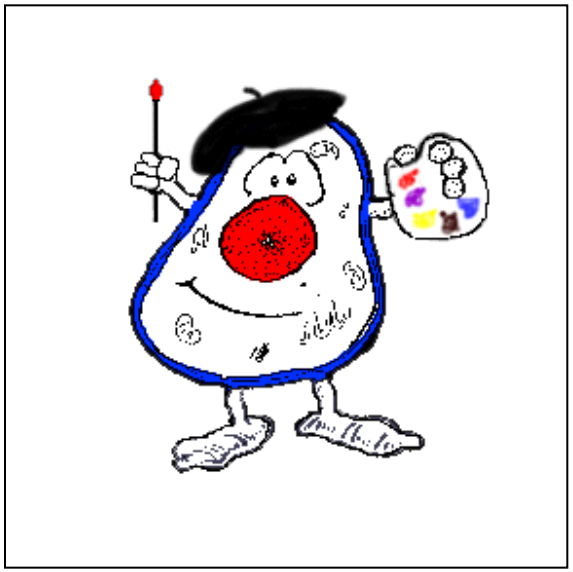
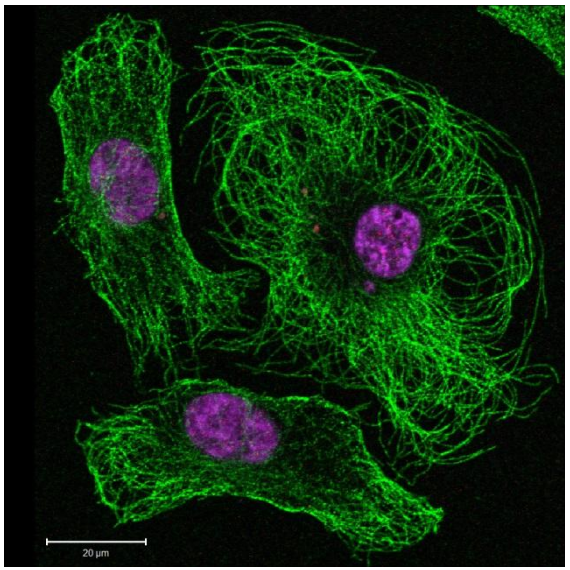
Visualising Biological Specimens & Microtechnique



Microtechnique

‘Staining’



ACA4 Morphological Research Methods & Imaging

Mike Mahon, 2020

Keele University

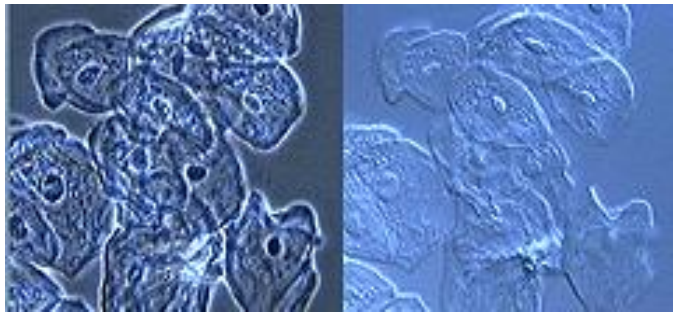
Visualisation

- Microscopy (BF,DF,Phase,Pol,DIC,Fluorescence)
- Vital Staining
- Tissue preparation (Microtechnique)
- Histology & Electron Microscopy
- Histochemistry
- Immunocytochemistry
- Lectin Cytochemistry
- In Situ hybridisation
- Tracer (Autoradiography,BrdU,Neuro)
- New (Transgenic, Ca^{++}, Super-res, expansion, …)

Microscopy

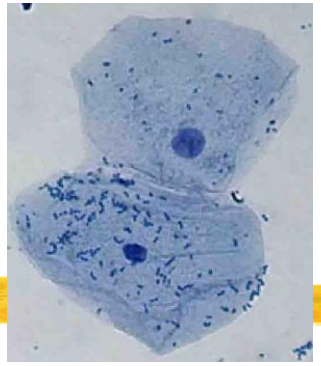
- Microscopy (BF,DF,Phase,Pol,DIC,Fluorescence)

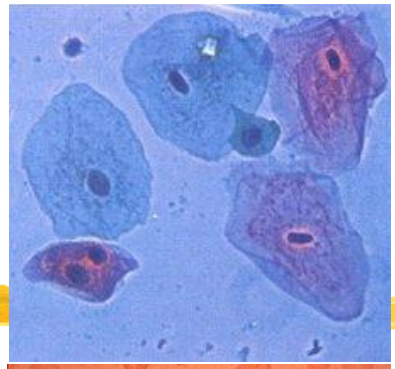
Thin biological specimens are generally transparent … so use alternative microscopies …. (See MICROSCOPY Later this week)

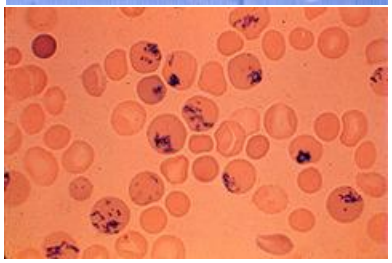


- **Very Important**
  – cells still alive and activities observable, 4th Dimension

Vital Staining







- Staining cells and tissues without killing them
- Living cells may absorb or phagocytose dyes
- Vital … eg Janus Green +O_2 > blue (‘stains’ mitochondria)
- Intravital … injected into organism, body
- Supravital … remove cells from body first
- Sometimes only dead cells stain (assess viability) eg Trypan Blue

Requirements of Specimens for BF Microscopy

- Well preserved
- Transparent
- Contrasty
- Informative
- Permanent
- Reproducible Technique

What do we want to know?

- General organisation & morphology
- Cell structure / variability
- Cell organelles, proteins, surfaces
- Associations/Closeness
- Tissue/Cell storage products & chemistry
- Tissue/cell activity, gene activity

Practical Requirements

- Living tissues
- Permanent preparations
- Quick look & record
- Rapid information (biopsies)
- Duplicate / serial samples
- Resolution, functional/chemical data
- Relative or Absolute quantitative data
- Costs

Tissue Preparation

- Is the tissue Hard / Opaque / Invisible / Fluid / Moving ?
- Then use one of ...
  - Drops on a slide / Grow on a slide
  - Smears, Teased
  - Whole mounts
  - Sections
  - Ground slices
  - Surface Etchings
- Need to pre-inject, pre-label ?
- Closeness ? – use expansion microscopy

Visualisation

- Microscopy (BF,DF,Phase,Pol,DIC,Fluorescence)
- Vital Staining
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Microtechnique

Living

Smear

Fix

Kill

Fix

Dehydrate

Clear

Embed

Section

Stain

Mount

View

Record/analyse

Kill / Living

Freeze

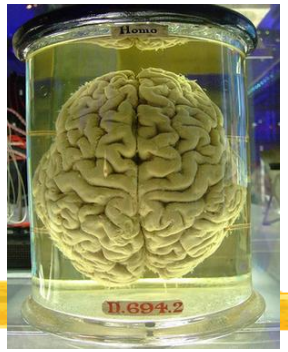
F/S

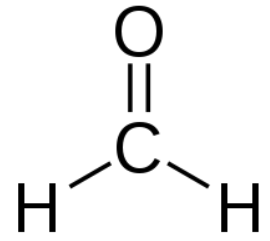
F/D

Fixation

- What if NO fixation?
  - Tissues dry, shrink, (swell), autolyse, putrefy
- Fixative AIMS
  - Preserve & stabilise constituents = in vivo
  - Prevent diffusion
  - ↑ hardness
  - Kill bacteria & moulds
  - Protect from subsequent treatments
  - Enable staining
  - Provide permanent preparations

Fixative types

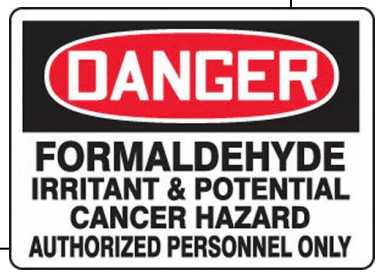




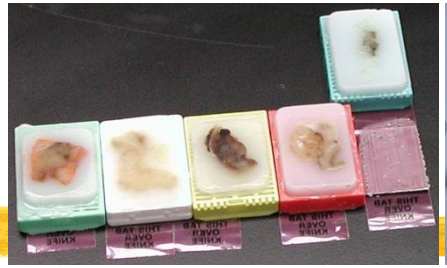
- Chemical (crosslinking, precipitating, oxidising)
  - Formaldehyde, Glutaraldehyde, acetone, alcohol, OsO_4
- Heat (microwaves)
- Freeze
- Freeze substitution
- Freeze dry

Chemical - factors to consider

- Block size - penetration time
- Shrinkage / Swelling
- Hardening
- Effects on staining
- Health & Safety

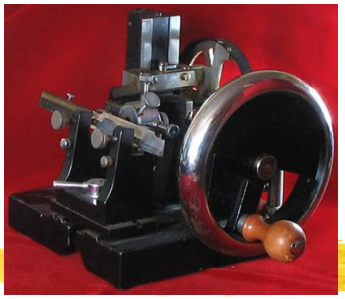


Embedding

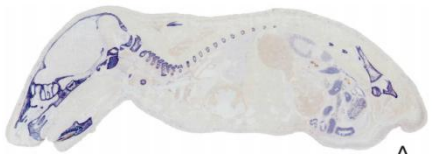


- Wax (paraffin, polyester)
- Celloidin, LVN
- Resin (methacrylate, epoxy)
- Water (frozen)
- Gels (polyacrylates)

Microtomy



- Microtomes
  - Rocker, Rotary, Sledge, Vibratome, Cryostat
- Knives
  - Disposable, Steel, Glass, Diamond
  - Shape, Angle



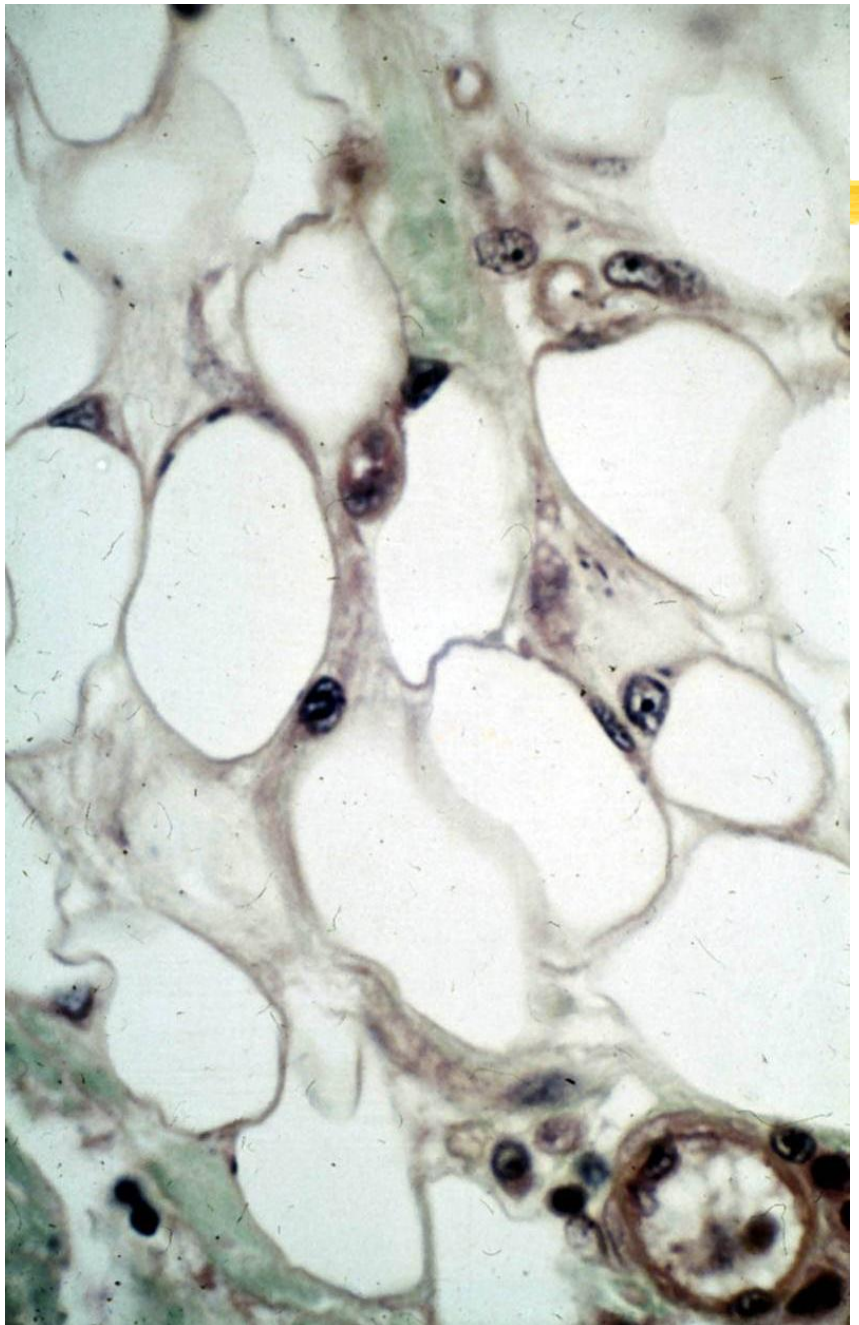
- Section Thickness
  - <2000 um Food Slicer
  - 20-200 um Gelatin Embedded
  - 8-30 um Frozen
  - 5-10 um Waxes
  - 0.5-3 um Resin
  - <0.1 um EM

Frozen Sections

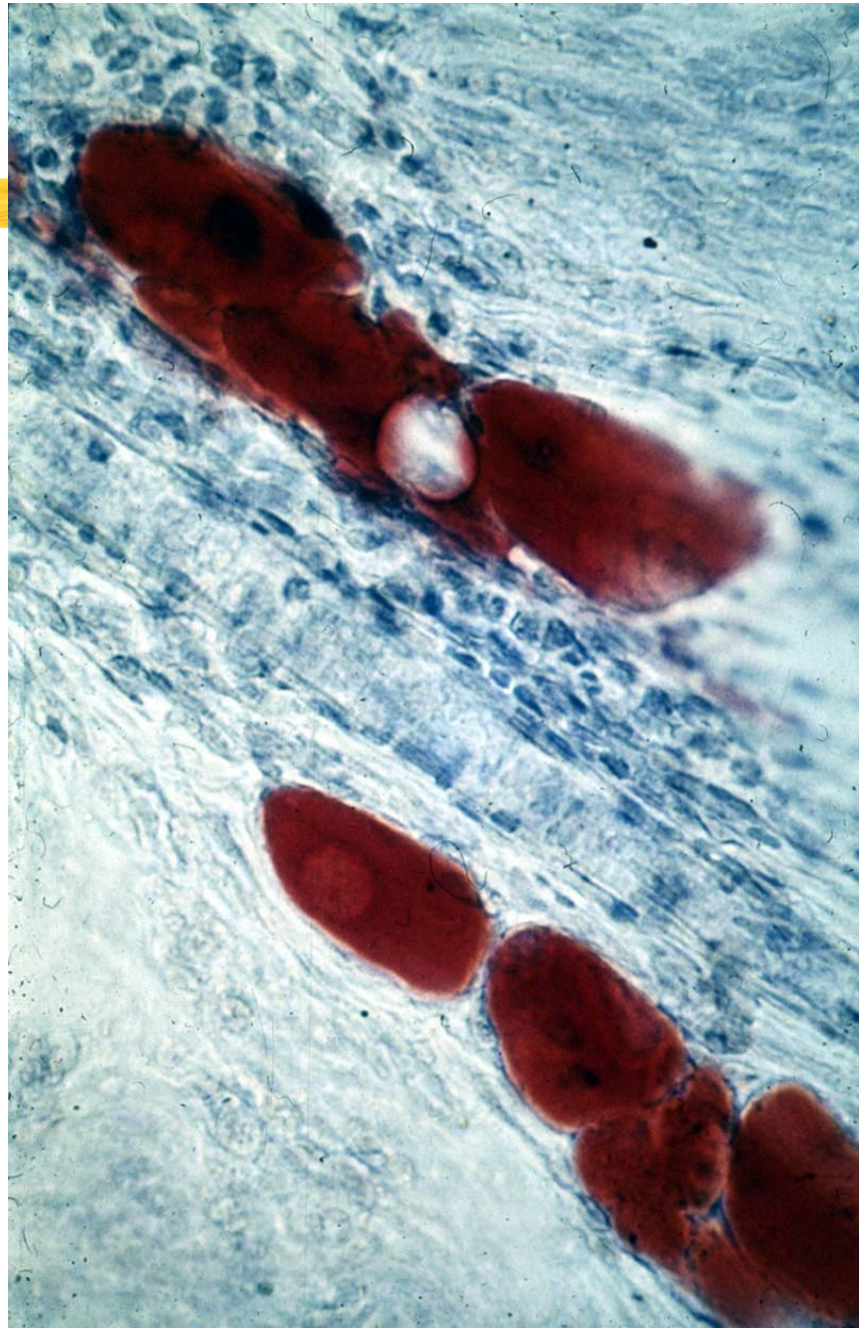
- Use for Histology & Histochemistry … (cryo-EM)
- +ve -ve
  - Quick & Cheap — Expense, Storage, Transport
  - No fixation — Permanency
    - √ morphology — Thick Sections
      √ morphometry — Size, Ice
      √ histochemistry … — Re-orientation
- Method
  - Aerosol (-50C), CO_2 (-70C), Liq N_2 (-190C)
  - Isopentane / Arcton
  - Mountants (water, liver, OCT, agar)
  - Cryostat -20C; Deep Freeze Storage -70C or Liq N_2

Adipose tissue

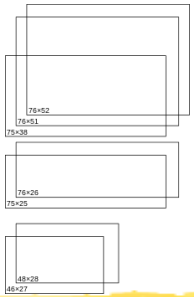
Fixed, embedded, dehydrated – fat lost (spaces)

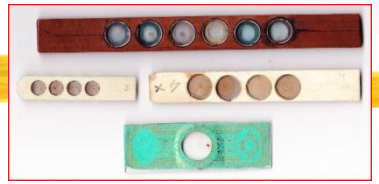


Frozen – lipids retained, stained red here



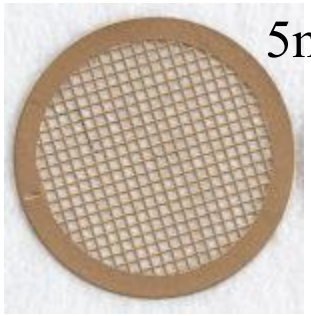
Mounting



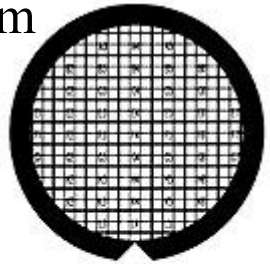


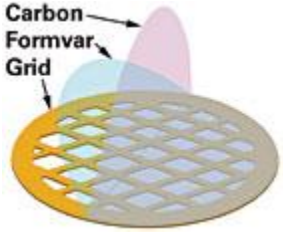
- Microscope Slides (size?)
  Standardized in 1840
  - Coverslips
  - Permanent
  - Mounting Media

- Copper Grids (EM)



5mm

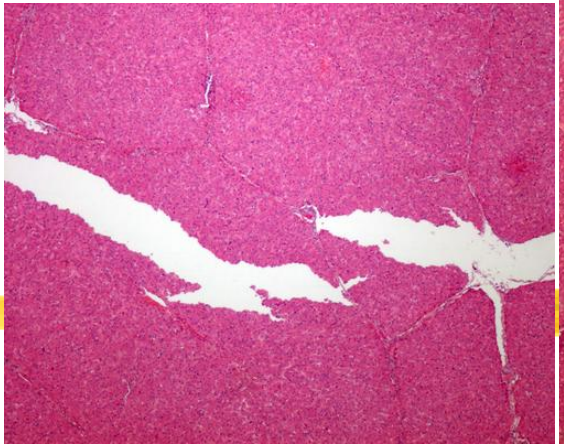
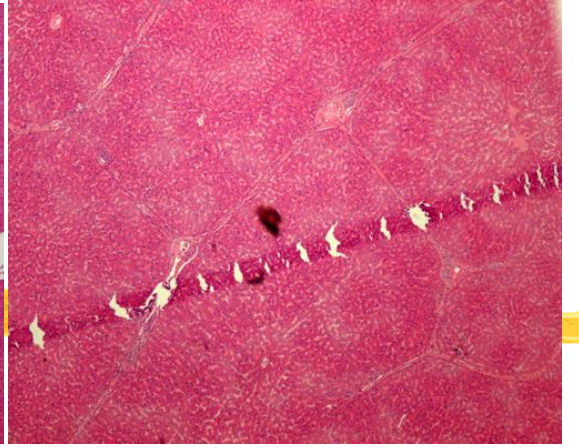
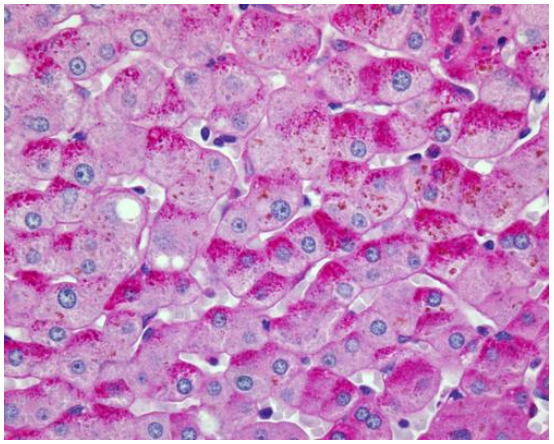
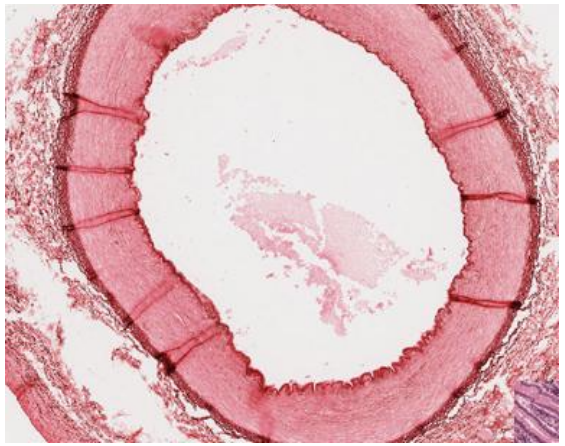
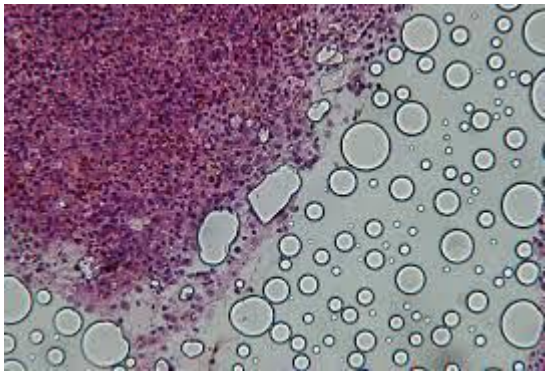
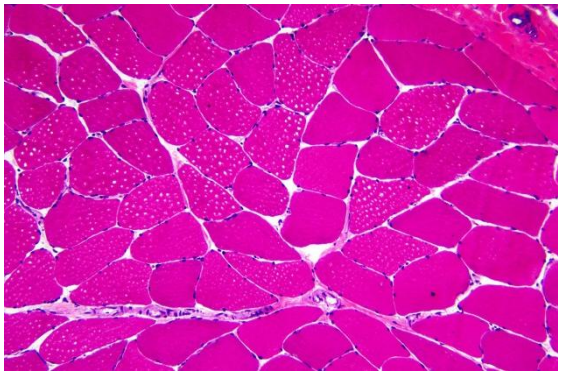




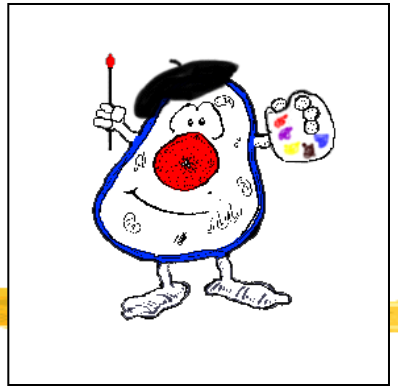
- Stubs (SEM)

Artefacts

- Fixation
- Freezing
- Microtomy
- Staining
- Mounting
- Microscopy

Staining



- Vital
- Physical
- Physico-chemical

} Histology & EM

- Histochemical
- Immunocytochemical
- Lectin cytochemistry
- In Situ Hybridisation
- Autoradiography / BrDU / Neuro/Gene Tracers

Histological Staining/Dyeing

- Why stain?
- What are stains/dyes?
- What gets stained?
- Why do dyes stick onto things
- Why don’t dyes stick to everything?
- Why do components remain stained?
- What does it all mean (interpretation)?
- What methods are used?

REF: Horobin RW (1978)

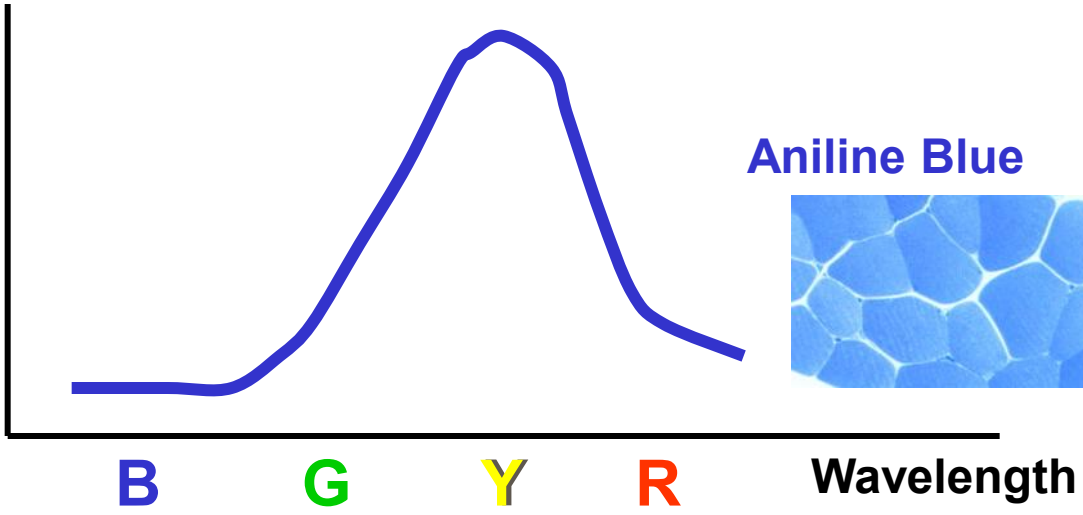
Histological Staining/Dyeing

- Why stain?
  - Improve visibility – absorption
  - Differentiate tissue components – selective absorption
  - Study previous cell activity – selective staining
- What are stains/dyes?
  - Ionic dyes D^+, D^- — aromatic rings, phenylated, sulphated, cationic, carboxylated, metallic cations
  - Reactive dyes
  - Metachromatic dyes
  - Non-dyes — metals, trappers, supravital

REF: Horobin RW (1978)

Dyes ....

• Saffron	Leeuwenhoek	1719
• Carmine	Cohn	1849
• Nuclear	Gerlach	1858
• H&E	Bohmer	1865
• Aniline	Germany/UK	19th 20th C



Aniline Blue

Mainly blue light passes through and reaches the eye

- Nomenclature / Chemical Index Number / Purity

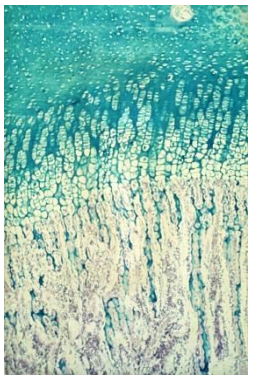
Histological Staining/Dyeing

- What gets stained?
  - Nothing
  - Everything
  - Selective (Carbohydrates or Lipids or Proteins)
  - Depends on prior fixation / preparation
    - Crosslinking
    - Coagulation
    - Exposure of hydrophobic surfaces
    - Change in potential of amino acids
    - Change in electric charges
    - Breakage of covalent bonds
    - Denature nucleic acids
    - Trap glycogen

REF: Horobin RW (1978)

Histological Staining/Dyeing

- Why do dyes stick onto things?
  - Dye-Tissue
    - Minimal use of free energy
    - Donnan membrane equilibrium
    - Van der Waals forces (attractive)
    - Energy of interaction
    - Electrolyte movement
    - Hydrogen bonding (electrostatic)
  - Solvent-Solvent
    - Hydrophobic bonding
    - Effects of pH, Salts, Critical Electrolyte Theory (Scott) *
  - Dye-Dye
    - Metachromasia



REFS: Horobin RW (1982), Bancroft & Stevens

Histological Staining/Dyeing

- Why don't dyes stick to everything?
  - They do but we don't notice – density effect

  - Dye-tissue interaction between substances
    - Affinity
  - Rate of staining differs with substrate
    - Permeability
    - Porosity
  - Everything stains but in different colours
    - Metachromasia

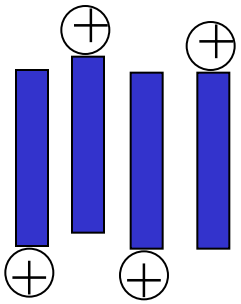
REFS: Horobin RW (1982), Bancroft & Stevens

Examples

- Metachromasia eg Toluidine blue
- Acid-Base Properties eg H&E
- Physical Properties eg Trichrome

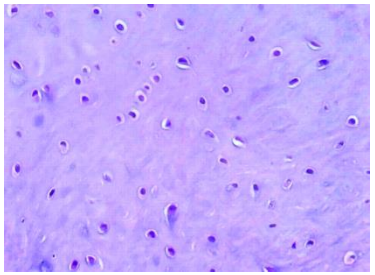
Examples 1

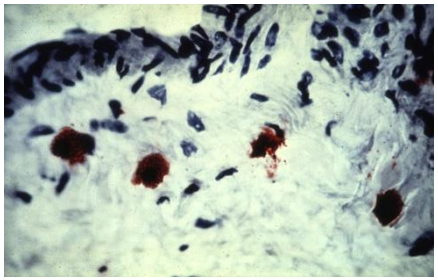
- Metachromasia eg Toluidine blue

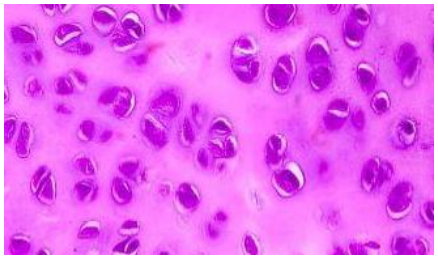


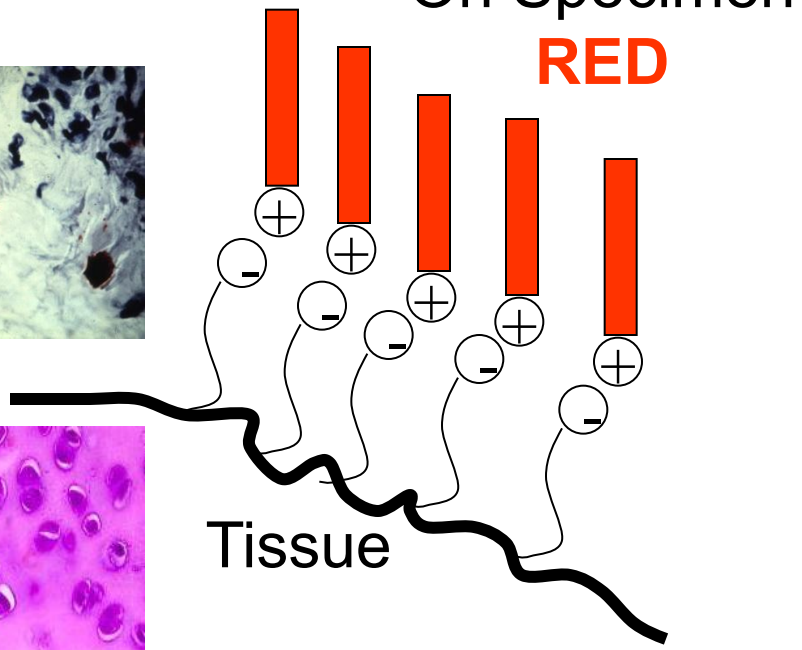
In Solution

BLUE



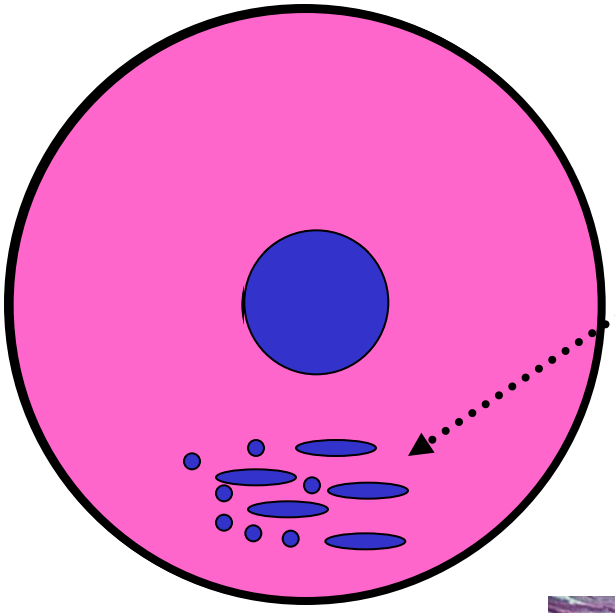






Examples 2

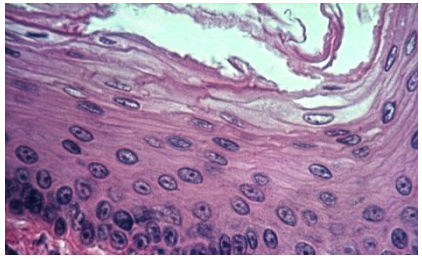
- Acid-Base Properties eg H&E

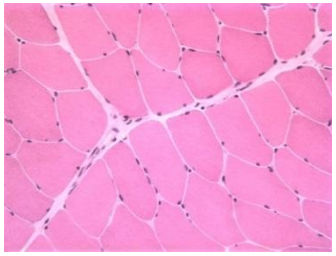


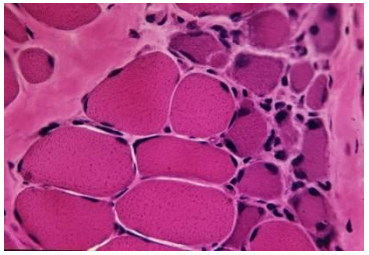
Haematoxylin – basic dye +++

Basophilia Useful to indicate regeneration or pathology

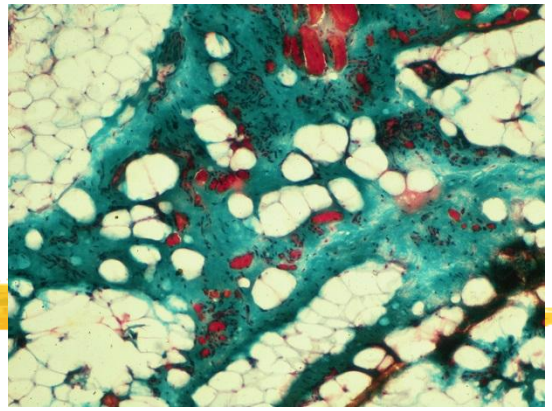
Eosin – acidic dye ---



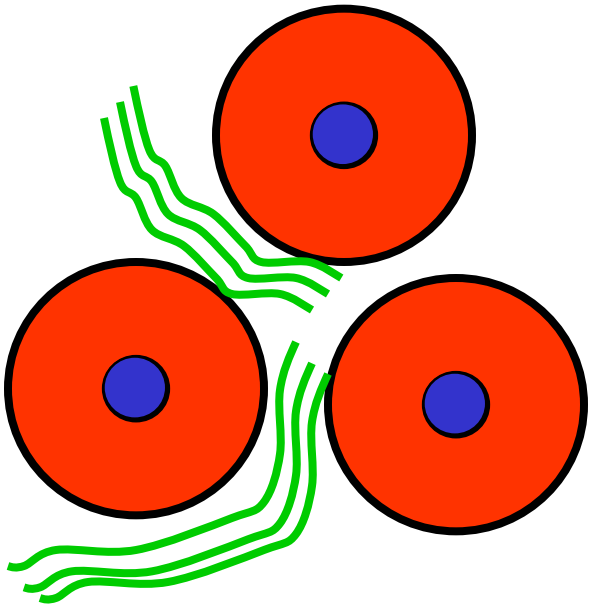




Examples 3



- Acid/Base + Physical Properties



eg Trichrome

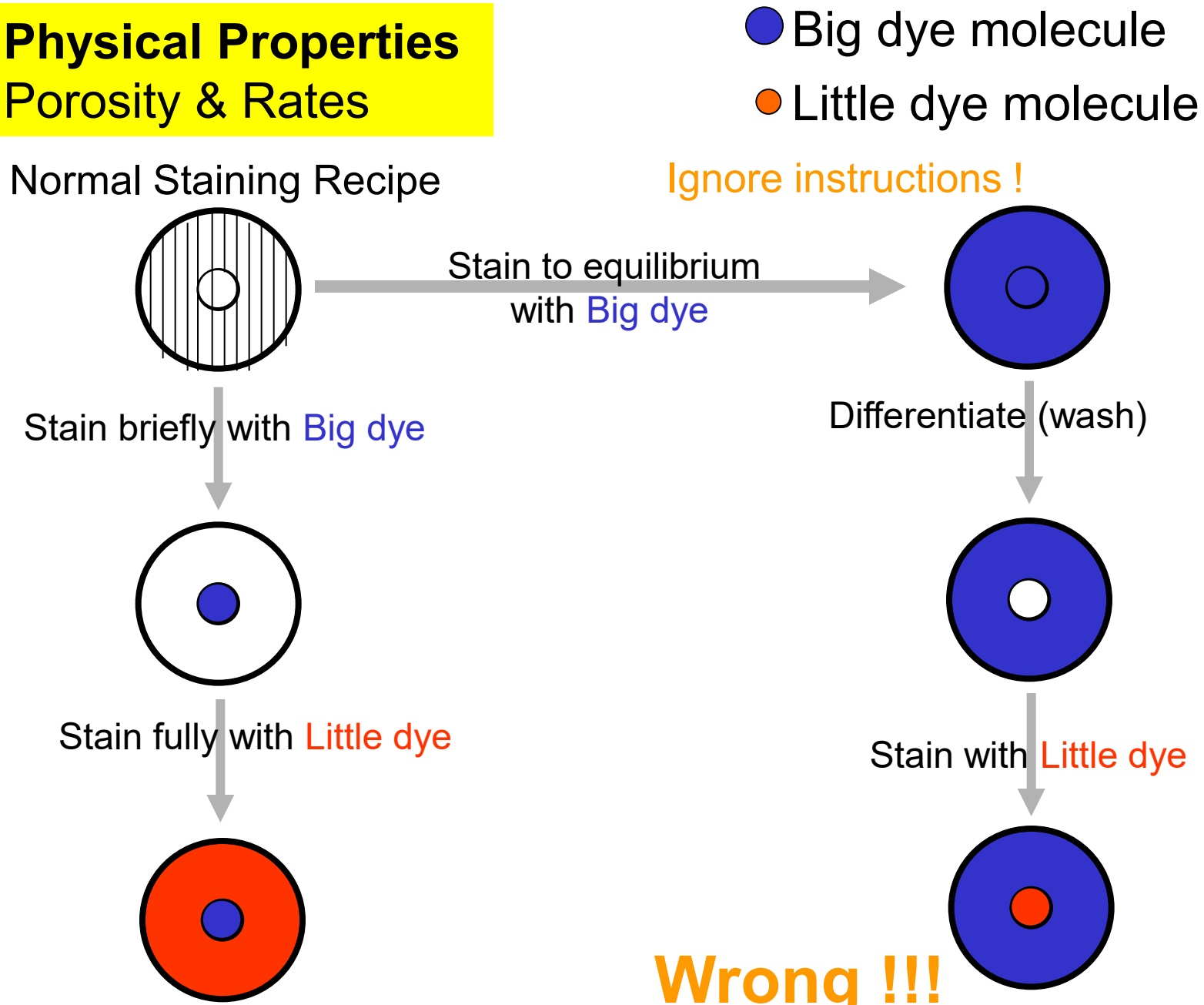
Haematoxylin – nuclei

Eosin – cytoplasm

Green - fibres

NB: rate dependant, preblocking, mordants

Physical Properties
Porosity & Rates



So, make sure you follow the recipe & don't take a fag or tea break!

Histological Staining/Dyeing

- Why do components remain stained?
  - They often don't !
    - Diffusion, Bleaching, Drying
  - Dehydrate rapidly
  - Air dry
  - Use mounting media with low affinity for dye
    - Hydrophobic – for ionic dyes
      - Canada balsam, DPX, xylene
    - Hydrophilic – for diazo formazans
      - Gelatin, Glycerol, Polyvinylalcohol
  - Use insoluble final reaction product
    - Ag, Au,

Histological Staining/Dyeing

- What does it all mean (interpretation)?

  What can you see?

  Are there any artefacts?

  Have the routines been followed exactly?

  Have adequate controls been done?

  Does it make biological sense?

  Does it agree with other workers?

Histological Staining/Dyeing

- What methods are used?
  - Subclasses
    - General
    - Connective tissues
    - Proteins
    - Nucleic acids
    - Carbohydrates & mucins
    - Lipids
    - Amyloid / Keratin / Fibrin
    - Pigments & Minerals
    - Neurohistology
    - Bacteria & Invertebrates

- **Typical Procedures**

Bring sections to water

Stain in Haematoxylin

Differentiate in acid-alcohol

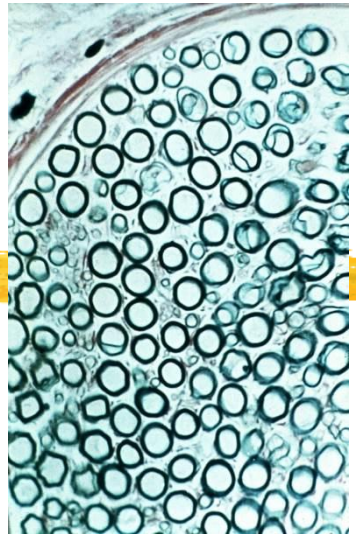
Blue in Lithium carbonate

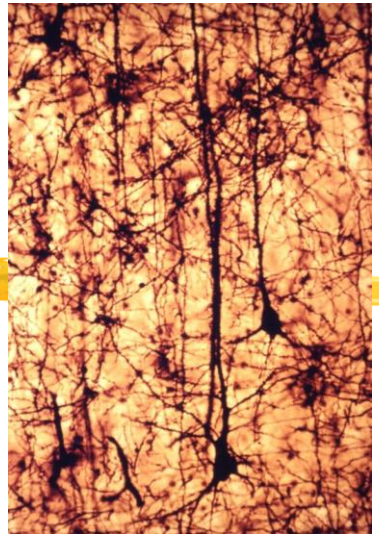
Stain in Eosin

Dehydrate, Clear, Mount

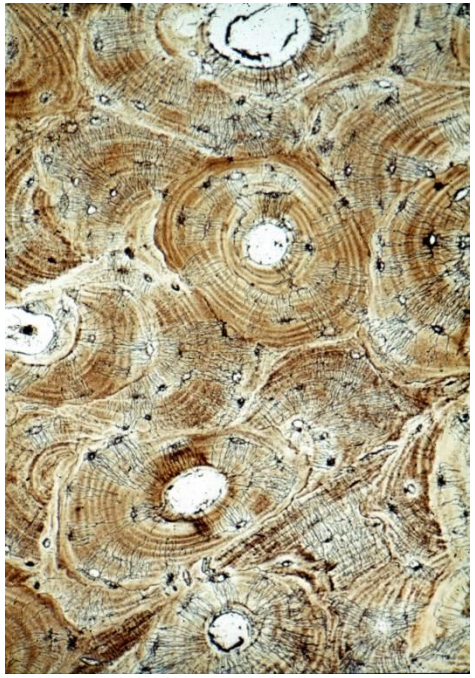
Dry, View, Record

Metallic Methods





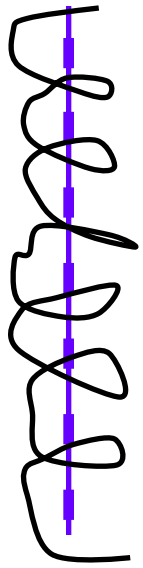
- Nerve
  - OsO_4 combines with –C=C-
- Silver
  - Hormones, gut cells, Reticular fibres, Nerve, BM, Collagen (gold/brown)
- Impregnation
  - Uptake reversible, irreversible
- Visualisation
  - Not required (Chromaffin)
  - Metallic Crystals, Ligand, Reactions (Cr, Fe)
    Care! Interactions with Formaldehyde



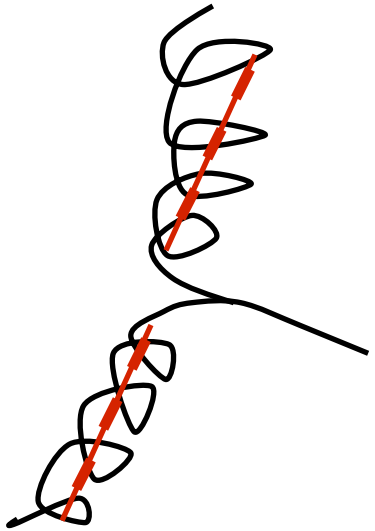
Golgi/Cajal – Nobel Prize

Trapping Methods

- Iodine for Polysaccharides
  (Long chains of dye appear blue, short chains red)



Starch



Glycogen

Stains

- Physical properties of tissue
  - **Acid & Base**: H&E (**Haematoxylin** & **Eosin**), Tol Blue
  - **Trichrome**: Masson (**Nuclei** - **Cytoplasm** - **Fibres)**
  - **Metallic**: Silver, **OsO_4** **Trapping:** Iodine
- **Histo-chemical** properties of tissue
  - PAS
  - Oil Red O, Sudan Black B
  - Feulgen
  - Enzyme reactions
- **Immuno-cytochemical** properties of tissue
- **Genomic-chemical** properties of tissue

HISTOCHEMISTRY

- Histological stains
  - EMPIRICAL (dyes)
- Histochemical reaction productions
  - RATIONAL (chemical reactions)
- AIMS of Histochemistry

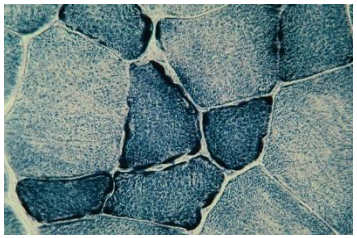
  To learn about tissue, cellular, organelle & chemical constituents, their activity & specific products in a SPATIALLY defined manner
- Biochemist's approach – fractionation, centrifugation, chemical analysis
- Histochemist's approach – freeze, section, stain, microscopy

Smash it up!

Preserve morphology!

HISTOCHEMISTRY

- Selective Uptake by substance X
- React X with Reagent
- Let X act as an enzyme
- Therefore Gives CHEMICAL & GEOMETRIC Information



…beats the biochemist,
geometric and no averaging !

HISTOCHEMISTRY

- Requirements
  - **Specificity** - single reactions
  - **Sensitivity** - small amounts
  - **Stoichiometry** - proportionality
  - **Localisaton** - diffusion absent
  - **Visibility** - no fading
  - **Reproducibility** - can redo
  - **Reliability** - can be controlled
  - **Simplicity** - one step procedure

- CONTROLS +ve / -Ve

HISTOCHEMISTRY

- Spot Test Histochemistry

  R + TS >>> TS/Coloured Product

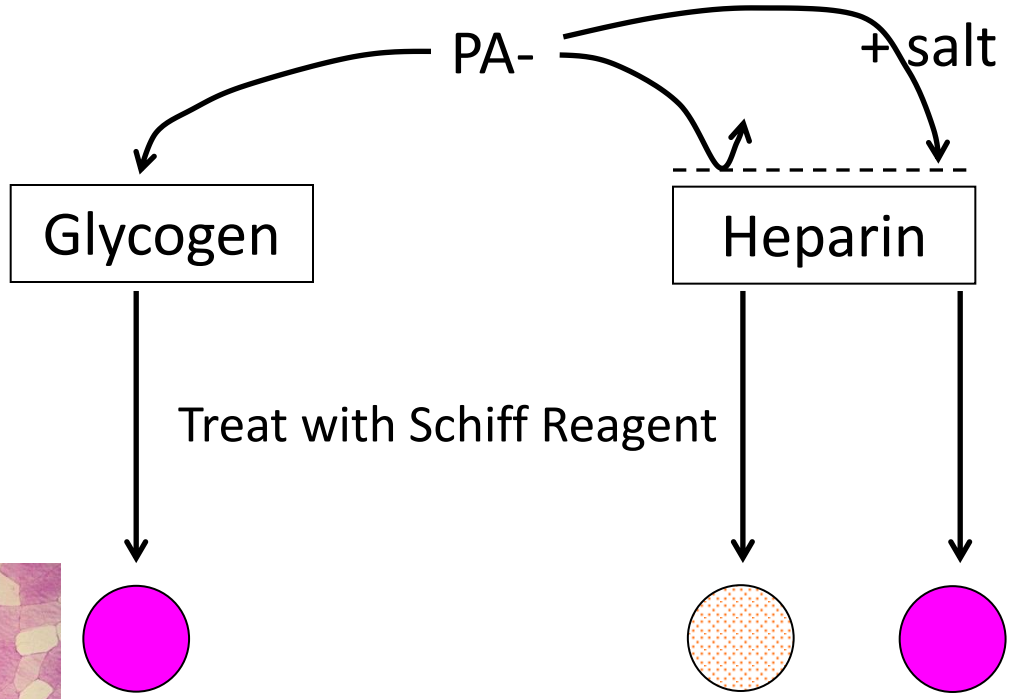
- Used to Identify …
  - A Class of compound, eg DNA (Feulgen)
  - A Particular Compound, eg Glycogen (PAS)
  - An Organic Group, eg NH2, -OH
  - An Inorganic Ion, eg Ca++, Fe+++, SO4--

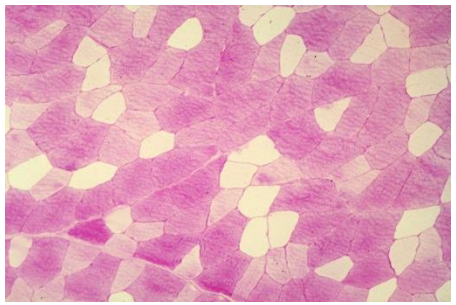
- Problems
  - Multistep, affinity, pH, electrostatic, absorption, rate fixation effects

Histochemistry (PAS)

- Electrostatic Effects

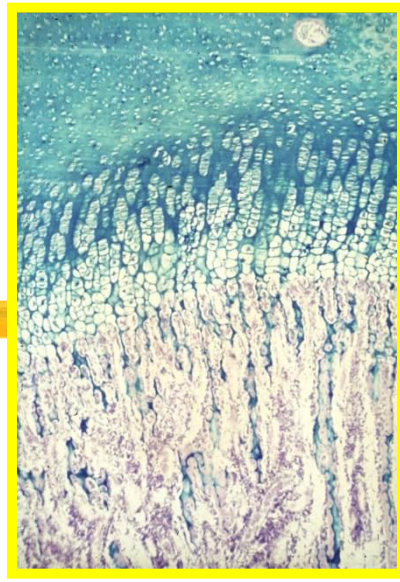
Periodic Acid oxidises H-C-OH to aldehydes (H-C=O)





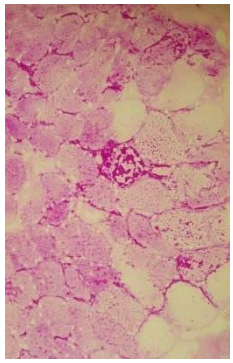
… also pH sensitive !!

HISTOCHEMISTRY



- Carbohydrates
  - PAS, Diastase, EH, Lectins, Metachromasia, Alcian Blue
- Glycogen
  - liver, cardiac muscle, skeletal muscle, hair follicles, endometrium, vaginal epithelium
    Carbohydrate----Enzyme--->Glycogen (stain, diastase digestion, controls)
- Glycogenases

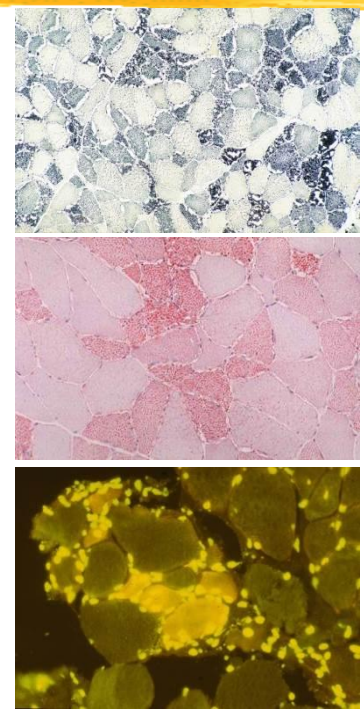
	Enzyme Deficiency	Eponym
I	G-6-phosphate	Von Giercke
II	Acid Maltase	Pompe
III	Debrancher Enzyme	
IV	Brancher Enzyme	Andersen
V	Myophosphorylase	McArdle
VI	Hepatic phosphorylase	
VII	Phosphoglutamase	
VIII	Phosphorylase	

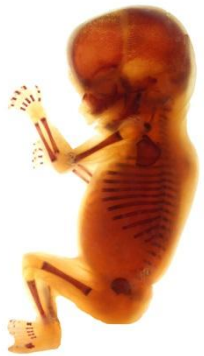


Mucins & Mucopolysaccharides – complicated !

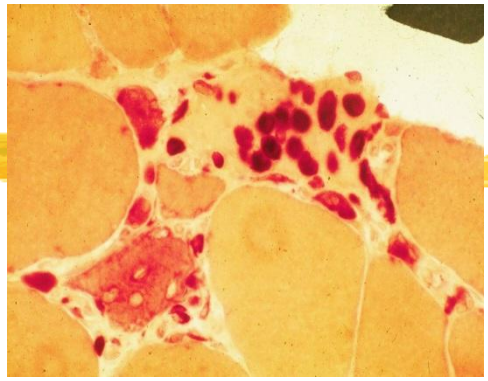
HISTOCHEMISTRY

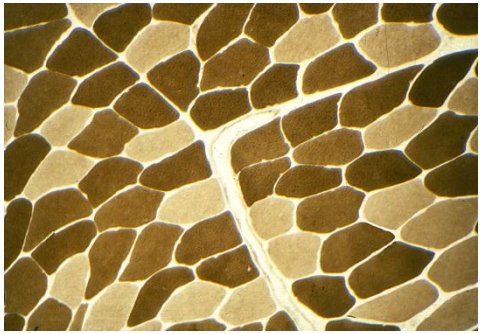
- Lipids
  - UnConjugated – fatty acids, cholesterol
  - Conjugated – Esters, phospholipids, sphingosines
  - Effects of preparation methods
  - OsO_4, Old Red O, Sudan Black, …
- Nucleic Acids
  - DNA feulgen + DNAase extraction
    RNA Methyl Green Pyronin + RNAase extraction
    Acridine Orange Autofluorescence
- Calcium
  - Von Kossa
  - Alizarin



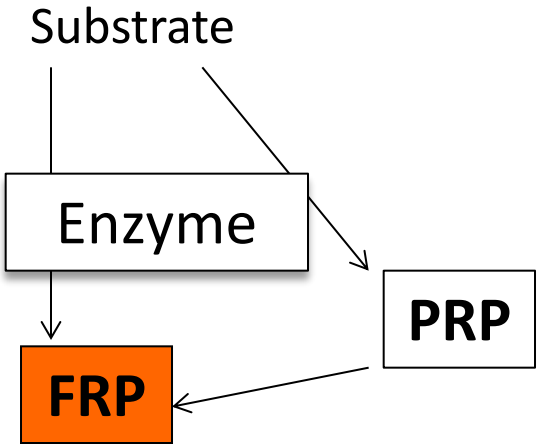


HISTOCHEMISTRY





- Enzyme Histochemistry

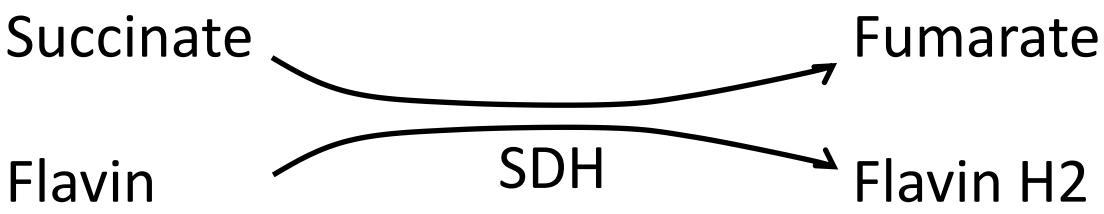


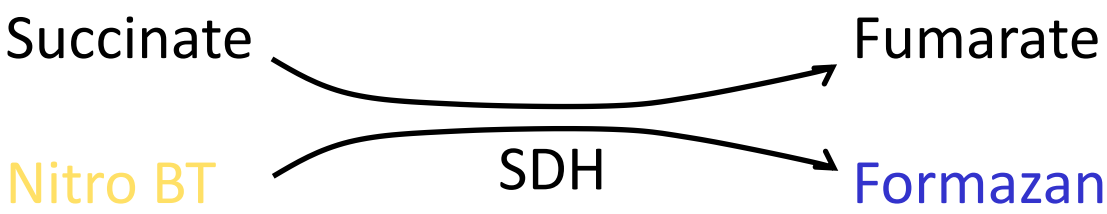
- Modes of Visualisation

•Simultaneous capture	S-En > PRP > FRP	Gomori
•Post incubation coupling	S-En > PRP > FRP	Diaz/Tetraz
•Self coloured substrate	S-En > FRP	

Example … Succinate dehydrogenase

Tetrazolium salts are colourless and water soluble and accept hydrogen released from substrates by the action of oxidative enzymes, they are then reduced to highly coloured water insoluble microcrystalline formazans





Enzyme Histochemistry Problems

- Preservation of Enzymes
  - Fixation inactivates enzyme
  - Substrate diffuses
  - PRP diffuses
  - Capture agent is inhibitory or dislikes pH
- Damage
  - Anoxia – damages mitochondria
  - Warmth or drying out inhibits enzyme
  - Freeze/Thaw cycles damage mitochondria
  - Osmotic damage – damages membranes
- Reaction sensitive to …
  - Temperature, pH, Inhibitors, Co-factors, substrate

Enzyme Histochemistry Usage

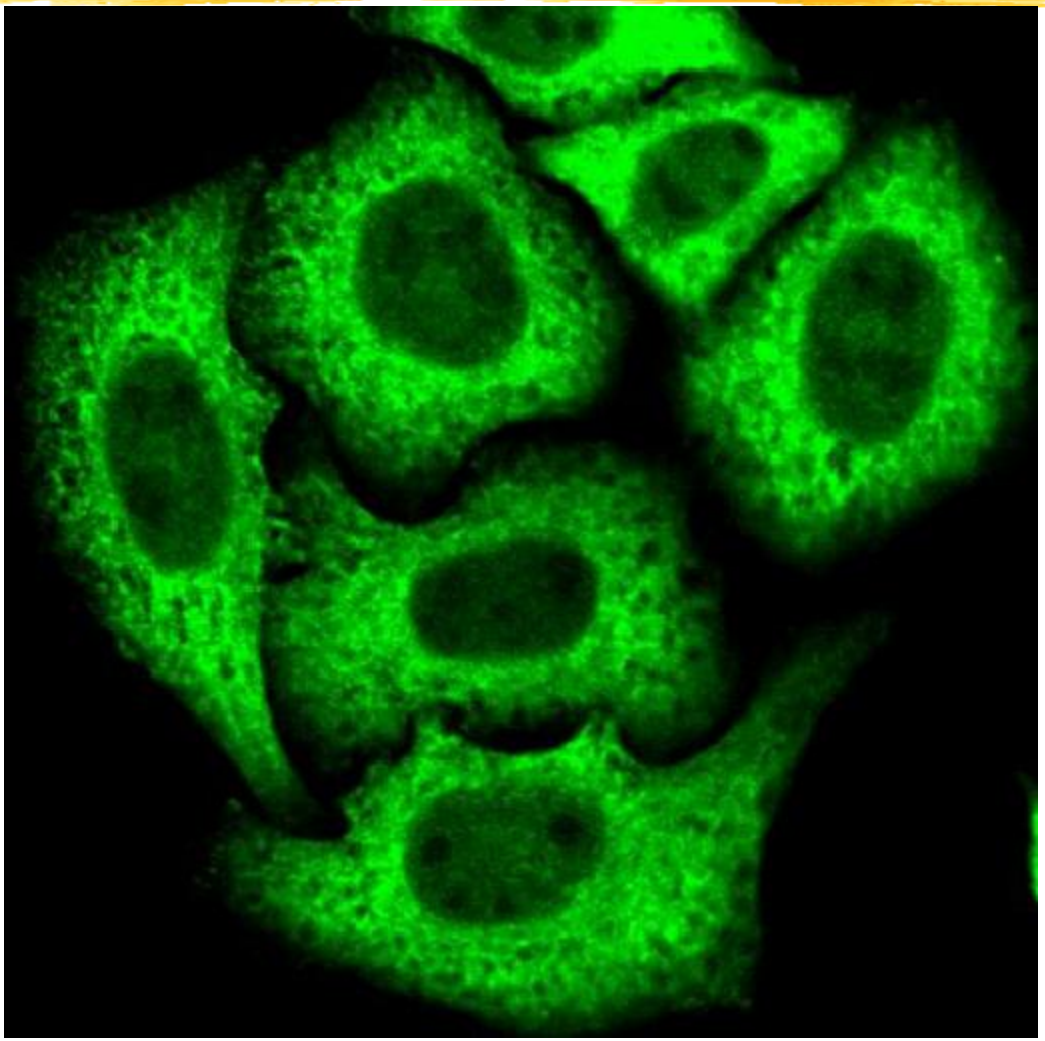
- Oxidoreductases — SDH, Cytochrome Oxidase, NADP
- Transferases — phosphorylase
- Hydrolases — acid phosphatase, alkaline phosphatase, ATPase, acetylcholinestarase
- Lyases
- Isomerases
- Ligases

- Important for Pathology of ..
  Skeletal Muscle, Mast Cells, WBC, Ganglia, Nerves, Liver, …

Immunocytochemistry

- Staining depends on biomolecular recognition & binding (detects small amounts of proteins)
  - Fluorescence, Confocal, BF, DF Microscopy
- History
  - 1941 Albert Coons Fluorescent dye Ab sticks to Ag in section
  - 1955 " " Indirect methods
  - 1960 various Enzyme & metallic methods
  - 1970s various Radioisotope methods
  - 1980s Cesar Milstein[NP] Monoclonal Antibodies
  - 1990s various Multiple staining, Realtime, 3D, Super-res

Visualisation



- **Fluorochromes**

FITC, TRITC, TR, AO

- **Enzymes**

Horse radish Peroxidase, AlkP, GO, beta galactosidase

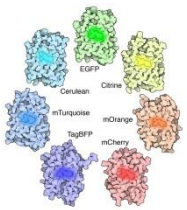
- **Dense Molecules**

Ferritin, OsO_4 DAB, Gold, Silver

- **Radioactive Isotopes**

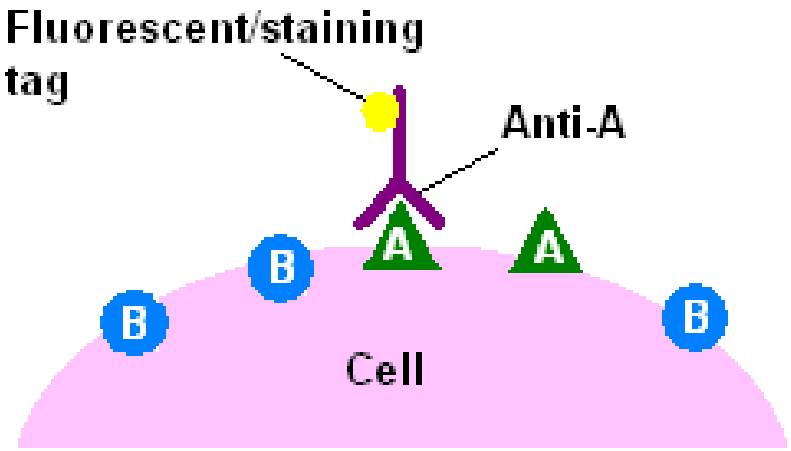
I, S

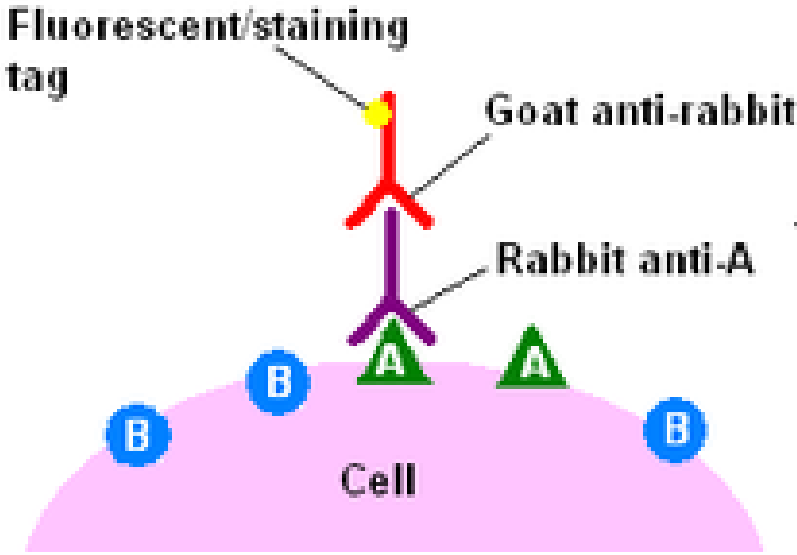
- **Natural** GFP



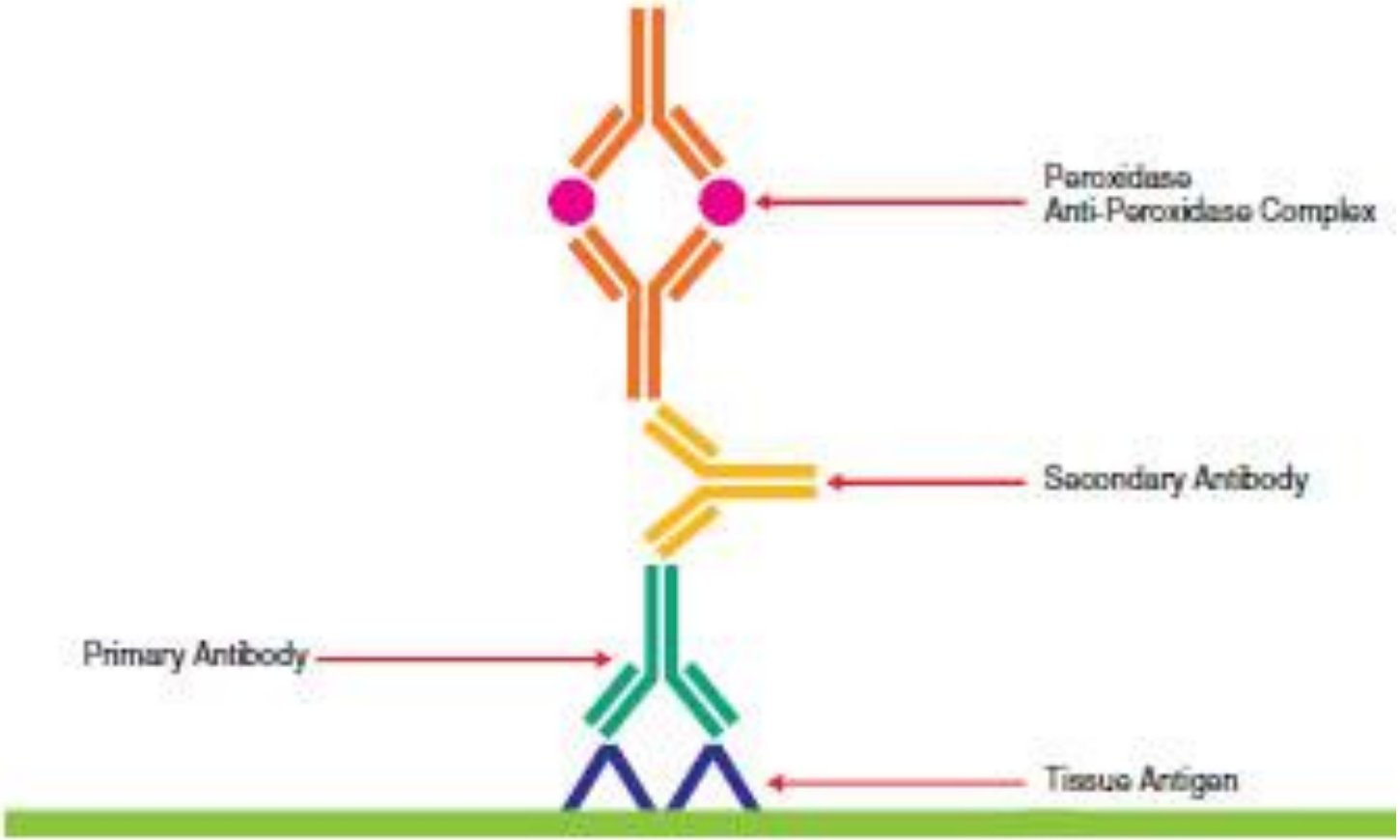
Direct Method

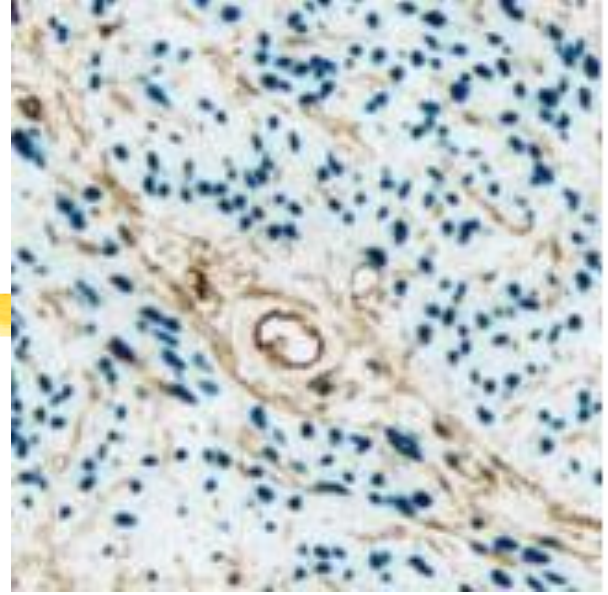
Indirect Method

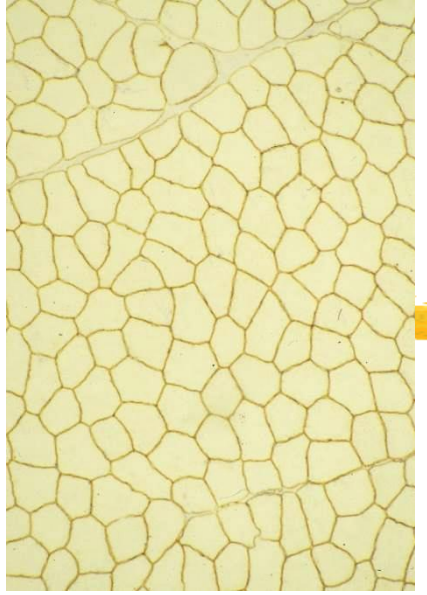




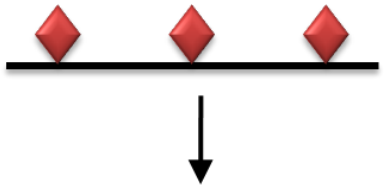
PAP Complex Method



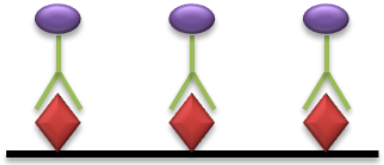




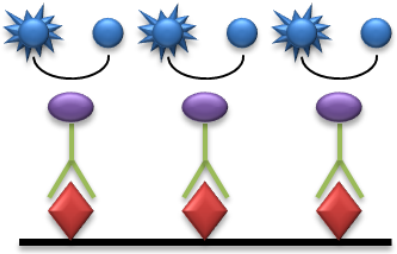
Virus Sample on Surface

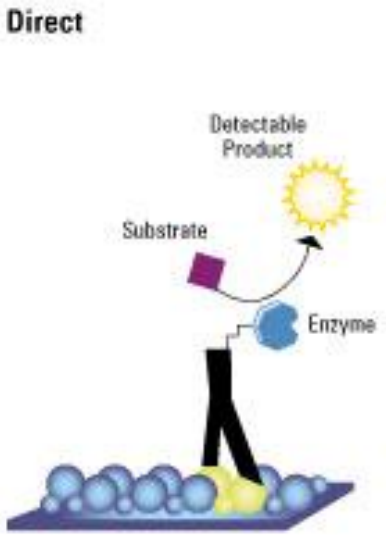


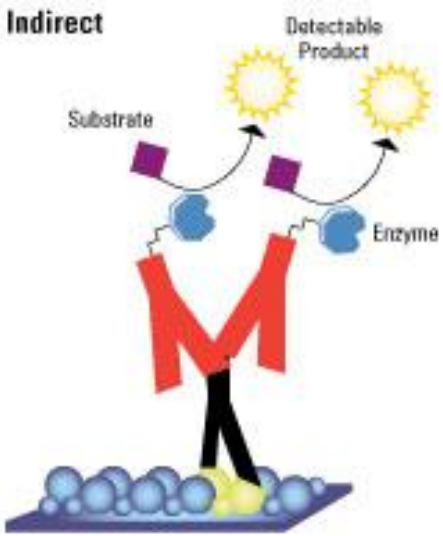
Antibody with enzyme conjugate attached to viral antigen



Substrate and enzyme interaction create color change for detection



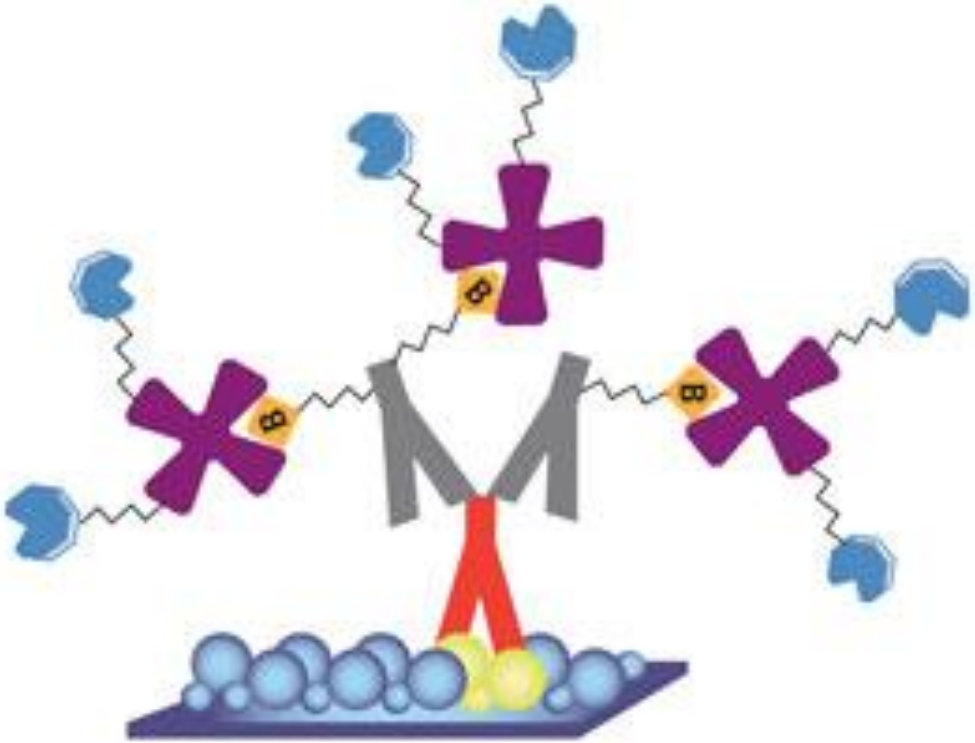




Biotin- Streptavidin Method

LSAB Method

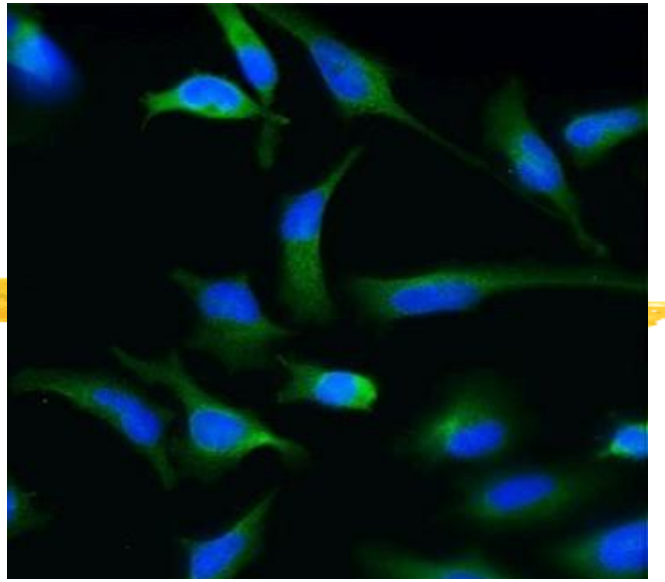
Lights up like a Christmas tree!

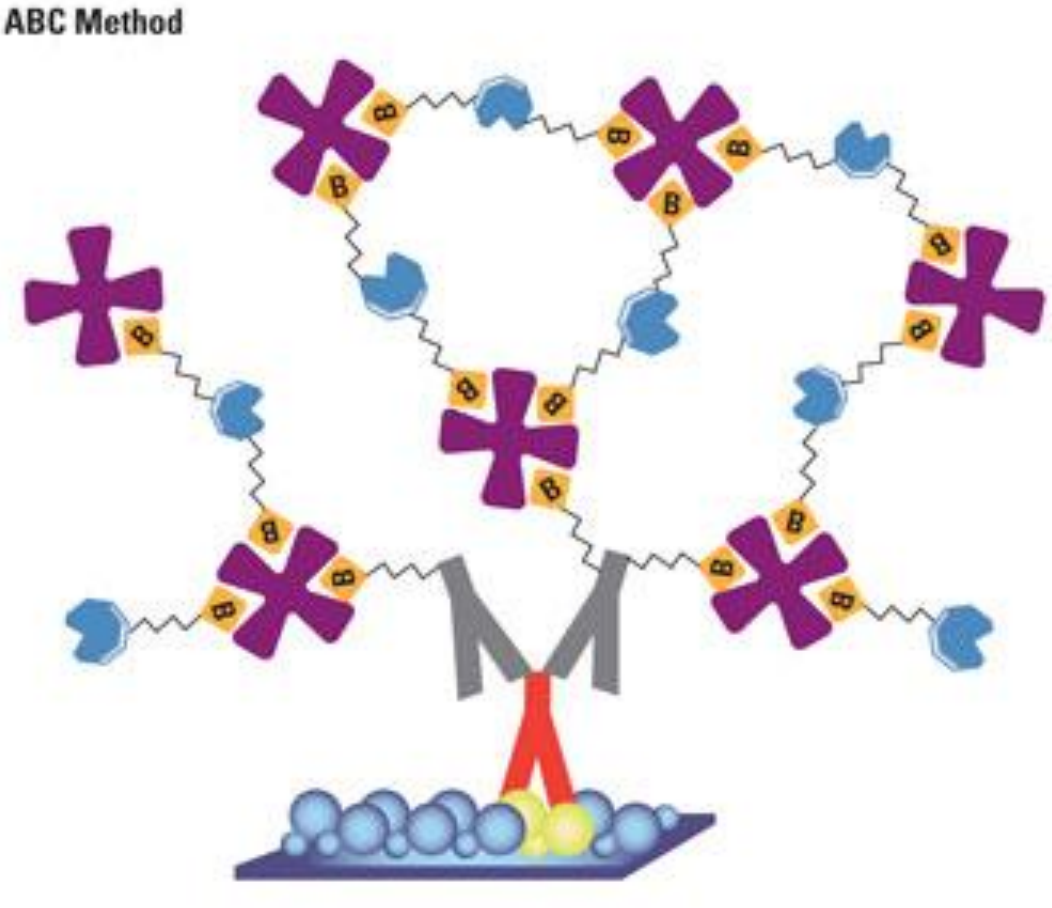


Legend

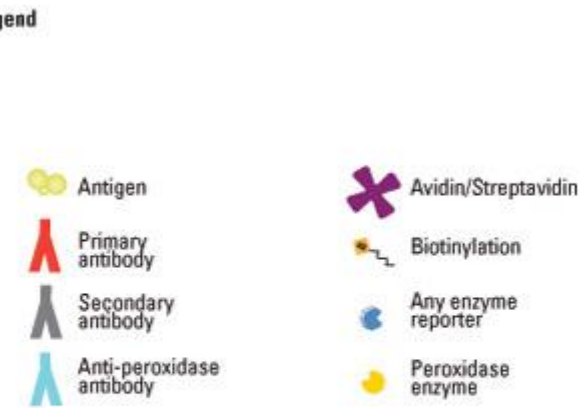
- Antigen
- Primary antibody
- Secondary antibody
- Anti-peroxidase antibody
- Avidin/Streptavidin
- Biotinylation
- Any enzyme reporter
- Peroxidase enzyme

Amplification – Blackpool Illuminations !!!



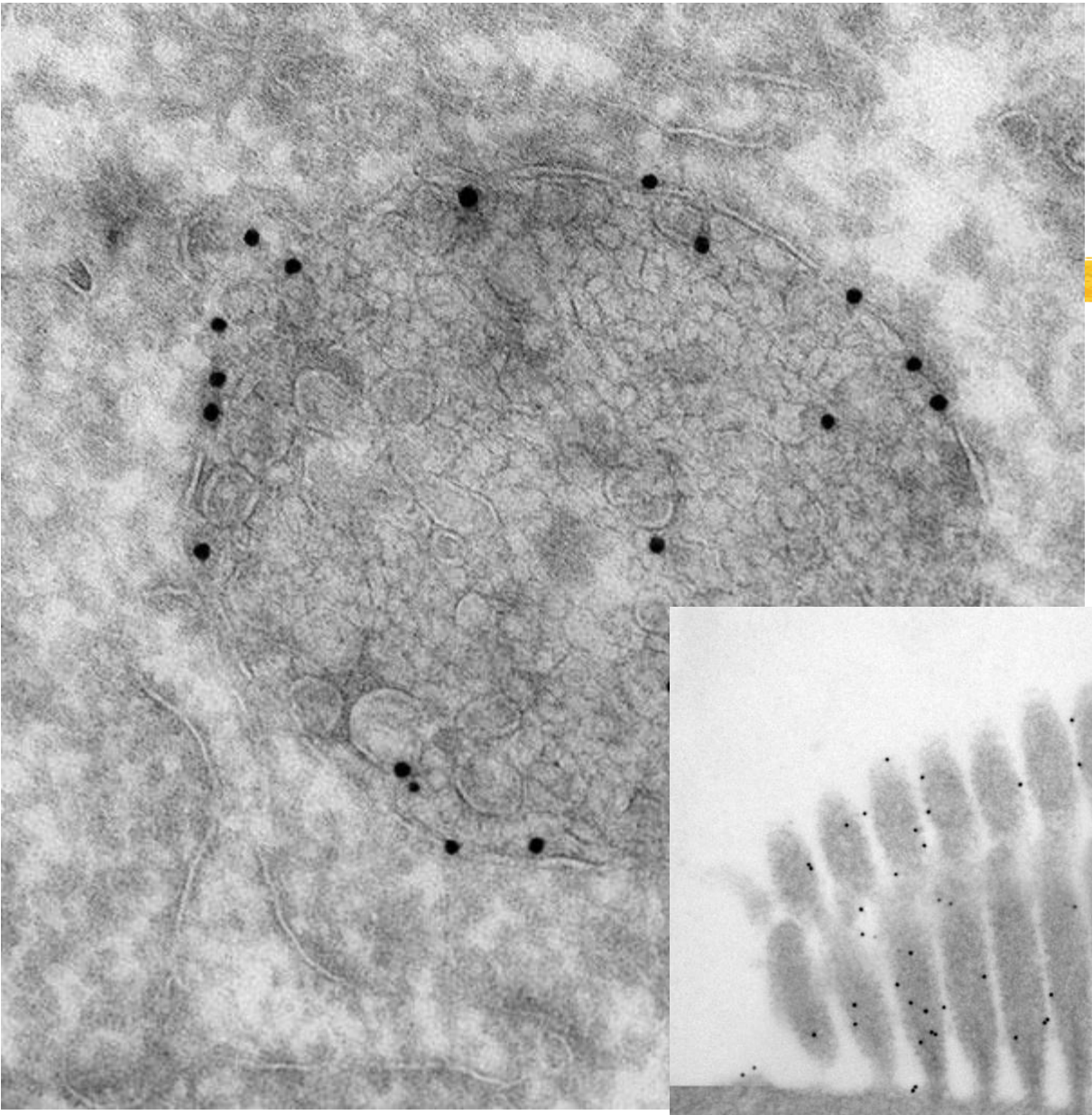


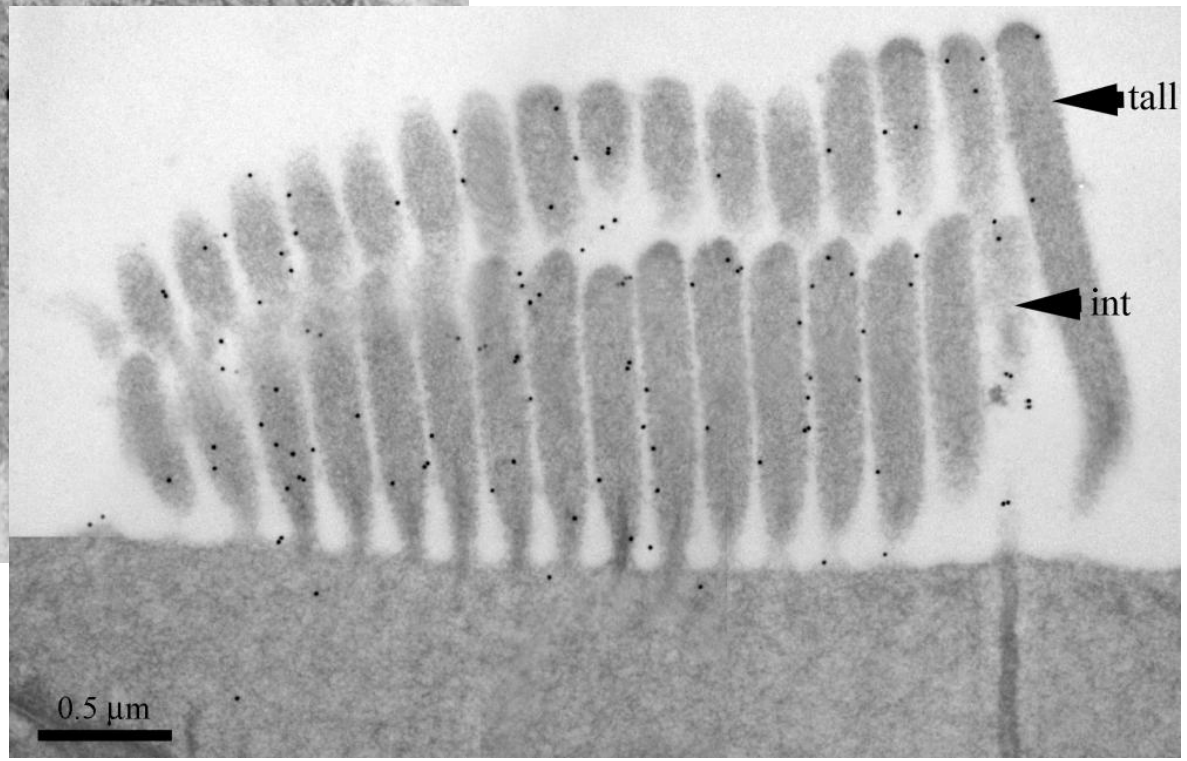
But …
spatial accuracy!



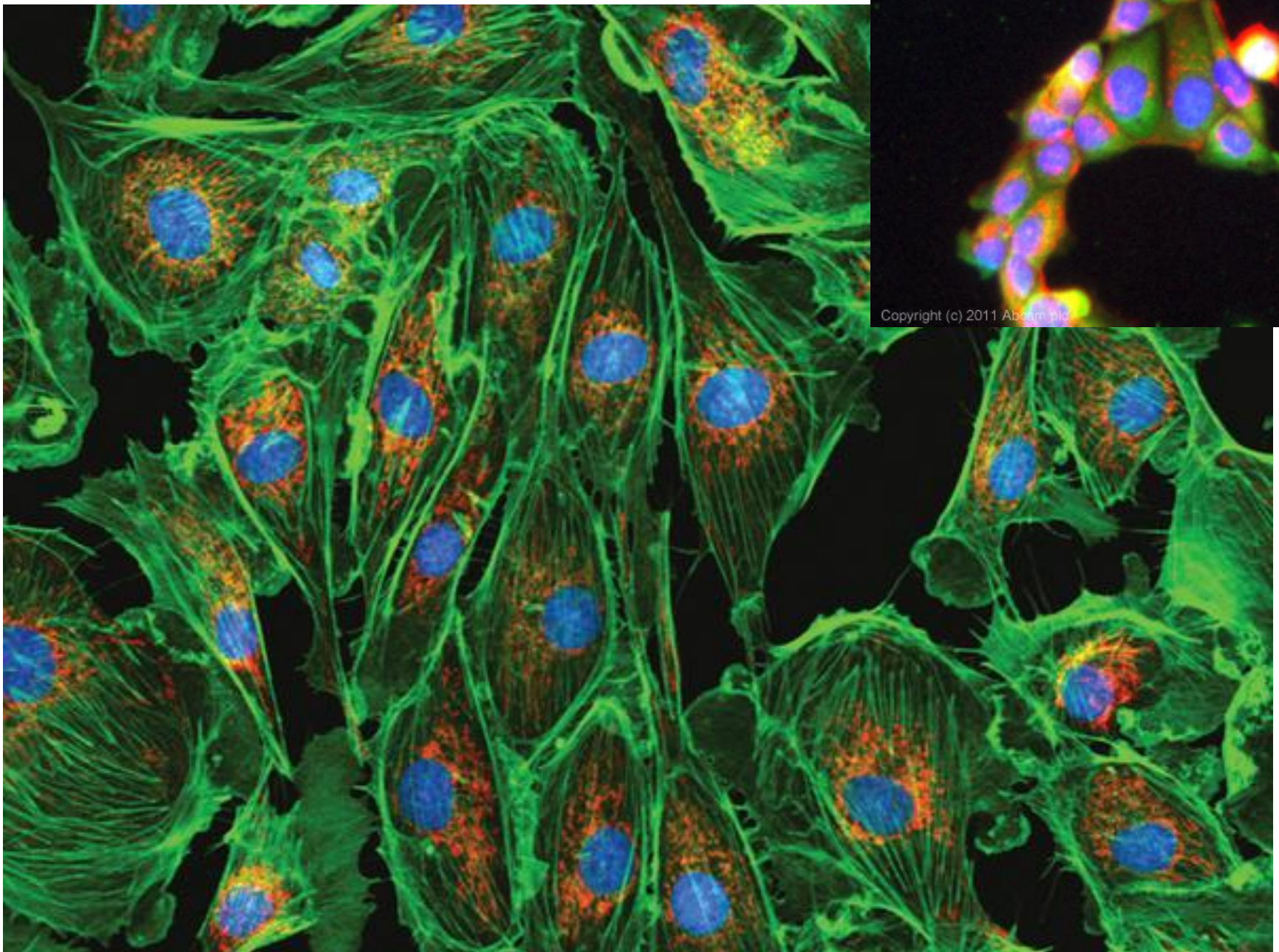
e.g. horse radish peroxidase biotinylated streptavidin rabbit anti mouse antibody for dystrophin

Electron Microscopy Immunocytochemistry with Gold





Double, Triple, Multiple Staining



Immunocytochemistry

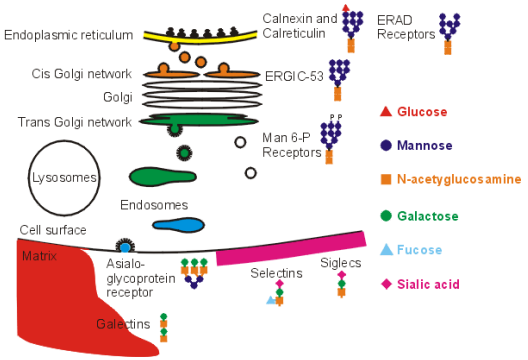
- Microscopy
  - Fluorescence (Epi)
  - Polarisation
  - Dark Ground
  - DIC
  - Confocal
  - Multiphoton
  - Super-resolution
- Problems & Treatments
  - Preservation (subbed slides, oxidation with H_2O_2, proteases)
  - Expansion
  - Fixation
  - Localisation
  - Non specific staining (Ab, Collagen (BSA blocking), loose dyes)
  - Steric Hindrance
  - Insufficient Antigen
  - Penetrance
  - Costs
- Controls + (ELISA, Radioimmunoassay, Immunoblotting)

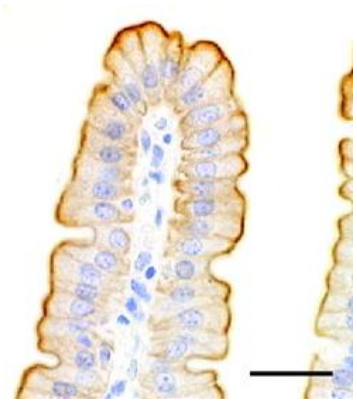
Immunocytochemistry - Applications

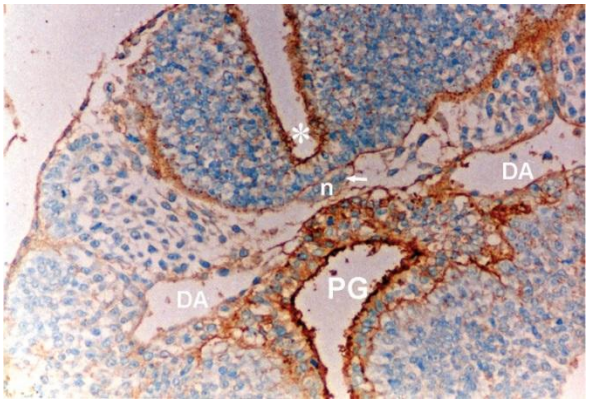
- Research
  - Morphology
  - Molecular Genetics
  - Cell Biology
  - Developmental Biology
  - Palaeobiology
- Diagnosis
  - Histopathology
  - Neuropathology
  - Cytology / Haematology (Tumour & White Cell markers, …)
    - Cytoskeletal
    - Cytokeratins
    - Peptides
    - Receptors
    - GFAP
    - Viruses & Bacteria & Toxins
    - Autoimmune diseases
- Evolutionary Studies
- Imaging
- Quantification
  - Flow Cytometry
  - Microscopy

Lectin Cytochemistry

- 1974 Etzler & Branstrator used Lectins (Plant products) as cytochemical tool
  - Bean, Pea, Wheatgerm, Peanut
  - Use Fluorescent markers, Gold, Ferritin
- Detects Specific Carbohydrates
  - Cell Surface
  - Secretions
- Applications
  - Developmental Biology
  - Epithelial Cell markers

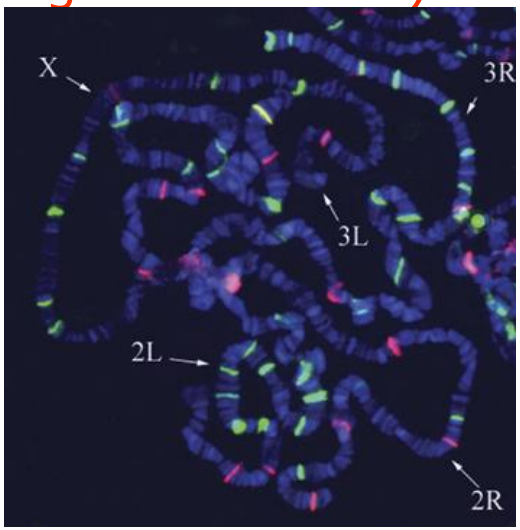




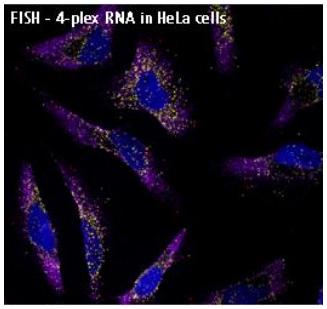


In Situ Hybridisation (ISH)

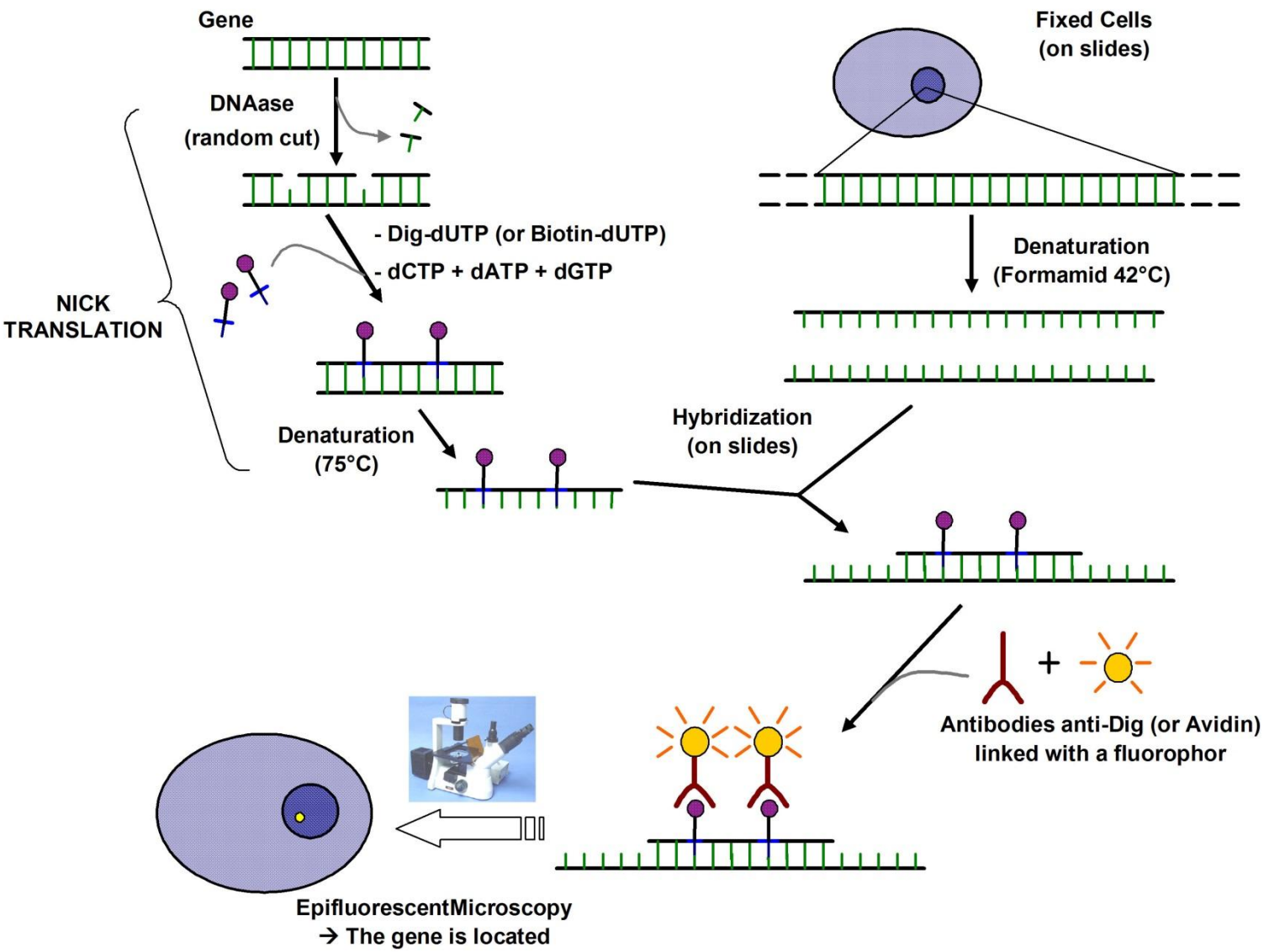
- Locate nucleic acid sequences in DNA & RNA
  - Reads the Genome (Southern & Northern Blotting + localisation)
  - Maps chromosomes & their aberrations
  - Spatial & Temporal expression of genes
  - Identify Viruses
  - Sex determination



- Procedure
  - Rapid Fix, section, RNAase
  - ~200base DNA probe (PCR), hybridise overnight at 37C
- Types
    - FISH, EISH,DISH, RISH

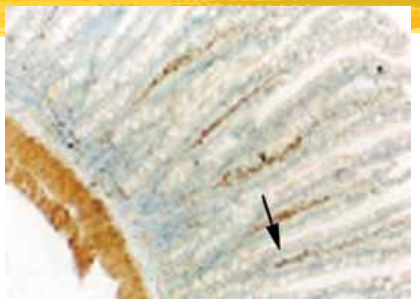


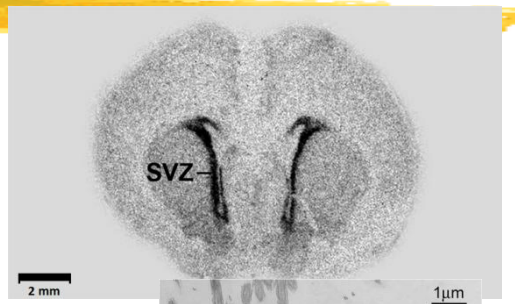
FISH (Fluorescent In Situ Hybridization)

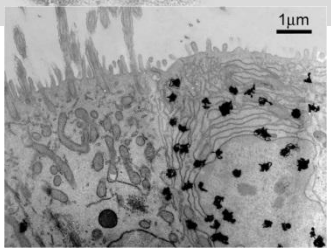


Tracer Techniques (+3D, +4D)

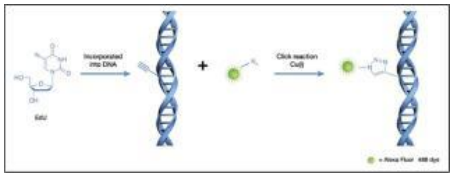
- Autoradiography
  - Tritiated thymidine



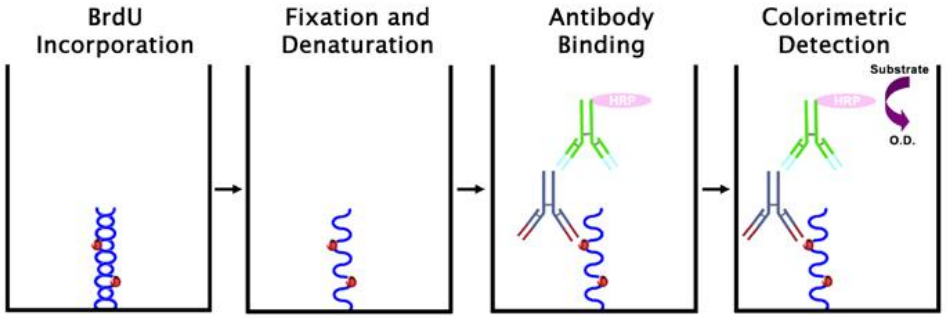


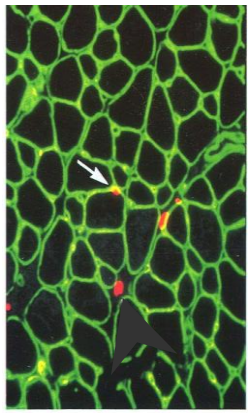


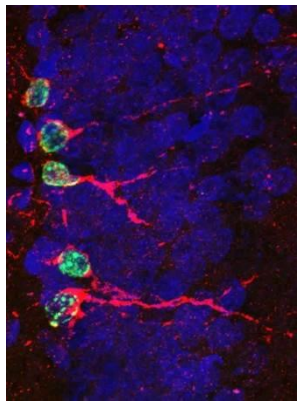
Inject into animal/tissue whilst alive



- BrDU
  - Substitutes for Thymidine
  - Identifies proliferating/replicating cells

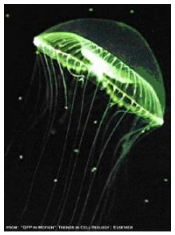
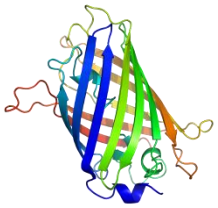


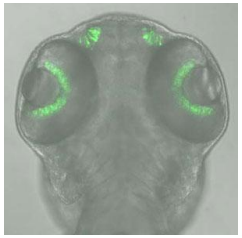
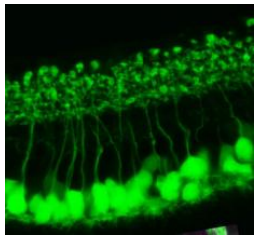




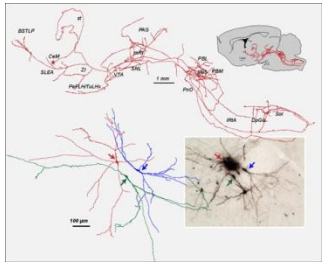
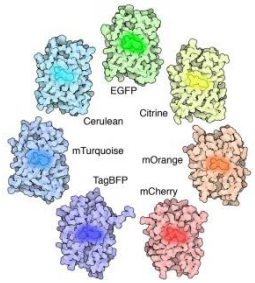
Other Techniques

- Transgenics
  - GFP (2008 Nobel Prize)

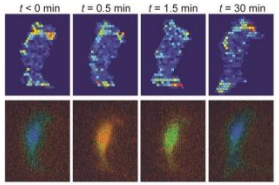



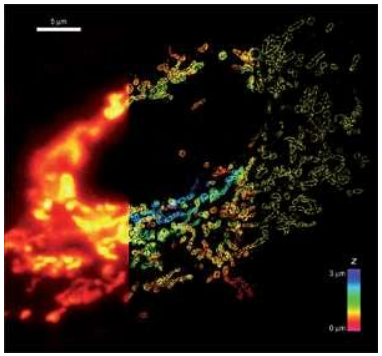



- Neuronal Tract Tracers

- Tunel (apoptosis)
- Antigen Retrieval Techniques
- Transient Calcium Detection



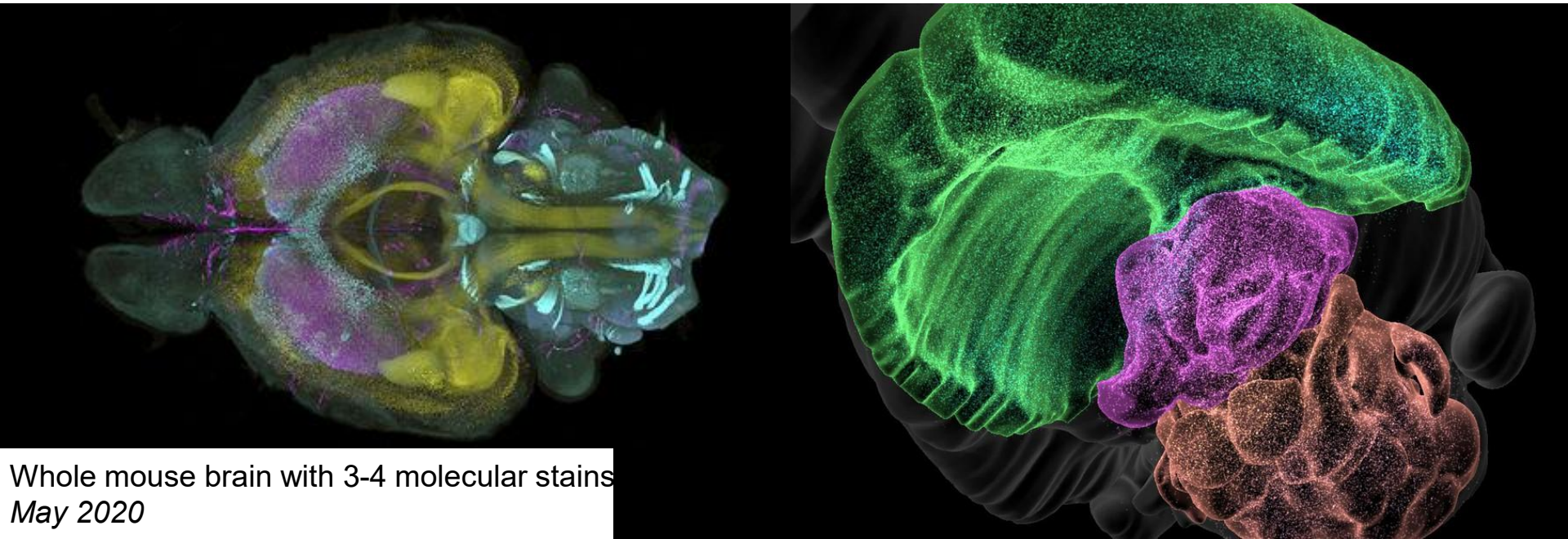


- Super-resolution microscopy of fluorophores (Nobel Prize 2014, 60 nm res)
- Expansion microscopy (2017) gels, brain, synapses, 25nm res

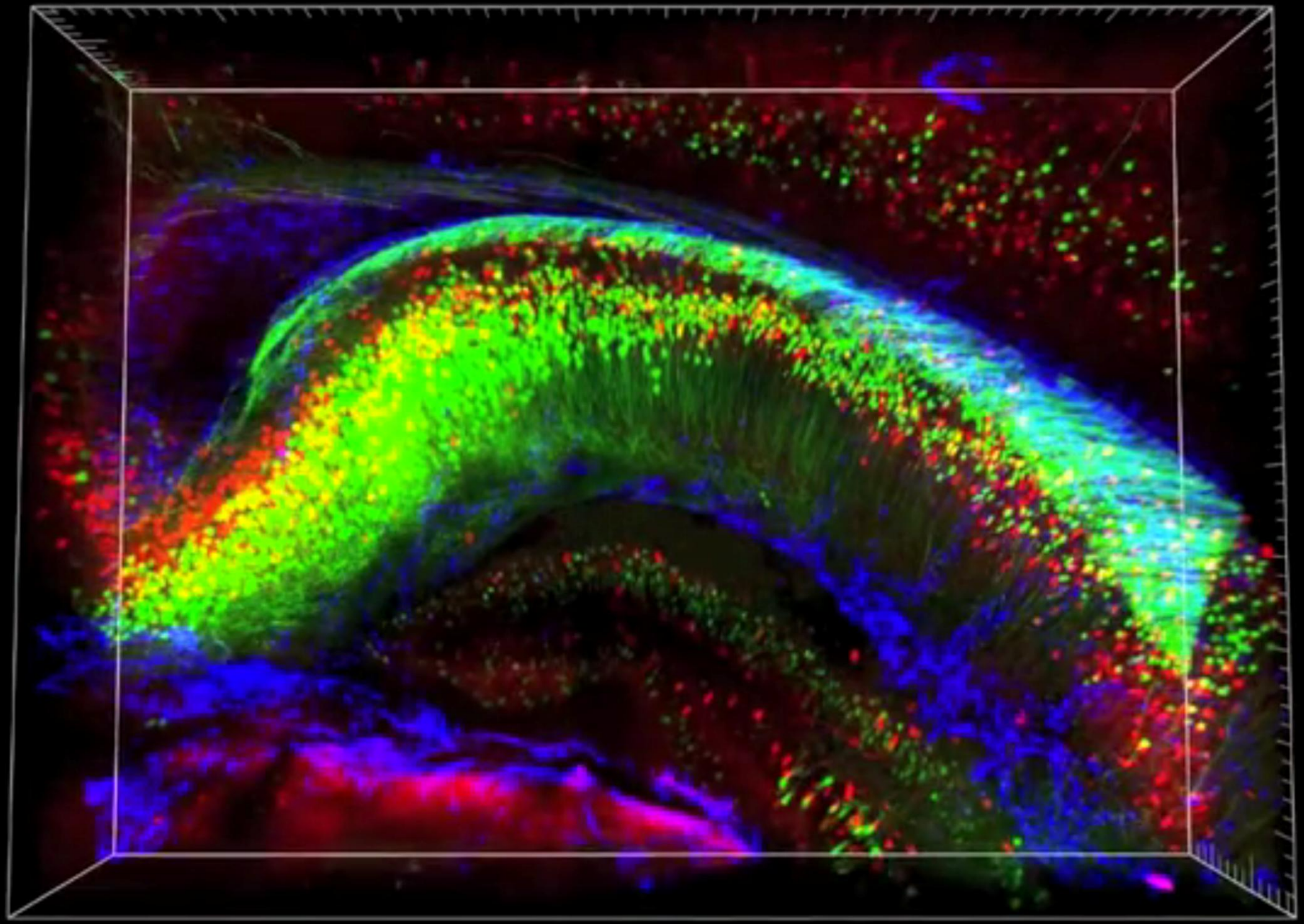
3D Staining Techniques

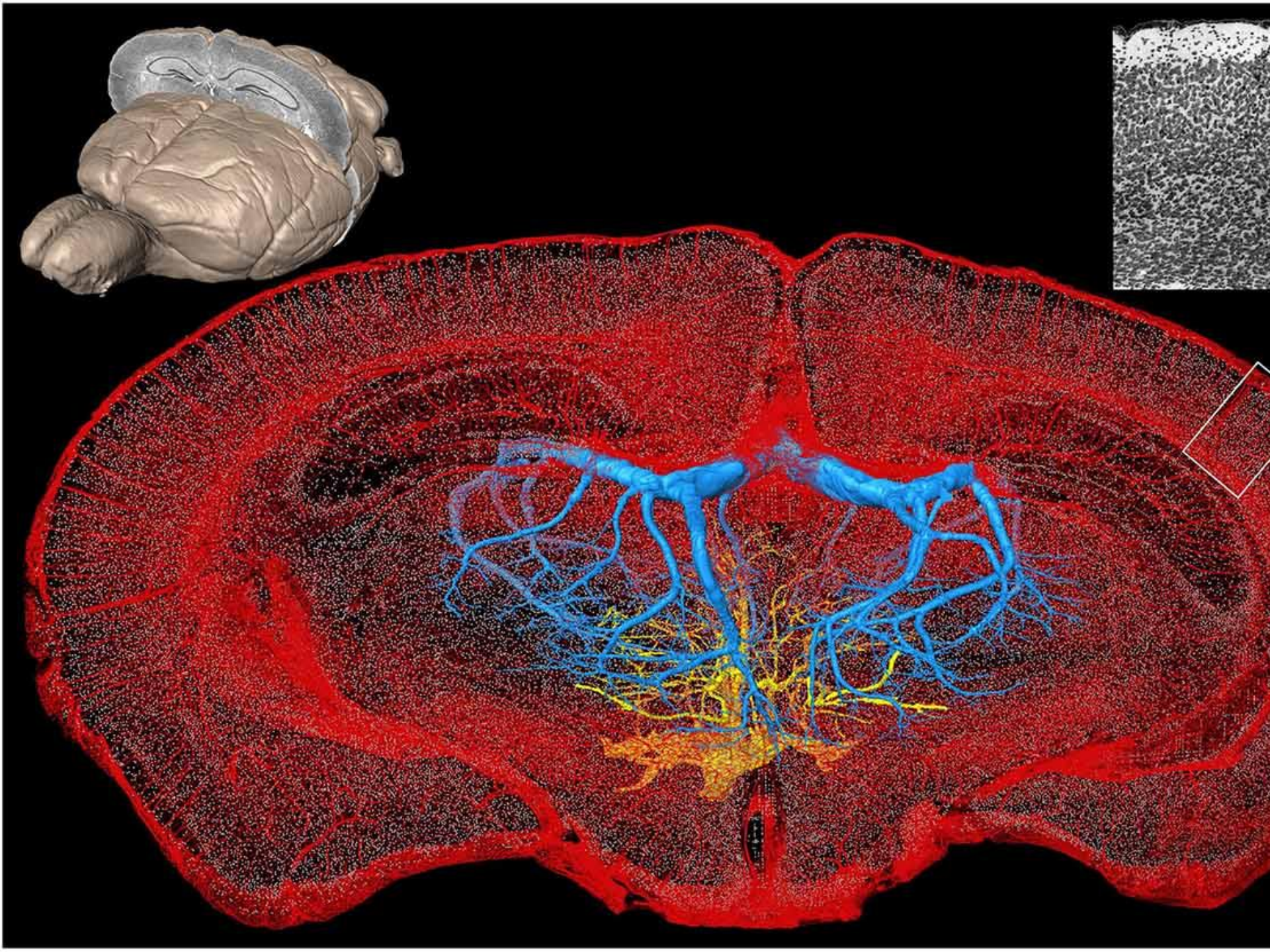
- Either serial section and computer reconstruct
  or
- Make transparent & infiltrate with ‘stains’ and 3D image
  DISCO, CLARITY,

video



Whole mouse brain with 3-4 molecular stains
May 2020





How has microscopy evolved to deal with these new staining techniques ?

… see tomorrow (MICROSCOPY)