SOCIETY OF
ELECTRON MICROSCOPE
TECHNOLOGY


# QUANTIFICATION

## MEASUREMENT & ANALYSIS IN MICROSCOPY

**Wednesday 22nd February 1995**

**EASTMAN DENTAL INSTITUTE**
**Grays Inn Road**
**London WC1X 8LD**

**2.00** **"Calibration in the electron microscope"**

Bill Clarke (Agar Scientific Ltd., Stansted)

**2.40** **"Quantification in scanning EM images"**

Philip Gaffney (Seescan Imaging, Cambridge)

**3.10** **TEA**

**3.30** **"Stereological quantification of microscope images: 3D quantities from 2D images"**

Prof. Terry Mayhew (Univ of Nottingham)

**4.10** **"Should we be counting gold particles in immunocytochemistry?"**

Paul Monaghan (Inst. Cancer Research, Sutton)

**4.50** **Final discussion.**

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Please reply to the Secretary - Dr Jill Lewis
19 Bellfield Avenue, Harrow, Middx. HA3 6ST
081 428 4264

**I hope to be present at the meeting on 22nd February -**

**Name** .....

**Address** .....

.....

# QUANTIFICATION
## MEASUREMENT & ANALYSIS IN MICROSCOPY

Abstracts of Papers

### Calibration and measurement in the Electron Microscope

Bill Clarke (Agar Scientific Ltd, Stansted, Essex)

*This talk will examine the needs for calibration and performance monitoring of electron microscopes and the factors which may contribute to errors. Illustrations will be given of the wide range of specimens which are available for use as calibration standards including those which are traceable to National Standards. The application areas and the useful magnification range for each of the specimens will be discussed. In conclusion there will be a short review of standards for calibration of energy dispersive X-ray analysers.*

### Quantification in scanning EM images

Philip Gaffney (Seescan Imaging, Cambridge)

### Stereological quantification of microscope images: 3D quantities from 2D images

Prof. Terry Mayhew (University of Nottingham)

1. *Often, structural information must be obtained from slices. These may be physical sections (knife, microtome), optical sections (confocal microscopy) or medical imaging slices (MRI, CT etc.).*
2. *Slicing leads to loss of dimensional information (3D to 2D) and this loss must be restored (2D to 3D) before meaningful science can be undertaken.*
3. *Stereology is state-of-the-art quantitation and the method of choice for obtaining hard (biologically-useful) 3D quantities (volumes, surface areas, lengths, numbers) from 2D slice images of arbitrary objects.*
4. *Stereology involves no measurement - it is based on counting or classifying chance events between image features and randomly applied probes (points, lines, areas, volumes).*
5. *Stereology has two major advantages over its competitors:*
   *it is practically unbiased (incurs zero or minimal systematic error) and*
   *it is very efficient (offers greater precision per unit of cost).*
6. *It is also cheap to apply (expensive measuring devices are not needed but it can be used with such devices) and it requires no assumptions about object shape, size or orientation. This is important because :*
   *"If you ASS-U-ME, you can make an ASS out of U and ME"!*
7. *Stereology relies on random (design-based) sampling in which the image is merely one sampling item in the overall design.*
8. *If all positions in the object have equal chance of being sampled, volumes and numbers can be estimated, and if all orientations (directions of sectioning) are also given equal chance of being selected, surface areas and lengths can be obtained.*
9. *Sampling designs and some of the new stereological methods will be introduced.*

Useful review articles:

Cruz-Orive LM, Weibel ER (1990) Am J Physiology **258**: L148-L156
Mayhew TM (1991) Exper Physiol **76**: 639-665
Mayhew TM (1992) J Neurocytol **21**: 313-328

**Should we be counting gold particles in immunocytochemistry ?**

Paul Monaghan (Inst of Cancer Research, Sutton, Surrey)

*The simple answer is of course, yes. The object of Immunocytochemistry is to determine the presence of a particular antigen in a specific location. Except for the situation where one is determining only the presence or absence of a target molecule, the quantification of the results is highly desirable and permits comparisons to be made, absolute numbers to be determined, and statistical analysis to be carried out. In light microscopy, the commonly used enzyme systems produce a reaction product which is difficult to quantify without relatively complex analysis systems. The particulate nature of colloidal gold, in contrast, is ideal for generating numerical data. So why do so few papers include statistical analysis of immunolabelling ? Manual counting is time consuming and laborious, but the electron dense colloidal gold markers are ideal for computerized image analysis. Images can be thresholded and areas defined easily giving data on particles/unit area and comparisons between cells or samples are readily made. Less easy to threshold are, for example, mitochondrial matrix vs mitochondrial membrane, or different regions of the golgi. What is more difficult to determine is what a precise number of colloidal gold markers per square micron actually means in terms of antigen density. The cumulative effects of each stage of the exact protocol used as well as the specificity of the reagents and the level of background labelling inherent in the system will profoundly influence the results obtained.*

S.E.M.T QUANTIFICATION 22 Feb.1995
Measurement &Analysis in Microscopy

**Calibration in the EM**
Bill Clarke
Agar Scientific, Stanstead

General reproducibility is good, but the accuracy is usually about 10%, and even this may be only for specific conditions. Variables are: hysteresis; specimen height; the image display and recording method. On an SEM, which screen does the magnification refer to? The image may have gone through a computer. Therefore:
- use micron marks on the image where possible
- re-calibrate regularly,

You should know the limitations of your EM, under the condditions of use, and how it changes with the conditions. You need to have standards for calibration and for resolution.
Test specimens must be clean and non-contaminating to the vacuum; mechanically stable and with a long shelf life; conducting or not subject to beam damage; useful areas should be available over large areas of the specimen.
For low magnification, use: grids and meshes; uniform spheres; grating replicas; for SEM, EB written patterns.
For medium magnification: grating replicas; spheres; for TEM, organic lattice plates.

Spheres can be 20µm - 100 ~~µm~~ nm; grids up to 2000 mesh - measure over 5 or 10 squares )= pitches). There is a new combined specimen from Agar Scientific, on silicon, which can be used for LM as well.

There are 7 different traceable standards for SEM; it is very difficult to get reliable standards for TEM. Gold, and graphitised carbon, are the best; even asbestos changes a bit, but is generally OK for 0.90 and 0.45 nm. (Copper phthalocyanin 1.0 nm)

Holey carbon films can be used for a "health check" for TEM; the holes fill in quickly if there is contamination.
Gold on carbon is good for resolution tests, and for astigmatism, especially on a gonio stage; also for microanalysis.

**Quantification in scanning EM images**
Philip Gaffney
Seescan Imaging, Cambridge

Older microscopes are more difficult to interface to image analysis equipment.
Image acquistion can be by:
a) cable - video rate !
or slow scan synchronisation - gives better resolution.
(if the interfacing is not intended by the manufacturer, e.g. on older EMs, it will invalidate the warranty)
b) by removable media
i) floppy disc - you can't get the whole of a high-magnification picture onto one disc !
ii) laser disc
read/write - fairly reliable, but need to back up frequently
WORM - these fail continuously, especially because of photocopier toner in the air system
CD-ROM - these are now affordable.
read/write optical discs - these are now good.
Calibration data etc must be stored with the images

Calibration by a scale bar - the bar must be in the video, not in the overlay plane; you may lose the overlay plane as you transfer to image analysis.
With an older, less stable, EM, you need to calibrate more frequently.
Have a standard operating procedure ! At the beginning of the day, get 3 within an acceptable picture.
Pseudo-colour is often super-imposed on the picture; therefore it is better to put it in <u>during</u> image analysis.

Image measurement:
1. Fixed aspect ratio
2. Manual measurement - counting
   linear measurement ( and zoom)
   trace measurement
   area defined by drawing

You are liable to have 10% instability on the EM.

Automatic measurement - segmentation = frame measurement
uniformity of field
statistical texture analysis (more likely of image or of background)

Errors can occur because of thresholding or stability.

Some parameters are very sensitive: number of pixels, perimeters, etc.

**stereological quantification of microscope images:** 3D quantities from 2D images
Prof. Terry Mayhew
University of Nottingham

If you want 3-dimensonal information by TEM, e.g. for volumes, surface areas, numbers, lengths, you require slices or slice-plane images. The images <u>must</u> be generated by random sampling, including the sectioning angle.

For lengths, $\Pi/4 \times I \times D$ where I = interstices, D = spacing

string and grid can be put on an overhead; if crumples, there will be equal orientation opportunities; the lattice should be thrown over the image.

Reproducible if there is a cluster of results; not reproducible if a big scatter.
Unbiased if it hits the bull; biased if it never hits the bull.

If you are put in the position of selecting for a particular type of image, the selection is <u>not</u> random and you are <u>not</u> giving all parts of the specimen an equal chance to be selected.
Starting from a stack of slices, the first should be selected randomly, then e.g. every 10. If one section is technically defective, move to one section back or forward, then return to the schedule.

"If you ASSUME, you can make an ASS out of U and ME."

e.g. if you assume that cells are spherical, you introduce a bias if they are not.
Estimate volumes by regular slices, as above.
For relative volumes, e.g. nucleus in cell, use the ratio of point counts on a random section.
If you are only counting objects completely within the field, select only small objects.
You can select the centre of the object in the field; then need a shield around it; or the upper tangent; or the "forbidden line" - don't count it if it touches the line. These methods may give different answers on a single section; you should do several modes on one section, or several sections separated by a known distance - parallel sections.
Number is useful is <u>some</u> circumstances, not in others.

**Should we be counting gold particles in immunocytochemistry?**

Paul Monoghan
Institute for Cancer research, Sutton

You should be able to compare, count, reproduce the results. It will be affected by: gold particles : antibody molecules
gold particle concentration
size of gold particles
no. of gold particles / unit area of section
no. of gold particles / unit antigen density

| e.g. | 5 nm | 15 nm |
|---|---|---|
| protein/particles | 1 - 2 | 6 - 8 |
| no. of particles | 50 | 1 |
| protein / ml | 16 | 4 |

eyeball analysis uses grid / dot + patience

numbers + areas

For image analysis you must first have image capture.

comparisons are easy; localisation is hard; estimating silver density is very hard numerically. If the particles are not over a clearly discrete area, you have problems.

Comparing the resins K4M and HM20: K4M is hydrophilic and should label more easily, to differentiate labelling of resin / cytoplasm / nucleus. With HM20 there is much less background labelling of the resin, some background on the nucleus; the cytoplasm is better.

Bcl2 is a gene for B-cell leukaemia / lymphoma gene 2, found on chromosome 18; the function is unknown; it over-expresses in leukaemia. When chromosomes 14 and 18 break, if Bcl2 is next to the cell death gene on 14, the cell becomes potentially immortal, i.e. cancerous. By the eyeball method, the level of labelling is low; on the outer mitochondrial membrane and rough ER.

Antigen density determination:
model system - with agar block etc; but influenced by processing
processing method - pre-embedding
or - thawed cryosections
or - fix + freeze
or - others

Processing variables: fixation and delay in fixation
fixation protocols
dehydration and infiltration
polymerisation (no known effect)
sectioning - may diffuse into water
labelling - antibodies
gold conjugate

Probably with Epon we section (smoothly) through the protein; with Lowicryls the section is rought and bits of protein may be hanging out. We must also consider the antibodies - poly/mono-clonal, epitope site; gold particle size and concentration; whether background is real; whther controls are adequate. Small gold particles give a higher label. Cross-reactions can occur where you do expect a reaction, too. A clean background is necessary. Routinely, he uses 5 nm gold with short silver enhancement.
A questioner commented that adjacent sections (serial) can give very different amounts of labelling.

**SEMT MEETING 22nd February 1995**

# QUANTIFICATION: MEASUREMENT & ANALYSIS IN MICROSCOPY

## List of Registrants

| | |
|---|---|
| Pauline Barber | EMU, Inst of Dental Surgery, Eastman Dental Hospital |
| Sue Barnes, | EMU, Natural History Museum, London |
| Terry Bull | Anatomy Dept, Charing Cross Hospital |
| John Bredl | Royal Veterinary College, London |
| Judith Brock | Oxford Instruments, Eynsham |
| Steve Cham | Leica, UK |
| Bill Cooley | EMU, CVL, Weybridge, Surrey |
| Terry Cooper | TAAB, Aldermarston, Berkshire |
| Heather Davies | Biology Dept, Open University, Milton Keynes |
| Barry Dowsett | Div of Pathol, CAMR, Porton Down |
| Ann Dewar | EMU, Royal Brompton Hosp., London |
| Anne Drewe | Microbiology Dept., Charing Cross Hospital |
| Colin Gagg | Materials Dept., Open University |
| David Gittens | PGT(UK), Peterborough |
| Bill Hanks | AEA Technology, Harwell |
| Roger Hockham | JEOL(UK) Ltd, Welwyn Garden City |
| Fiona Holt | Wye College, Asford, Kent |
| Joanne Hunt | Materials Dept., Open University |
| Chris Jones | Dept Mineralogy, Natural History Museum |
| Louisa Jones | EMU, Natural History Museum |
| Mike Kelly | Dept Oral Pathology, London Hospital Med School |
| Tania King | Dept of Paleontology, Natural History Museum, London |
| Jill Lewis | EMU, Eastman Dental Hospital |
| Patricia Lovell | Institute of Zoology, Regents Park, London3 |
| David McCarthy | EMU, School of Pharmacy |
| Linda Moran | Anatomy Dept, Charing Cross Hospital |
| Nicky Mordan | Inst of Dental Surgery, Eastman Dental Hospital |
| Janina Penn | Morgan Materials Tech, Stourport-on-Severn |
| Chrissie Prychid | Jodrell Lab, Royal Botanic Gardens, Kew |
| Shelagh Reardon | EMU, Wye College, Kent |
| Andrew Rogers | NHLI, Royal Brompton Hosp., London |
| Padmini Sarathchandra | Royal National Orthopaedic Hospital, Stanmore |
| Sue Sims | Cranfield University, Beds. |
| Wendy Tynan | Cortecs Res Lab, School of Pharmacy |
| Alison Wildman | Coates-Lorilleux Research, Orpington |
| Naomi Williams | Materials Dept., Open University |