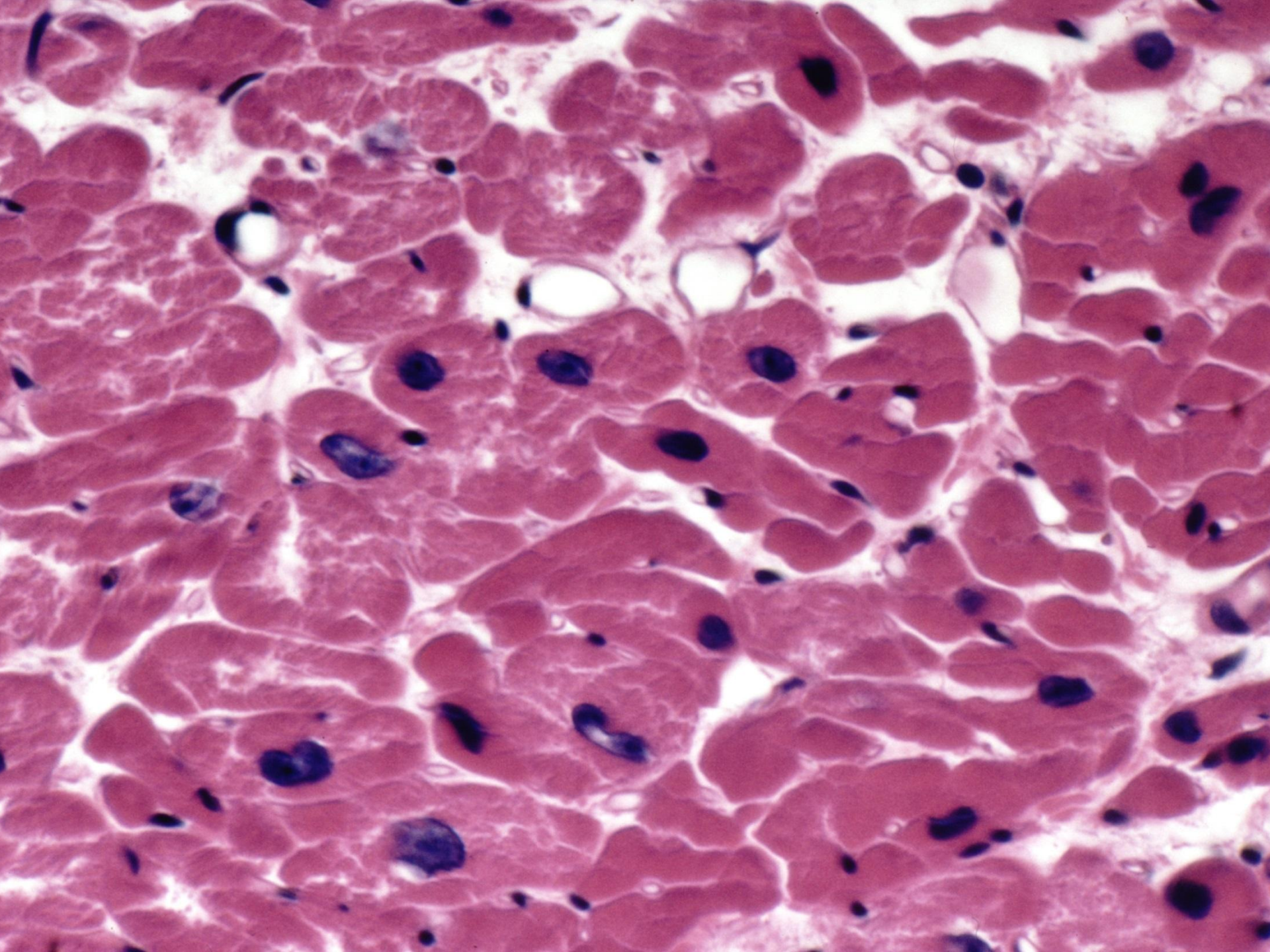


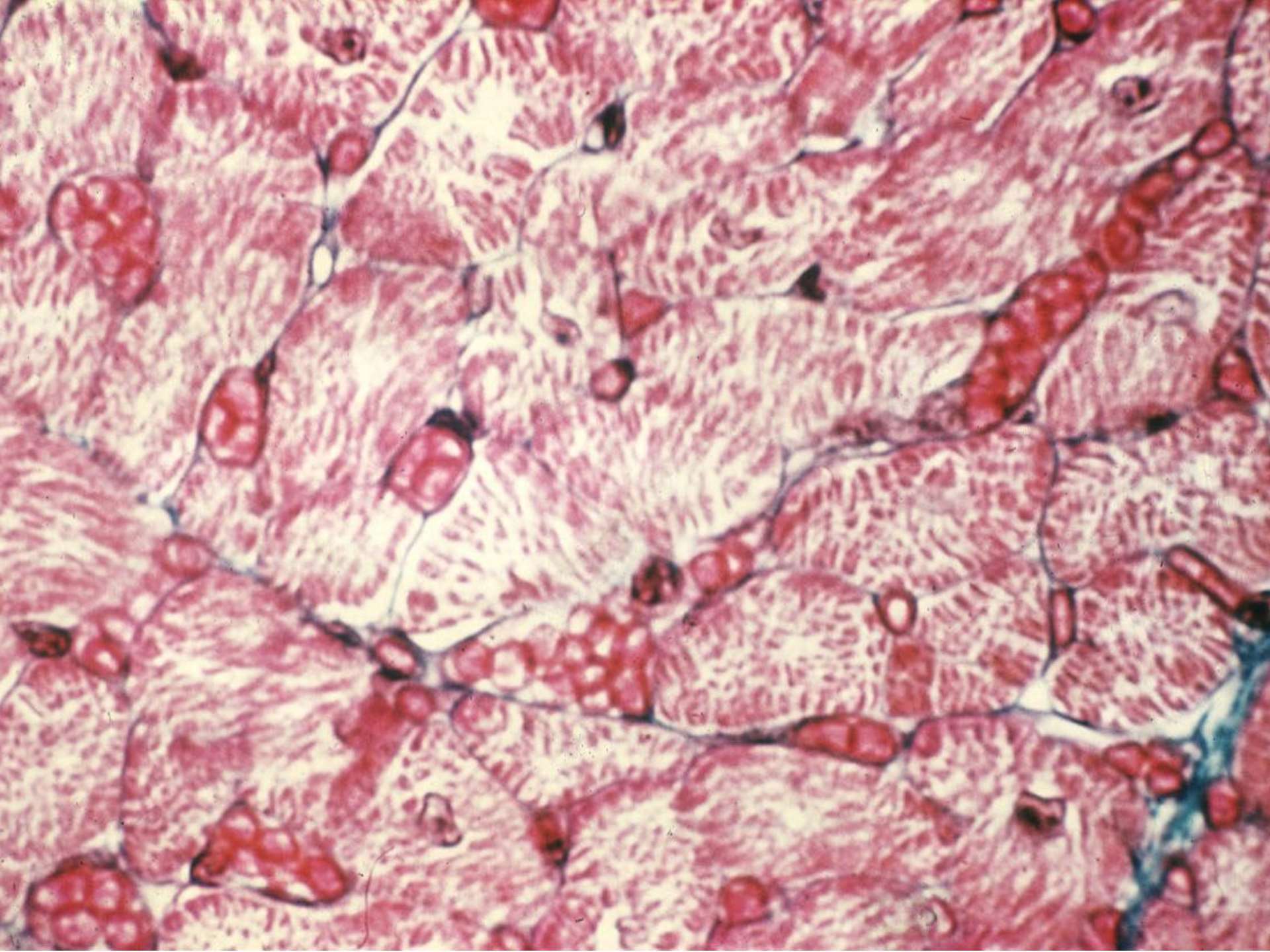


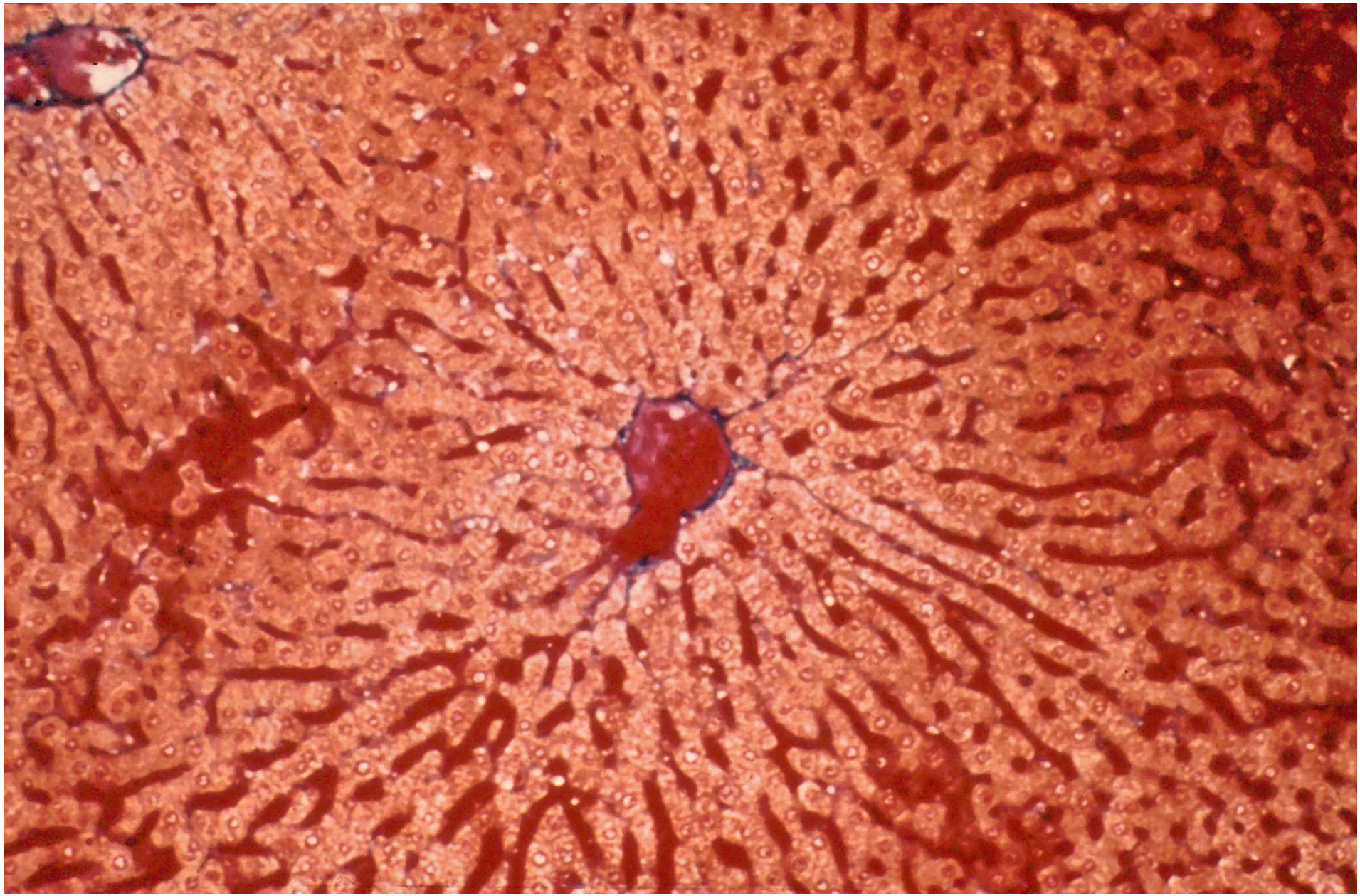


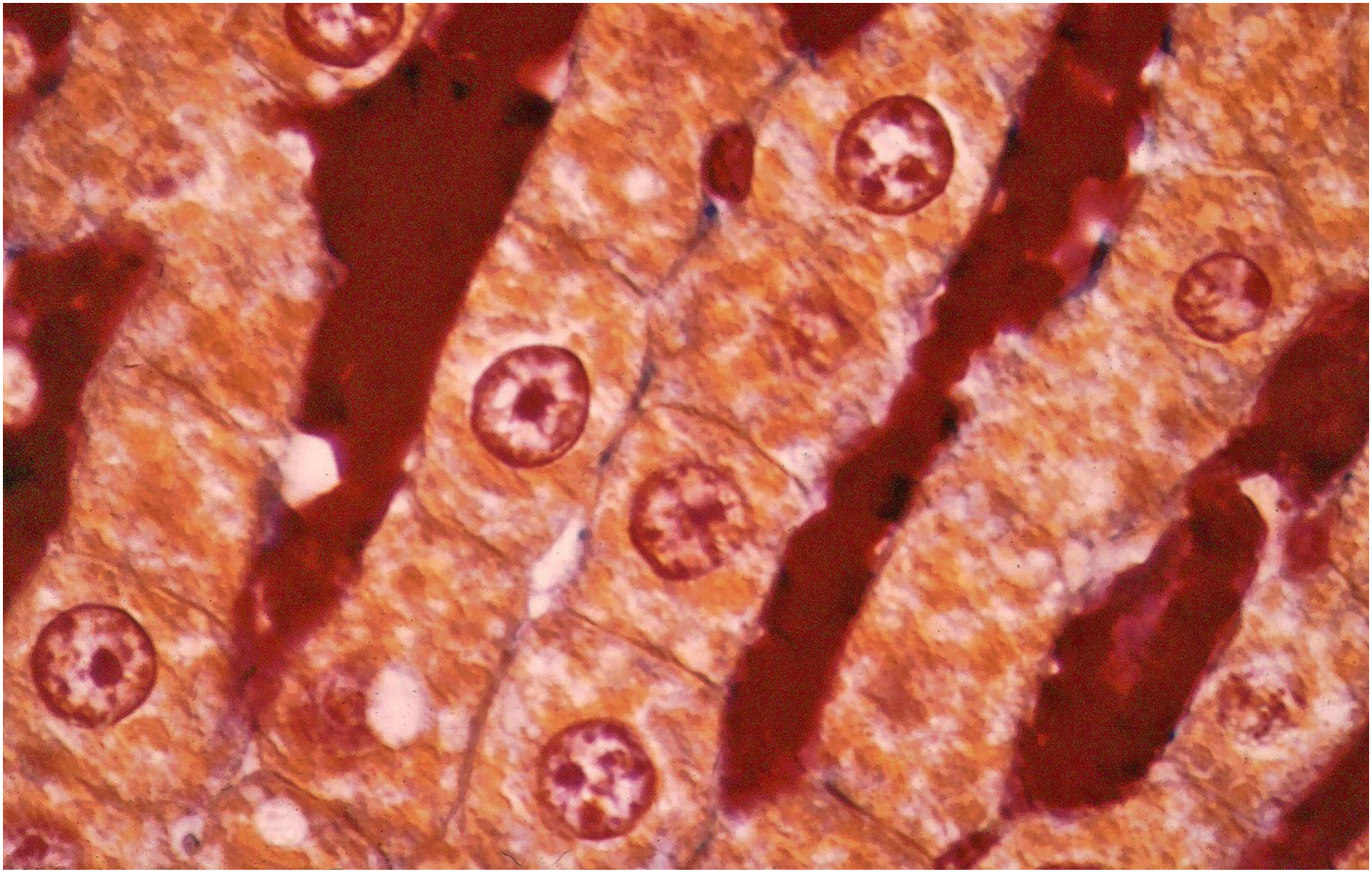










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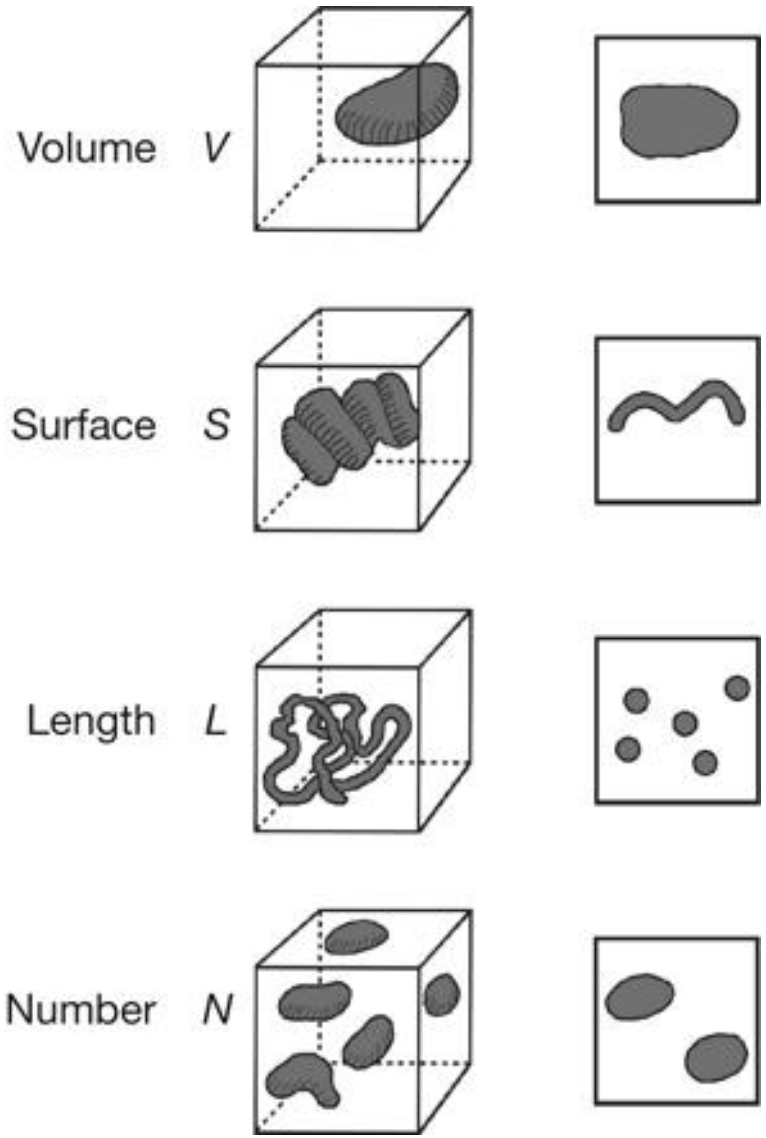
CHINA



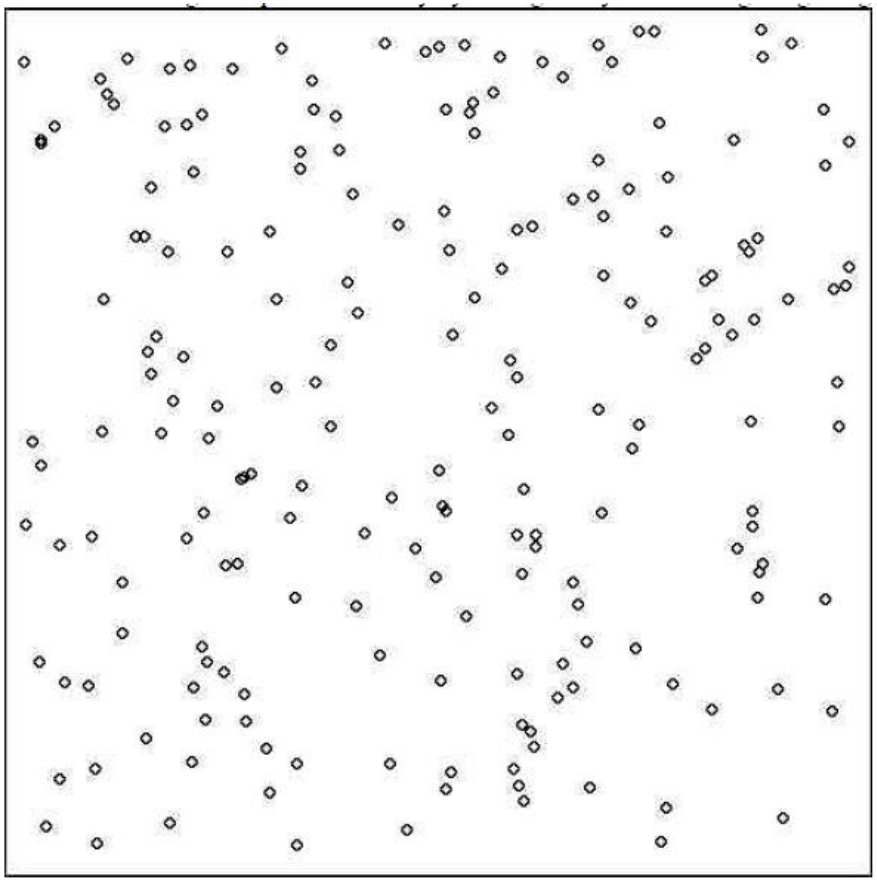
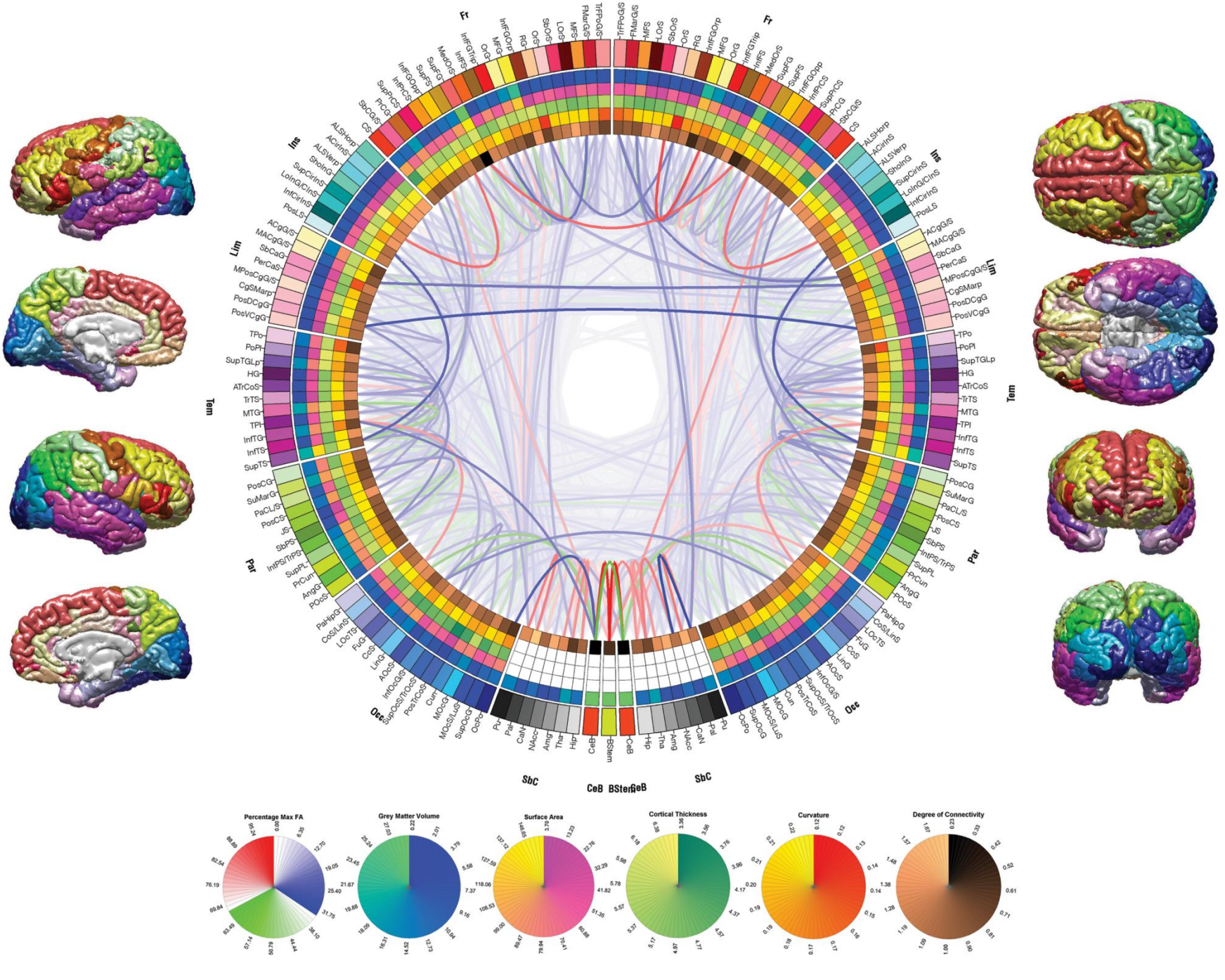



Figure 2. An example of 200 random points. Note the presence of clusters of points that oversample some regions while large gaps exist elsewhere.

a b

c

Figure 11. Examples of clustering (a), self-avoidance(b) and a random distribution of features (c).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Ins
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Percentage Max FA
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Surface Area
Cortical Thickness
Curvature
Degree of Connectivity