Stereology

or

Counting leaves on a tree, and cells in the brain

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Plan

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5. Stereology: counting
1. A story about counting
The Mahabharata

(1000 BC)
The story of Nala
(interpolated in 400 AD)

Nala was a king addicted to gambling.
He lost everything, except for his faithful wife and his skill with horses.
Humiliated, Nala went into service as chief horseman to the virtuous king Rtuparna.
Rtuparna admired Nala’s horsemanship and wanted to learn from him.

To induce Nala to reveal his secret, Rtuparna decided to demonstrate his skill with numbers.
They galloped past a Vibhitaka tree.

Rtuparna grabbed a few twigs, examined them, and declared

“this tree has 2095 fruit
and 100,000 leaves.”
Nala insisted that they stop and count the fruit on the tree. After a long time, he arrived at almost the same number.

Astonished, Nala asked how Rtuparna achieved this feat.
Rtuparna answered:

“I possess the science of dice and so am skilled at number.”
(Nala and Rtuparna agree to exchange secrets, but in a whisper.)
What Rtuparna probably did:

- randomly selected some twigs representing $\frac{1}{m}$ of the tree.
- counted the fruit on these twigs.
- estimated

$$\text{total fruit on tree} = m \times \text{fruit counted}$$
2. Sampling principles
We’re studying a population of distinct units.

We take a *sample* — a selection of units out of the population.
Systematic random sampling

To draw a systematic random sample of period $m$ from a population:

1. Order the units in any arbitrary sequence.
2. Choose a random number between 1 and $m$.
3. Using this number as the starting point, count off every $m$th item.
Cluster sampling

Suppose it is convenient to gather the population into “groups” or “clusters”.
Cluster sampling:

1. Group the population into clusters (arbitrarily).

2. Treat the clusters as the sampling units, and take a systematic random sample of clusters.

3. Take all the units in the selected clusters as the sample.
Example:

Leaves on a tree are naturally grouped into branches.

We can take a sample from the leaves on a tree by taking a sample of branches.
Two-stage cluster sampling

1. Group the population into clusters.

2. Treat the clusters as the sampling units, and take a systematic sample of clusters.

3. Within each selected cluster, take a systematic sample of the units in the cluster.
Estimating the number of words in a book

- Take every 4th chapter.
- Take every 10th page in the selected chapters.
- Take every 5th line on the selected pages.
- Count all the words on the lines selected, and multiply the total by 200.
What Rtuparna probably did (#2):

1. sampled every $m$th branch of the tree.
2. sampled every $k$th twig from the selected branches.
3. counted the fruit on these twigs.
4. multiplied by $m \times k$ to estimate the total.
But, in the story:

\[ 2095 = 5 \times 419 \]

and 419 is a prime number ...
There were probably 5 fruit in Rtuparna’s sample. If he did not count them as being ‘on’ the tree after they were removed, then altogether there were

\[ 2095 + 5 = 2100 \]

fruit. Since \( 2100 = 5 \times 420 \), and \( 420 = 2 \times 2 \times 3 \times 5 \times 7 \) is composite, the sampling fraction was probably \( 1/420 \).
3. Counting cells in the brain
Dementia and other organic disorders of the brain are a major cost to public health. The 1990’s were declared the “Decade of the Brain” (with handsome funding)
Major motivation:

“The brain progressively loses neurons (nerve cells) with age.

Alone among all organs, the brain appears to be unable to regenerate tissue.”
durchschnittlichen Person während des Lebens.

Abbildung 1: Das mittlere Hängewicht steigt in der Ordinate und das Alter.
Numerous studies confirmed that neuron density (cells per cm$^3$) declined with age.
These studies were based on small “samples” of brain tissue.
Ein morphometrischer Beitrag zu dieser Frage

Gibt es Nervenzellverluste während der Alterung in

der menschlichen Hirnrinde?

Aus Wissenschant und Forschung
H. Haug (1985)

- fresh tissue is prepared for microscopy by fixation, staining and embedding.
- it is well known that soft tissue shrinks under fixation
- all previous measurements of cell density were for the treated (fixed & embedded) tissue
- shrinkage is less pronounced in older tissue ("younger brain tissue shrinks more")
Individual estimates of cell number
(approximately corrected for shrinkage)

All previous ‘evidence’ of a loss of neuron number was overturned — Haug showed it was an artefact of shrinkage.
Haug’s critique was dismissed by neuroscientists who continued to assert that it was “well established” that neurons are lost with age.
In the mid-1990’s experiments demonstrated neuron proliferation in the human brain.

(Earlier experiments by J. Altman had demonstrated neuron proliferation in rats).
4. Stereology
Stereology originated as a technique for obtaining 3-D information from 2-D microscope images.
Delesse (1847): microscopy of polished plane sections of rocks & minerals.

Idea: the composition of the plane section is “representative” of the composition of the rock.
Say we want the fraction of quartz by volume

\[ V_V = \frac{\text{total volume of quartz in rock}}{\text{total volume of rock}} \]

We observe the proportion of quartz by area on section,

\[ A_A = \frac{\text{observed area of quartz on section}}{\text{area of section}} \]

Delesse derived the relation

\[ V_V = A_A \]
Example:

White phase occupies 13% of section area (using computer/image analyser)

\[ A_A = 0.13 \]

By Delesse’s formula, we estimate \( V_V = 0.13 \)
— we estimate the white phase occupies 13% of volume of rock.
Brief justification (for non-mathematicians)

A plane section is like a very thin slice:

The volume of each phase in the slice is equal to its section area times the slice thickness: \( V = A \times t \).

Volume fractions in the slice are the same as area fractions in the section.
Brief justification (for mathematicians)

Fubini's theorem: for $X \subset \mathbb{R}^3$

$$\text{volume}(X) = \int \text{area}(X \cap T_h) \, dh$$

where $T_x = \{(x, y, z) : z = h\}$. 
Delesse’s formula $V_V = A_A$

- relies on basic principles of geometry/sampling
- does not depend on shape of objects/phases
- is not specific to rocks & minerals, can be applied to
  - biological tissue
  - ceramics
  - food
  - ...

- does not require reconstruction of 3-D shape
Cavalieri’s principle:

Two solids with identical cross-sections on every horizontal plane have equal volume.
In the 1840’s, the area fractions $A_A$ were measured by

1. tracing the section onto tracing paper
2. cutting out shapes in cardboard
3. weighing shapes

There had to be a more practical way...
Rosiwal (1889): superimpose a grid of “test lines” on the section:

Measure the length fraction

\[ L_L = \frac{\text{total length of lines over shaded area}}{\text{total length of lines over section}} \]
Then determine $A_A$ from

$$A_A = L_L$$
Example:

White phase occupies 15% of total length of test lines (using computer or ruler)

\[ L_L = 0.15 \]

We estimate \( V_V = 0.15 \) — the white phase occupies 15% of volume of rock.
Glagoleff (1943): superimpose a regular grid of “test points” on the section:

\[
\begin{array}{cccccccccc}
+ & + & + & + & + & + & + & + & + \\
+ & + & + & + & + & + & + & + & + \\
+ & + & + & + & + & + & + & + & + \\
+ & + & + & + & + & + & + & + & + \\
+ & + & + & + & + & + & + & + & + \\
\end{array}
\]

Measure the point count fraction

\[
P_P = \frac{\text{number of grid points falling on shaded area}}{\text{number of grid points falling in section}}
\]

Then

\[
A_A = P_P
\]
Example:

2 out of 12 test points hit the white phase:

\[ P_p = \frac{2}{12} = 0.17 \]

We estimate \( V_V = 0.17 \) — the white phase occupies 17% of volume of rock.
Summary

Stereological methods for determining volume fraction:

\[ V_V = A_A = L_L = P_P \]

“The percentage of one phase in the solid rock is equal to its percentage representation in plane sections, on line transects and in points.”
A test system is a kind of systematic random sample.
Test systems are often seen as “old fashioned” in microscopy — and replaced by digital image processing.
Variance paradox

Test systems may be **more efficient**: it is possible that

$$\text{var}(A_A) > \text{var}(P_P)$$

and occurs in real applications.

A consequence of the positive correlation between nearby pixels.

Stereology also includes “test system” techniques for measuring

**SURFACE AREA** of membranes, interfaces, boundaries;
**LENGTH** of filaments, tubules, fibres, dendrites;
**THICKNESS** of membranes, layers;
**AVERAGE SIZE** of cells, grains, particles;
**CONNECTIVITY** of a network of tubes, fibres.
Stereology is an application of sampling theory.

Miles & Davy, JRSS B 1976
5. Stereology — counting
Classical stereology does not include techniques for estimating the total number of cells, particles, grains etc. with the same generality.
Typical errors

- “The average cell volume is 234 square microns”
- “The cell density is 234 cells per $\mu m^2$ of tissue”
In fact there is no general relationship between the number of particles in 3-D and the number of particle profiles seen on a plane section.
“Tomato salad problem”

Imagine making a tomato salad out of 3 large red tomatoes and 3 small green tomatoes.
Slice the tomatoes evenly ...
The salad is **not** 50% red slices and 50% green slices!
Visualise a population of cells.
Take serial sections.
Select one section uniformly at random.
These are the cells that have been sampled.
A plane section is a biased sample of a population of particles/cells/grains.

Taller particles are overrepresented.
The number of particle profiles seen on a section depends on \textbf{both}

- the number of particles
- the sizes (heights) of the particles

There is no easy way to reverse this relationship, in general.
Rhines-DeHoff relation

\[ N_A = \bar{H} \, N_V \]

where

\[ N_V = \text{number of particles per unit volume} \]

\[ N_A = \text{number of particle profiles per unit area} \]

\[ \bar{H} = \text{average particle height} \]

(for convex particles, spatially homogeneous)
To determine the density $N_V$ of neurons in the brain, standard practice was to count the frequency $N_A$ of neuron profiles in a plane section, and "correct" it.
A solution — the dissector rule

1. Divide the containing space into thick sections (slabs) by a series of section planes.

2. Take a uniform random sample of slabs, with known sampling fraction \( f \).

3. For each sampled slab, find all those particles which intersect the slab (in three dimensions) wholly or partly, and do not intersect any higher slab in the sequence.

4. The chosen particles taken together form the sample.
The 3D dissector is essentially a way of sampling the “tops” of particles. Each particle has a unique “top” and the rule samples a particle if its “top” falls in the sampled slab.

Clearly this gives us a uniform sample of the “tops” of particles, hence of the particles themselves.
Implementation using physical thin sections

Nominate one section plane as the ‘counting’ plane and the next section plane as the ‘forbidden’ or ‘lookup’ plane.

Sample any particle which is cut by the counting plane but not by the lookup plane.
Invention of the disector

- W.R. Thompson (1926) — Iowa dentist
- D.R. Cox, H.E. Daniels (1940’s) — textile sampling
- H. Gundersen (1986) — microscopy
- special case of adaptive cluster sampling
  (S.K. Thompson, 1990)
How to count cells in the brain:

1. cut *entire* brain into pieces

2. take systematic sample of pieces

3. cut sampled material into smaller pieces

4. take systematic sample of these pieces

... ... ... ...

\( n \) count cells using dissector principle. Multiply total count by inverse sampling fraction.
This sampling procedure yields unbiased estimates of total neuron number in the brain (or in a specific brain region).
The estimates are unbiased, regardless of spatial inhomogeneity, shrinkage, etc.
Conclusions

- some standard techniques in microscopy give rise to biased samples.
- fundamental principles of random sampling make it possible to estimate the number of cells in an organ (volume, surface area, ...)
- systematic sampling is practical and efficient
- special protocols must be adopted to avoid multiple counting bias.
Problems

- Variance of estimators
  - largely unknown
  - existing theory is unrealistic, but suggests huge advantages in systematic sampling
  - data-based variance estimation

  J. Chia 2002 PhD thesis

- Optimal sample design
  - largely unknown
  - nested ANOVA suggests biological (between-animal) variation dominates; hence, sample sparsely
Controversies

- Spirited defence of 2-D correction methods in neuroscience  
  Trends in Neuroscience, Feb–June 2002

- ASTM grain size standard
http://www.maths.uwa.edu.au/~adrian