PRINCIPLES OF STEREOLOGY
AND ITS APPLICATION IN ANIMAL
MODEL DEVELOPMENT

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EVERY STEP OF THE WAY

ROUTINE HISTOPATHOLOGY
The current “gold standard”

- The human eye is great for pattern recognition.
- However, changes in particle quantity are often below the sensitivity of the eye alone.
- The change must usually reach 25-40% before it can be appreciated.

2-DIMENSIONAL MORPHOMETRY
Quantitative Pathology

Measurements or counting are performed on single or multiple 2-dimensional histologic tissue sections.

- Often efficient and cost-effective.
- Increased sensitivity over qualitative evaluation.
- Can be manual, semi-automated, or fully automated (using image analysis software).
- Can be useful as a screening tool.
- Sensitivity to detect large differences between groups — useful in early animal model development, to screen for large differences in density/quantity that are missed by routine histopathology.
- However, this technique lacks the sensitivity to detect subtle differences between groups due to numerous sources of bias.
WHAT IS BIAS?

Bias in the context of data or sampling: Systematic error that is introduced into sampling or testing by selecting or encouraging one outcome or answer over others

- Type I error
  - An effect is detected that is not really present (false positive)
- Type II error
  - An effect that is present is undetected (false negative)

NOT the same as precision!
- Precision is the reproducibility of the data (amount of variability)
- Data can be precise (closely clustered together) but still biased (far from the true population mean)
- In biological studies, the true population mean is most often unknown!

BIAS VS. PRECISION

Unbiased Data  |  Biased Data
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Precise Data  |  Precise Data
Imprecise Data  |  Imprecise Data

SOURCES OF BIAS 2D MORPHOMETRY

Assumptions:
- Homogeneity throughout organ or region of interest
- Section being analyzed is truly representative of the tissue as a whole
- No change in organ size or volume during tissue processing
- Definitely not the case, particularly with paraffin processing
- Control and test article-treated tissue respond equally to shrinkage

Density estimates (such as those obtained through 2D morphometry) are very sensitive to the effects of tissue shrinkage
- Increased shrinkage causes over-estimation of number and under-estimation of volume
**SAMPLING BIAS**

Sample is collected in such a way that some members of the intended population are less likely to be included than others.

- Several stages where this can be introduced:
  - Organ sampling
  - Most optimum tissue sections are captured and analyzed (nonrandom tissue samples)
  - Block sectioning (microtomy)
  - Microscopic field selection
  - Inherent user bias when selecting fields
  - Region of interest selection within chosen microscopic field

Statistical analysis of intergroup differences is not completely valid!

- All statistical methods for hypothesis testing presume random sampling

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**GEOMETRICAL BIAS**

Profile counts are not an accurate estimate of cell or object number!

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**CONSEQUENCES OF BIAS**

Estimates can be in the opposite direction of the truth!

- Estimation of trabecular bone number in treated minipigs was found to be higher than controls when 3D methods were used but lower than controls when 2D morphometry was used (Boyce et al., 1995)

Methods may fail to show a difference between groups when there really is one

- No difference was found in Leydig cell number estimates between groups when 3D methods were used but estimates were lower than controls when 2D methods were used — due to differences in shrinkage between control and treated groups (Mendis-Handagama, 1992)

Cell/object number may be highly over-estimated

- Neuron number by 2D profile counting is overestimated by 30-40% (Pakkenberg et al., 1991)
- Ratios obtained through 2D profile counting are statistically significantly higher than 3D estimates (Bratu et al., 2014)
POSITIONS BY SOCIETIES AND JOURNALS

American Thoracic Society / European Respiratory Society (Hsia et al., 2010)
- In quantitative assessment of lung structure, alveolar number and size cannot be accurately measured from simple profile counts; 3D methods such as the disector must be used.

American Society of Nephrology (Madsen, 1999)
- Beginning in 1999, appropriate stereologic methods were requested to be used to quantify structures in all manuscript submitted to the journal.

Journal of Comparative Neurology (Saper, 1996)
- Expectation that any papers using simple profile counts or correction factors would provide adequate justification for the methods.

UNBIASED STEREOLOGY

Design-based vs Assumption-based

Utilizes sensitive and stringent sampling methods based on statistics and stochastic geometry (combines statistical sampling principles with geometric analysis of tissue microstructure)
- Can be applied to any tissue from any species — highly standardized!
- Can be applied at any level from whole tissue to electron microscopy
- Results are absolute estimates (rather than densities or ratios)
- High sensitivity to detect subtle intergroup differences
- Samples objects based on their presence — independent from size, shape, or orientation in section
- Precision can be estimated (calculation of CE).

STEREOLOGY ENDPOINTS

- Number
  - Sub-compartment (i.e. pancreatic islets, renal glomeruli)
  - Cells (cell type, cells expressing a certain marker)
  - Cellular inclusions/organelles (even at ultrastructural level)

- Volume
  - Total tissue volume
  - Sub-compartment
  - Cells
  - Cellular inclusions/organelles

- Surface Area
- Length
ENDPOINTS FOR ANIMAL MODELS

CNS Diseases (Parkinson's disease, Alzheimer's disease)
- Neuron number (specific neuron types or locations)
- Number and/or size of amyloid plaques

Lung Disease (emphysema, pulmonary fibrosis, acute lung injury, bronchopulmonary dysplasia)
- Total lung volume, parenchymal volume
- Alveolar number and size
- Alveolar surface area
- Alveolar septal thickness (calculated from other values)

Other diseases
- Active bone surface area in models of osteoporosis
- Glomerular volume in diabetic nephropathy models
- Beta cell number and mass in diabetes models
- Number and size of cardiomyocytes, capillary length in heart failure models

How does stereology minimize sampling bias?

SYSTEMATIC UNIFORM RANDOM SAMPLING

SURS

Stereological sampling principle in which every structure has equal probability of being sampled
- Organ or region of interest is sectioned at regular intervals (T) with random start (RS) between 0 and T
- Goal of obtaining 8-10 sections through the tissue

For small tissues, this can be accomplished at microtomy or for larger tissues, one or more sub-sampling steps may be needed

SURS OF SMALL TISSUES

Automated Microtome

SURS OF MEDIUM-SIZED TISSUES

Paraffin Embedding


SURS OF MEDIUM-SIZED TISSUES

Plastic Embedding
How does stereology minimize geometrical bias?
COUNTING RULES FOR STEREOLOGY TEST SYSTEMS

(A) (B) (C)

EVERY STEP OF THE WAY


UNBIASED ESTIMATION OF CELL/OBJECT NUMBER

The Disector Principle

EVERY STEP OF THE WAY


PHYSICAL VS OPTICAL DISECTORS

Two ways to estimate cell or object number

EVERY STEP OF THE WAY

What about shrinkage?

**CORRECT FOR SHRINKAGE**

Method

- Not an issue for number estimates!
  - All sampling fractions are kept track of (Fractionator sampling) and applied to final estimate calculations

- Calculate 3D global shrinkage
  - Weigh tissue before processing
  - Weigh tissue after processing
  - 3D global shrinkage = 1 – Wpost/Wpre

- Correct reference space volume estimate for amount of shrinkage
  - V (corrected) = V / [1 – 3D global shrinkage]

How can sensitivity be measured in stereology?
ESTIMATION OF PRECISION
Coefficient of Error

- Precision can be estimated in any stereology study by calculating the Coefficient of Error (CE)
- The CE and CV can be measured in a small pilot study to determine whether the analysis is precise enough
  - PROBE = CV² / CE²
- If the analysis is not precise enough, you can sample more to improve the precision!
  - Increase the density of the test systems (probes) on the sampled fields of view
  - Sample more fields of view
  - Take more sections through the tissue
  - Include more animals in the study
- Precision cannot be accurately estimated with 2D methods
TOTAL LUNG VOLUME

Cavalieri’s principle

\[ V = \sum P \times T \times A(p) \]

SURFACE AREA

Lung and bone disease models

ESTIMATION OF ALVEOLAR NUMBER

NEURON NUMBER IN SUBSTANTIA NIGRA
Mouse model of Parkinson’s Disease

NEURON NUMBER IN DENTATE GYRUS
Mouse model of Alzheimer’s disease

MULTIPLE ESTIMATES IN ONE SAMPLING
SUMMARY

• Quantitative analysis is extremely useful in detecting changes in density or quantity that cannot be detected by the human eye alone
• 2D morphometry is helpful as a screening tool, but often lacks sensitivity to detect small intergroup differences due to sampling and geometrical bias
• Unbiased stereology utilizes stringent, standardized sampling and counting techniques that can be applied to any tissue and any species (it is design-based) which minimizes bias (particularly sampling and geometrical bias)
• Precision of the estimate can be measured and improved upon to increase the sensitivity of detection of small intergroup differences
• Stereological endpoints such as volume, number, and surface area can be useful for detection of subtle differences between groups in a variety of study types

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REFERENCES