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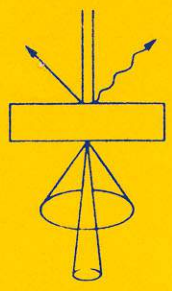
SEMT

Society of Electron Microscope Technology

Affiliated to the Royal Microscopical Society



You are cordially invited to a meeting of SEMT on Friday, Febuary 23rd. entitled

NUCLEIC ACIDS, PROBES and E.M.

Programme

2.00 p.m.	Chairman's Introduction.
2.05	"In situ hybridization : the how and the why."
	Dr. Gary Coulton (Biochem. Dept., Charing Cross Hospital Med. Sch.)
2.45	"Unravelling the genomes of higher organisms using in situ hybridization."
	Dr. Andrew Leitch (Institute of Plant Science and Research, Cambridge)
3.15	TEA
3.35	"Methods and applications of in situ hybridization in pathology".
	Dr. Ken Fleming (Nuffield Dept. of Pathol., John Radcliffe Hosp., Oxford)
4.05	Fourth speaker to be arranged.
4.35	Chairman's summing up and general discussion.

The meeting is to be held in the premises of the Imperial Cancer Research Fund, Lincoln's Inn Fields. If you hope to be able to come, please complete the form below and send it as soon as possible to the Secretary,
Dr. Jill Lewis, Electron Microscope Unit, St. Bartholomews Hospital Medical School, Charterhouse Square, London, EC1M 6BQ.

.....

Name .....

Address .....

.....

Phone no. .....

I hope to be present at the meeting on Friday, Feb. 23rd., 1990

NUCLEIC ACIDS, PROBES AND E.M. - SEMT Meeting, Feb. 23rd. 1990

Abstracts

In situ hybridization: the How and the Why.

Dr. Gary Coulton,(Dept. Biochem., Charing Cross and Westminster Med. Sch.)

Specific intracellular localization of nucleic acid sequences using complementary nucleic acid probes is possible by in situ hybridization histochemistry. This can be done on cytogenetic preparations, cultured cells or tissue sections. Electrophoretic methods can only indicate the identity and quantity of a target but do not provide spatial information regarding the cellular site of, for example, particular mRNA's or viral particles. This may be critical fortthe investigation of pathogenic processes. The power of in situ hybridization is that, although inherently less sensitive than blotting techniques, it relies on the presence of "local depots" of high target concentration within nuclei or cytoplasm. The spatial dimension means that any area not containing target can be ignored. When tissues are homogenized the result is dilution of the target by a vast excess of non-target nucleic acid.
I will describe the basic characteristics of nucleic acid hybridization as they relate to the practicalities of in situ hybridization. I will also discuss probe production and labelling (radioactive and non-radioactive), choice of label, tissue pretreatments, hybridization conditions and controls.
Finally I will show a range of examples of the techniques atlight and electron microscope levels.

Unravelling the genomes of higher organisms using in situ hybridization.

Dr. Andrew Leitch (Institute of Plant Science Research, Cambridge)

In situ hybridization using biotinylated DNA and avidin detection systems was used to examine the location of DNA sequences at the light and electron microscope level. DNA-DNA hybrids were visualised at the LM level by Texas Red fluorescence or the enzymatic precipitation of Diaminobenzidine (DAB) and at the EM level by DAB or colloidal gold. The use of sectioned material allowed the location of the probe hybridization to be established unequivocally in both metaphase and interphase nuclei. In situ hybridization on sectioned material of first generation hybrid plants between cultivated barley and wild rye enabled the location of the two parental genomes at all stages of the cell cycle. The two genomes did not intermix but occupied distinct domains even in cells many divisions after the initial fusion that formdd the zygote. In situ hybridization on sections of this hybrid plant, and human lymphocytes, enabled the position of rDNA sequences within the nucleolus of interphase cells to be demonstrated.

Methods and applications of in situ hybridization in pathology.

Dr. Ken Fleming (Nuffield Dept. of Pathol., John Radcliffe Hosp., Oxford)

The recent increase in the use of insitu hybridization in pathology has largely resulted from two methodological developments of the early 1980's.
i, non-isotopic labelling systems and enzymatic detection
ii, digestion protocols for formalin-fixed, paraffin-embedded tissues.
To date, the major limiting factor of these systems has been relative lower sensitivity compared with frozen tissues and isotopic probes. This has now been largely overcome, as will be shown by detection of less than 10 copies of a human gene in transgenic mouse tissue. Althpugh many factors are involved in this increased sensitivity, all result in lowered background, allowing prolonged incubation in substrate.
The ranbe of applications of ISH in pathology is expanding. Currently the most frequent clinical use is in Virology. Thus, 1) ISH may allow more sensitive and rapid detection of virus, especially non-culturable virus, than conventional techniques. 2) Viral typing on small clinical samples. 3) Investigation of viral-host interactions, e.g. identification of cell type infected and correlation with protein synthesis and with inflammatory response. Uses outside Virology, which are rapidly developing, include investigation of interphase cytogenetics in normal, premalignant and invasive cells, cell lineage studies in chimaeric tissues and studies of epithelial-stromal relationships in tumours and in differentiation. Aspects of each of these areas will be illustrated.

3-D in situ hybridization in plants.

Dr. David Rawlings (John Innes Institute, Colney Lane, Norwich)

In order to address the question "where and in what conformation are genes in the interphase nucleus ?", we have used in situ hybridization to thick tissue slices where the three-dimensional structure of cells is maintained. cDNA probes are hybridized into these slices and are visualized by immunofluorescence and confocal optical microscopy. The confocal microscope allows thin optical sections to be obtained which are stored on computer as digital grey scale images. Computer display of projections through stacks of optical sections allows the 3-D arrangement of the probed genes to be examined. Studies already completed have given detailed information about the arrangement of ribosomal genes in pea root nuclei (Chromosoma, in press) and we are currently working on methods to increase the sensitivity of detection down to single copy genes. These methodd include the use of 1 nm gold-conjugated antibodies and digoxygenin-labelled probes. Silver enhancement of gold-labelled antibodies may allow visualization at the light and electron microscopic level of the same nucleus.

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Specific intracellular localization of nucleic acid sequences using complementary nucleic acid probes is possible by in situ hybridization histochemistry. This can be done on cytogenetic preparations, cultured cells or tissue sections. Electrophoretic methods can only indicate the identity and quantity of a target but do not provide spatial information regarding the cellular site of, for example, particular mRNA's or viral particles. This may be critical fortthe investigation pathogenic processes. The power of in situ hybridization is that, although inherently less sensitive than blotting techniques, it relies on the presence of "local depots" of high target concentration within nuclei or cytoplasm. The spatial dimension means that any area not containing target can be ignored. When tissues are homogenized the result is dilution of the target by a vast excess of non-target nucleic acid.
I will describe the basic characteristics of nucleic acid hybridization as they relate to the practicalities of in situ hybridization. I will also discuss probe production and labelling (radioactive and non-radioactive), choice of label, tissue pretreatments, hybridization conditions and controls.
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To localise specific DNA or RNA sequences within tissues. We can get the spatial information: where? cell specificity? does the distribution change? multiple sequence detection; and perhaps quantification.

Target sequence -- complimentary probes -- reporter molecule

Types of duplex:

DNA - DNA
DNA - RNA
RNA - RNA

↓ increasing stability at any particular temperature

It is possible to manipulate the stability of the duplexes.
Hybrid stabilisation temperature Tm = Temp.$^{\circ}$C where half the population of duplex molecules become dissociated to single strands.

$$Tm = 81.5^{\circ}C + 16.61 \log M = 0.41(\%GC) - \frac{820}{L} - 0.6(\%F) - 1.4\%\%MIS$$

M = ionic structure L = probe length
%GC = (% guanine/cytosine)
%F = % formamide (duplex destabiliser)
%MIS = % non-complementary BP

Increase in M stabilises the duplex.

GC uses 3H bonds; AT uses 2H bonds.

Most in situ hybridisations are optimum at -25°C, i.e. low stringency.

Hybridisation time is related to probe length and probe complexity, i.e. the number of repeats. The most rapid hybridisation is with simple long probes at high (probe); but these long probes cannot penetrate tissues.

Types of probe:

- double-stranded DNA
- single-stranded DNA
- single-stranded RNA (riboprobes)
- synthetic oligonucleotides

cDNA (double) probes

easy	but	tend to reanneal
form networks		vector present
high specific activity		

RNA probes

single-stranded, so can't re-anneal	but	sticky
stable hybrids		in vitro transcription
high specific activity		
sense-strand controls		

Oligonucleotide probes

single-stranded	but	DNA synthesiser
simple synthesis		must know sequence
designed		less stable hybrids
can penetrate tissues because small		

Probe labelling methods

1. Enzymatic incorporation of labelled nucleotides
   a. Nick translation
   b. oligonucleotide primed extens.
   c. oligonucleotide synthesis
   d. polymerase chain reaction
2. Non-enzymatic chemical modification of nucleic acid
   a. mercuration
   b. sulphonation
   c. photobiotinylation

There are numerous reporter systems, e.g.

biotin-avidin conjugates

bromodeoxyuridine - antibody

direct fluorochromes

Non-radioisotopic labels have the advantages of safety, ease and expense, rapidity, and possibility of multiple labelling.

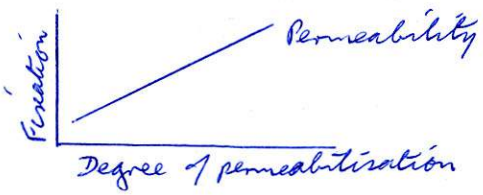
The bioin-avidin system can form massive complexes; it can be bound to colloidal gold; and is compatible with immuno-histochemistry.

In situ hybridisation can be used on cultured cells, cryosections, fixed frozen material, wax sections. Briefly, the protocol is:

1. select tissue
2. Fix
3. Permeabilisation - to allow entry of probe
4. Prehybridisation - buffer without probe
5. Hybridisation
6. Post-hybridisation - to increase specificity
7. Visualisation of probe

The overall aims are to prevent target loss, preserve morphology, and allow penetration of probe. Fixation can be by precipitators (acetone, ethanol/acetic acid), or cross-linkers (formaldehyde etc). Selection of the optimum fixative and schedule is by trial and error: it is essential to go through the whole series of optimisations of time, concentration etc for each fixative.

Permeabilisation can be done with proteinase K, HCl, pepsin/HCl, or pronase.



Hybridisation mixture for DNA probe:

1. Buffer, 10 - 20 ul/slide
   - 50% Formamide
   - 2 x SSC
   - dextran sulphate - increases specificity & signal strength
2. Carrier DNA e.g. salmon sperm, to block non-specific reaction
3. Probe 10 - 50 ng/ slide.

Double-stranded DNA: first step is to denature probe & target at above 95°C for 10 minutes; then hybridise for 3 hours or overnight.

Post-hybridisation, use:

1. stringency washes - formamide etc, at various temperatures
2. enzyme digestions, eg RNAse if seeking DNA

The blocking solution can be e.g.

0.1 M Tris-HCl pH 7.5

1 M NaCl

2 mM MgCl

0.5% Tween 20

0.05% Triton X-100

low-fat milk, etc.

Specificity controls are essential, and can be:

1. Positive tissue
2. Negative tissue
3. Hybridise with unrelated probe
4. Hybridise with no probe
5. High salt stringency wash
6. DNAse pretreatment
7. RNAse pretreatment
8. Competition with cold probe
9. Thermal stabilisation
10. Northern blot
11. Check by immuno-chemistry

(At Micro 90, Jeff MacFadden will be speaking on July 6)

Sections can be stuck on slides with chromalum, poly-l-lysine, etc.

For EM, only picogram quantities are needed.

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With the barley/rye cross, the chromosomes were of noticeably different sizes between the two parents enabling them to be identified in situ, even on serial sections for EM. In metaphase, the long barley chromosomes tended to be located in the middle of the cluster, surrounded by the shorter rye chromsomes. Interphase is the more important phase, for gene expression. rDNA for ribosomes could be localised and identified within the nucleolus for the two species, by in situ hybridisation.

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Clinical material is usually rather less than optimal for fixation etc, so most work is done on formalin-fixed paraffin-embedded tissue. Radio-active markers are expensive, slow, and not very good resolution. Non-isotopic markers are therefore generally used; but the best sensitivity is with radio-active markers on frozen tissue.

Transgenic mice have been produced, carrying a known number of copies of a human gene, e.g. mouse with 10 copies/ cell. On "a good day", one can detect only 2 copies/cell.

High sensitivity depends on low background, probe, digoxigenin, dextran sulpahte. Digoxigenin gives a cleaner background than biotin, because tissues contain endogenous biotin. Hybridisation can be continued for up to 24 hours, because the signal production is linear.
high signal/noise ratio ≡ clean background

The probe should consist of less than 400 base pairs: the critical number is 100 - 400 pairs. Some days the probes prepared are good, other days bad, for no known reason.

Batches of dextran sulphate vary, too. It is best to get a selection, scfeen them, then buy up the batch (by code number) of the best one. The same goes for the formamide.

In situ hybridisation is very labour-intensive, and all stages are critical.

Uses of in situ hybridisation:
chromosomal abnormalities
monitor progress of epithelium becoming invasive
chromosomal disorganisations
marker for malignancies
mRNA versus protein synthesis/secretion/storage
monitor transplant chimeras eg renal transplant, bone marrow transplant, male/female transplant; HLA mis-match increases rejection rate.

A few elukaemic patients have a successful bone marrow transplant but later become leukaemic again, apparently with the donor cells.
Epithelium/stroma interactions in tumours - the stroma gives the message to the epithelium as to what sort of tissue to become; eg basal cell carcinoma.

Viral applications
eg for HIV, CMV, Hepatitis B, adeno, parvo, HPV. For simple diagnosis, in situ has probably been superceded by PCR.

It is possible to identify the current infection (serology demonstrates previous infections); it is not necessary to have fresh tissue; the viruses can be typed; cytological location is possible; there is no need for high replication/expression of protein, ie latent infection can be shown; virus in only a few cells can be shown; retrospective analysis is possible; the technique is fast.

CMV is especially important in liver transplants (Daco now has an anti-early-CMV pack).
Of fetal infections with human parvovirus, 1 in 4 lead to fetal death, mostly from hydrops. The virus can be found primarily in the erythrocytes, and therefore in all other tissues; the hydrops is probably caused by infection of the myocardial cells. They are about to start doing EM on these.
There are over 50 types of human papilloma virus; types 6 and 11 cause sexually-transmitted anogenital warts, types 1 2 etc ordinary skin warts. Types 6 and 11 may befound in children under 5 years old, possible transmitted in utero.

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3-D in situ hybridisation in plants - Arabidopsis. Used silver-enhanced gold-conjugated antibodies with reflection microscopy some double-labelling. Optical sectioning.

Rawlins & Shaw 1990 J Microsc 157 83-89

Agard 1980 Ann Rev Biophys. Bioeng.

Agard 1989 Meth Cell Biol 30. 353-377.

SEMT Meeting, Feb. 23rd. 1990

List of Registrants .

Ms. Sue Barnes	Natural History Museum, London
Mr. Don Claugher	"
Mr. Kevin Jones	"
Mr. John Spratt	"
Miss Anne Drewe	Microbiol. Dept., Charing Cross Hospital
Mr. John Paul Cassella	Inst. Dermatol., St. Thomas' Hosp. Med. Sch.
Mr. Tony O'Grady	"
Dr. Hohin Eady	"
Akemi Yamamoto	"
Mr. David Gunner	"
Dr. John Beswetherick	Reta Lila Inst. Of Neurological Studies, UCH
Dr. Lynda Bonner	Dept. Botany, Univ. of Reading
Dr. Jennifer Plummer	Royal Vet. College, London
Mr. John Bredl	"
Dr. J.Moss	Dept. Histopathol., Ch.X and WMS
Mr. I.Shore	"
Miss Fiona Holt	Physiol. Dept. Wye College
Miss Shelagh Reardon	"
Miss Rosemary Suswillo	Anatomy Dept., Royal Vet. Coll.
Dr. Swati Majumdar	Lung Pathology, NHLI, Brompton Hosp.
Dr. Pater Jeffery	"
Mr. Phil Coates	Histopathol., St. Bartholomews' Hospital
Dr. Jill Lewis	EMU, St. Bartholomews' Med. Coll.
Prof.Max Weinreb	Inst. Dental Surgeond, Eastman Dental Hosp.
Joseph Aduso-Opoku	Hunterial Dental Res. Unit, Eastman Dental Hosp.
Mrs. Pauline Barber	EMU, Eastman Dental Hospital
Miss Charlene Africa	"
Mrs. Nicola Mordan	"
Mr. Simon Lumb	London Hosp. Med. Coll.
Ms Jane MacDougall	"
Mr Roy Russell	"
Miss Ann Dewar	EMU, Nat. Heart and Lung Inst., London
Miss Fiona Nelson	"
Miss Judith Mac	"
Miss Nicki Thurlow	"
Dr. Richard Blackburn	Sch. of Environmental Sci., Thames Polytechnic
Mr. Mikke Wombwell	Biorad Microscience Ltd.
Mr. Alan R Gray	Pathol. Dept., London Hospital
Dr. Gisele Hodges	Imperial Cancer Research Fund
Mr. Brian Murphy	William Harvey Hospital, Ashford
R.Jenkins	London Sch. of Hygiene and Tropical Medicine
Dr. D.Dourmashkin	London Hospital