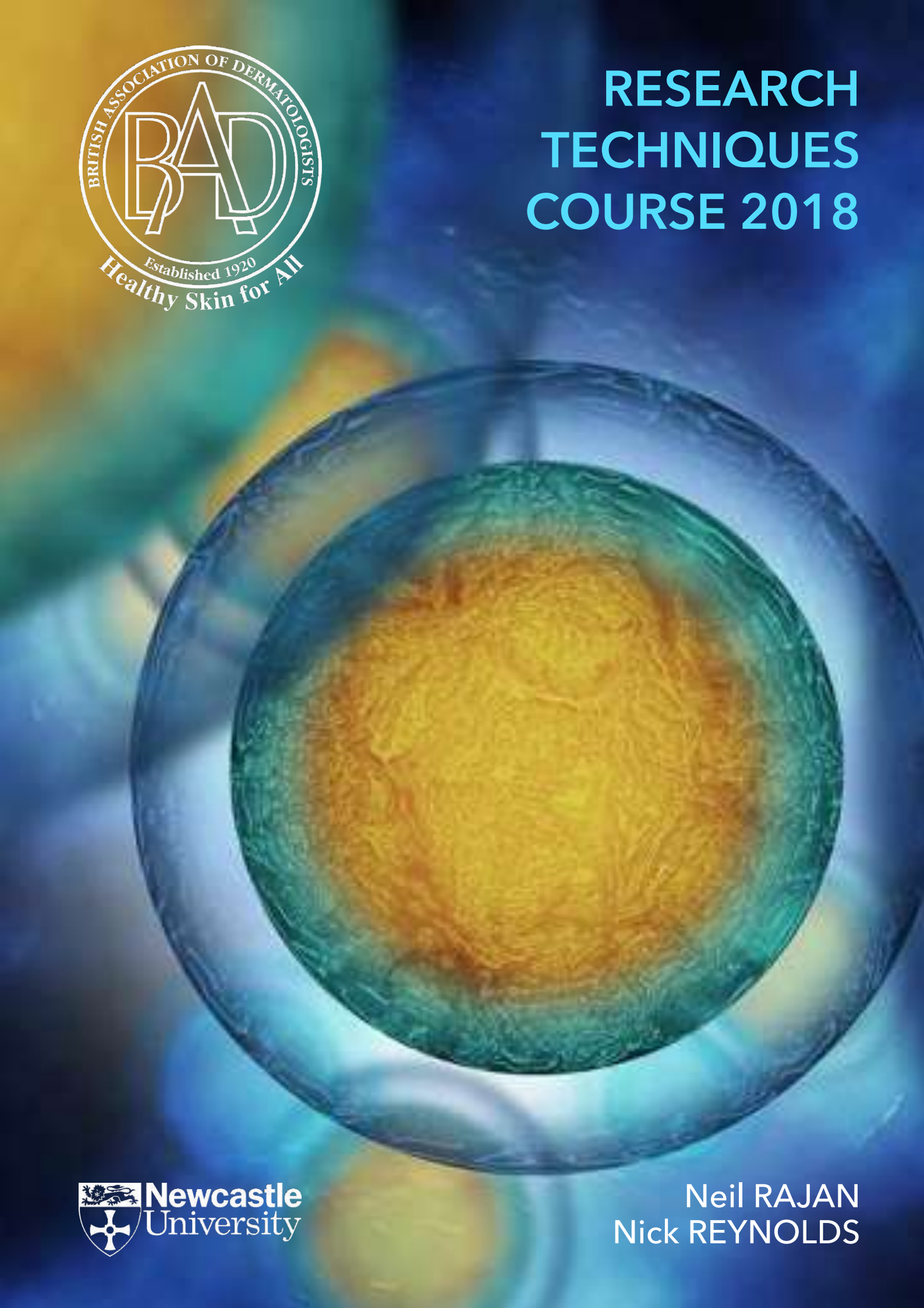


RESEARCH TECHNIQUES COURSE 2018

Newcastle University

Neil RAJAN
Nick REYNOLDS

Course Faculty

Mark Birch-Machin

Simon Cockell

Jonathan Coxhead

Rob Ellis

Andy Filby

Pawan Gulati

Muzz Haniffa

David Hill

Kirsty Hodgson

Alex Laude

Penny Lovat

Ashleigh McConnell

Siobhan Muthiah

Beth Poyner

Neil Rajan

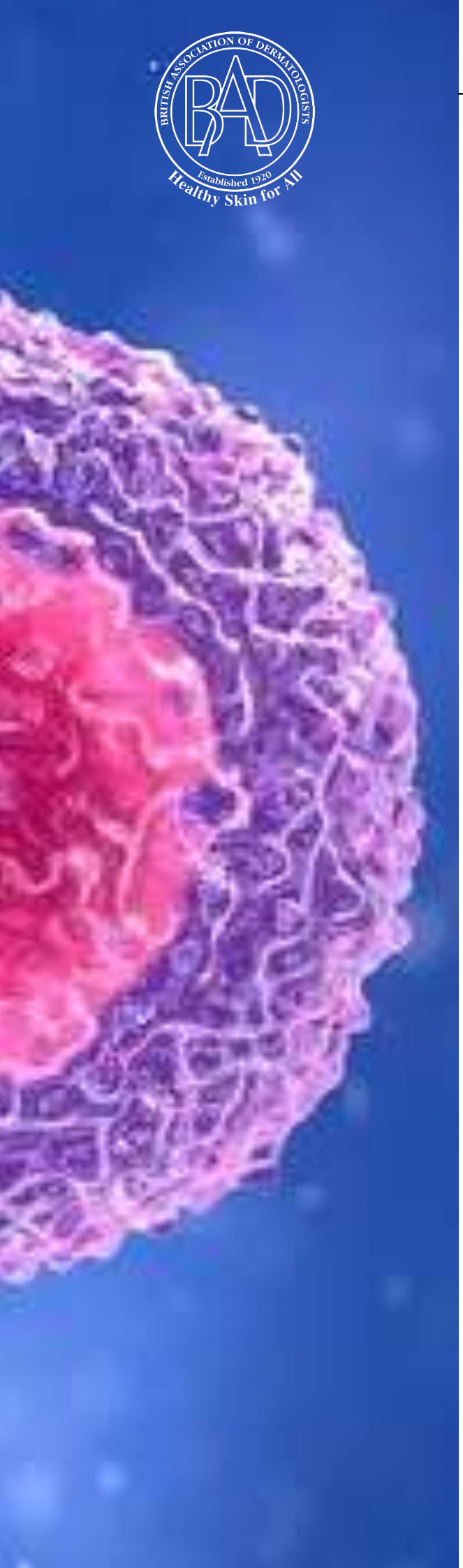
Nick Reynolds

MONDAY

Start	End	Activity	Speaker/Lead	Location
850	900	Arrival and Registration	Neil Rajan	Biomedicine West Reception
900	920	Introductions and overview	Neil Rajan	Biomedicine West Lecture Theatre
920	930	Lab Health and Safety	Julie Taggart	Biomedicine West Lecture Theatre
930	950	DNA extraction and PCR	Kirsty Hodgson	Biomedicine West Lecture Theatre
950	1000	Transfer to labs, bag storage	NR/KH/SM	Rajan/Veltman Labs
1000	1045	Practical - PCR setup	NR/KH/SM	Rajan/Veltman Labs
1045	1115	Coffee		B235 Boardroom
1115	1130	Introduction to animal cell culture and skin disease models	Pawan Gulati	B235 Boardroom
1130	1145	3D culture and zebrafish models for studying melanoma metastasis	David Hill	B235 Boardroom
1145	1200	Transcriptomics in psoriasis	Nick Reynolds	B235 Boardroom
1200	1215	Human cell atlas and the skin	Muzz Haniffa	B235 Boardroom
1230	1330	Lunch		B235 Boardroom
1330	1415	Zebrafish practical	David Hill	Fish room
1420	1430	Birthmarks and genetic mosaicism	Siobhan Muthiah	B235 Boardroom
1430	1500	Massively parallel sequencing technologies	Jonathan Coxhead	B235 Boardroom and Sequencing facility
1500	1520	Coffee		B235 Boardroom
1520	1540	Bioinformatics	Simon Cockell	B235 Boardroom
1540	1620	Bioinformatics practical:Heatmaps!	Simon Cockell	B235 Boardroom
1620	1700	PCR gel practical	NR/KH/SM	Rajan/Veltman Labs
1900	Late!	Course dinner	Delegates and Faculty	Earl of Pitt street 1900 for 1930 start

TUESDAY

Start	End	Activity	Speaker/Lead	Location
900	930	Skin ageing in the 21st century	Mark Birch-Machin	B235 Boardroom
930	1000	Biomarkers for melanoma	Rob Ellis/ Penny Lovat	B235 Boardroom
1000	1045	Practical analysis of a novel immunohistochemical prognostic biomarker for AJCC stage 1 melanoma	Ashleigh McConnell/RE/ PL	B235 Boardroom
1045	1110	Coffee+Cake		B235 Boardroom
1115	1130	"Cytometry": How to become a cell detective	Andy Filby	B235 Boardroom
1130	1140	Multi-modal microscope based imaging: From the organelle to the organ	Alex Laude	B235 Boardroom
1140	1230	FACS, live cell imaging demos and single cell analysis (4x4 group)	Filby/Laude/ Poyner	FACS Facility/Bioimaging/ B235
1235	1300	Analysis of FACS data	Andy Filby	B235 Boardroom
1300	1400	Lunch and feedback	Rajan/ Reynolds	B235 Boardroom



DNA EXTRACTION AND PCR

Kirsty Hodgson

DNA extraction and PCR

BAD Research Techniques Course
International Centre for Life
12 Nov 2018

Overview

- What is PCR?
- Core reagents
- DNA extraction
- Primers
- Thermal cycling stages
- Gel Electrophoresis
- Applications

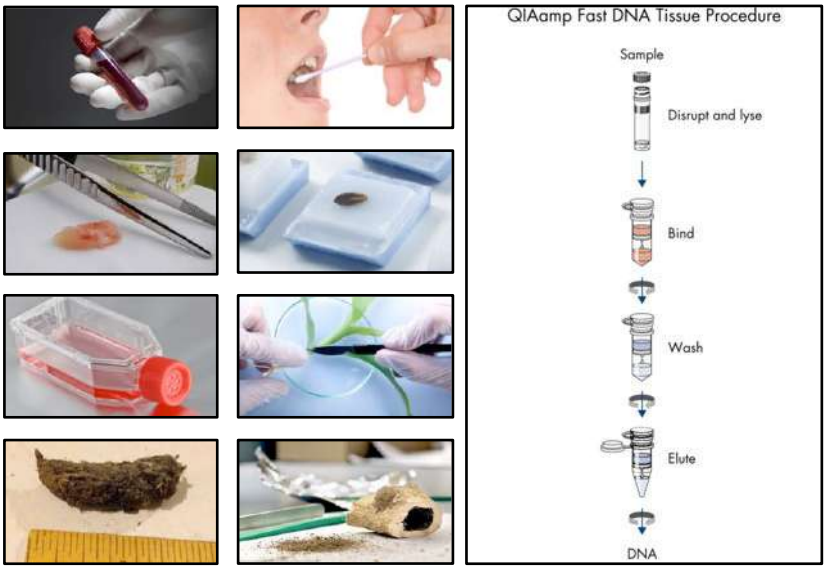
What is PCR?

- The polymerase chain reaction (PCR) is a technique widely used to study DNA
- Developed in 1983, it is a rapid way of amplifying a specific target DNA sequence from a mixed pool of DNA (often total genomic DNA)
- Numerous applications in basic and clinical research

Six core PCR reagents

- Template DNA
- Primers
- DNA nucleotide bases (dNTPs)
- Magnesium Chloride ($MgCl_2$)
- Taq polymerase enzyme
- Buffer

DNA extraction



Primer design

Template DNA

5’ GCTGGAAAAATCAAGCAGTTTTGTAAAACCTGCAACACTCAAGTGAGCTTCCCTTCACTTAAT 3’
3’ CGACCTTTTTAGTTCGTCAAAACATTTTGGACGTTGTGAGTTCACTCGAAGGGAAGTGAATTA 5’

Region to be copied

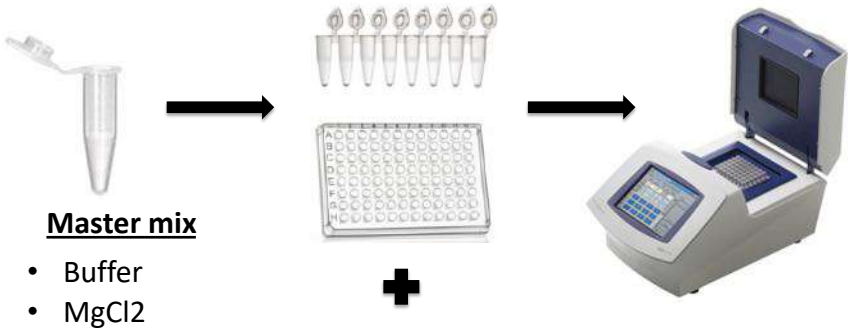
5’ GCTGGAAAAATCAAGCAGTTTTGTAAAACCTGCAACACTCAAGTGAGCTTCCCTTCACTTAAT 3’
3’ ACTCGAAGGGAAGTGAATTA 5’

Primer 2

Primer 1

5’ GCTGGAAAAATCAAGCAGTT 3’
3’ CGACCTTTTTAGTTCGTCAAAACATTTTGGACGTTGTGAGTTCACTCGAAGGGAAGTGAATTA 5’

PCR sample preparation



Master mix

- Buffer
- MgCl2
- Taq polymerase
- Primers
- H2O

Template DNA

Thermocycler

Step 1. Denaturing

Double-stranded template DNA is heated to split it into two single strands

Template DNA

5’ GCTGGAAAAATCAAGCAGTTTTGTAAAACCTGCAACACTCAAGTGAGCTTCCCTTCACTTAAT 3’
3’ CGACCTTTTTAGTTCGTCAAAACATTTTGGACGTTGTGAGTTCACTCGAAGGGAAGTGAATTA 5’

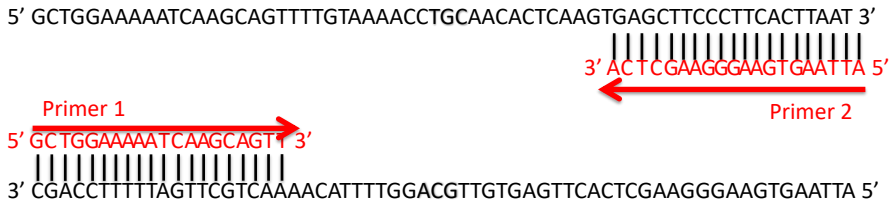
94-95°C

5’ GCTGGAAAAATCAAGCAGTTTTGTAAAACCTGCAACACTCAAGTGAGCTTCCCTTCACTTAAT 3’

3’ CGACCTTTTTAGTTCGTCAAAACATTTTGGACGTTGTGAGTTCACTCGAAGGGAAGTGAATTA 5’

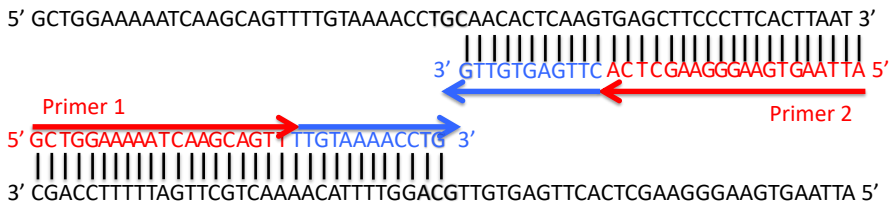
Step 2. Annealing

The temperature is lowered to allow the primers to anneal to the single strand template DNA

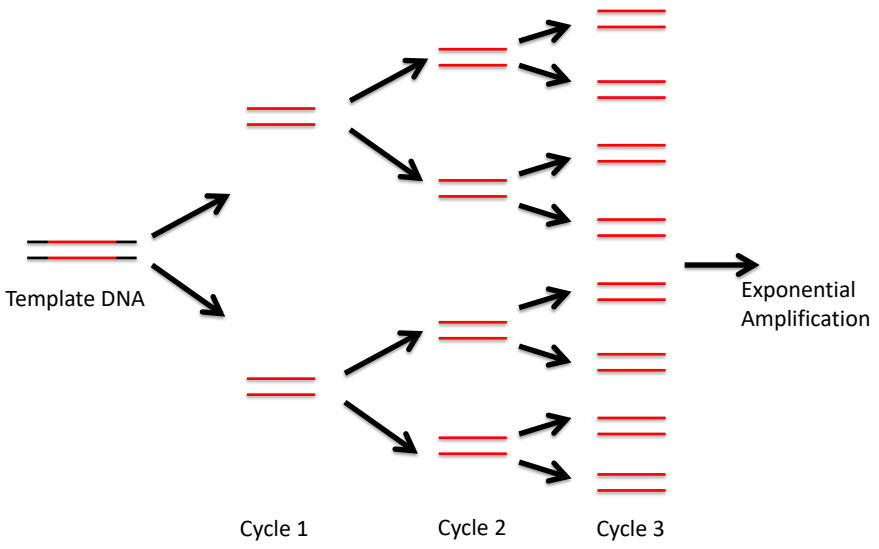


Step 3. Extension

The temperature is raised and new strands of DNA are made by the taq polymerase enzyme



30 – 40 cycles of steps 1 - 3



Gel electrophoresis

Applications

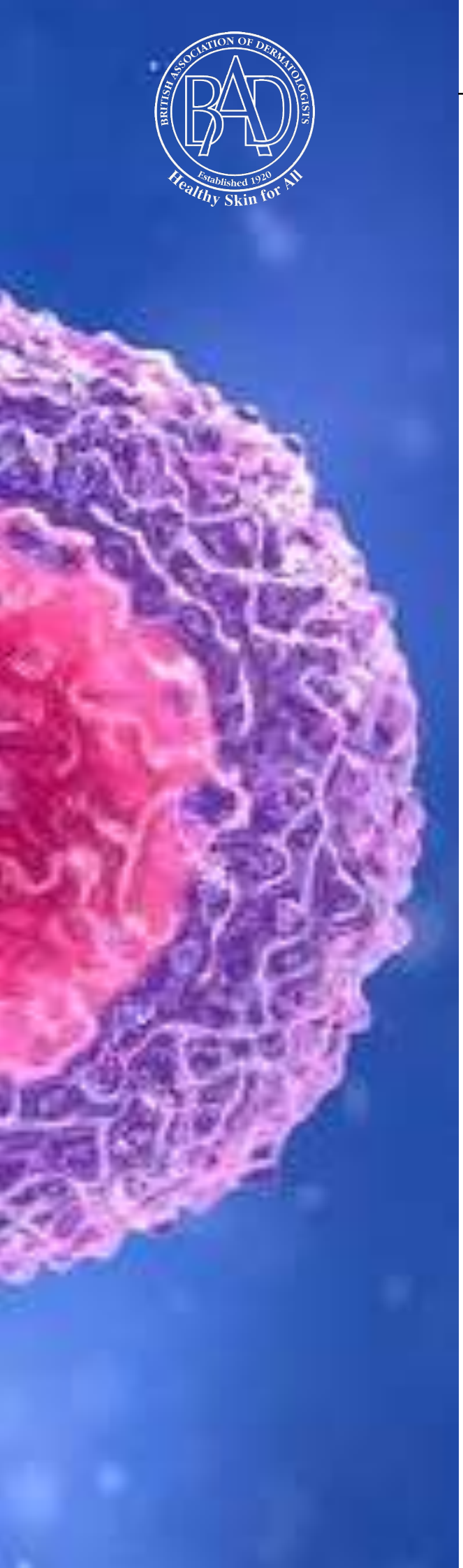
- CRISPR
- DNA Barcoding
- Forensics
- Sequencing
- Genotyping
- Cloning
- Mutation detection
- Paternity testing etc

Further reading/Resources

Strachan T & Read A (2010) Human Molecular Genetics (4th Ed). Garland Science (pages 182 – 190).

UCSC Genome Browser
https://genome.ucsc.edu

Primer BLAST
https://www.ncbi.nlm.nih.gov/tools/primer-blast



INTRODUCTION TO ANIMAL CELL CULTURE AND SKIN DISEASE MODELS

Pawan Gulati

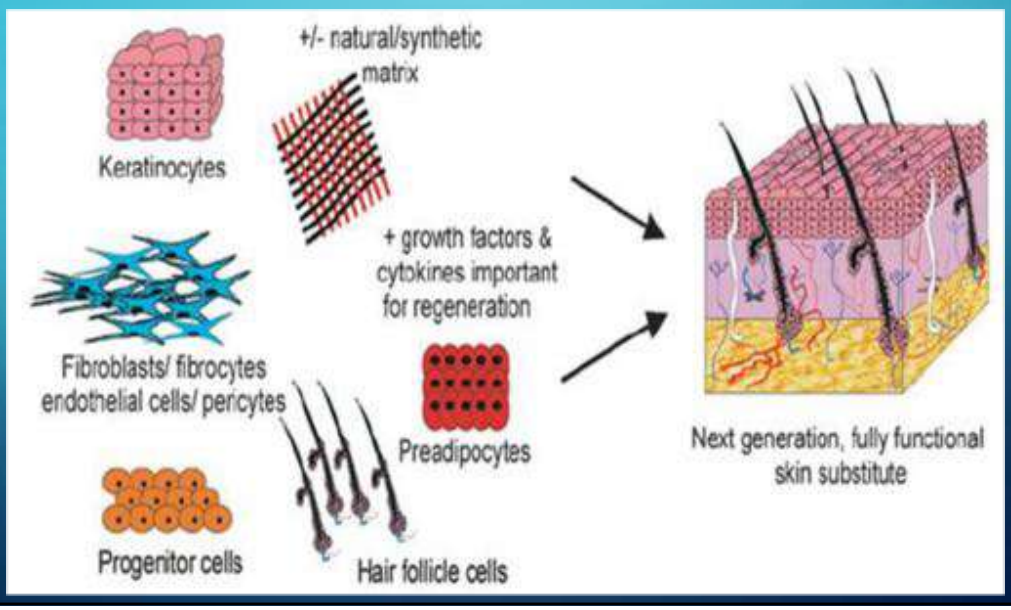
Introduction to Animal cell culture and Skin disease Models

(Highlight slides)

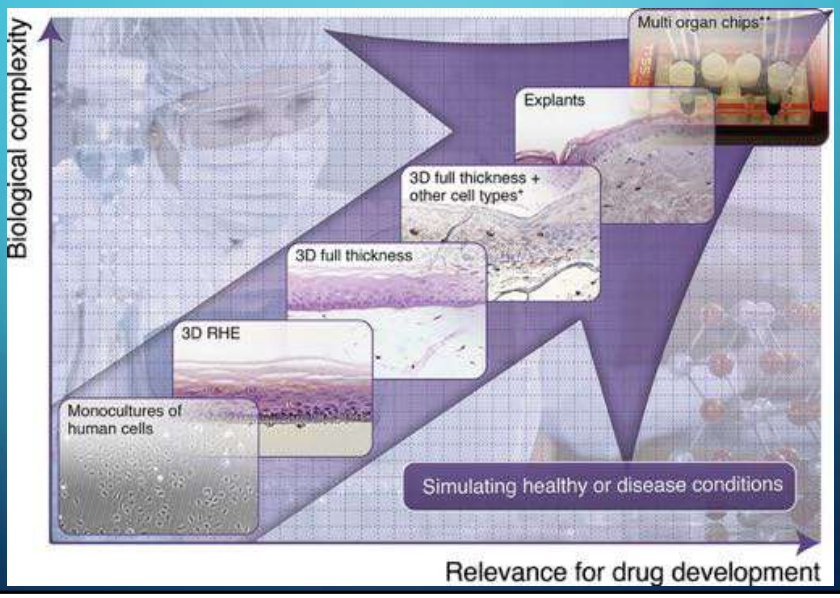
Pawan Gulati

British Association of Dermatologists Research technique course
Newcastle University
12-13th November 2018

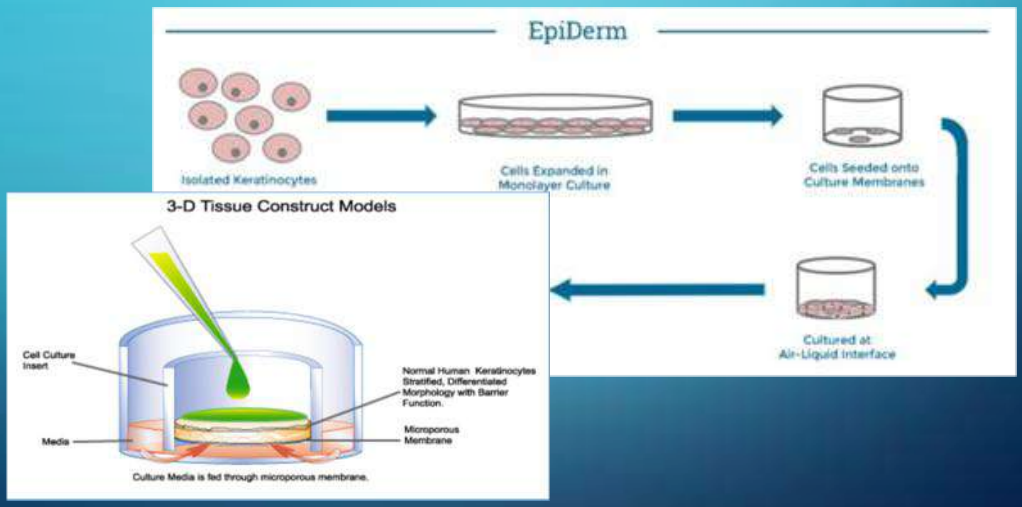
An ideal skin model



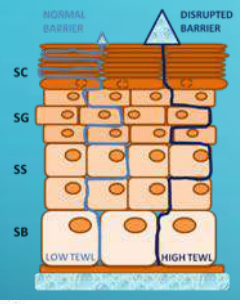
Models of skin diseases

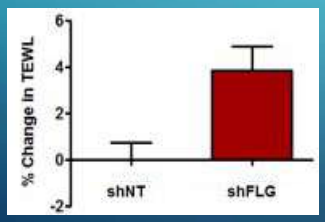


3-D model of skin disease

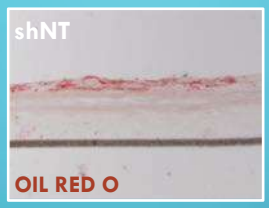


Filaggrin knockdown epidermal model displays functional hallmarks of AE

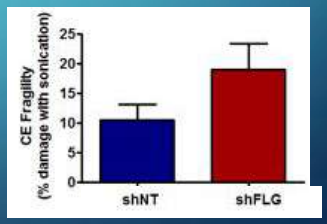




↑ Trans Epidermal Water Loss



↓ Epidermal lipid



↑ Cornified Envelope fragility

Applications of cell culture in human diseases and beyond

Areas where cell culture technology is currently playing a major role

- Model systems for

  Studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies

- Virology

  Cultivation of virus for vaccine production, also used to study there infectious cycle.

- Genetic Engineering

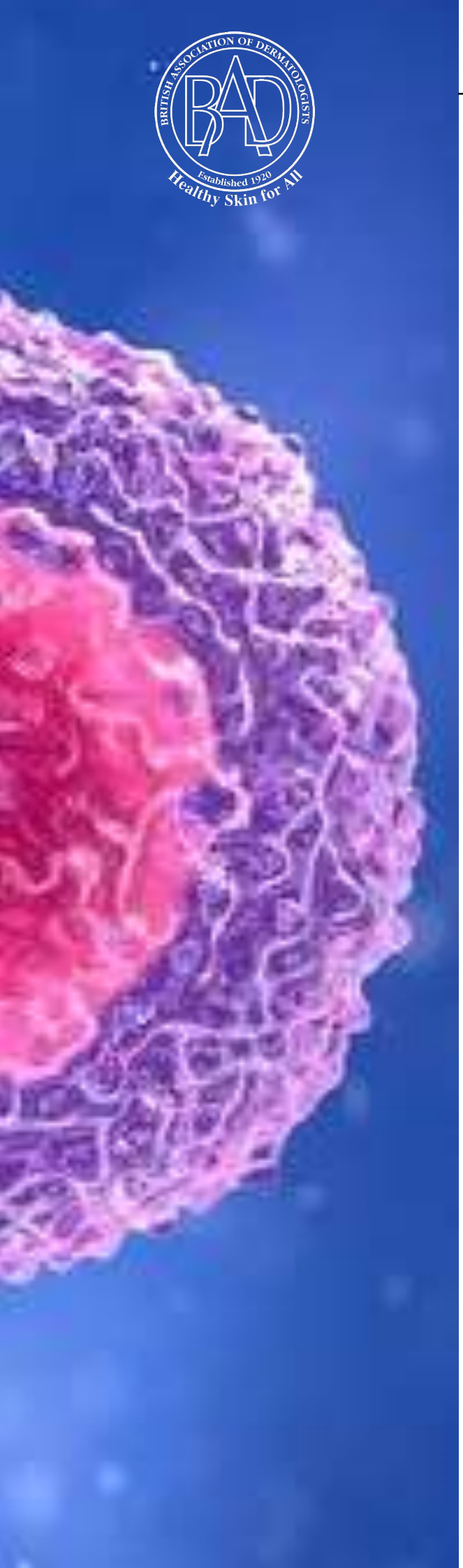
  Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

- Gene therapy

  Cells having a functional gene can be replaced to cells which are having non-functional gene

- Toxicity testing

  Study the effects of new drugs



3D CULTURE AND ZEBRAFISH MODELS FOR STUDYING MELANOMA METASTASIS

David Hill

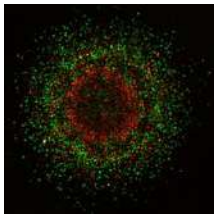
3D culture and zebrafish models for studying melanoma metastasis

Dr David Hill david.hill5@newcastle.ac.uk
Stratified Medicine, Biomarkers, & Therapeutics,
Institute of Cellular Medicine,
Newcastle University, UK

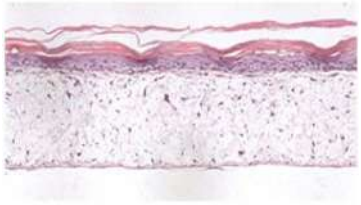
The Newcastle upon Tyne Hospitals NHS Foundation Trust
LANDSFORENINGEN FORSØGSDYRENES VÆRN
ALTERNATIVFONDET
Newcastle University
NC 3Rs National Centre for the Replacement Refinement & Reduction of Animals in Research
NESRF North Eastern Skin Research Fund
The Company of Biologists
Journal of Cell Science

3D culture and zebrafish models for studying melanoma metastasis

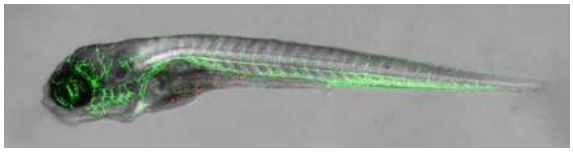
- 3D collagen-embedded spheroids



- Full-thickness skin organoid model

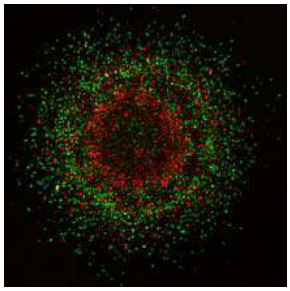


- Embryonic zebrafish xenograft of human melanoma



3D collagen-embedded spheroids

Untreated



Day -1: Grow melanoma cells to 60-70% confluence

↓

Day 0: Add 5000 melanoma cells to 96 well plates coated with 1.5% agarose

↓

Day 3: Transfer melanoma spheroids to 24 well plates in 1 ug/ml collagen gel

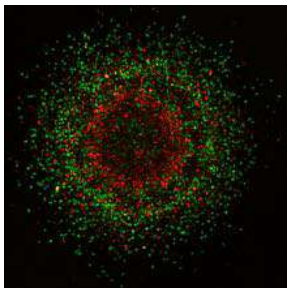
↓

Day 3-10: Use microscopy to monitor growth and invasion of melanoma cells into collagen for up to 7 days

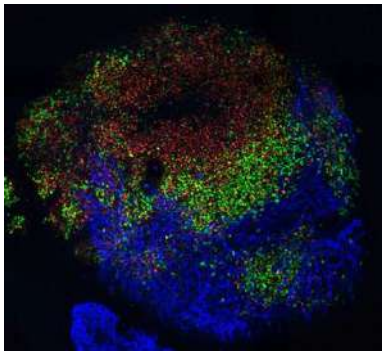
Hill DS, and Beaumont KA, *et al* (2016)
Cell cycle phase-specific drug resistance as an escape mechanism of melanoma cells.
J Invest Dermatol; 136:1479-1489.

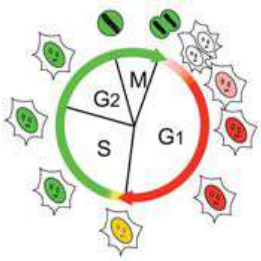
3D collagen-embedded spheroids

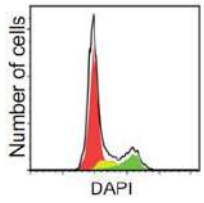
Untreated

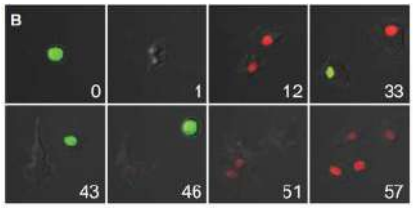


Mouse xenograft melanoma stained with anti-CD31









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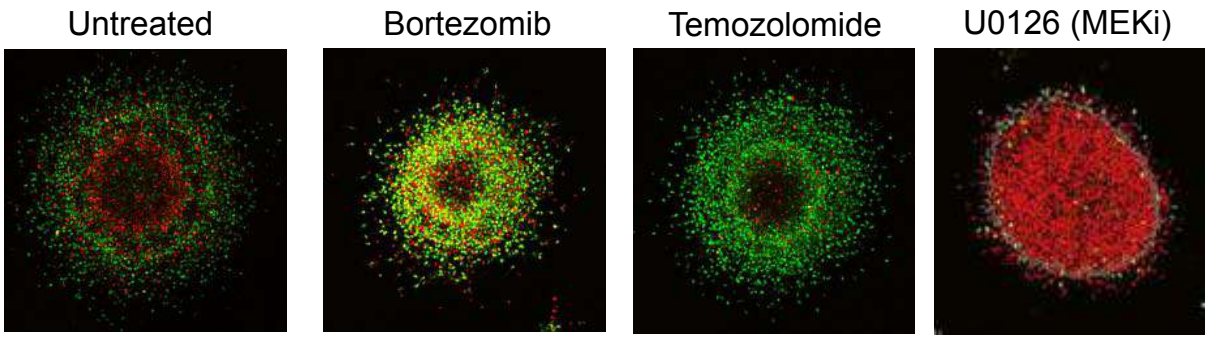

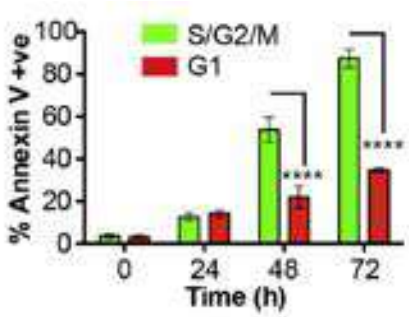

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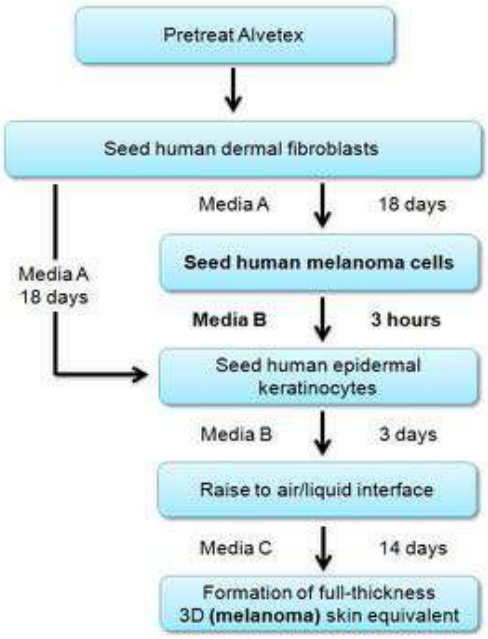
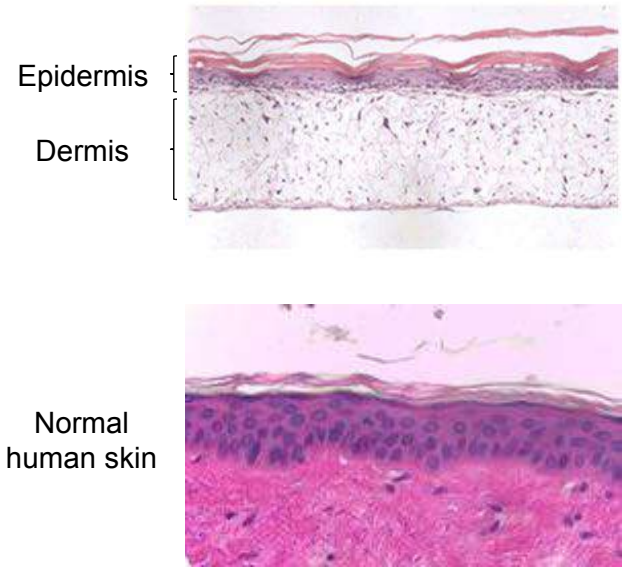
Full-thickness skin organoid model





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Molecular Cancer Therapeutics Nov;14(11):2665-73.

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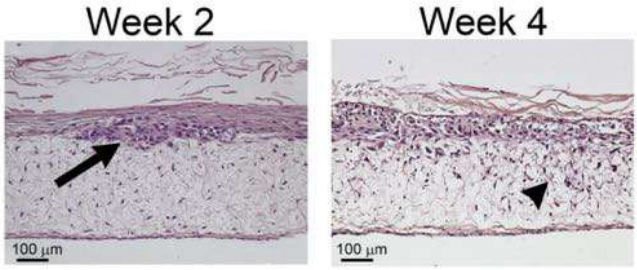

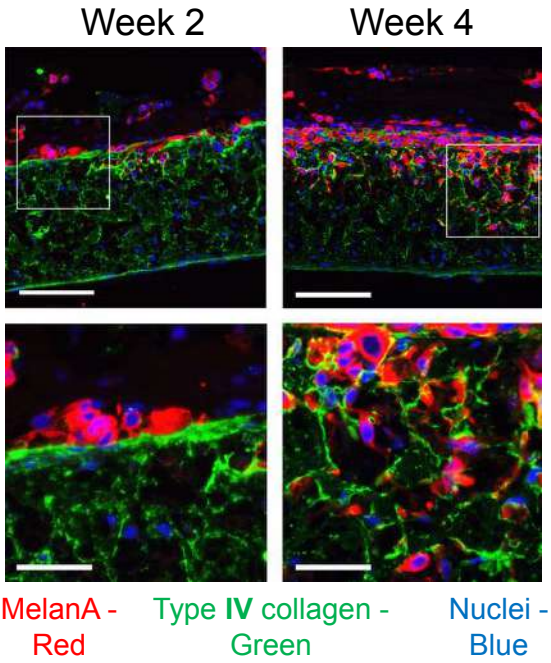

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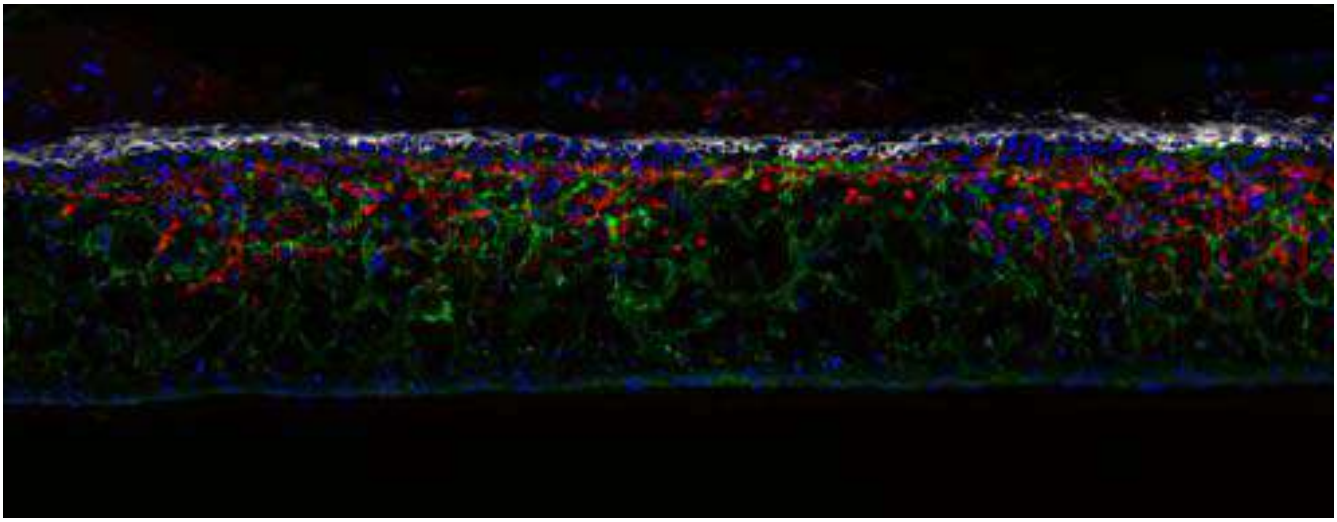
Full-thickness skin organoid model

Invasion of metastatic melanoma cells into full-thickness skin organoid

- Red = SKmel28 melanoma cells
- White = Cytokeratin 14, marker of keratinocytes
- Green = Type IV collagen, marker of ECM/basement membrane
- Blue = DAPI, marker of cell nuclei



Embryonic zebrafish xenograft of human melanoma

Day -1: Pair up fish for breeding

↓

Day 0: Collect fertilised eggs

↓

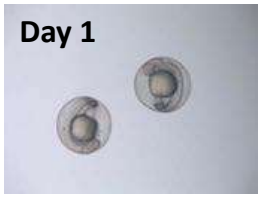
Day 2: Stain melanoma cells with DiI (Red fluorescent dye)

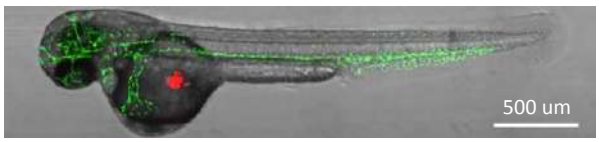
↓

Day 2: Micro-inject melanoma cells into the yolk sac, or circulation of 2-day old anaesthetised zebrafish embryos

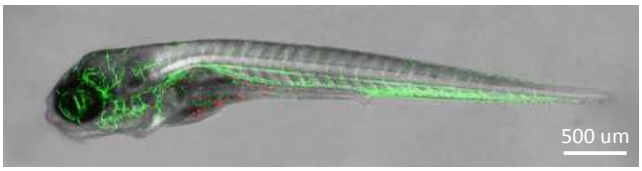
↓

Day 2-5: Image live zebrafish by microscopy for up to 72 hrs



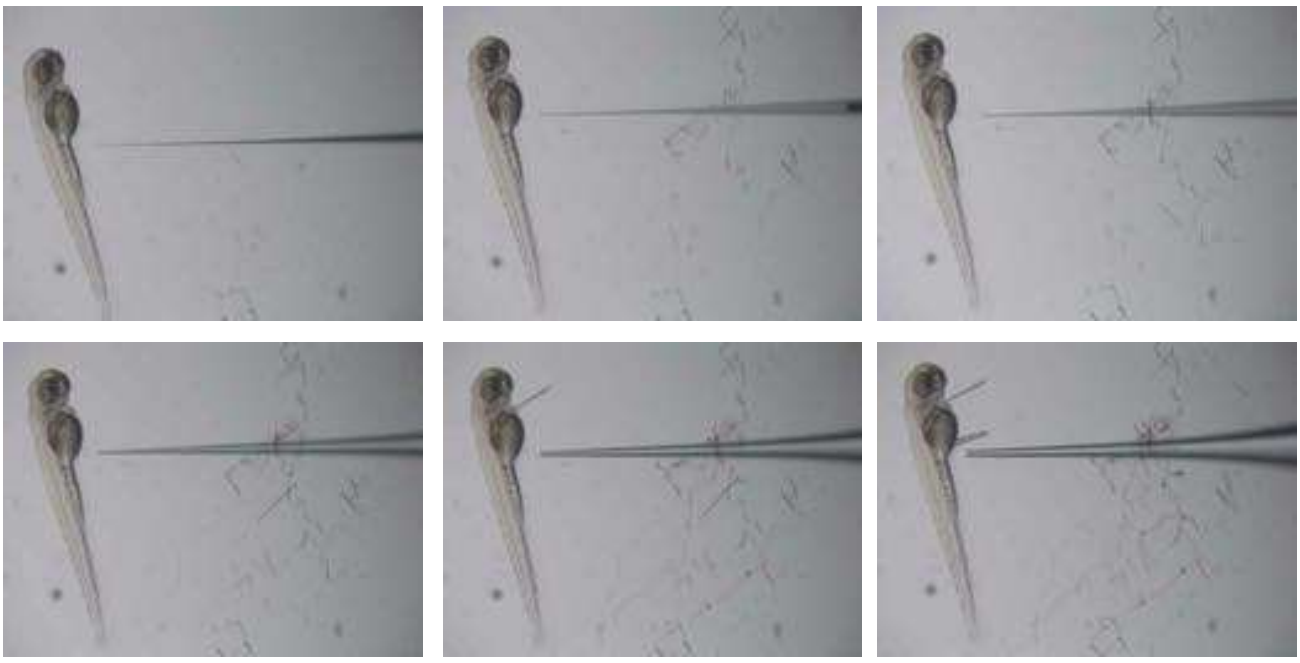


↓ 72 hours



Hill DS, *et al* (2018)
Embryonic zebrafish xenograft assay of human cancer metastasis.
F1000Research; Oct, 7:1682-1693.

Embryonic zebrafish xenograft of human melanoma



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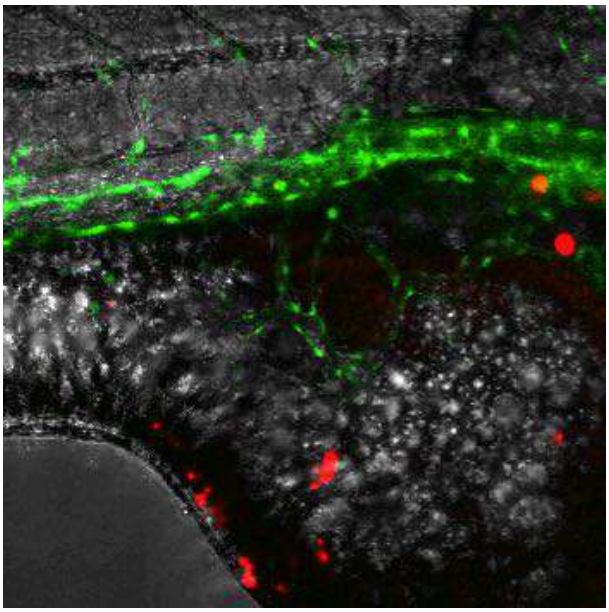

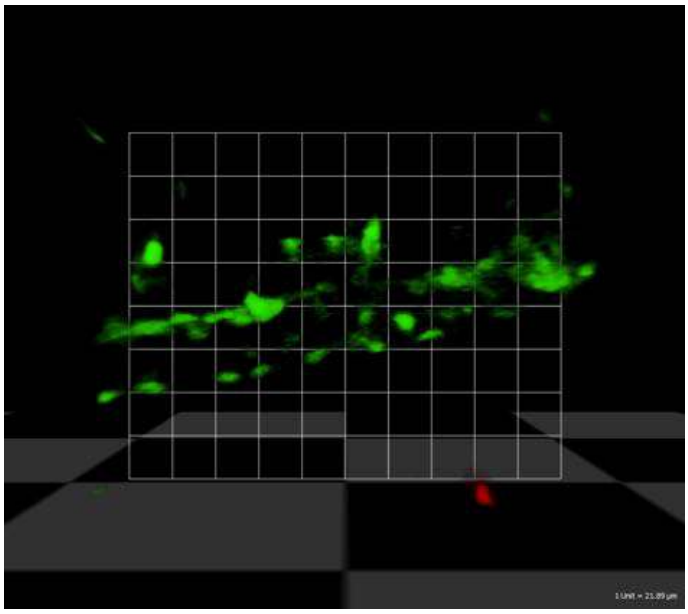

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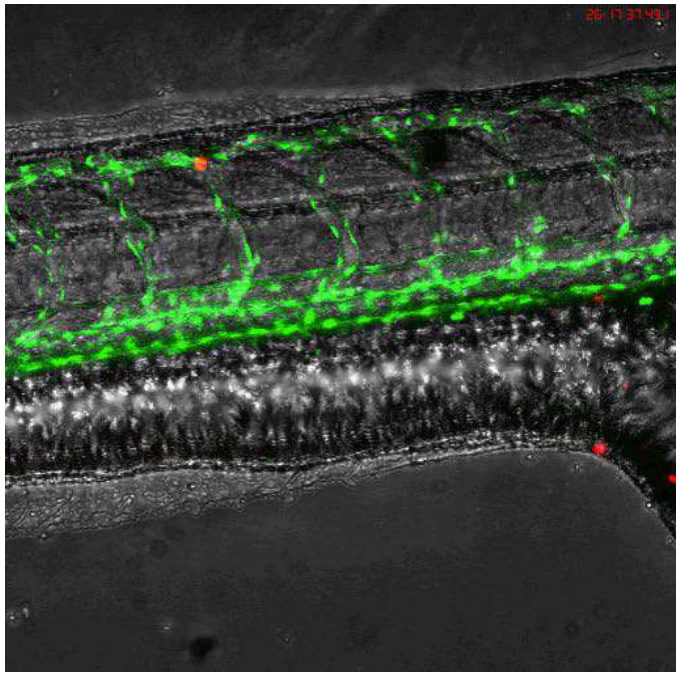

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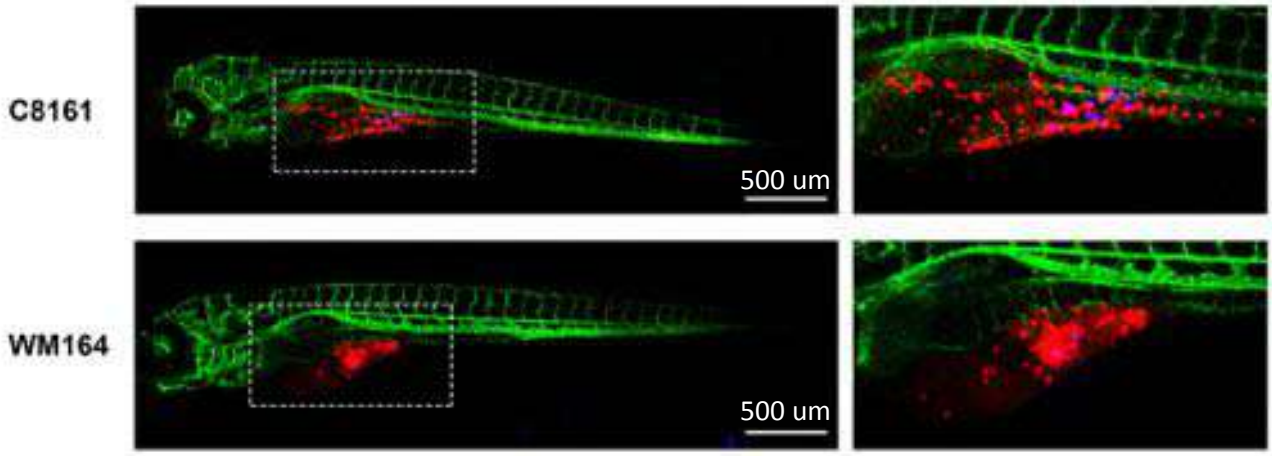
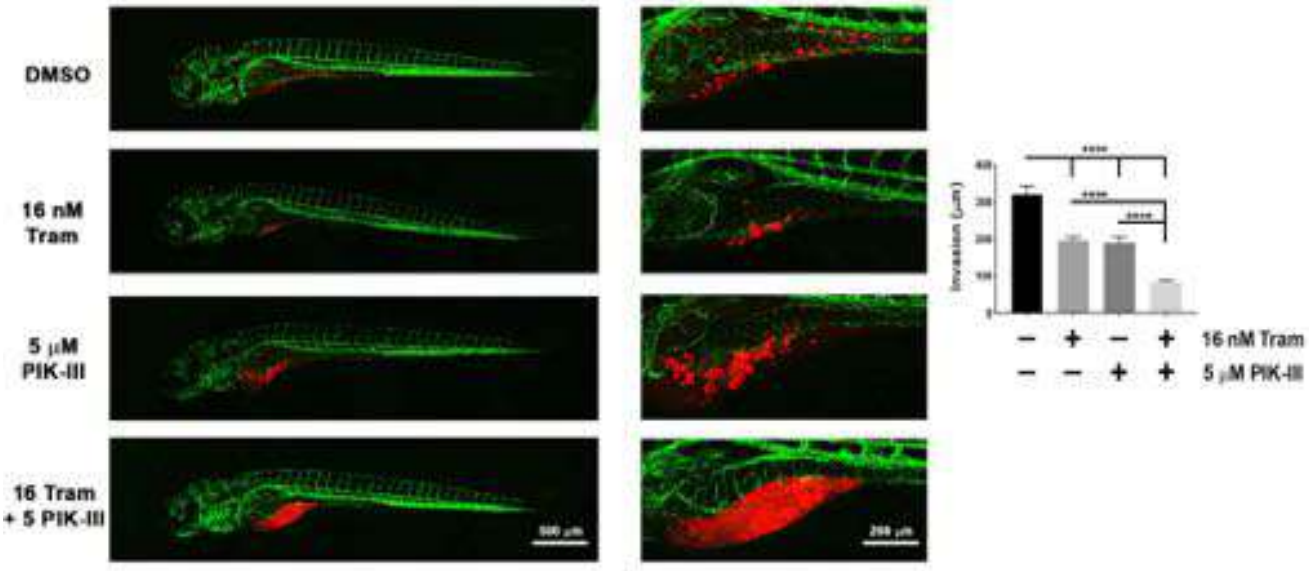
F1000Research; Oct, 7:1682-1693.

Embryonic zebrafish xenograft of human melanoma

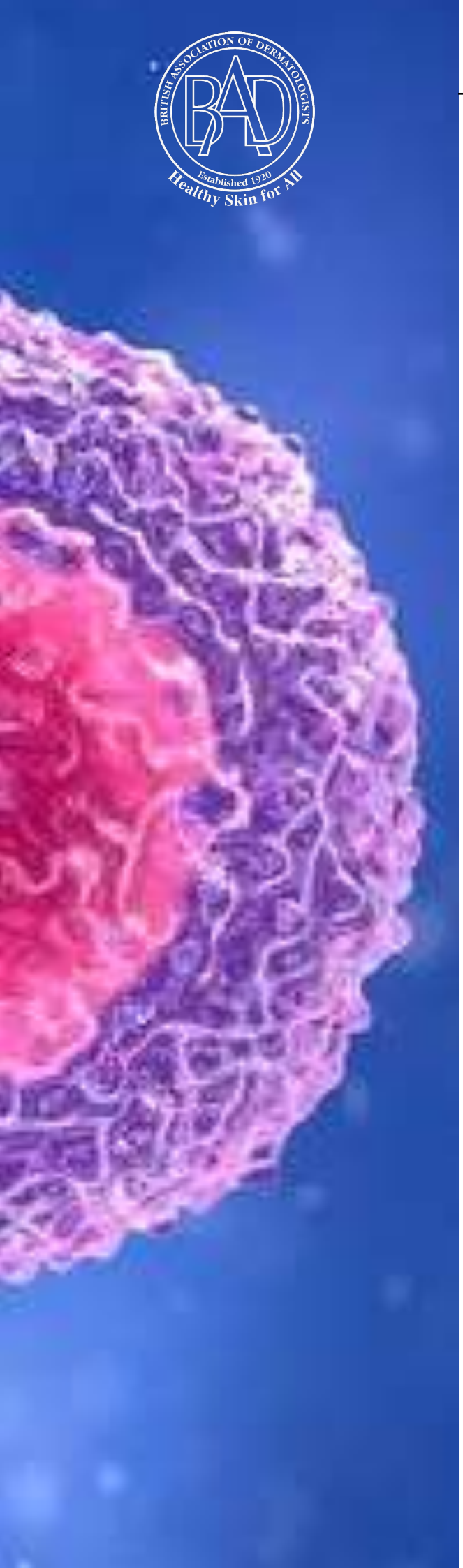


Hill DS, *et al* (2018)
Embryonic zebrafish xenograft assay of human cancer metastasis.
F1000Research; Oct, 7:1682-1693.

Embryonic zebrafish xenograft of human melanoma



Verykiou *et al* (2018)
Harnessing autophagy to overcome MEKi induced resistance in metastatic melanoma.
Brit J Dermatol; *in press*.



TRANSCRIPTOMICS IN PSORIASIS

Nick Reynolds

Transcriptomic analysis of psoriasis following biologic therapy

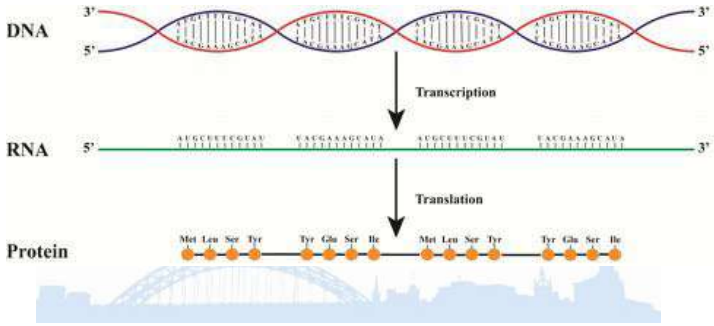
Nick J Reynolds
Newcastle University and Royal Victoria Infirmary,
Newcastle upon Tyne, UK

Outline of talk

- Translational research
  - Identifying important questions and unmet clinical need
- Development of analytical techniques (transcriptomics and other omic technologies) that can be usefully applied to human tissue samples to address:
  - Disease and therapeutic endotypes (psoriasis)

A pilot omics study of etanercept response in psoriasis

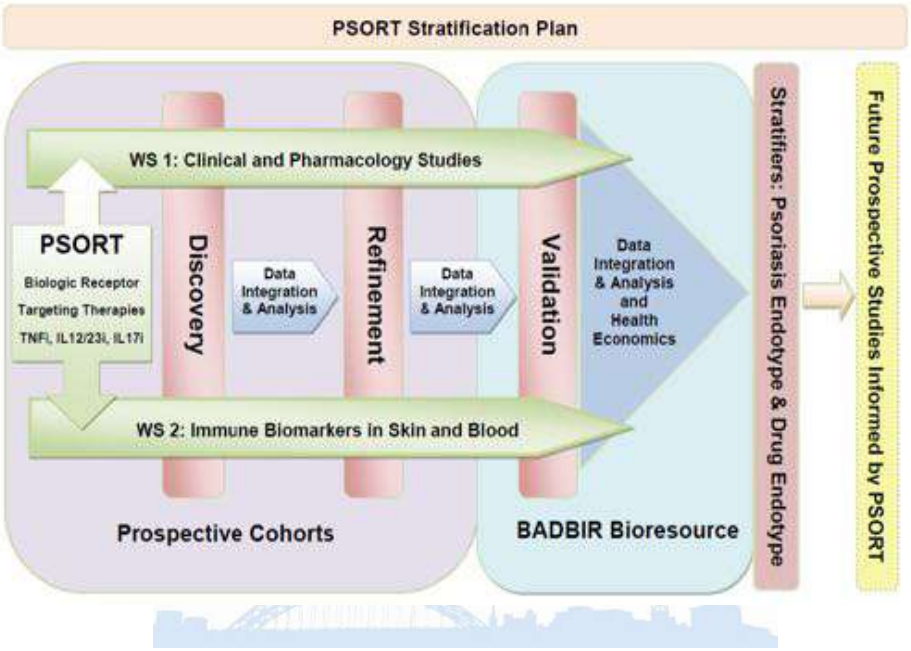
- Lead: Dr Amy Foulkes, Manchester
- GSK-funded pilot study of 10 biologic naïve, psoriasis patients initiating etanercept (TNF inhibitor)
- Multiple omic measures at 0wk, 1wk and 12wks
  - mRNAseq (Skin biopsies - involved & uninvolved skin)



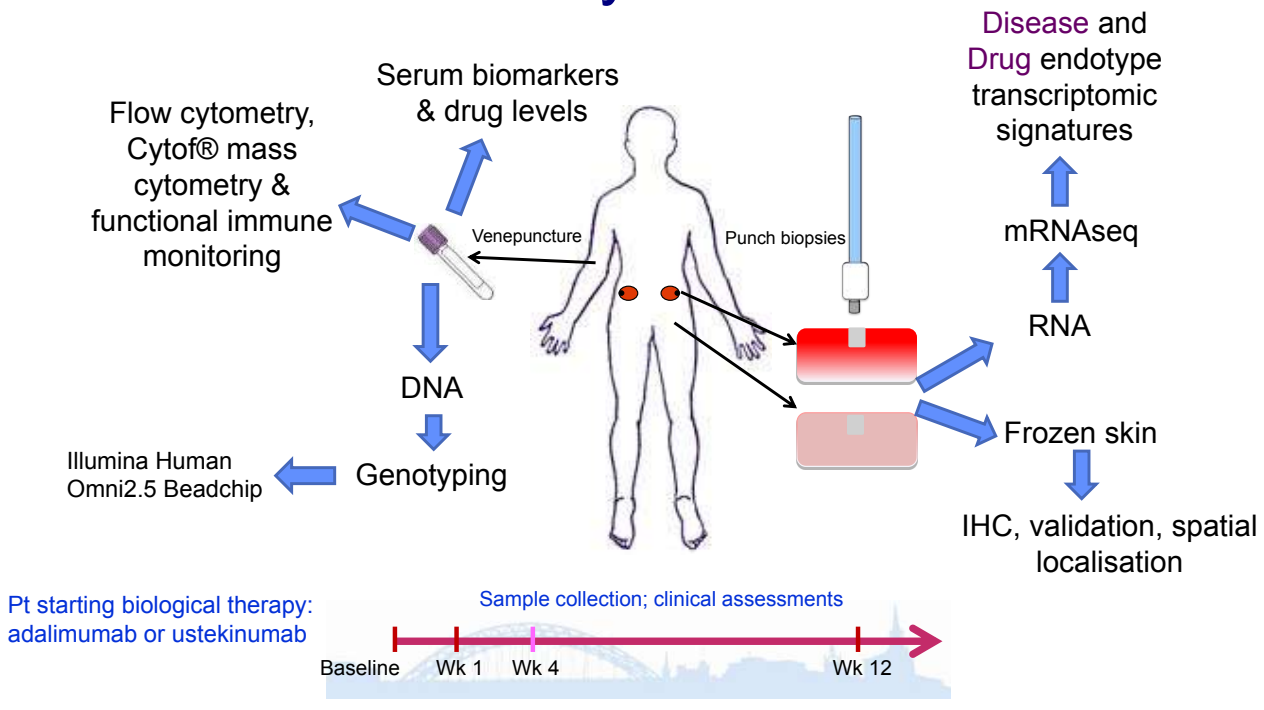
Psoriasis Stratification to Optimise Relevant Therapy (PSORT)

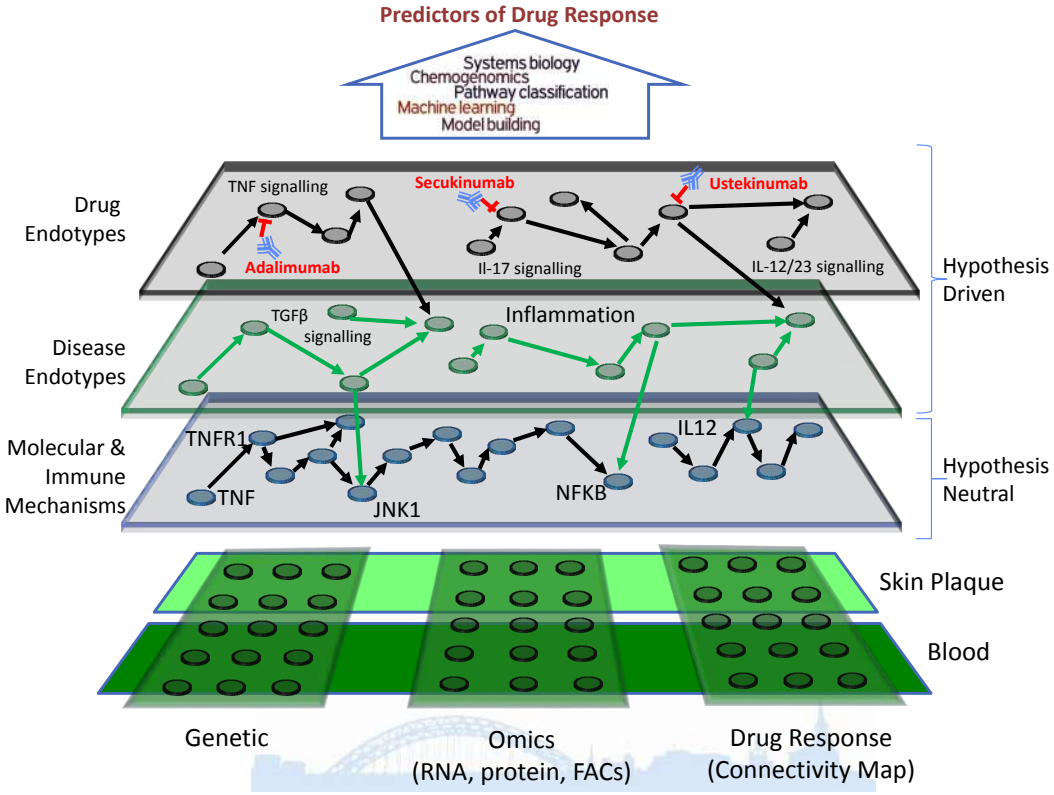
- Multi-disciplinary consortium
  - Based around British Association of Dermatologists Biologics Intervention Registry (BADBIR)
  - Understand determinants of response to biologic therapies
- Stratification of therapy
  - Clinical
  - Pharmacological
  - Genetic
  - Immune

PSORT Integrative Structure



Discovery Cohort





A pilot omics study of etanercept response in psoriasis

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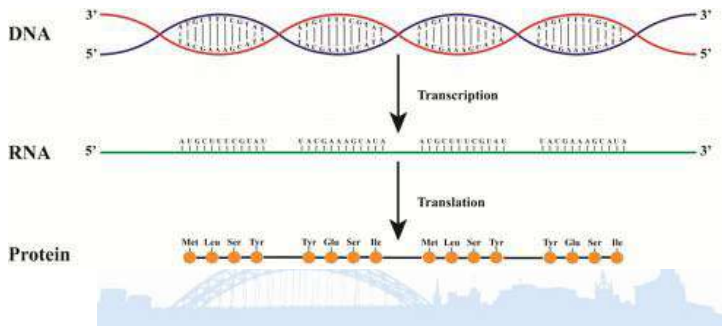
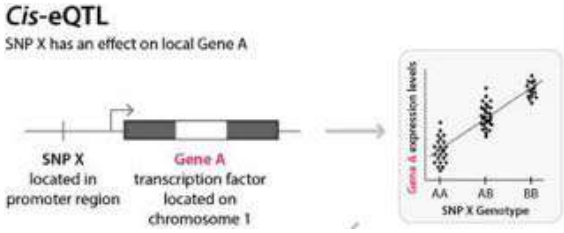
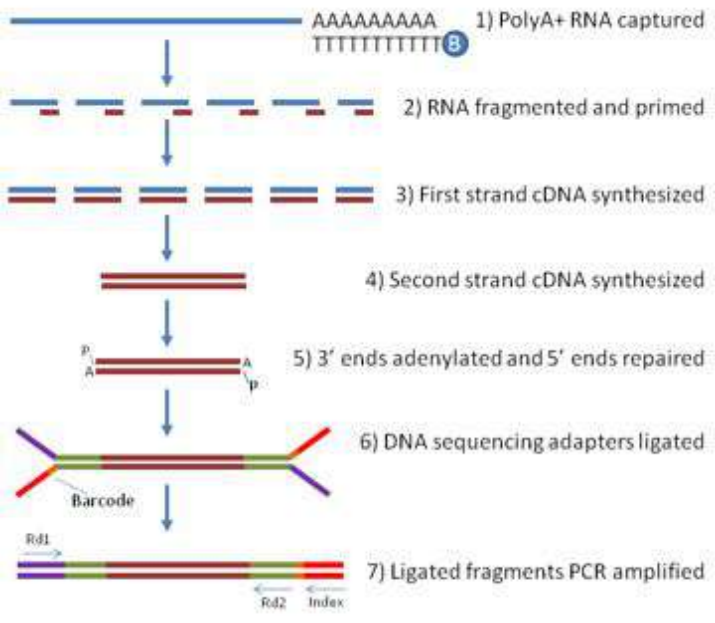


RNAseq

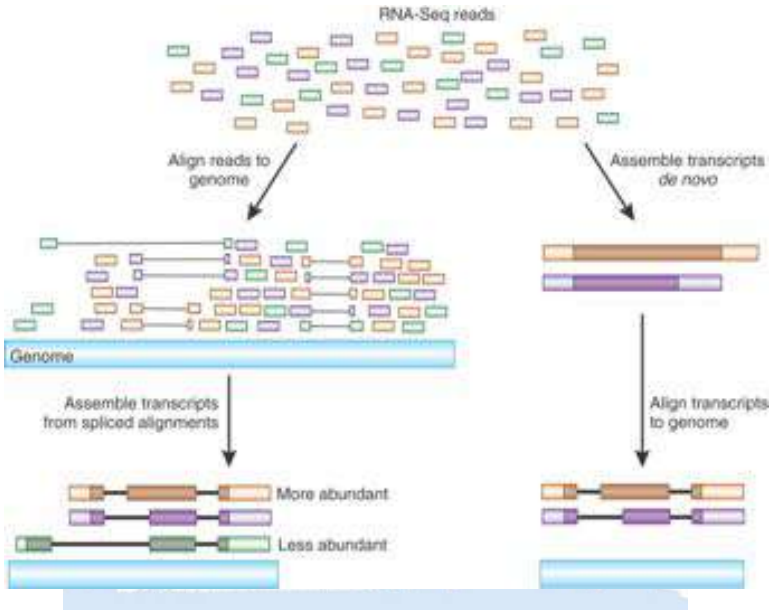
- Advantages compared to gene arrays
- Increased sensitivity (low abundance transcripts)
- Isoform specific expression
- Integration with genetic data yields information on *cis* and *trans* eQTL



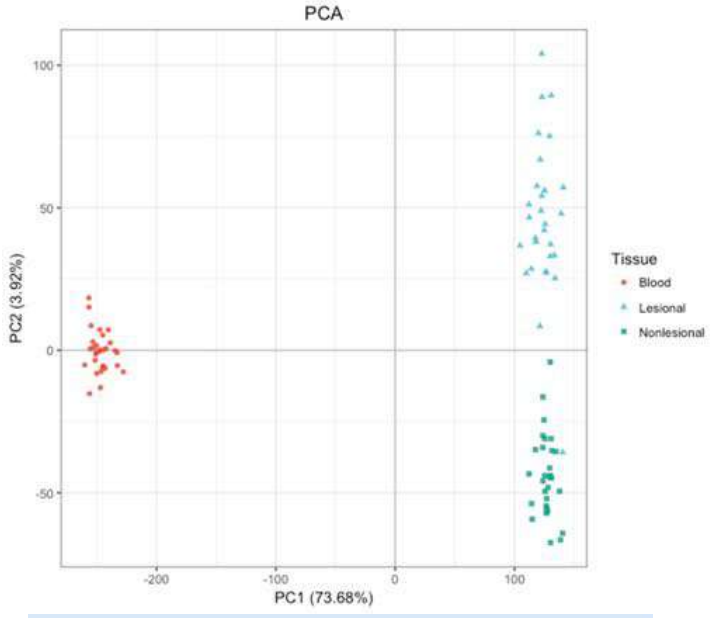
RNAseq

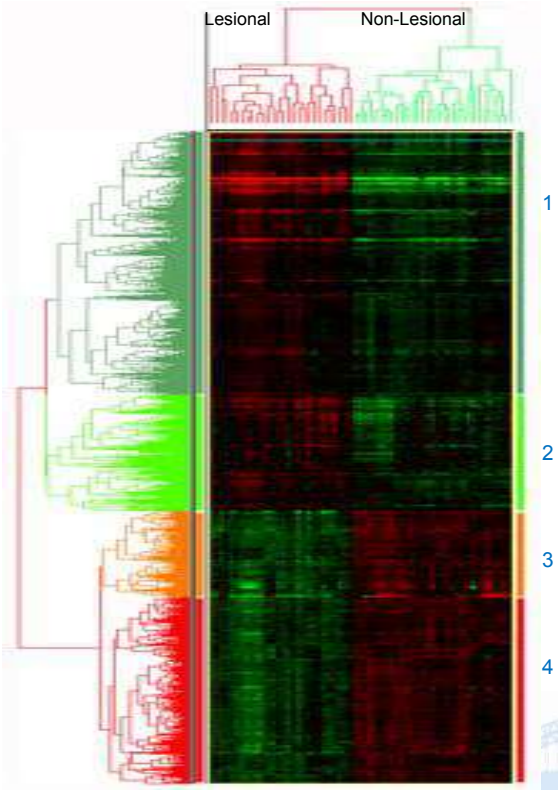


RNAseq



Principal component analysis of all skin and blood transcriptomic samples across all time points





RNAseq

↓

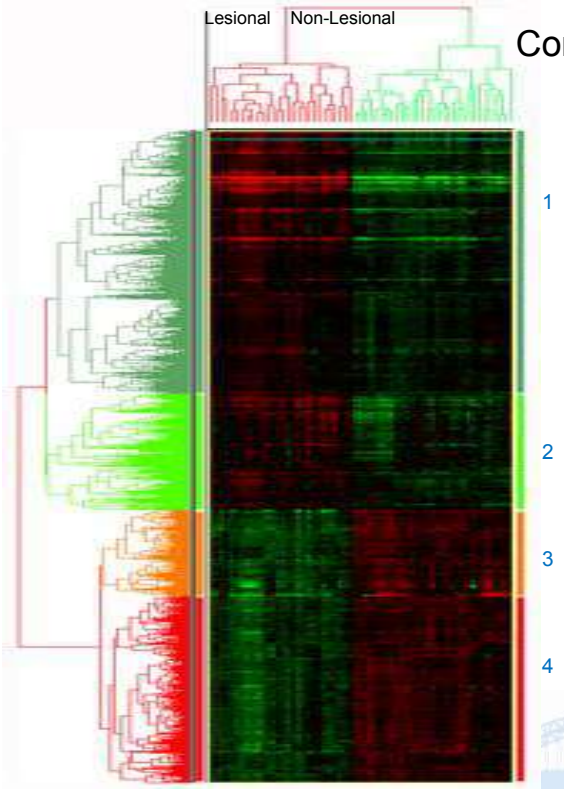
Levels of RNA from individual genes

↓

Red = upregulated (increased)
Green = down-regulated (decreased)

↓

Computer cluster analysis



Comparison of lesional and non-lesional skin at baseline:
Connectivity map analysis

Cluster 1: 1789 genes up-regulated in psoriasis plaque
- Which drugs down-regulate these genes?

↓

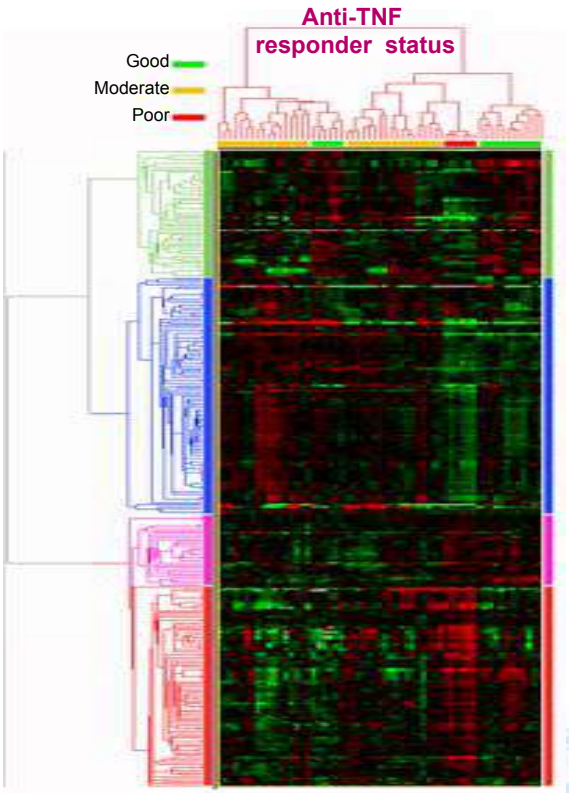
Data base computer search for similar patterns

↓

Methotrexate (one of top 3)
- *First line treatment* for psoriasis

↓

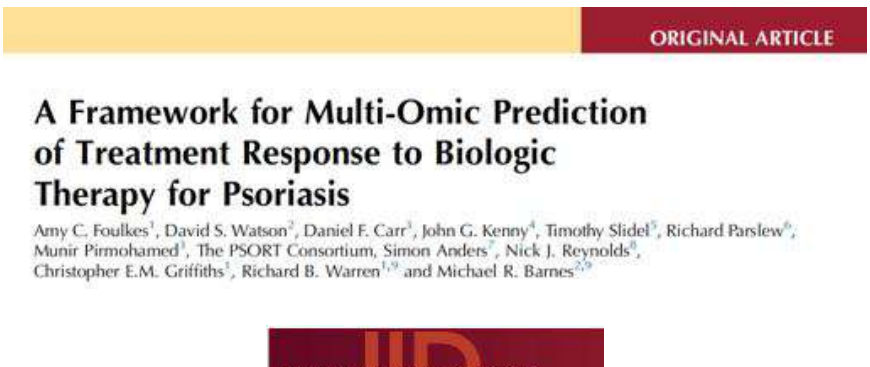
Supports concept of *disease endotype*



Time course analysis of anti-TNF response

- 295 transcripts with different levels of expression between responders and non-responders

Precision medicine for pso



ORIGINAL ARTICLE

A Framework for Multi-Omic Prediction of Treatment Response to Biologic Therapy for Psoriasis

Amy C. Foulkes[1], David S. Watson[2], Daniel F. Carr[3], John G. Kenny[4], Timothy Slidel[5], Richard Parslew[6], Munir Pirmohamed[3], The PSORT Consortium, Simon Anders[7], Nick J. Reynolds[8], Christopher E.M. Griffiths[1], Richard B. Warren[1,9] and Michael R. Barnes[2,9]

JOURNAL OF INVESTIGATIVE DERMATOLOGY JID

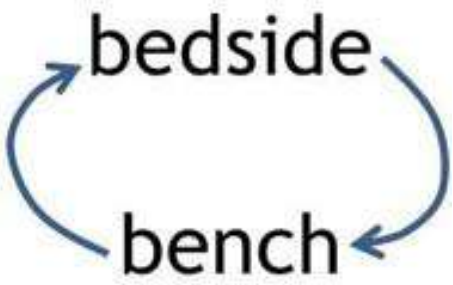
PSORT

MRC Medical Research Council

psoriasis association

Perspectives

- Value of combining hypothesis driven research with omic analysis and data interpretation
- New analysis techniques readily applicable to carefully phenoptyed clinical samples
- Exciting time to be entering the field



Acknowledgements

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PSORT
Chris Griffiths
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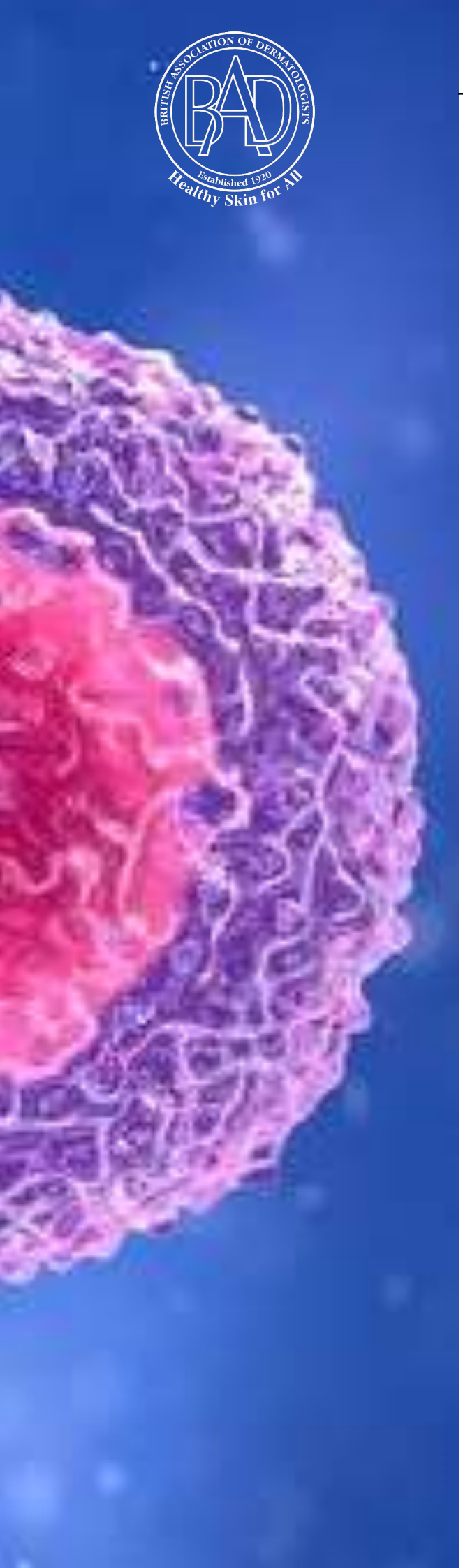
Catherine Smith
Mike Barnes
Amy Foulkes
Simon Cockell
Graham Smith
Deborah Stocken
Richard Parslew

GSK
Michael Donaldson
Sequencing hub, Stevenage

Patients
Psoriasis clinics
Research nurses
Technicians
Data managers

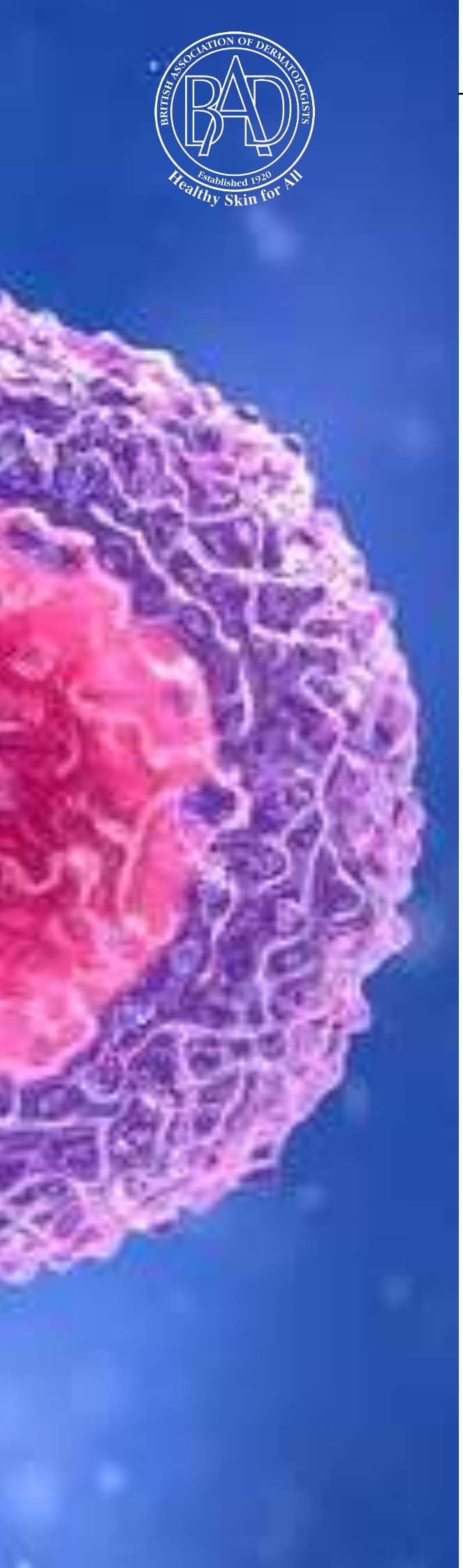
NHS Newcastle Dermatology

Newcastle Biomedical Research Centre



HUMAN CELL ATLAS AND THE SKIN

Muzz Haniffa



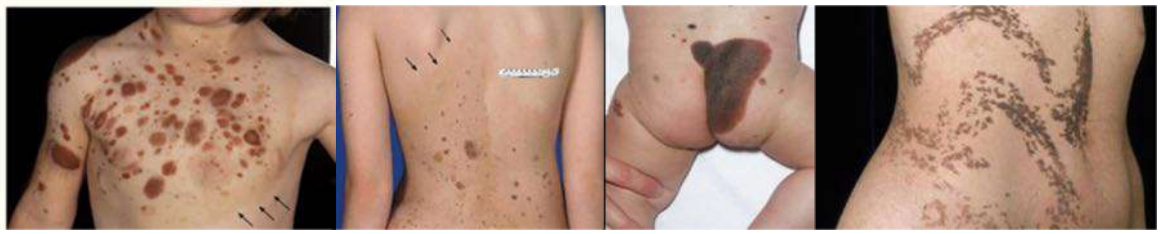
BIRTHMARKS AND GENETIC MOSAICISM

Siobhan Muthiah

Birthmarks and genetic mosaicism

BAD Research Techniques Course

2018

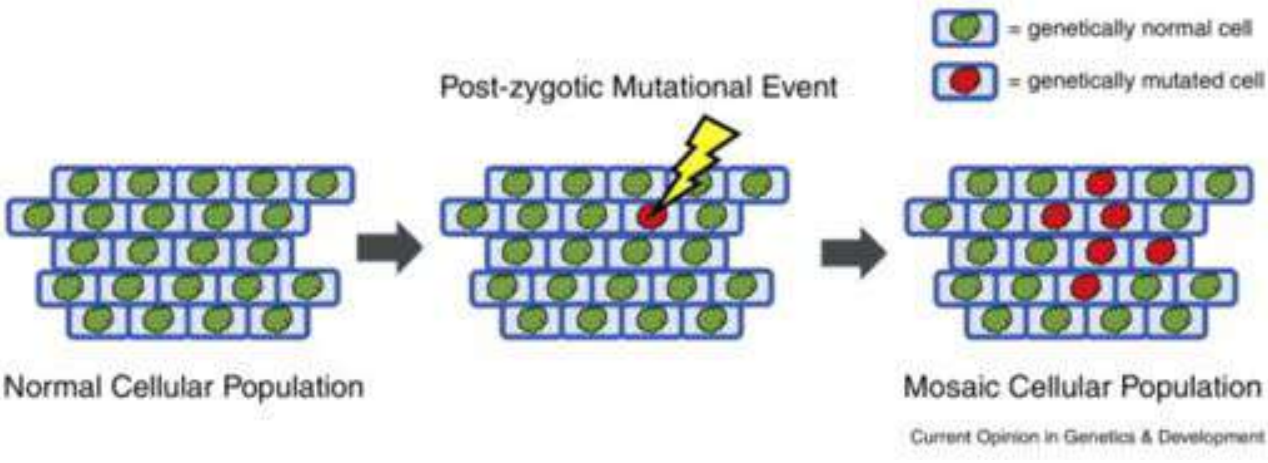


Aims

- Discuss cutaneous mosaicism and its patterns
- Explore current understanding of naevi in the spectrum of mosaic RASopathies

Cutaneous mosaicism

Mosaicism



Machiela MJ, Chanock SJ. The ageing genome, clonal mosaicism and chronic disease. Current Opinion in Genetics & Development. 2017 Feb 1;42:8–13.

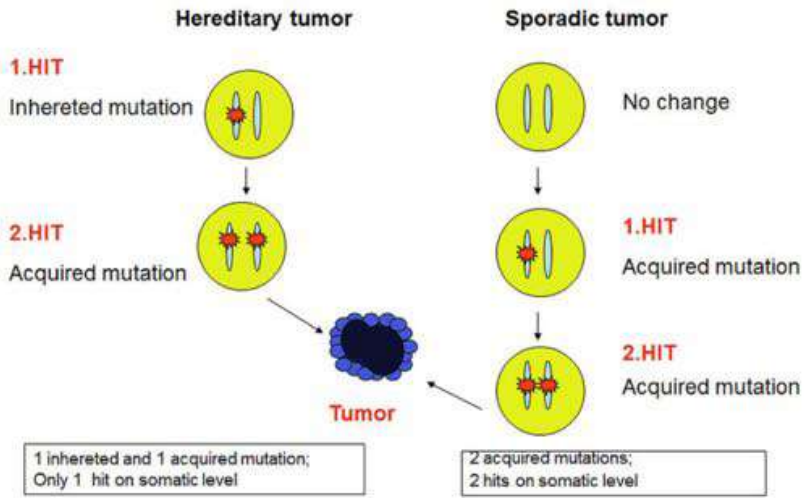
Cutaneous mosaicism

- Mosaicism can involve all organs but is most easily apparent in the skin in the form of birthmarks
- It can arise through a number of ways
  - Loss of heterozygosity
  - Lethal mutations
  - Nonlethal mutations

Loss of heterozygosity

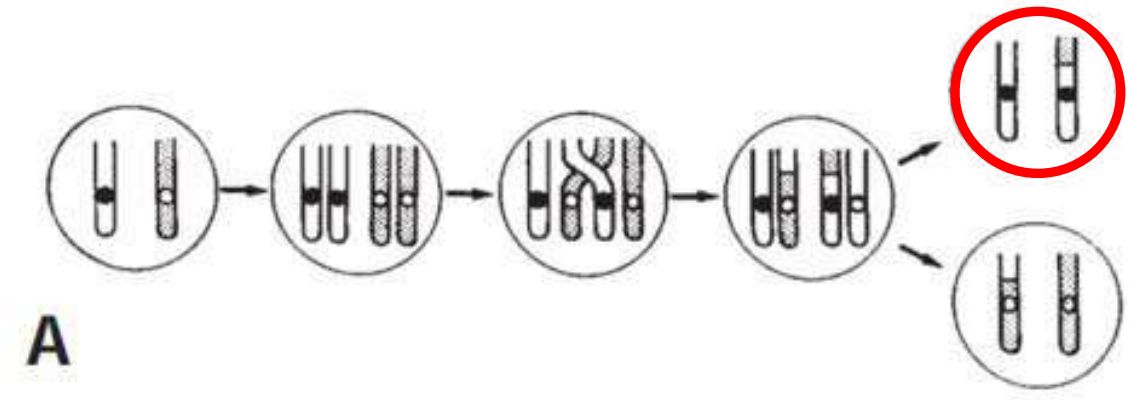
- In an individual heterozygous for a mutation, post-zygotic loss of the corresponding wild-type allele may result in a mosaic phenotype
- Benign and malignant skin tumours can be seen as examples of cutaneous mosaicism.
- This idea is known as the "two-hit" hypothesis, and it was first proposed by geneticist Alfred Knudson in 1971.
- LOH may arise from a variety of mutational events

Loss of heterozygosity



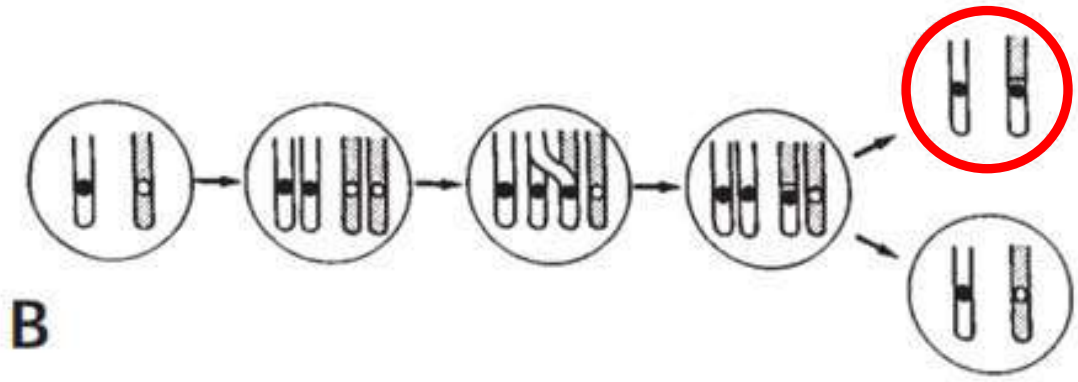
Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Mitotic crossing-over



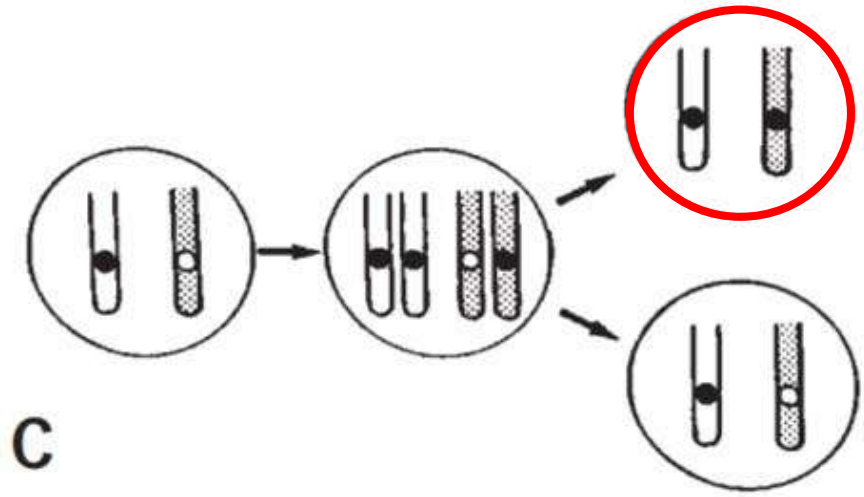
Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Gene conversion



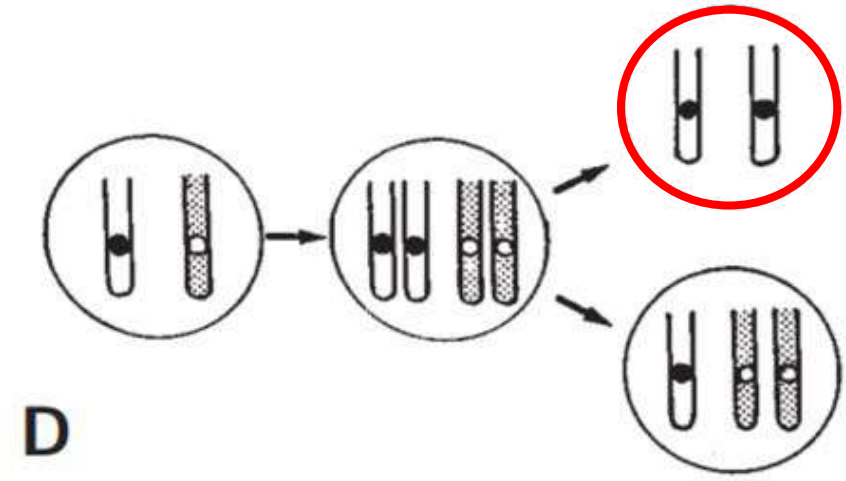
Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Point mutation



Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Mitotic non-disjunction



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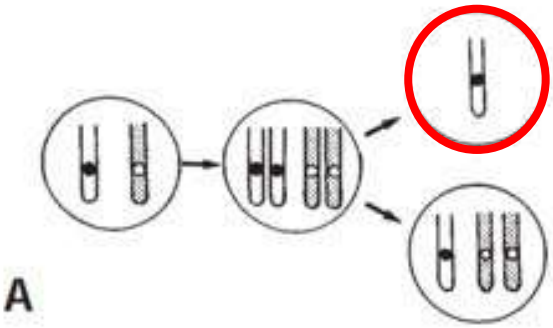
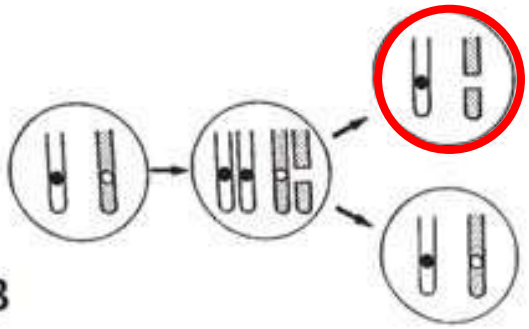


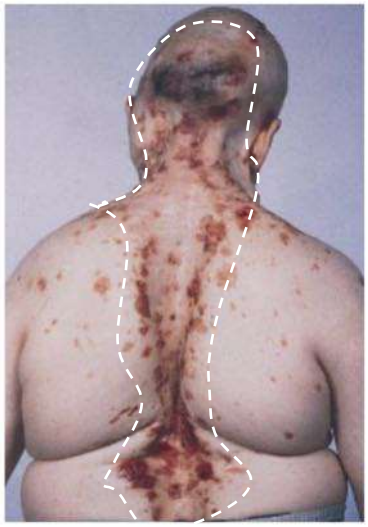
Deletion



Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Loss of heterozygosity

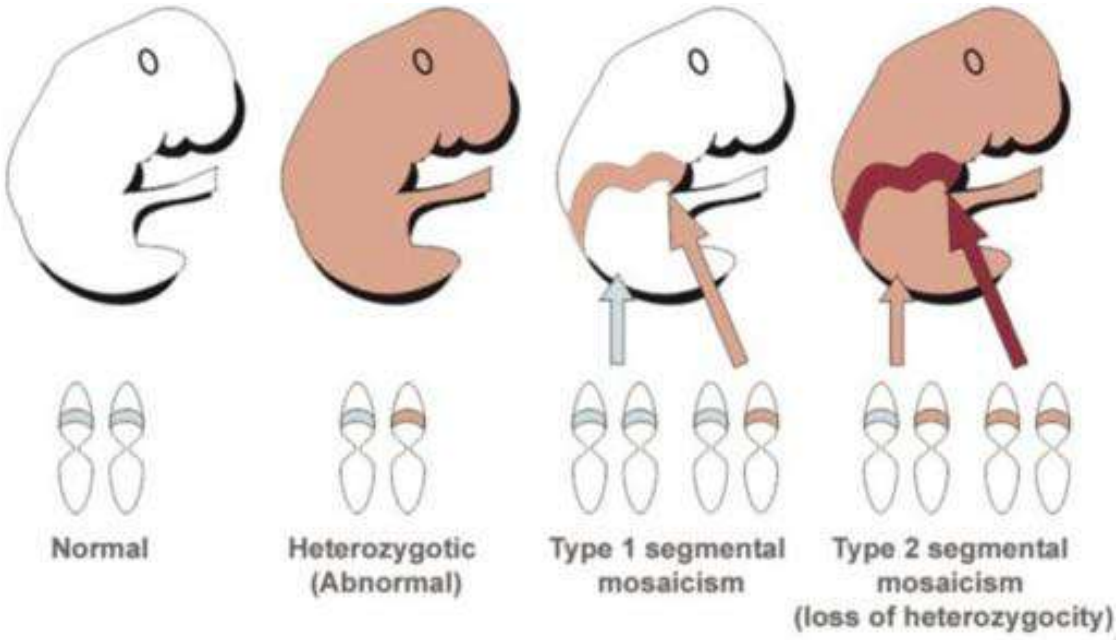
- Malignant skin tumours originating from LOH
  - Nevoid basal cell carcinoma originates from loss of the wild-type allele at 9q22.3



Happle R. Loss of heterozygosity in human skin. Journal of the American Academy of Dermatology. 1999 Aug 1;41(2):143–61.

Mosaicism of Nonlethal Mutations

- Happle described patterns of non-lethal mutations as
  - Type 1 segmental
  - Type 2 segmental



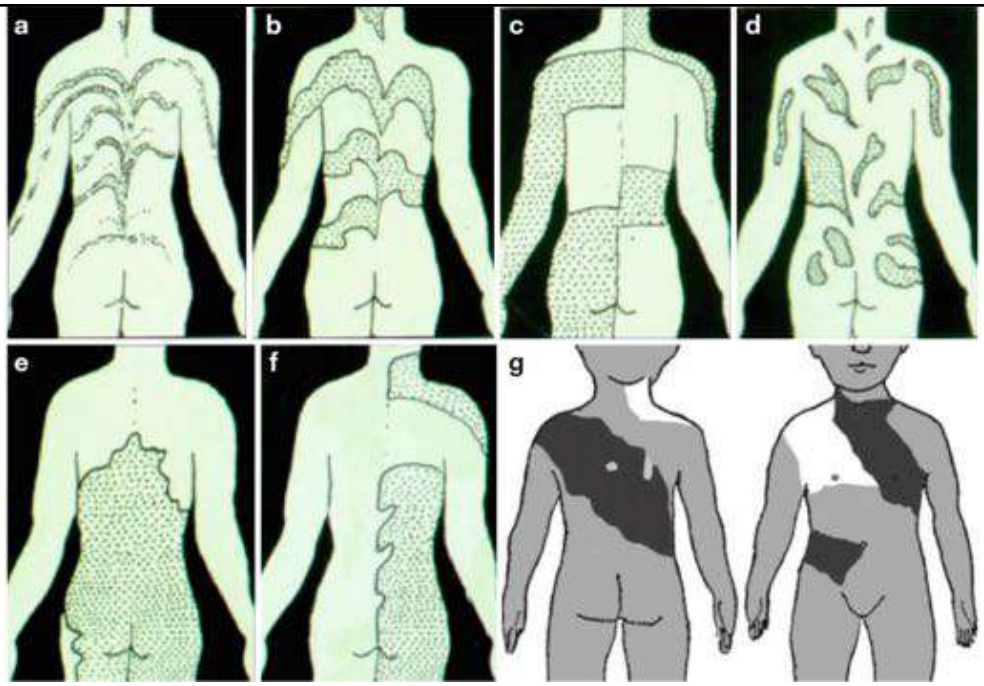
Happle, Rudolf. Mosaicism in Human Skin: Understanding Nevi, Nevoid Skin Disorders, and Cutaneous Neoplasia. 2013. 229 p

Patterns of cutaneous mosaicism

Patterns of cutaneous mosaicism

6 archetypical patterns of cutaneous mosaicism can be distinguished

1. Lines of Blaschko
2. Checkerboard pattern
3. Phylloid pattern
4. Patchy pattern without midline separation
5. Lateralisation pattern
6. Sash-like pattern



Happle, Rudolf. Mosaicism in Human Skin: Understanding Nevi, Nevoid Skin Disorders, and Cutaneous Neoplasia. 2013. pg 46

Naevi

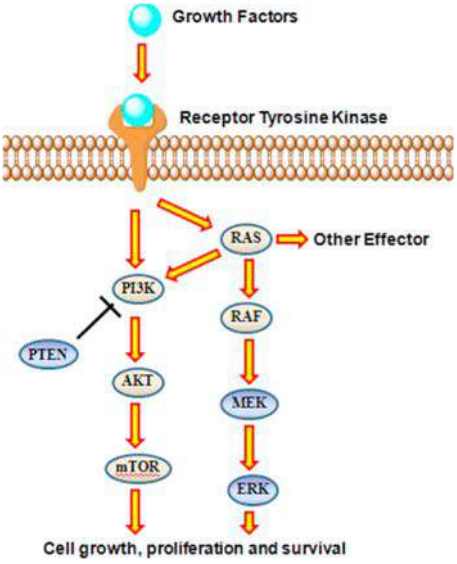
Naevi

- Naevoid proliferations make obvious mosaic presentations particularly when they are extensive. Examples include:
  - Systemised keratinocytic epidermal naevi (KEN)
  - Schimmelpenning syndrome
  - Phacomatosis pigmentokeratotica
- The molecular basis for these naevi **continue to be elucidated and recently <u>RAS signalling appears to be an important pathway in the formation of some naevi</u>**

RASopathies and mosaic RASopathies

RAS proteins

- RAS proteins transduce extracellular growth factor stimuli into the intracellular environment
- 2 RAS dependent pathways
  - Ras-Raf-MEK-ERK
  - PI3K-Akt



Asati V, Mahapatra DK, Bharti SK. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. European Journal of Medicinal Chemistry. 2016 Feb 15;109:314–41.

RAS proteins

- RAS proteins are encoded for by 3 genes
  - ***HRAS, KRAS and NRAS***

- Activating mutations in *RAS* genes are found in 30% of human cancers

RASopathies and mosaic RASopathies

- **"RASopathies** are a group of syndromes with overlapping clinical symptoms due to GERMLINE mutations in the Ras/MAPK signalling pathway"
  - Noonan, NF1, Costello syndrome, cardio-facio-cutaneous syndrome

- **Mosaic RASopathies** are due to mutations in the Ras/MAPK signalling pathway that occur in the mosaic state presenting with a congenital syndrome distinct to that seen in the corresponding germline mutation

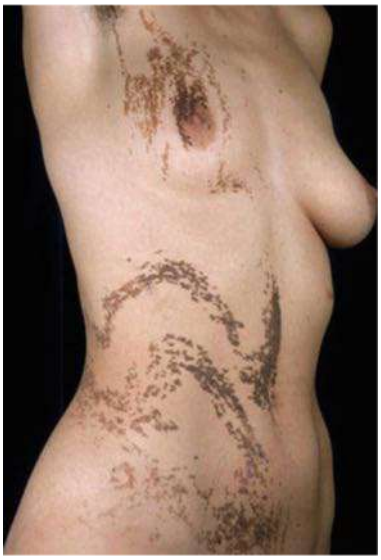
Hafner C, Groesser L. Mosaic RASopathies. Cell cycle (Georgetown, Tex). 2013 Jan 1;12(1):43–50

Mosaic RASopathies

- Epidermal naevi
  - Represent a unique paradigm for cutaneous mosaic disorders
  - Follow Blaschko's lines
  - Become visible at birth or in first few years of life

Mosaic RASopathies

- Keratinocytic Epidermal Naevi (KEN)
  - Can occur as a result of mosaic mutations in *FGFR3 and PIK3CA*
  - More recently KEN has been reported to occur as a result of **mosaic *RAS* mutation**
  - The *RAS* mutation were seen in the lesion BUT not in the epidermal tissue adjacent and in blood leukocytes



Happle, Rudolf. Mosaicism in Human Skin: Understanding Nevi, Nevoid Skin Disorders, and Cutaneous Neoplasia. 2013. pg 80

Mosaic RASopathies

- Sebaceous Naevus
  - A type of epidermal naevus which can develop secondary tumours in later life (25%), preferentially affecting the scalp and face
- Schimmelpenning syndrome
  - Is the presence of sebaceous naevus and extracutaneous abnormalities

Figure 1: Clinical, histological and molecular analyses of nevus sebaceous.

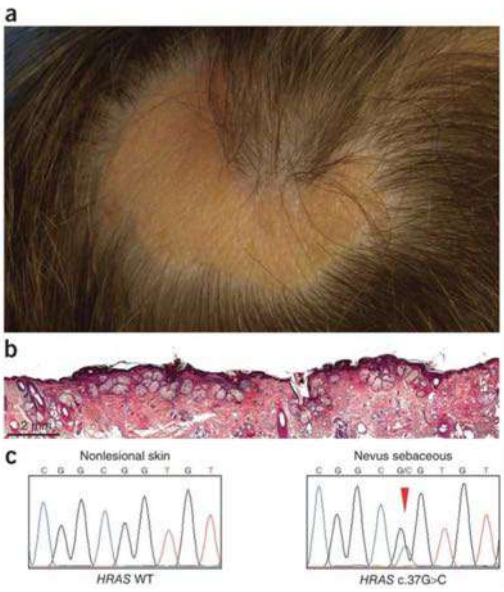
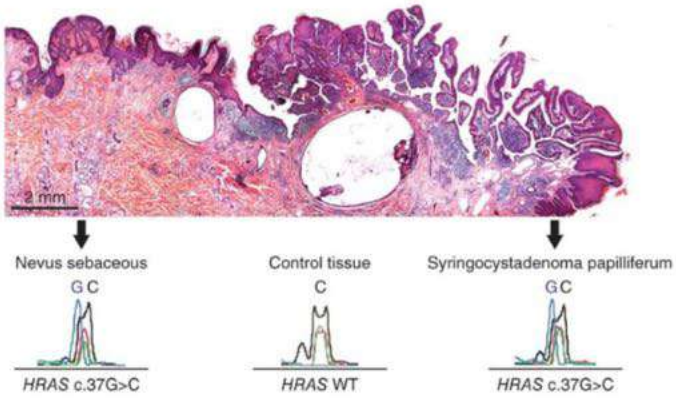
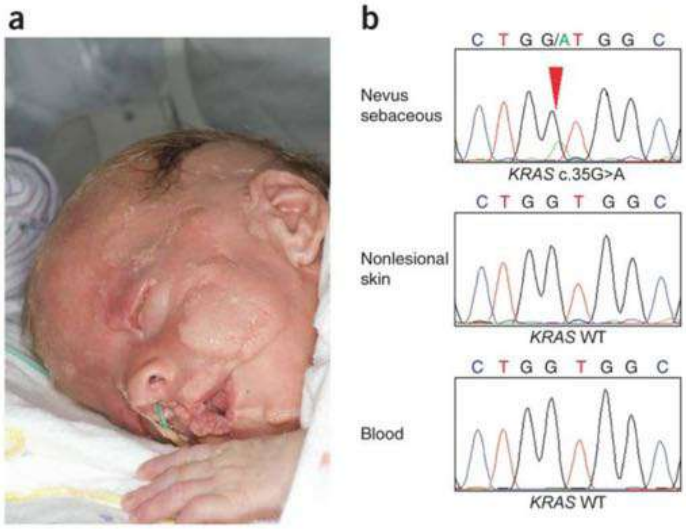


Figure 3: Molecular analysis of a syringocystadenoma papilliferum associated with a nevus sebaceous.



Groesser L, Herschberger E, Ruetten A, Ruivenkamp C, Lopriore E, Zutt M, et al. Postzygotic HRAS and KRAS mutations cause nevus sebaceous and Schimmelpenning syndrome. Nature Genetics. 2012 Jun 10;44:783.

Figure 2: Clinical manifestations and molecular analysis of Schimmelpenning syndrome in a newborn.



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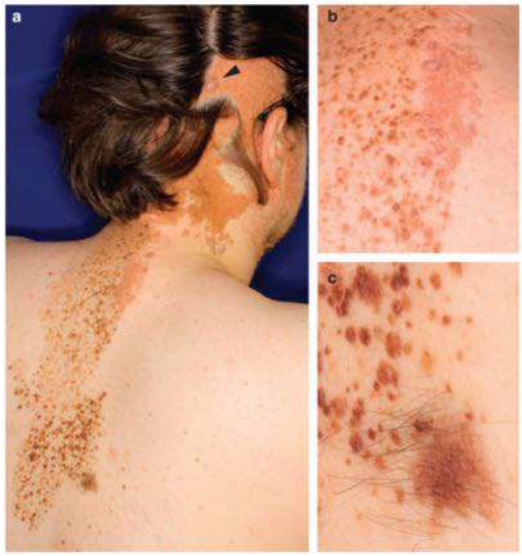
Mosaic RASopathies

- Sebaceous Naevus
  - Underlying genetic cause of this is a post zygotic mutation in HRAS c.37G>C of the epithelial cells
- Schimelpenning syndrome
  - **Extensive mosaicism for activating HRAS and KRAS** (HRAs c.37G>C and KRAS c.35G>A) mutations involving the skin, skeletal, ocular and CNS is the **genetic cause for this syndrome**

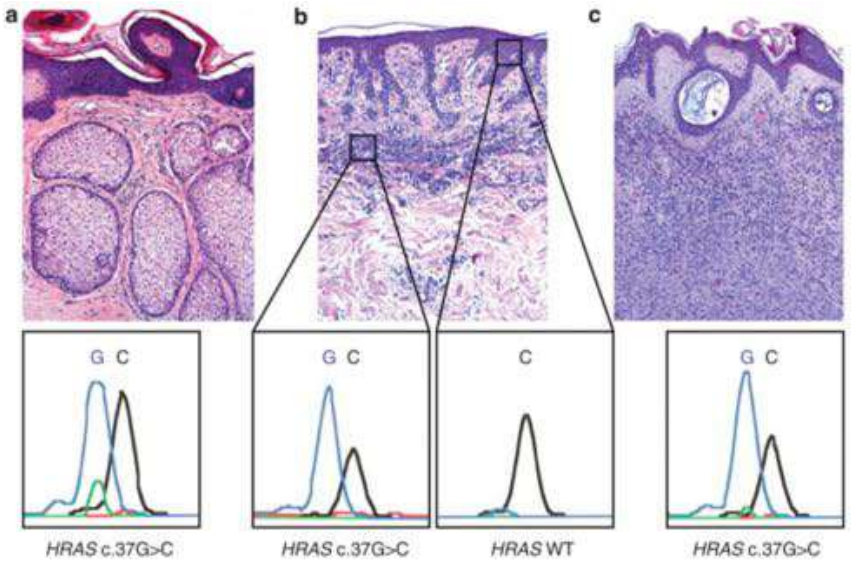
Mosaic RASopathies

- Phacomatosis pigmentokeratotica
  - Epidermal naevus syndrome
    - Sebaceous naevus
    - Speckled lentiginous naevus (naevus spilus)



Groesser L, Herschberger E, Sagrera A, Shwayder T, Flux K, Ehmann L, et al. Phacomatosis Pigmentokeratotica Is Caused by a Postzygotic HRAS Mutation in a Multipotent Progenitor Cell. Journal of Investigative Dermatology. 2013 Aug 1;133(8):1998–2003

Mosaic RASopathies



Groesser L, Herschberger E, Sagrera A, Shwayder T, Flux K, Ehmann L, et al. Phacomatosis Pigmentokeratotica Is Caused by a Postzygotic HRAS Mutation in a Multipotent Progenitor Cell. Journal of Investigative Dermatology. 2013 Aug 1;133(8):1998–2003

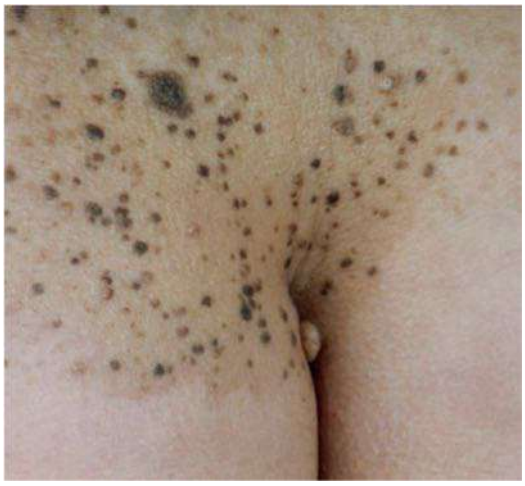
Mosaic RASopathies

- This study showed that, sebaceous naevi, speckled naevi (papular type) and congenital melanocytic naevi **all harbour same HRAS mutation within each patient**
- Highlights the pleiotropy (single gene influences 2 or more unrelated phenotypic traits) of HRAS mosaic mutation in the context of an **ectodermal progenitor cell**

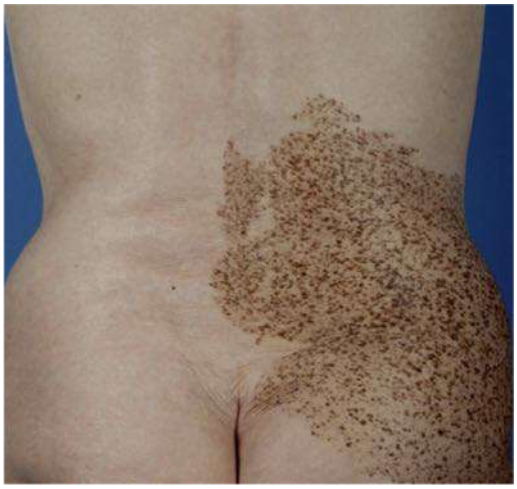
Speckled lentiginous naevi (naevus spilus)

SLN

Papular type



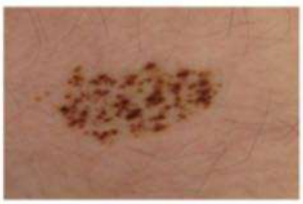
Macular type

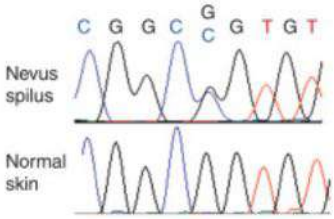


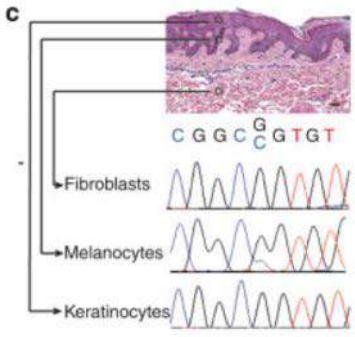
Happle, Rudolf. Mosaicism in Human Skin: Understanding Nevi, Nevoid Skin Disorders, and Cutaneous Neoplasia. 2013.

Small naevus spilus

- Khavari group in 2014 published work looking at HRAS in SLN/ naevus spilus



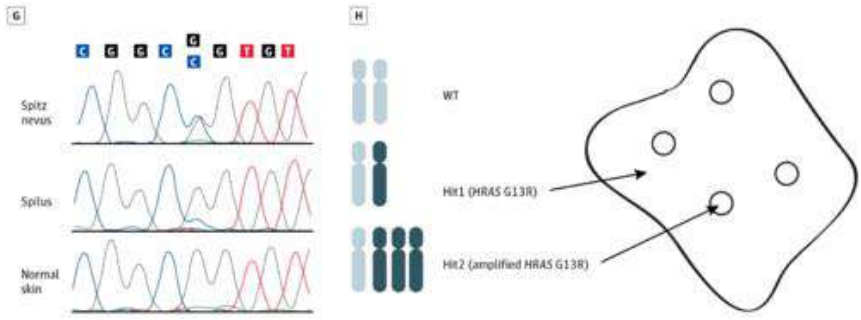




Sarin KY, McNiff JM, Kwok S, Kim J, Khavari PA. Activating HRAS Mutation in Nevus Spilus. Journal of Investigative Dermatology. 2014 Jun 1;134(6):1766–8

Naevus spilus and spitz naevi

- Khavari group in 2013 identified agminated spitz naevi in a naevus spilus
- They identified HRAS in the naevus spilus and a copy number increase in HRAS in the agminated spitz naevi



Sarin KY, Sun BK, Bangs CD, et al. Activating hras mutation in agminated spitz nevi arising in a nevus spilus. JAMA Dermatology. 2013 Sep 1;149(9):1077–81.

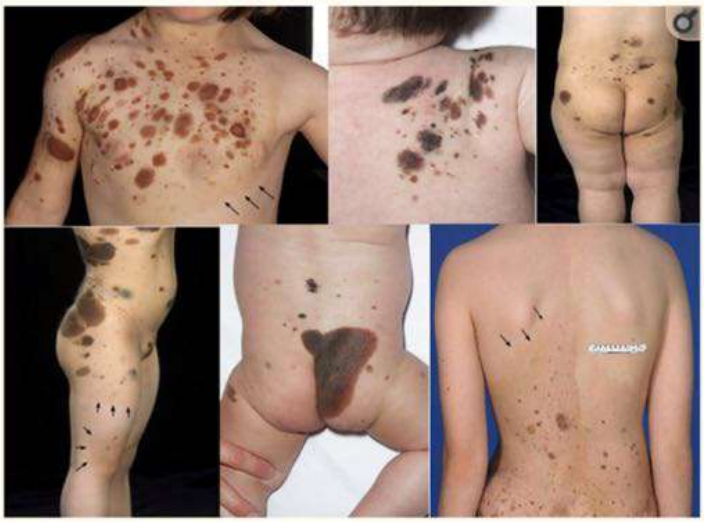
Naevus spilus/SLN and spitz naevi

- However they have yet to identify the secondary mutations that give rise to the diverse melanocytic neoplasms seen in SLN
- Or how HRAS mutations are represented differentially amongst the different types of SLN
  - Papular versus macular

Nevus Spilus-Type Congenital Melanocytic Naevus

Naevus Spilus-Type CMN

- Phenotype that has been described with café au lait macule and medium/large CMN



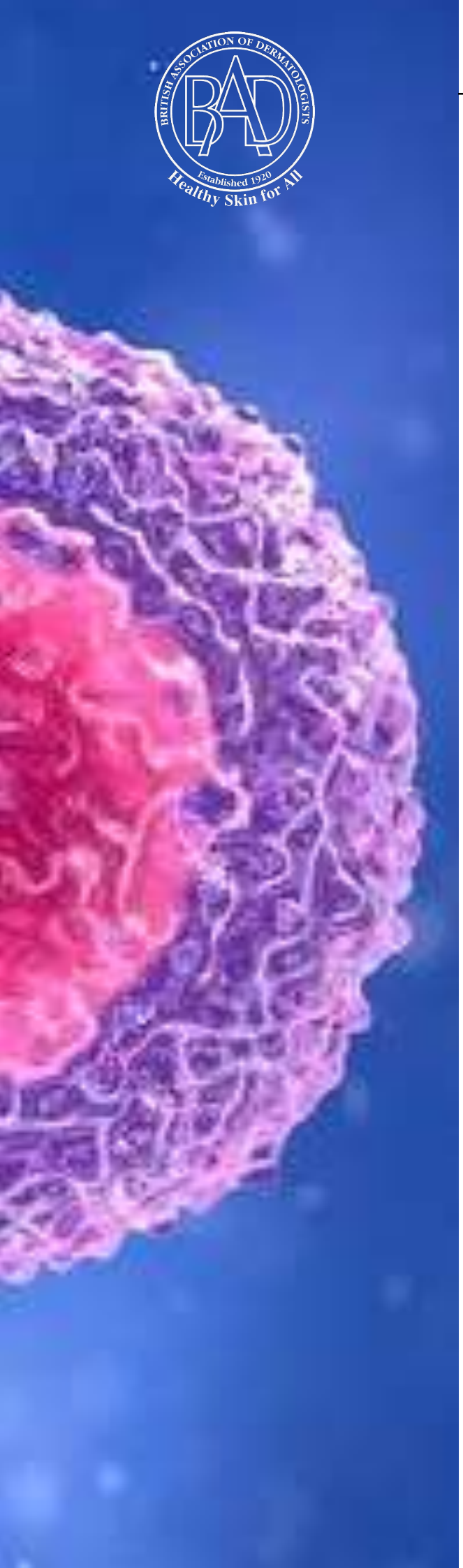
Kinsler VA, Krengel S, Riviere J-B, Waelchli R, Chapusot C, Al-Olabi L, et al. Next-Generation Sequencing of Nevus Spilus–Type Congenital Melanocytic Nevus: Exquisite Genotype–Phenotype Correlation in Mosaic RASopathies. The Journal of Investigative Dermatology. 2014 Oct;134(10):2658–60.

Naevus spilus-type CMN

- A single *NRAS* mutation was found in the café-au-lait macule and the superimposed CMN
- The missense activating mutation in *NRAS* was identified in the skin and absent from the blood

Summary

- Various patterns found on the skin, such as the lines of Blaschko, checkerboard and phylloid patterns, are well-known manifestations of mosaicism
- Nevoid proliferations make obvious mosaic presentations, particularly when they are extensive
- The molecular basis for these phenotypic expressions are continually being elucidated



MASSIVELY PARALLEL SEQUENCING TECHNOLOGIES

Jonathan Coxhead

Massively parallel sequencing *technologies (2018 overview)*

Dr Jonathan Coxhead

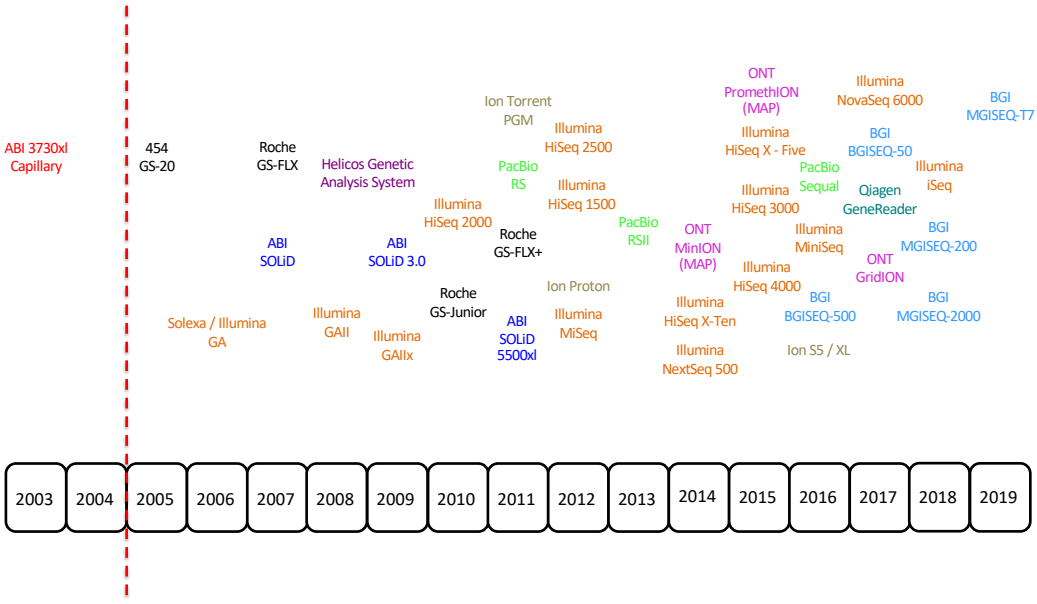
12th November 2018

Overview

- NGS glossary and terms
- Importance of sample QC
- Commercially available NGS technologies
- Massively parallel sequencing is here to stay – keeping up to date

[***Use of images in this presentation*** *– unless otherwise stated, the images related to the technologies described in this presentation have been taken from publicly available marketing material associated with the relevant technology manufacturer*]

Technology >10 years of NGS

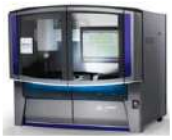


Technology - commercial platforms

NGS Past

Helicos
Bankruptcy 2012

Roche 454
Discontinued 2016



Life Technologies SOLiD

NGS Present

Illumina

http://www.illumina.com

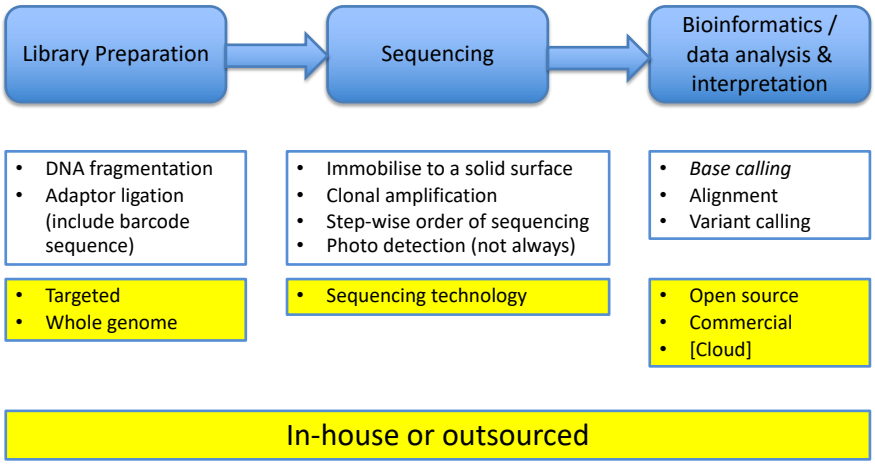
Life Technologies Ion

http://www.lifetechnologies.com/uk/

Pacific Biosciences

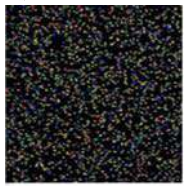
http://www.pacificbiosciences.com

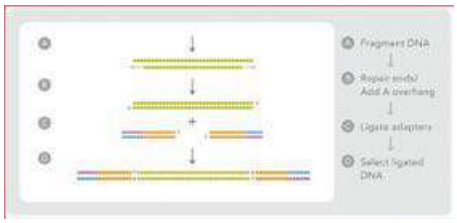
Sequencing pipeline - broken down

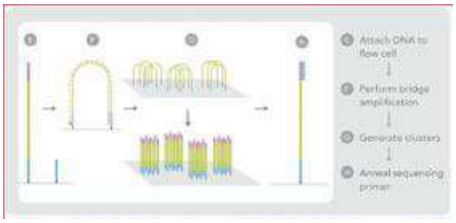


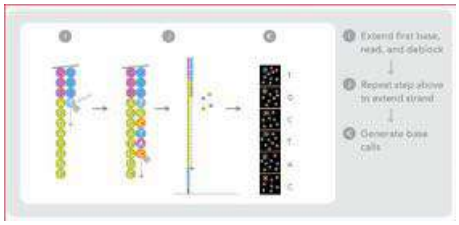
Illumina sequencing chemistry

- Fragment DNA
- Adaptor ligation
- Cluster formation
- Sequencing









http://youtu.be/womKfikWlxM

Illumina throughput specifications

Instrument / Format	Read Length (bp)	No. Reads	Output	Run time	Fluorophore chemistry	Flow cell Architecture
iSeq 100	2 x 150	4 million	1.2 Gb	17.5 hours	2 colour	Patterned
MiniSeq High	*2 x 150*	*25 million*	*7.5 Gb*	*24 hours*	*2 colour*	*Random*
MiniSeq Mid	*2 x 150*	*8 million*	*2.4 Gb*	*17 hours*	*2 colour*	*Random*
MiSeq v3	2 x 300	25 million	15 Gb	65 hours	4 colour	Random
NextSeq 500 High	2 x 150	400 million	100 Gb	29 hours	2 colour	Random
NextSeq 500 Mid	2 x 150	130 million	36 Gb	26 hours	2 colour	Random
HiSeq 2500 v4 High	*2 x 125*	*4 billion*	*1 Tb*	*6 days*	*4 colour*	*Random*
HiSeq 2500 v4 Rapid	*2 x 250*	*600 million*	*300 Gb*	*60 hours*	*4 colour*	*Random*
HiSeq 3000	*2 x 150*	*2.5 billion*	*750 Gb*	*3.5 days*	*4 colour*	*Patterned*
HiSeq 4000	*2 x 150*	*5 billion*	*1.5 Tb*	*3.5 days*	*4 colour*	*Patterned*
*HiSeq X * (x5 or x10)*	*2 x 150*	*6 billion*	*1.8 Tb*	*3 days*	*4 colour*	*Patterned*
NovaSeq S1	2 x 150	1.6 billion	1 Tb	25 hours	2 colour	Patterned
NovaSeq S2	2 x 150	4.1 billion	3 Tb	36 hours	2 colour	Patterned
NovaSeq S4	2 x 150	10 billion	6 Tb	45 hours	2 colour	Patterned

Illumina sequencing capacity

MiniSeq and MiSeq v4
- Amplicon
- Gene panels
- Microbial genomes
- Metagenomics
- mtDNA sequencing

NextSeq 500 Mid Output / High Output
- 40 Gene Expression
- 3 - 10 mRNA-Seq
- 20 Gene panels
- 3 – 9 exomes
- 1 genome

HiSeq 2500 Rapid Run / High Output
- 40 – 260 ChIP-Seq
- 15 - 100 mRNA-Seq
- 20 - 150 exomes
- 1 – 10 genomes

HiSeq 3000/4000
- 50 - 100 transcriptomes
- 90 - 180 exomes
- 6 -12 human genomes

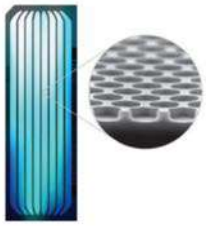
HiSeq X
- 16 human genomes / per instrument

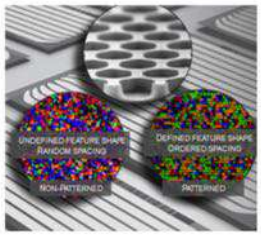
Pros / Cons

- Range of platform options
- Widely adopted (USA colleges etc.)
- Where's the competition?
- Poor for amplicons
- Substitution error

Illumina – $1000 genome and population scale sequencing

- 18k genomes / year
- Patterned flow cell





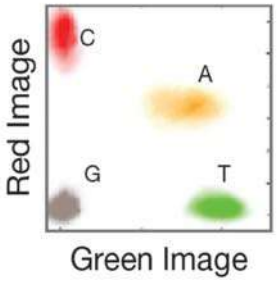
- "Genomics England machine"

- Exclusion amplification chemistry
- Up-graded camera and lasers

Only cost effective if run consistently!

Illumina – Flexible affordable platforms

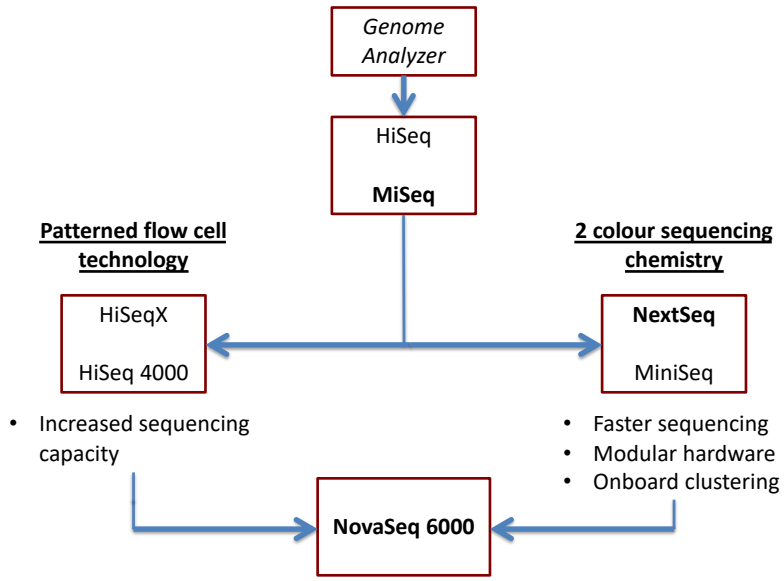
- Whole genome bench-top sequencing



- Budget bench-top sequencing

- Tumour profiling
- Germline testing
- Expression profiling

Illumina instrument evolution



Illumina NovaSeq 6000

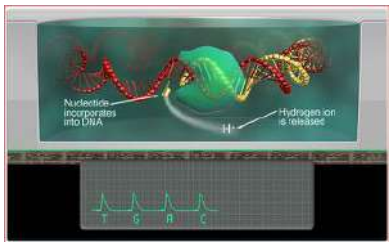
†48 human genomes per run at 30x

- Launched January 2017
  - Large dataset applications –†WGS, Ultra-deep WES and Tumour-Normal profiling
  - Large sample projects - WES, RNA-Seq and Single Cell studies
  - Single or dual flow cells; 3 flow cell sizes S1, S2 and S4; 1.6 – 10 billion reads or max output 0.5 – 3 Tb per flow cell.
  - Read length 2 x 50 bp, 100 bp or 150 bp

Ion Torrent & Proton sequencing chemistry (Life Technologies)

http://youtu.be/WYBzbxIfuKs

- Fragment DNA
- Adaptor ligation
- Bead Capture
- emPCR
- Semi-conductor sequencing



Ion Torrent throughput specifications

Instrument / Format	Read Length (bp)	No. Reads	Output	Run time
PGM 314	400	600 thousand	100 Mb	4 hours
PGM 316	400	3 million	1 Gb	5 hours
PGM 318	400	5 million	2 Gb	7 hours
Proton PI	200	82 million	10 Gb	4 hours
Ion S5 510	400	3 million	1 Gb	4 hours
Ion S5 520	400	6 million	2 Gb	4 hours
Ion S5 530	400	20 million	8 Gb	4 hours
Ion S5 540	200	80 million	15 Gb	2.5 hours

Ion Torrent sequencing capacity

- Amplicon
- Small genomes
- Small gene panels
- Gene expression (targeted RNA)

- Amplicons
- Gene panels
- 2 exomes
- 48 – 384 16S metagenomics

- Gene expression (targeted RNA)
- 1 – 3 exomes
- 1 – 4 transcriptomes
- 2 – 4 ChIP-Seq

Pros / Cons

- Fast run time (doesn't take into account prep)
- Main instrument relatively simple – no expensive optics
- Diagnostic market
- Small user base – bioinformatics & technical expertise
- Homopolymer error
- At the moment limited for human WGS

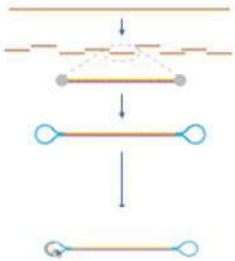
PacBio sequencing chemistry – SMRT (single molecule real-time)

DNA sample

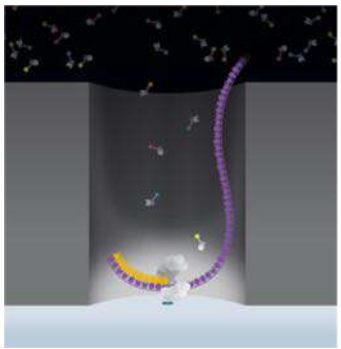
Fragmentation

End Repair

Adaptor Ligation

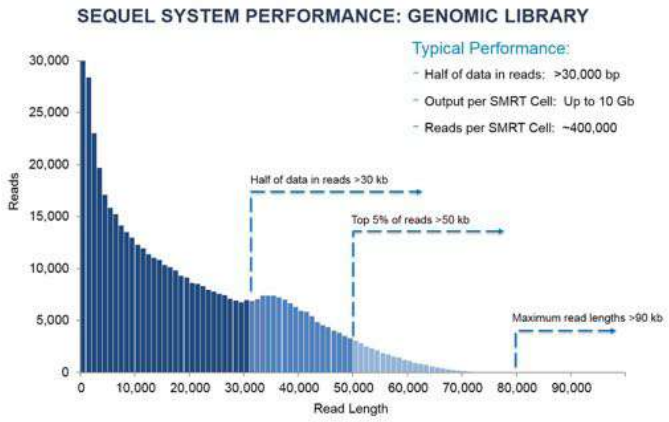


http://youtu.be/NHCJ8PtYCFc



Primer Annealing and Polymerase Binding to SMRTbell Template

PacBio throughput specifications



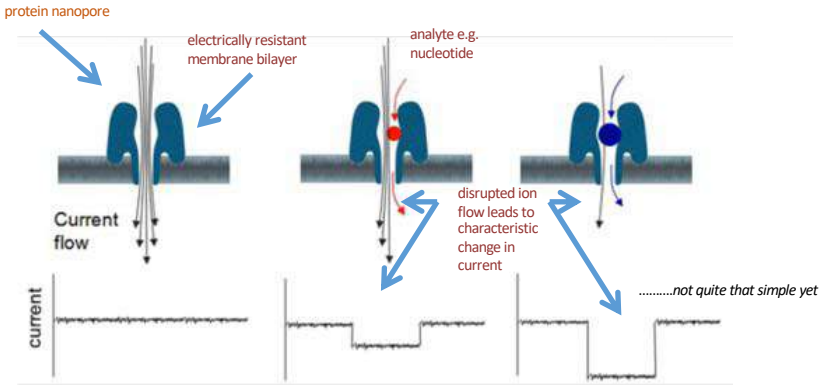
- V2 Chemistry 30 min – 20 hour run time, 1 – 12 SMRT Cells per run
- Continuous Long Read (CLR) 85-89% accuracy
- Stochastic error rate
- Circular Consensus Sequence (CCS) >99.999% accuracy (at 30x)

PacBio (RSII and Sequel) - applications

Modest number of long reads (as opposed to a large number of short reads)

- **De Novo Assembly**
  - genome finishing
- **Targeted Sequencing**
  - Haplotype phasing
  - Full length transcripts
- **Base Modification detection**
  - epigenetics
- **Microbial genomes**
- **Emerging technology in cancer genomics**
- **…human WGS?**

Oxford Nanopore Technologies



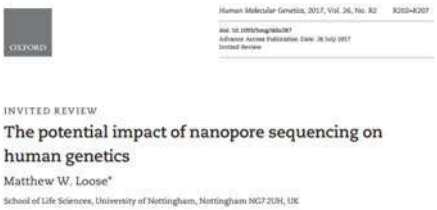
- Biological nanopores in the form of propriety pore-forming proteins, similar to those found in nature that transport ions and molecules through cell membranes
- Each nanopore embedded in a polymer membrane across the top of a microwell with its own electrode
- Multiple microwells per array chip – each nanopore sequencing independently
- Technology designed so that data analysis is simultaneous - sequencing can be stopped when enough data has been collected
- https://nanoporetech.com/applications/dna-nanopore-sequencing

Nanopore sequencing technologies

MinION
- Run time 1 min - 48 hrs
- Output 10-20 Gb
- Up to 512 channels

GridION
- Run time 1 min - 48 hrs
- Output 50-100 Gb
- Up to 2560 channels

PromethION
- Run time 1 min - 64 hrs
- Output 4.3-6 Tb
- Up to 144k channels



INVITED REVIEW

The potential impact of nanopore sequencing on human genetics

Matthew W. Loose*

School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, UK

Necessary to evaluate based on personal experience with relevant application / sample, for instance accuracy could be 85%-95% (or lower)

- Sample input 10 pg – 1 µg
- Min sample prep time = 10 mins
- Time to first usable read = 2 mins
- Read length = fragment length
- Raw read accuracy = up to 99%

End-to-end solution

Roche Diagnostics · illumina · ThermoFisher SCIENTIFIC · QIAGEN

Clinical Market
$$$$$$$

DNA extraction >>> Candidate gene list


Ion PGM Dx


MiSeq Dx


NextSeq 550 Dx


Qiagen GeneReader

Technology - keeping up to date

- Press releases - news outlets - social media

GEN Genetic Engineering & Biotechnology News
Biotechnology from bench to business
http://www.genengnews.com/
@Genbio

Bio-IT World
Next-Gen Technology • Big Data • Personalized Medicine
http://www.bio-itworld.com/
@bioitworld

genomeweb
https://www.genomeweb.com/
@DailyNewsGW

- Journals

nature biotechnology

nature methods
Techniques for life scientists and chemists

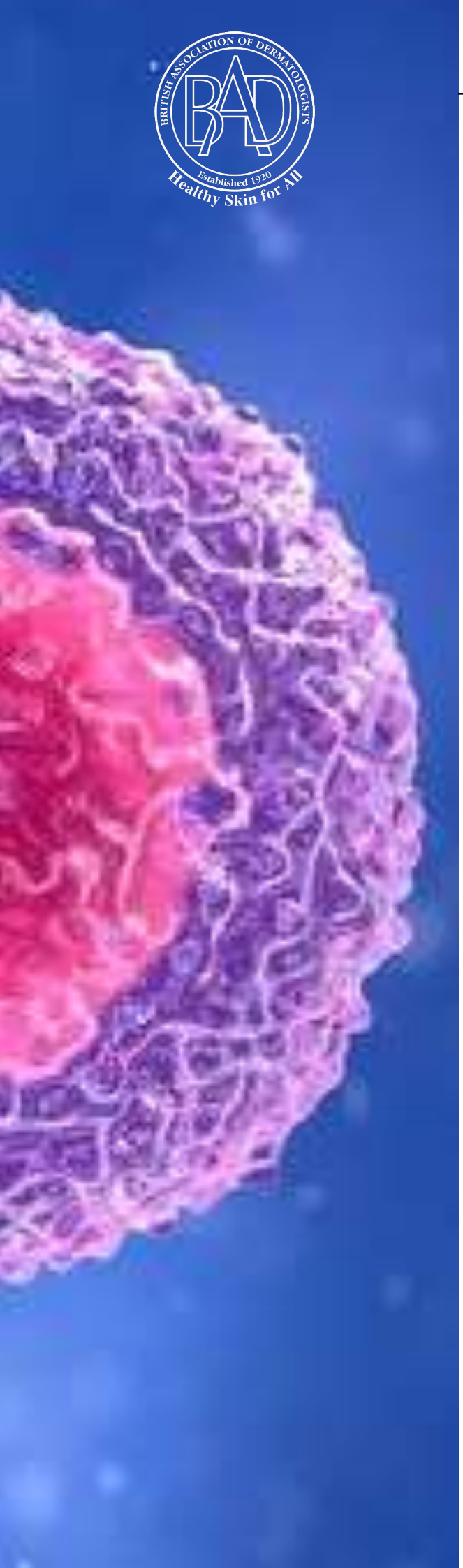
News story

Matt Hancock announces ambition to map 5 million genomes

The NHS Genomic Medicine Service is the first national genomic healthcare service in the world and will allow faster diagnosis and personalised care.

Published 2 October 2018
From: Department of Health and Social Care

https://www.gov.uk/government/news/matt-hancock-announces-ambition-to-map-5-million-genomes



BIOINFORMATICS

Simon Cockell

Research Techniques Course 2018

Simon Cockell

08 November, 2018

Introduction to R

R is a programming language and environment for statistical analysis and data visualisation. It is used extensively in bioinformatics because it enables the manipulation and analysis of very large data sets.

R is an extensible environment and new functionality can be added to the core language via installation of packages. There are large existing repositories of these packages for R, including the Comprehensive R Archive Network (CRAN)[1] which includes thousands of packages covering many use-cases, and Bioconductor[2] which caters specifically for the analysis of biological data.

Bioconductor

Bioconductor is an open source software project for R. It is a collection related R packages, which you can install and use in your code. These packages are tailored to perform a set of specific tasks based around a particular type of analysis.

As well as packages, the Bioconductor site also provides documentation for each package, often a brief vignette and a more comprehensive user guide.

Tidyverse

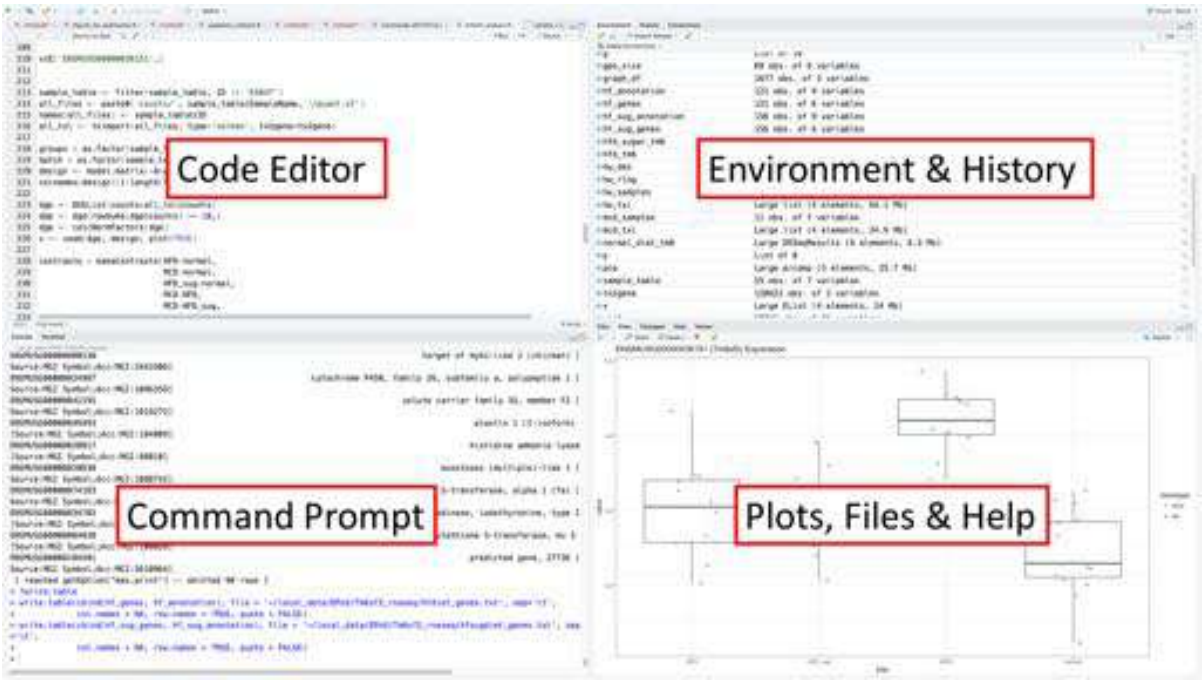
Tidyverse[3] is set of packages available through CRAN. It describes itself as "an opinionated collection of R packages designed for data science". Components of the Tidyverse provide convenient methods for reading, writing, visualising and organising data.

RStudio

RStudio[4] is an Integrated Development Enviornment (IDE) which supports the development and running of R programs. It has a number of convenience features that make developing R code much more user-friendly.

The RStudio interface is split into four panes as shown:

[1] https://cran.r-project.org
[2] https://bioconductor.org
[3] https://www.tidyverse.org
[4] https://www.rstudio.com



Using R

We will be using R as a command-driven data analysis environment. R is a Turing-complete[5] programming language capable of much more than will be demonstrated today, but that is beyond the scope of this course.

The commands we will use in R take a general form:

```
command(arguments)
```

The name of the command to be executed is followed by a paranthetic, comma-separated list of arguments that provide input, or modify the behaviour of that command. The results of a command can be stored as a *variable* by using the assignment operator (`=`):

```
x = command(arguments)
```

Help on a command, and the arguments it takes, can be found using the `?` operator before the name of the command:

```
?command
```

This help will open in the bottom right-hand pane of RStudio.

Installing Packages

Packages provide vital extensibility to the core functionality of R. We will be using Bioconductor's package installer as a universal interface to a large number of package repositories (including Bioconductor, CRAN and Github). To install the installer, simply execute the following command in the R Console (bottom left pane of RStudio).

```
install.packages('BiocManager')
library(BiocManager)
```

[5] https://en.wikipedia.org/wiki/Turing_completeness

Now we have the `BiocManager` package, we can use this to install all of the other packages we need for today's exercises.

```
install(c('devtools', 'pheatmap',
          'Bioinformatics-Support-Unit/bsudata',
          'DESeq2', 'tidyverse'))
```

In order to make use of these packages, they must be included in our session with the `library()` command.

```
library(tidyverse)
library(DESeq2)
library(pheatmap)
library(bsudata)
```

Introduction to RNA-Seq

RNA-Seq, or Whole Transcriptome Shotgun Sequencing (WTSS) refers to the sequencing of cDNA in order to profile the RNA content of a sample. The technique takes advantage of a simple assumption – that the number of reads that map to a particular gene in a sequencing experiment is in direct proportion to both its length, and the amount of that molecule that is present in the sample. So if two RNAs of equal length are present at a ratio of 2:1 in the sample, then we will retrieve twice as many reads for one as we will for the other when we sequence the sample. So, given two samples we can normalise for transcript lengths and total number of reads, and work out which species are differentially expressed between the two samples.

RNA-Seq is often seen as a replacement for the very popular microarray approach to studying gene expression, and while the two approaches have different strengths and weaknesses, it is generally true that the data from RNA-Seq correlates better with qPCR data (the "gold standard" for measuring gene expression data) and the sheer depth of the data generated makes RNA-Seq the better choice in many circumstances - assuming the budget allows.

This also means that many of the mistakes, with regards to experimental design, that were made in the early days of microarrays are being repeated in earnest with RNA-Seq experiments. Good experimental design is out of the scope of this course, but come and speak to the BSU – as we can help you with experimental design. One of the most common false assumptions is that with RNA-Seq you don't need replicates. This is simply untrue, we still need to model the variance of the population in order to return meaningful statistics and consequently it's advisable to do at least 4 biological replicates per condition minimum for cell lines, more for mice/rats and many more for humans. Unlike with microarray analysis however technical replicates are seldom required, as despite the stochastic nature of sequencing runs they tend to be highly reproducible.

TPM

TPM stands for Transcripts per Million (mapped reads), and it is a way of expressing the normalised read counts for each transcript in an RNA-Seq experiment. In order to calculate TPMs for a sample, you do the following:

1. Divide the read counts by the length of each gene in kilobases. This gives you reads per kilobase (RPK).
2. Count up all the RPK values in a sample and divide this number by 1,000,000. This is your "per million" scaling factor.
3. Divide the RPK values by the "per million" scaling factor. This gives you TPM.

In practice, however, we rarely need to compare the expression levels of different genes to one another, we are usually comparing the expression of Gene A in sample 1 to Gene A in sample 2 and so on. Therefore, normalisation based on gene length as well as total number of reads is unnecessary. Most packages in Bioconductor normalise based on read count across samples.

The Dataset

For this session we will be working with a human cell line RNA-Seq dataset in R. The dataset has six conditions, 3 treatments at each of 2 timepoints. For each of these we have four replicates (except one condition, with only 3). The sequencing used in this experiment is 100-bp paired-end data from an Illumina sequencer (the HiSeq 2500).

Quantification of RNA-Seq Data

Modern RNA-Seq analysis procedures tend to avoid an alignment step, which is computationally costly, slow, and results in very large output files. These output files are largely redundant, given that they replicate the contents of the raw FASTQ files.

Rather than alignment, we have used Salmon[6], a tool which performs 'quasi-alignment' to quantify transcript expression via read abundances. For completeness, the commands used to generate the quantification data are included below, as a BASH script.

```
#!/bin/bash
wget ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_27/gencode.v27.transcripts.fa.gz
salmon index -i salmon.idx -t gencode.v27.transcripts.fa
vals=($(seq 1 1 23))
for i in ${vals[@]}; do
  salmon quant -i /data/rna-seq-course/salmon.idx -l A \
    -1 fastq/Sample${i}_R1_001.fastq.gz \
    -2 fastq/Sample${i}_R2_001.fastq.gz \
    -o ~/rna-seq/counts/Sample${i}
done
```

This raw count data is then imported into R as a gene-level count matrix, via the Bioconductor package `tximport`.

```
library(tximport)
# where count_files is a named vector of file names:
quant_table = tximport(count_files,
                       type="salmon",
                       tx2gene=gene_map,
                       ignoreTxVersion=TRUE)
```

To analyse the count data for differentially expressed genes, we use the `DESeq2` package[7]. `DESeq2` uses a model based on the negative binomial distribution for testing differential expression among RNA-Seq expression data.

Again, we provide a reference implementation here, for information.

```
library(DESeq2)
deseq_data = DESeqDataSetFromTximport(txi=quant_table,
          colData=sample_table, design = ~ treatment)
deseq_data = DESeq(deseq_data)
```

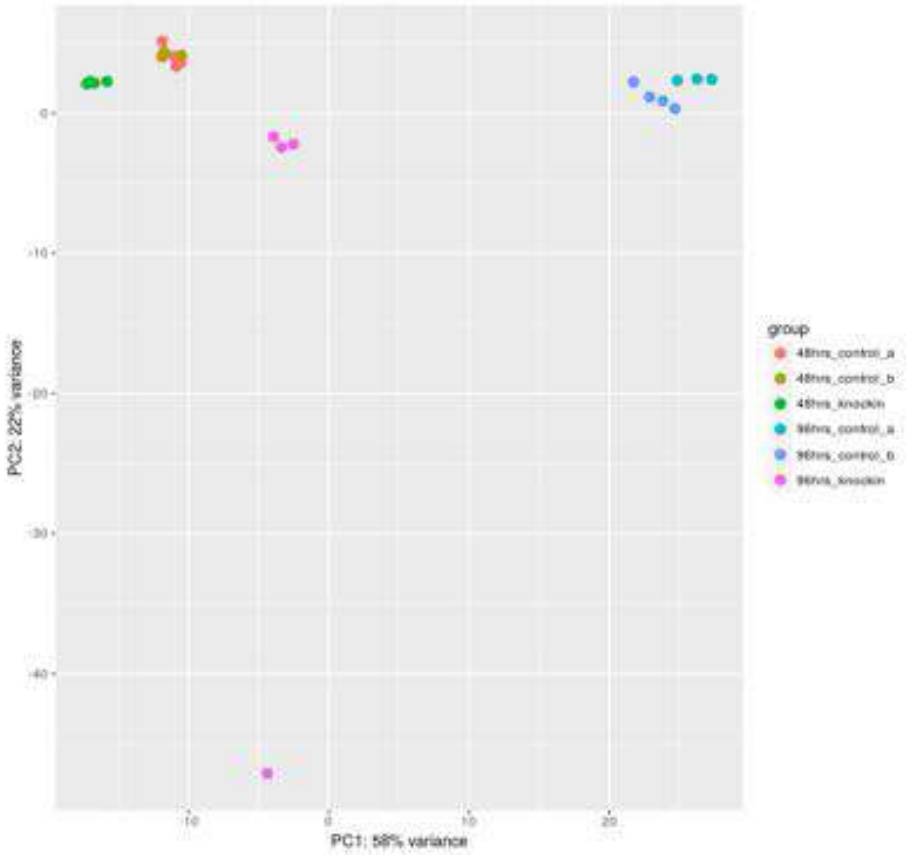
Principal Component Analysis

At this point we can examine the ordination of the samples, to get some idea of how similar samples are to one another, and whether any need to be excluded as outliers. In general, dimensionality reduction techniques

[6] https://www.nature.com/articles/nmeth.4197
[7] https://genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0550-8

such as Prinicpal Component Analysis (PCA) are used for this[8].

```
rlog_data = rlogTransformation(deseq_data, blind=TRUE)
plotPCA(rlog_data, intgroup=c("treatment"))
```



The PCA plot above demonstrates that we have a clear outlier among our samples - the magenta point at the bottom of the plot is almost entirely responsible for the variation we see in the second principal component (22% of the variation in the entire dataset), and clusters nowhere near the other samples with which it belongs in a group. For all analysis going forward, we will remove this sample from the raw data, and re-process.

Determining Differentially Expressed Genes

`DESeq2` includes a `results` function, which we can use to extract differentially expressed genes for the comparisons defined by our experimental design. The primary use of this function involves 2 arguments:

1. The dataset you want to analyse (created as per the above)
2. A list of the conditions you want to analyse - in the simplest form this consists of 3 things:
    i. The column of the sample table containing the conditions you care about
    ii. The numerator condition for calculating differential expression
    iii. The denominator condition for calculating differential expression (by convention the control)

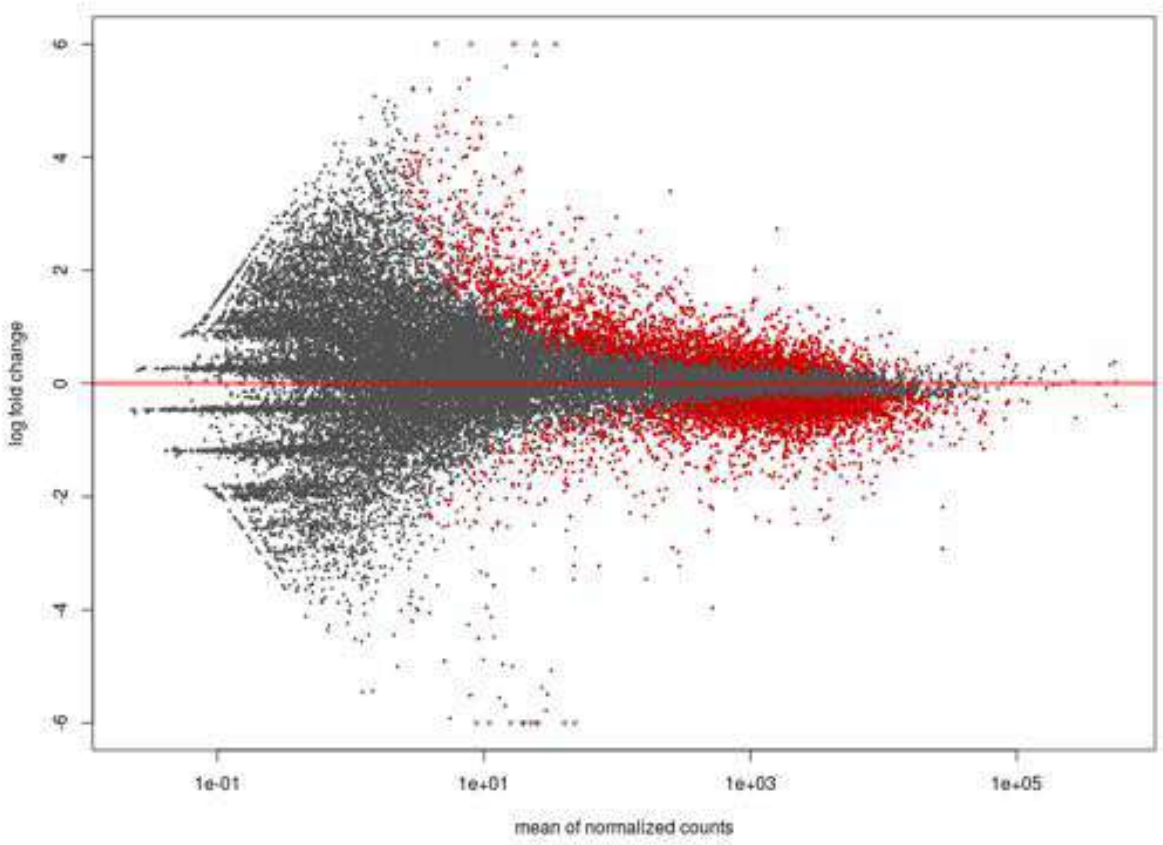
```
result_table = results(deseq_data,
                       contrast = c("treatment", "96hrs_knockin", "96hrs_control_b"))
```

Finally for this introduction, an MA plot provides a useful overview of a two-group comparison (such as we have here). The MA plot has mean normalised count on the x-axis, and log2 fold change on the y, with

[8] https://www.nature.com/articles/nbt0308-303

points coloured according to whether they are significantly differentially expressed (depending on defined cut-offs). `plotMA` is a handy `DESeq2` function for producing the plot:

```
plotMA(result_table, alpha=0.05, ylim=c(-6,6))
```



Making Heatmaps from RNA-Seq data

The Data

All the data we will need for today's exercise is provided in the `bsudata` package you installed above. You can list, attach, and take a look at any of the data in the package:

```
library(bsudata)
# list available data
data(package="bsudata")
# attach specific dataset
data("count_table")
# take a peek at this data
head(count_table)
```

Gene Expression Heatmap

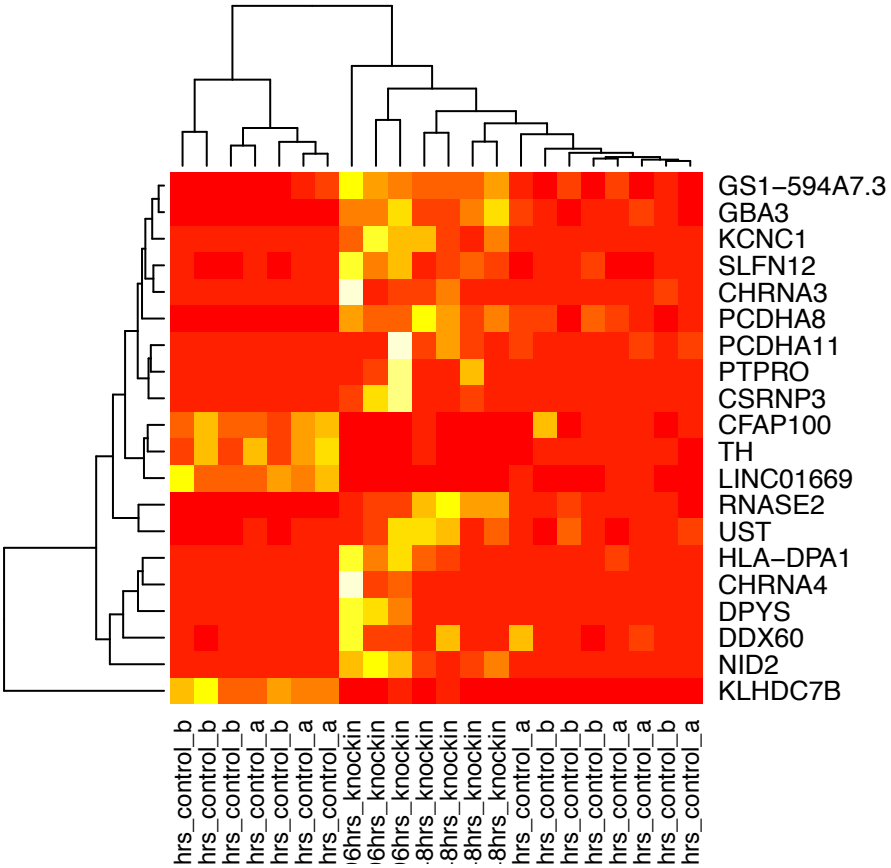
We're going to use data, analysed according to the workflow detailed above, to make heatmaps of gene expression values and fold changes for the top 20 differentially expressed genes for one comparison (96 hrs knock-in vs control B). All the data we need for producing these heatmaps is provided in the `bsudata` package. Briefly, this is how these objects are prepared:

```
# Get the normalised count table
data("count_table")
# Get the full list of differentially expressed genes
data("T96_vs_B_sig")
# Select the 'top 20' - the genes are ordered by fold change
top20_genes = slice(T96_vs_B_sig, 1:20)
# Extract the counts for the top 20 genes
top20_ensembl = pull(top20_genes, ensembl_geneid)
top20_counts = slice(count_table, match(top20_ensembl, count_table$ensembl_geneid))
# Transform these counts into a matrix, for making heatmaps
top20_matrix = select(top20_counts, -starts_with("ensembl")) %>% as.matrix
rownames(top20_matrix) = pull(top20_genes, symbol)
```

This matrix is available in the `bsudata` package as the object `top20_matrix`. We can use this directly for drawing heatmaps.

To begin with, use R's built in `heatmap` function to produce a default plot.

```
data("top20_matrix")
heatmap(top20_matrix)
```

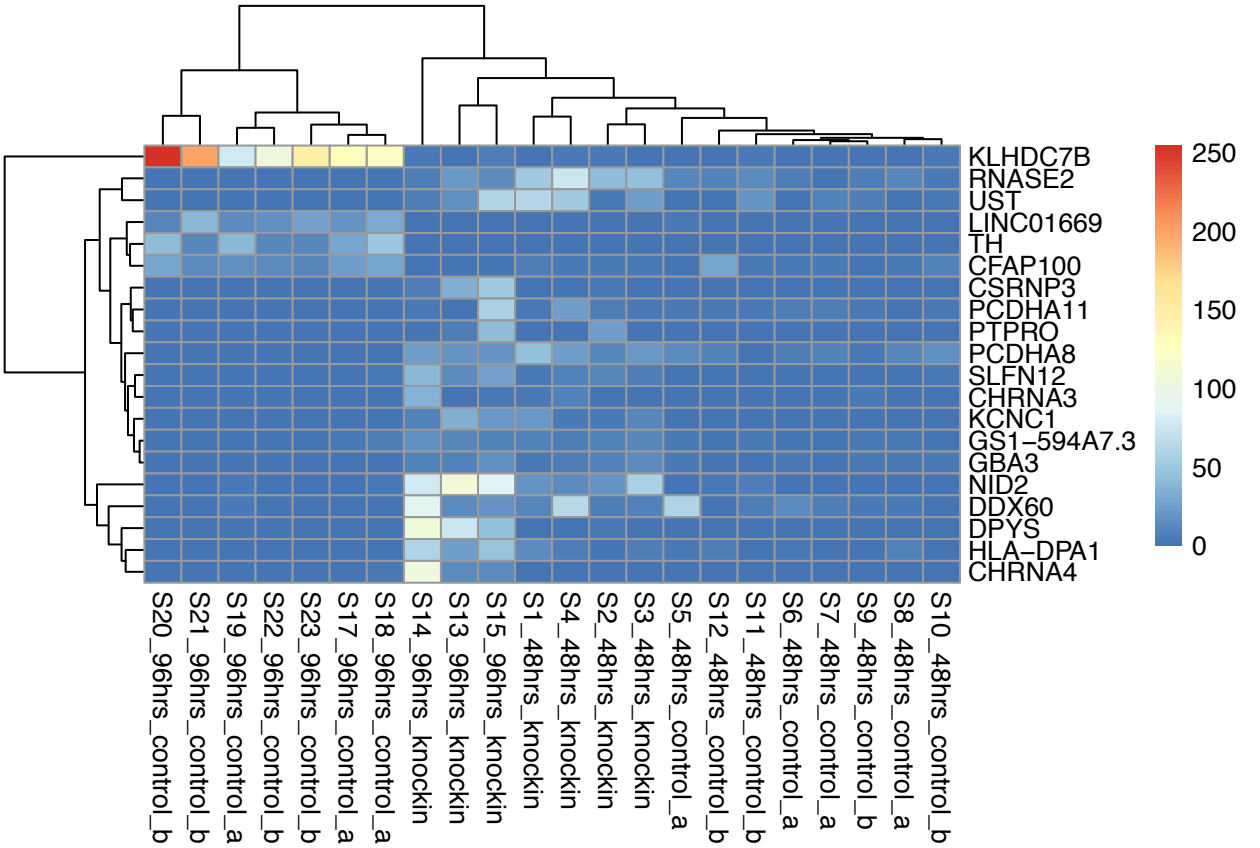


This default function has several problems that it would be good to resolve.

1. Every row is scaled to its own minimum and maximum, making comparisons between rows impossible
2. There is no colour scale provided. How do we know what each colour in the plot represents?
3. The `heatmap` function doesn't provide us with easy ways of resolving these issues (it is difficult to configure)

For these reasons, we will use an alternative heatmap implementation for the remainder of the session. There are many of these available to R users, including `heatmap.2` (in the `gplots` package)[9], `ComplexHeatmap`[10] and `superheat`[11]. For the purposes of this tutorial, we're going to use the `pheatmap` package[12], which you should have already installed.

```
library(pheatmap)
pheatmap(top20_matrix)
```



The defaults of `pheatmap` are preferable to base `heatmap`, but we still have a couple of issues to address here. Primarily:

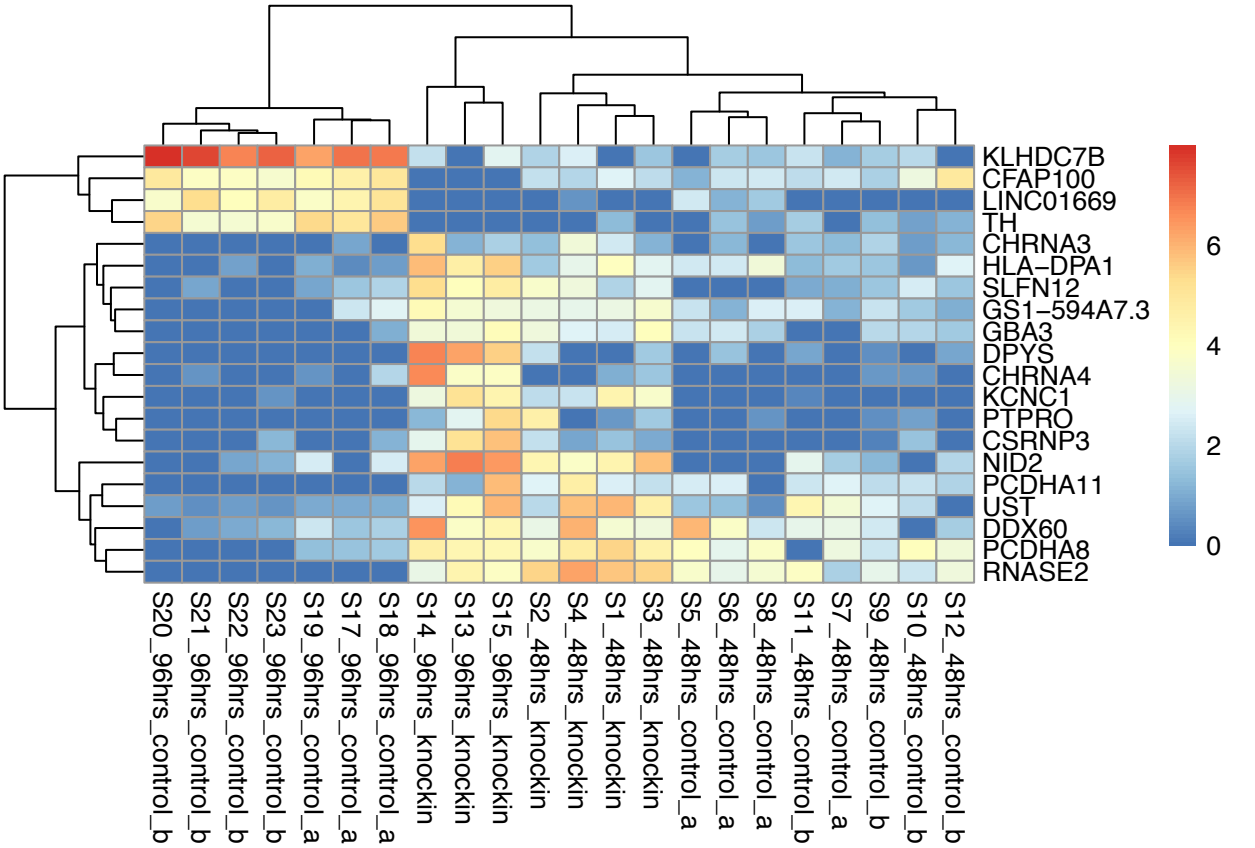
1. The range of the data is very skewed - 1 gene (KLHDC7B) has some very large expression values compared to the rest and this dominates the colour scale
2. `pheatmap` uses a diverging colour scale by default - this is good if the data centres around an inflection point and has 'up' and 'down' directionality, but not so good for this kind of numerically sequential data.

To address the first of these issues, we can apply a simple transformation to the data. We are going to use a logarithmic transform (`log2`). In order that this doesn't create problems where we have zeros in our data we

[9] https://cran.r-project.org/web/packages/gplots/index.html
[10] https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html
[11] https://rlbarter.github.io/superheat/index.html
[12] https://cran.r-project.org/web/packages/pheatmap/index.html

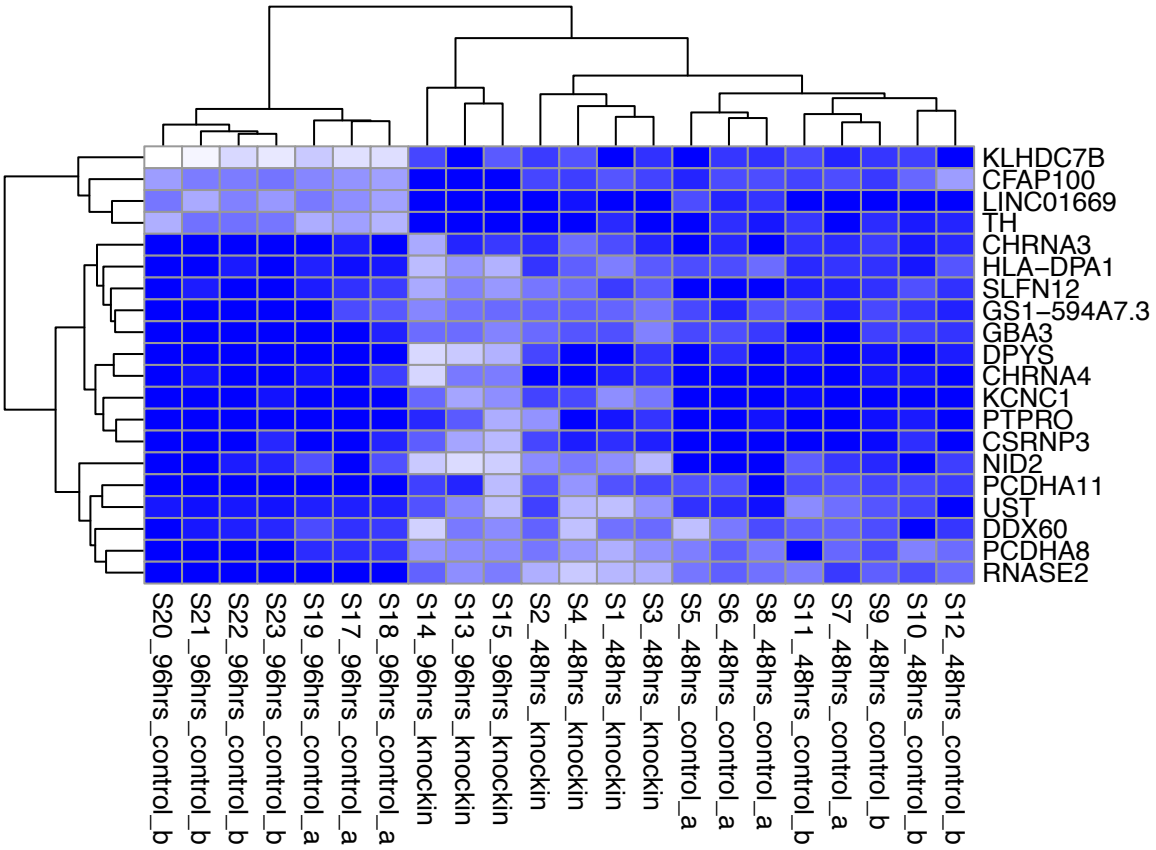
will add 1 to every observation:

```
transformed_counts = log2(top20_matrix + 1)
pheatmap(transformed_counts)
```



This transformation reveals more detail in our data. Now, in order to address the colour scheme, we must create a new list of colours, using the `colorpanel` function from the `gplots` package. This lets us rapidly create simple colour schemes from 2 or three named colours ('low', 'mid' and 'high' - mid is optional). R has a large number of named colours, you can get a list of them using the `colors()` function.

```
library(gplots)
mycolours = colorpanel(n=100, low='blue', high='white')
pheatmap(transformed_counts, color = mycolours)
```

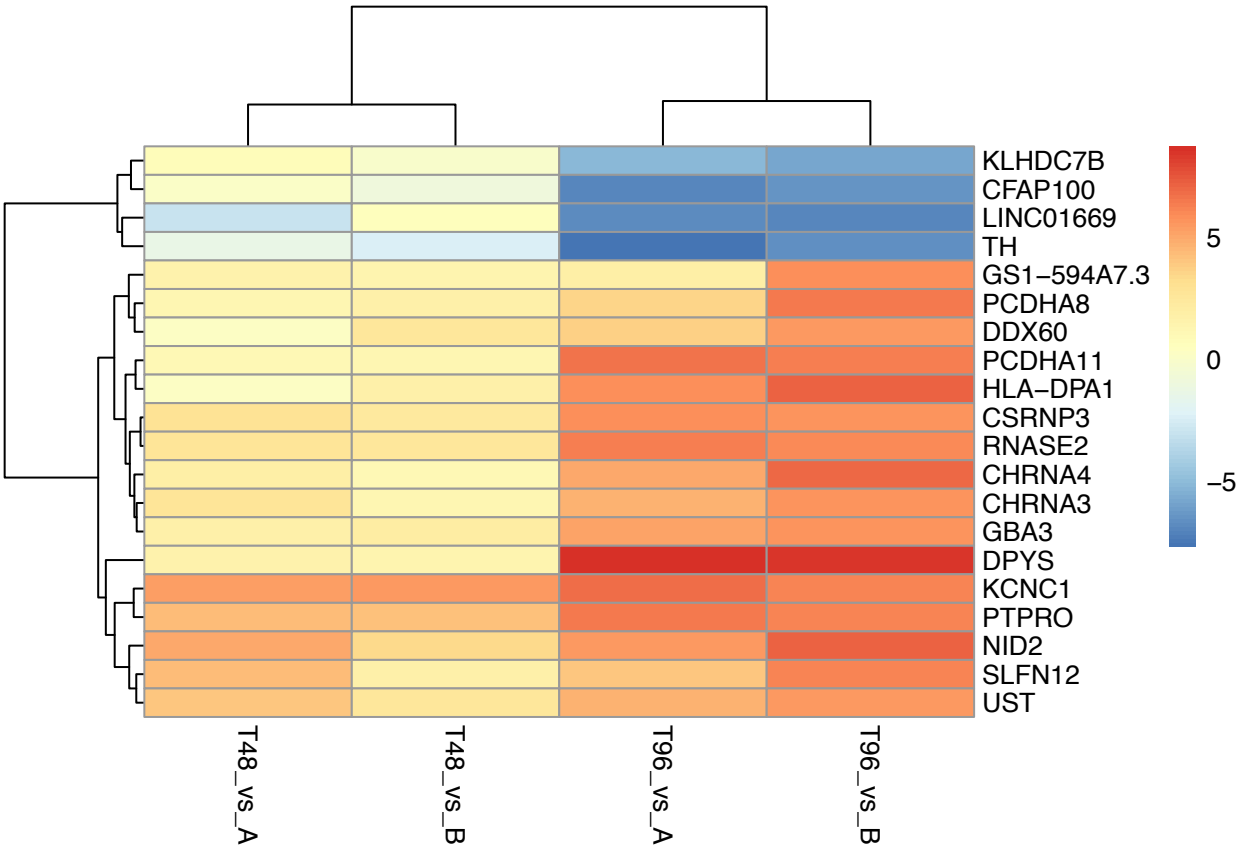


Fold Change Heatmap

In an experiment with many comparisons, it can be useful and informative to see how a set of genes are varying across all the analyses. For this, a fold change heatmap can be very useful. In the `bsudata` package, there is a matrix that contains the fold changes for the top 20 genes plotted above, across 4 comparisons in the experiment (knock-in vs control A and control B at 48 and 96 hours).

To plot a basic heatmap from this data:

```
data("fc_matrix")
pheatmap(fc_matrix)
```

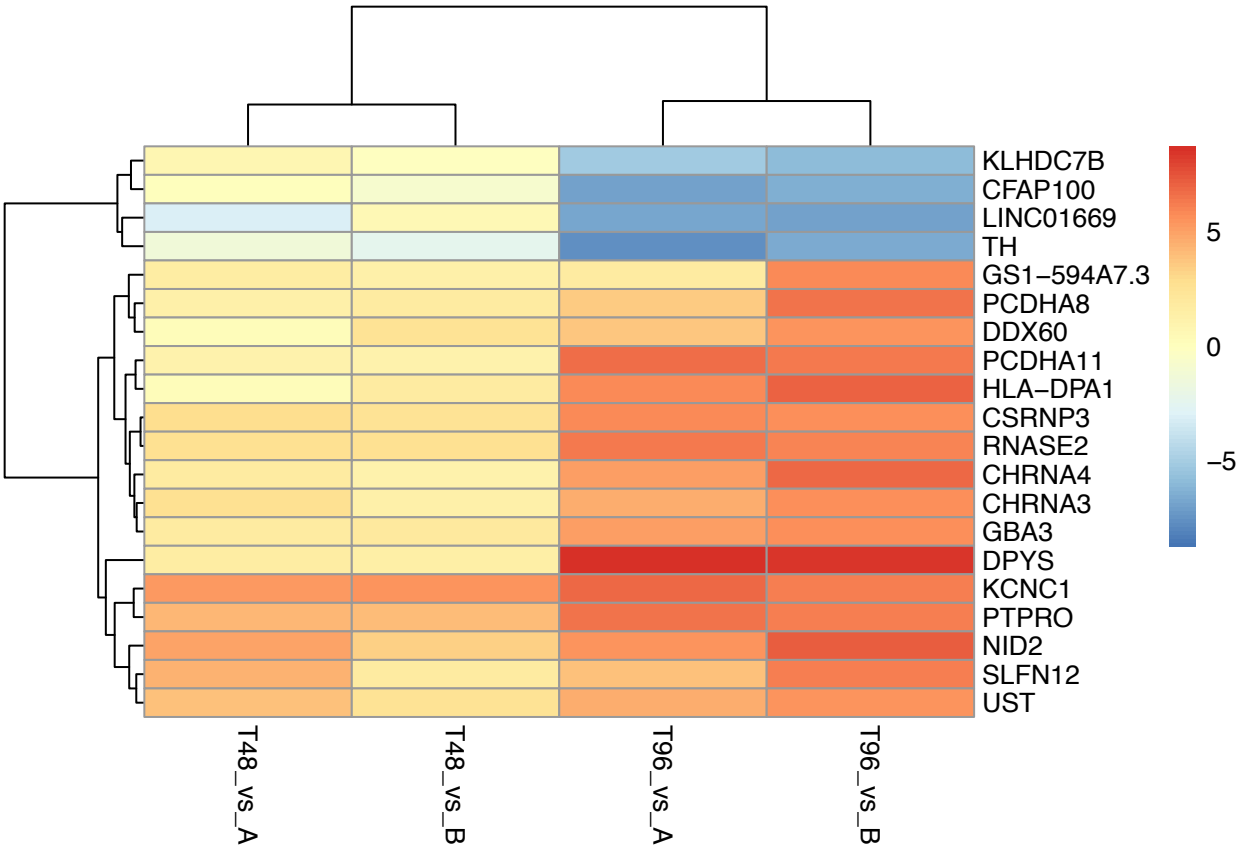


Again, this requires some configuration to improve it. There are two main things we will address:

1. The 'inflection point' - the scale is not symmetrical about 0, so the midpoint of the colour scale is also not falling at 0.
2. The colour scheme - the default is OK, but it can be useful to know how to change it.

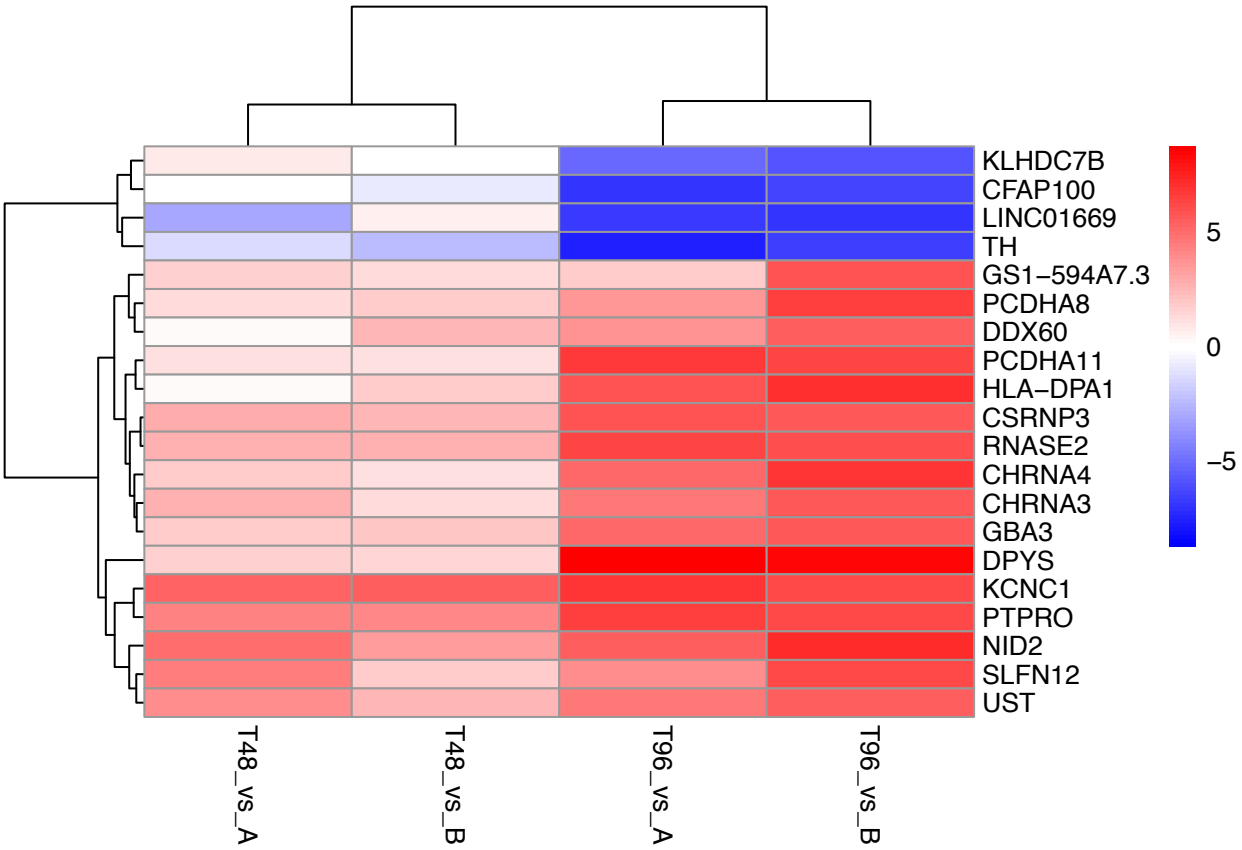
To address the first point, we have to set what's known as the 'breaks' - this sets the breakpoints in a scale, in the case of a heatmap, the breaks are the point along the numerical scale at which the colour being used changes. By default, `pheatmap` uses 100 colours, so we need 101 breaks:

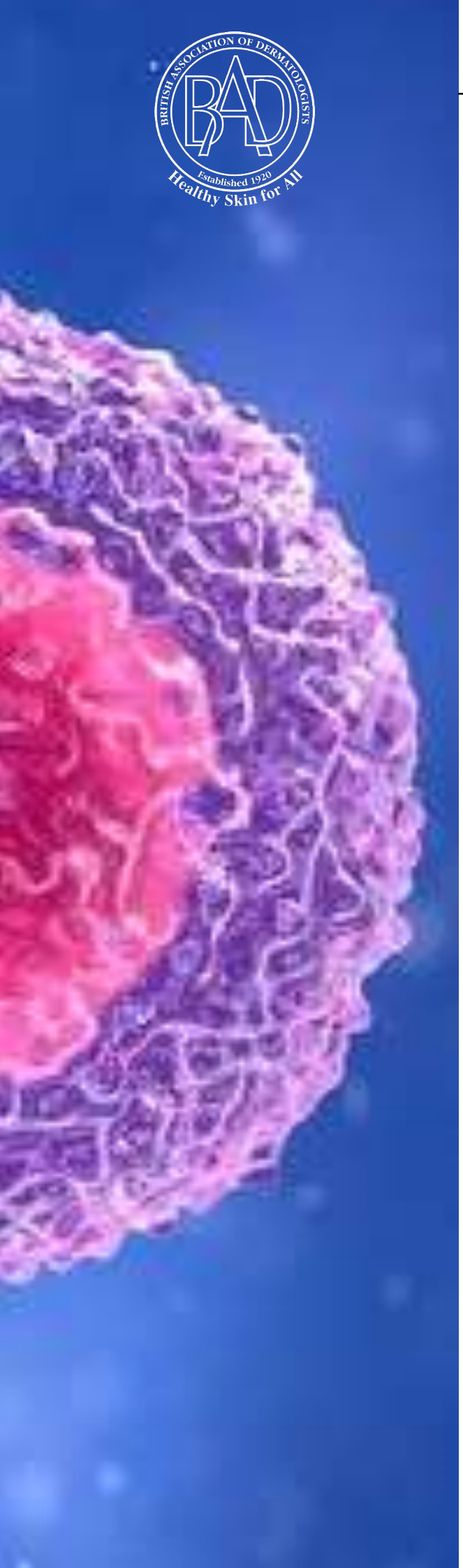
```
# get the largest number in the fold change matrix
big_fc = max(abs(fc_matrix))
# set the breaks to move evenly between
# the negative and positive versions of this number
hm_breaks = seq(-big_fc, big_fc, length.out = 101)
# now use these breaks to draw the heatmap
pheatmap(fc_matrix, breaks=hm_breaks)
```



Finally, we'll set a bolder colour scheme, running from blue for negative fold change to red for positive.

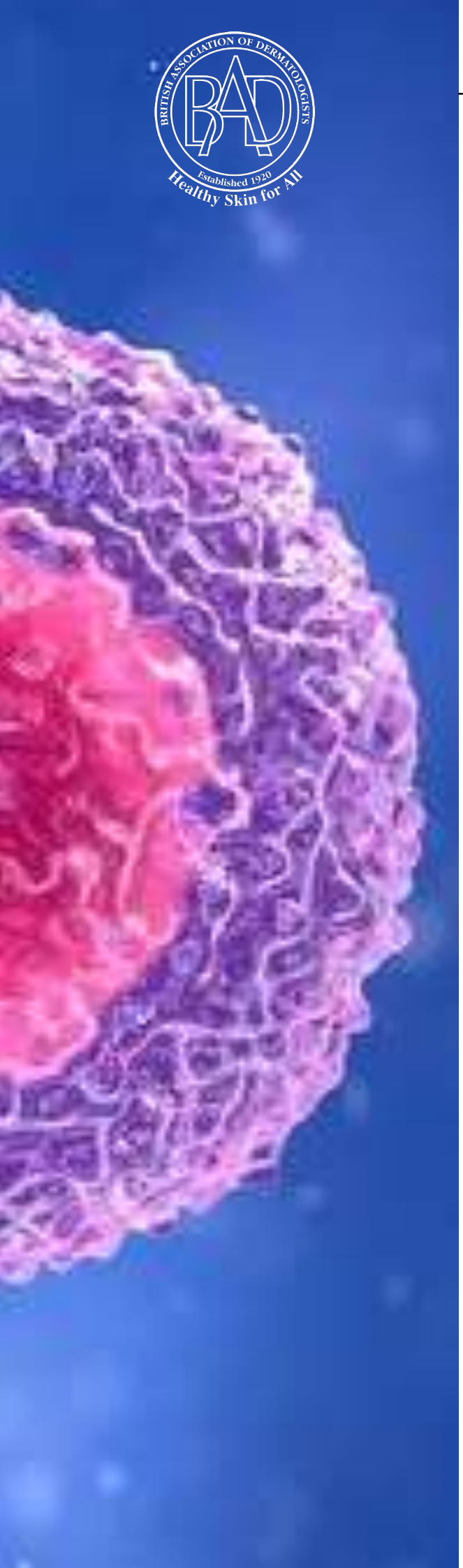
```
mycolours2 = colorpanel(n=100, low='blue', mid='white', high='red')
pheatmap(fc_matrix, breaks=hm_breaks, color = bluered(100))
```


KLHDC7B
CFAP100
LINC01669
TH
GS1−594A7.3
PCDHA8
DDX60
PCDHA11
HLA−DPA1
CSRNP3
RNASE2
CHRNA4
CHRNA3
GBA3
DPYS
KCNC1
PTPRO
NID2
SLFN12
UST
5
0
−5
T48_vs_A
T48_vs_B
T96_vs_A
T96_vs_B



SKIN AGEING IN THE 21ST CENTURY

Mark Birch-Machin



BIOMARKERS FOR MELANOMA

Rob Ellis

Penny Lovat

Ashleigh McConnell

Biomarkers in Melanoma

Dr Rob Ellis

Consultant Dermatologist, County Durham and Darlington NHS FT
Honorary Clinical Senior Lecturer, Newcastle University
Chief Medical Officer, AMLo Biosciences Ltd.

Nov 2018

What is a biomarker?

- Portmanteau of “biological marker”
- National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.
- Objective, quantifiable characteristics of biological processes.

What is a biomarker?

- **Diagnostic**
  - Blood Sugar in diabetes

What is a biomarker?

- **Diagnostic**
  - Blood Sugar in diabetes
- **Prognostic**
  - PSA in prostate cancer

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What is a biomarker?

- **Diagnostic**
  - Blood Sugar in diabetes
- **Prognostic**
  - PSA in prostate cancer
- **Predictive**
  - ER positive breast cancer

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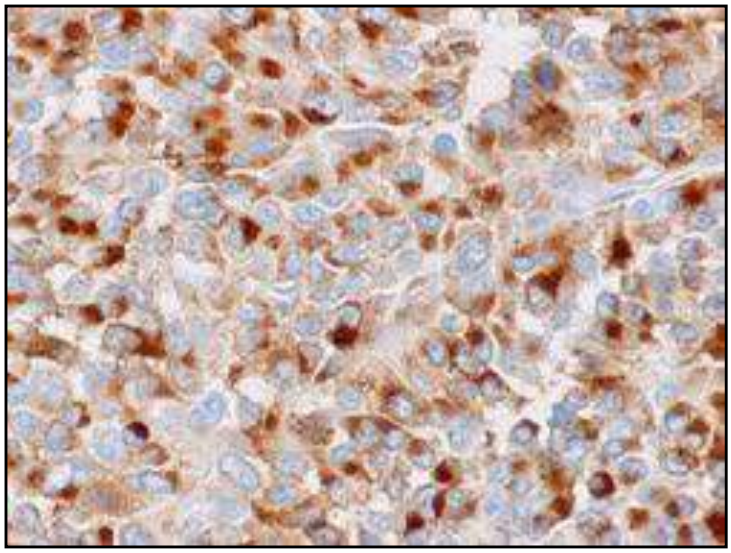
Melanoma examples

- **Diagnostic**
  - HMB45



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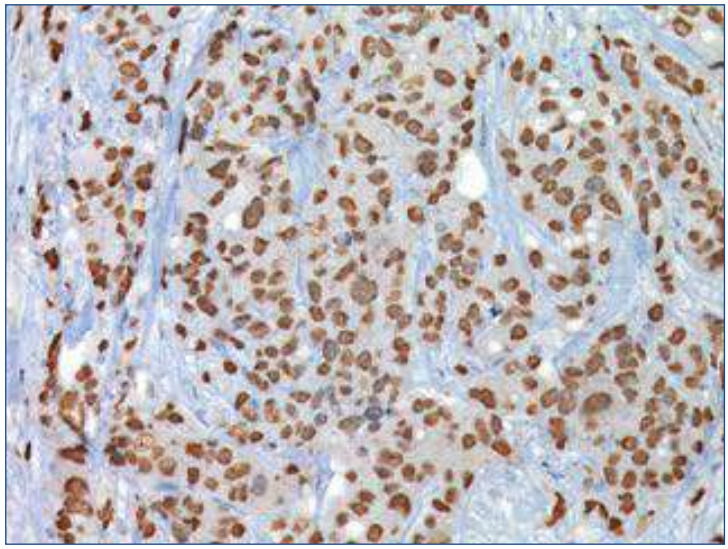
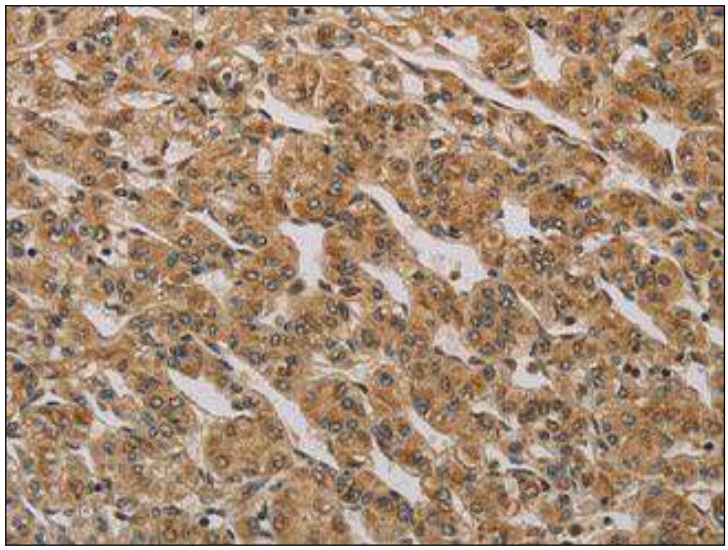
Melanoma examples

- **Diagnostic**
  - HMB45
- **Prognostic**
  - Ki-67



Melanoma examples

- **Diagnostic**
  - HMB45
- **Prognostic**
  - Ki-67
- **Predictive**
  - BRAF



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South Tees Hospitals NHS Foundation Trust Dermatology Group

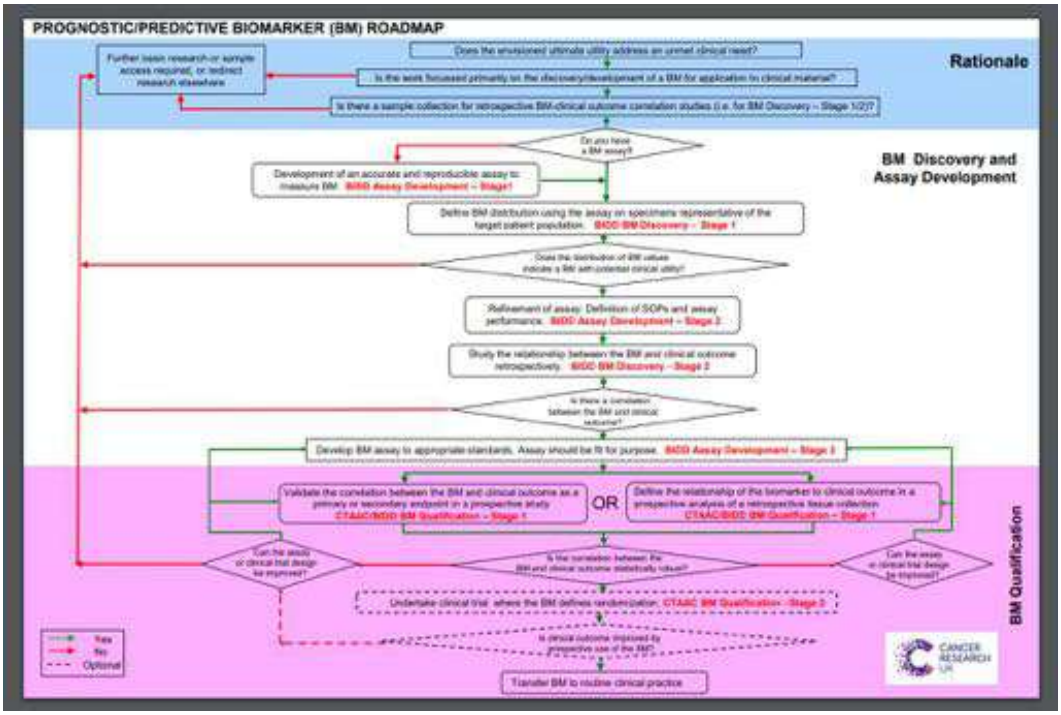
Current role of Biomarkers in Melanoma

- **Currently very limited**
- **Diagnostics performed by Pathologist**
- **Prognostics incorporated in AJCC staging (SLNB?)**
- **Predictive only BRAF at present**
- **Most have either been shown to be surrogate markers of Breslow Depth, or studies not undertaken rigorously enough**

Which type of biomarker is most useful?

- **Diagnostic?**
  - Pathologists' eyes are very good
  - MLUMPs/Spitzoid tumours?
- **Prognostic**
  - "Is my melanoma going to spread?"
  - Help design further management
- **Predictive**
  - Which is the best drug to give to this patient?

Cancer Research UK Biomarker Roadmap



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REMARK Guidelines

British Journal of Cancer (2005) 93, 387 – 391

www.bjcancer.com

Guidelines

REporting recommendations for tumour MARKer prognostic studies (REMARK)

LM McShane*,[1], DG Altman[2], W Sauerbrei[3], SE Taube[3], M Gion[4] and GM Clark[5] for the Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics[6]

[1]US National Cancer Institute, Bethesda, MD 20892, USA; [2]Cancer Research UK Medical Statistics Group, Centre for Statistics in Medicine, Wolfson College, Oxford OX2 6UD, UK; [3]Institut fuer Medizinische Biometrie und Medizinische Informatik, Universitaetsklinikum Freiburg, 79104 Freiburg, Germany; [4]Centro Regionale Indicatori Biochimici di Tumours, Ospedale Civile, 30122 Venezia, Italy; [5]OSI Pharmaceuticals Inc., Boulder, CO 80301, USA

Despite years of research and hundreds of reports on tumour markers in oncology, the number of markers that have emerged as clinically useful is pitifully small. Often initially reported studies of a marker show great promise, but subsequent studies on the same or related markers yield inconsistent conclusions or stand in direct contradiction to the promising results. It is imperative that we attempt to understand the reasons that multiple studies of the same marker lead to differing conclusions. A variety of methodological problems have been cited to explain these discrepancies. Unfortunately, many tumour marker studies have not been reported in a rigorous fashion, and published articles often lack sufficient information to allow adequate assessment of the quality of the study or the generalisability of the study results. The development of guidelines for the reporting of tumour marker studies was a major recommendation of the US National Cancer Institute and the European Organisation for Research and Treatment of Cancer (NCI-EORTC) First International Meeting on Cancer Diagnostics in 2000. Similar to the successful CONSORT initiative for

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Current situation

Diagnostic and Prognostic Biomarkers in Melanoma

[a]DAVID WEINSTEIN, MD; [a]JENNIFER LEININGER, MD; [b]CARL HAMBY, PhD; [a]BIJAN SAFAI, MD
[a]Department of Dermatology, [b]Department of Microbiology and Immunology, New York Medical College, New York and Valhalla, New York
(*J Clin Aesthet Dermatol.* 2014;7(6):13–24.)

CONCLUSION

Currently most diagnostic biomarkers of melanoma rely on detection of melanocytes rather than melanoma itself. Newer biomarkers depend on cytogenetic markers of carcinogenesis and signatures of mutations utilizing panels of biomarkers. There are no current serologic markers for the early detection of melanoma, and there may never be. Such evidence may be possible only in advanced stage disease that has metastasized from the primary site. As such, current serologic biomarkers detect circulating melanoma cells or secondary evidence of advanced disease, such as LDH.

Future research into serological and histological methods to detect early stages of melanoma hopefully will improve prognosis through earlier intervention. Such research might investigate markers of melanoma stem cells or markers of melanoblast differentiation that indicate a survival advantage and progression toward neoplasia.[194] Microarrays could be utilized to screen for similarities between melanoma and stem cells. Genes in common among melanoma and stem cells could be investigated to design better diagnostic and prognostic assays, perhaps using FISH, as well as to suggest new therapeutic targets.

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New Prognostic Biomarkers in Melanoma

CASTLE BIOSCIENCES INCORPORATED
MELANOMA DECISIONDx

31-gene expression profile

AJCC I - III

MELAGENIX Precision Medicine

8-gene expression profile

AJCC II+

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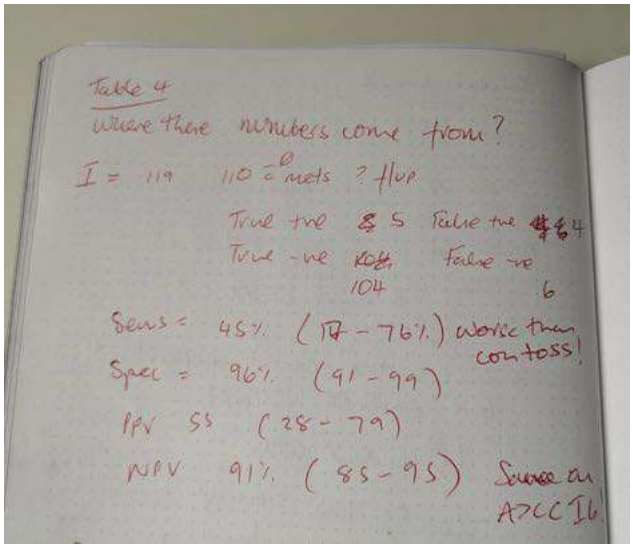
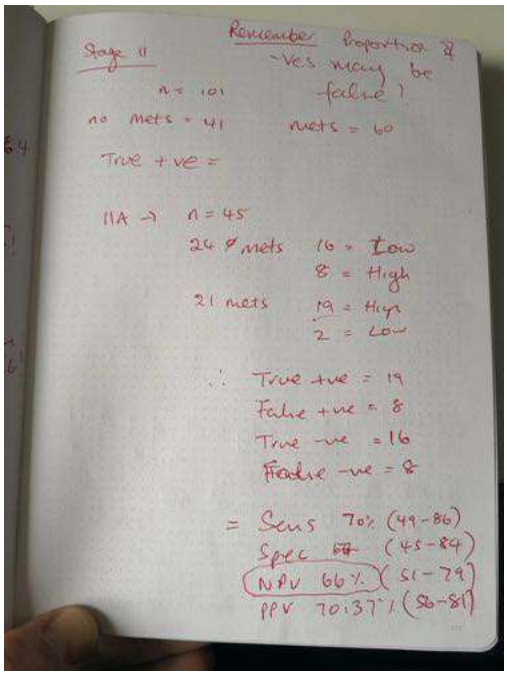
New Prognostic Biomarkers in Melanoma

MELAGENIX
Precision Medicine





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Ideal Prognostic Biomarker

- **Accurate**
- **Reproducible**
- **Fits into current pathway**
- **Cheap**
- **Quick**
- **Health Economic Assessment**

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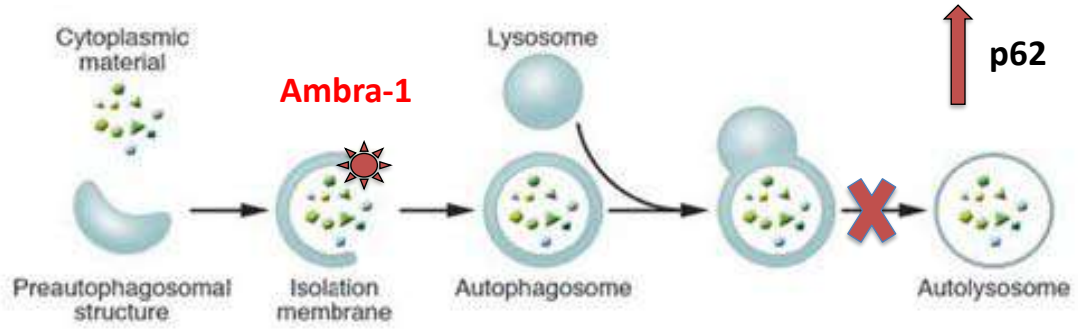
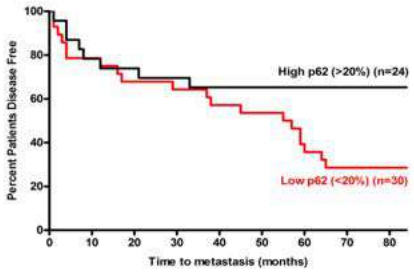
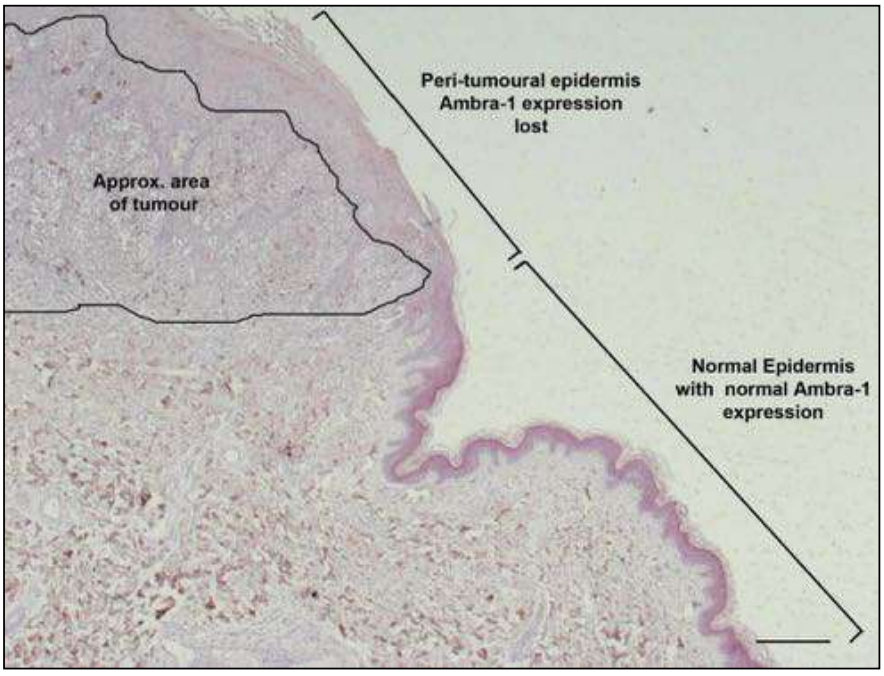
Autophagy and Melanoma



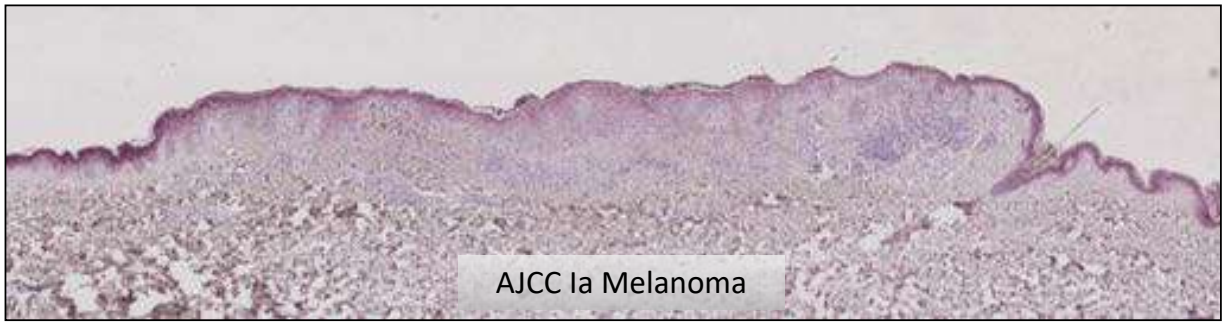


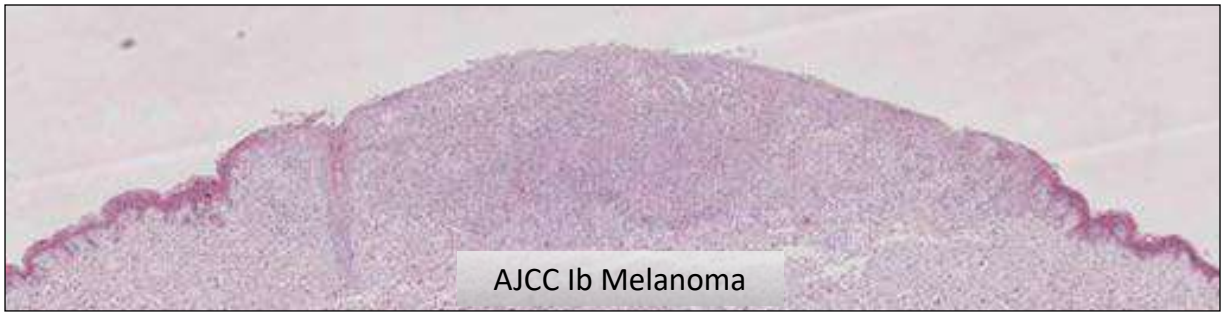
Ellis *et al.* J Invest Dermatol 2014

Epidermal AMBRA1



Epidermal AMBRA1





Newcastle University

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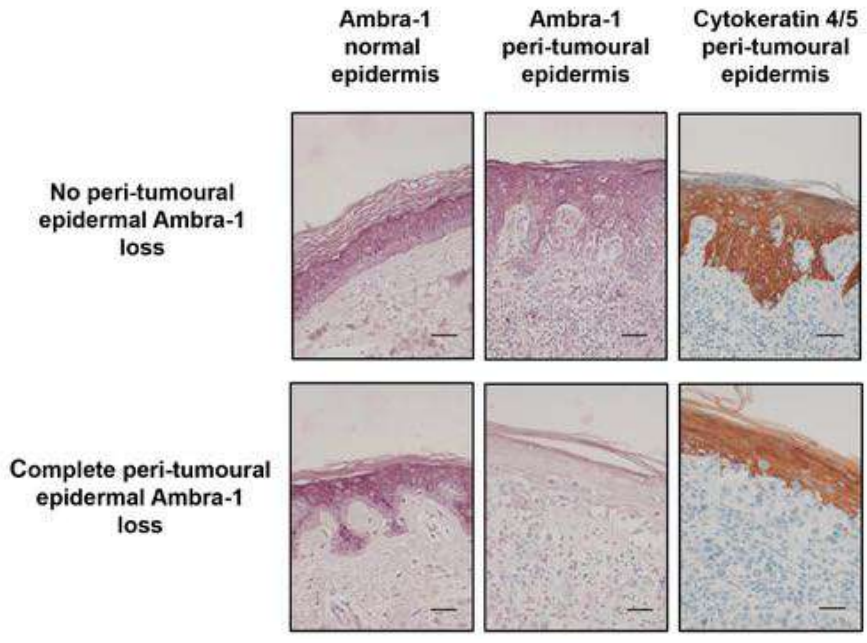
Epidermal AMBRA1 does not correlate with degree of melanoma invasion



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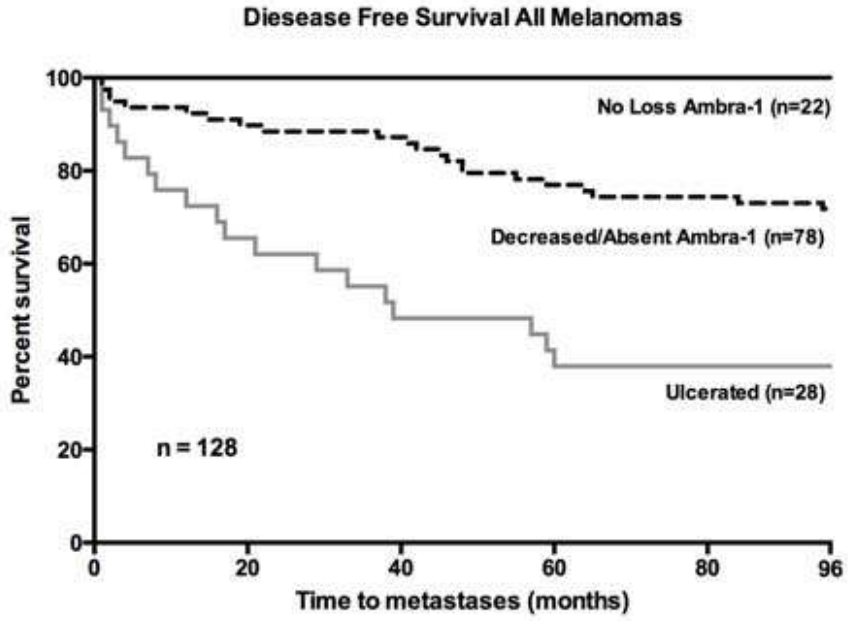
South Tees Hospitals NHS Foundation Trust Dermatology Group

AMBRA1 as a Prognostic Biomarker



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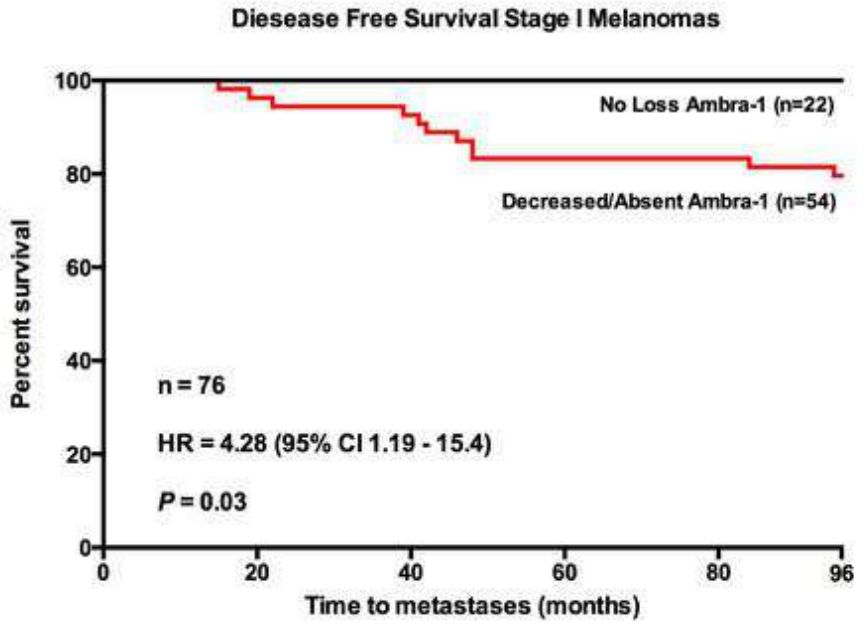
AMBRA1 as a Prognostic Biomarker



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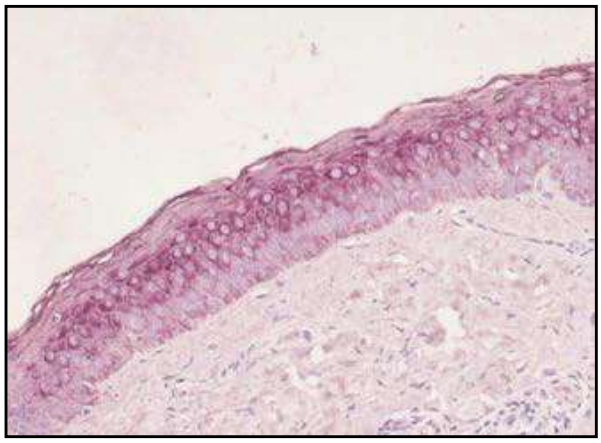
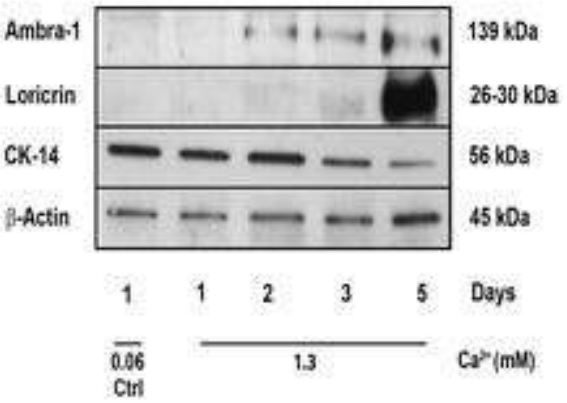
Epidermal differentiation is associated with increased AMBRA1





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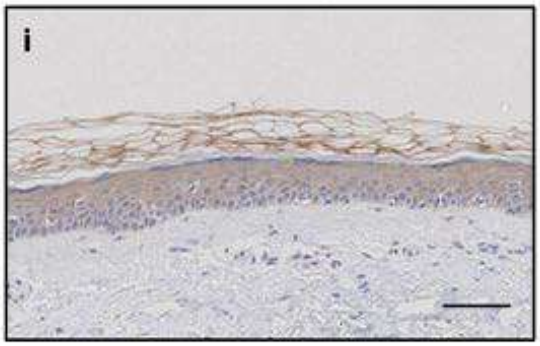
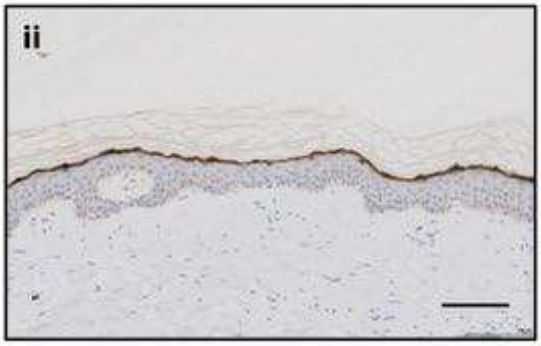
The AMLo Biomarker





AMBRA1 and loricrin optimized on James Cook Hospital clinical platform with DAB counterstaining

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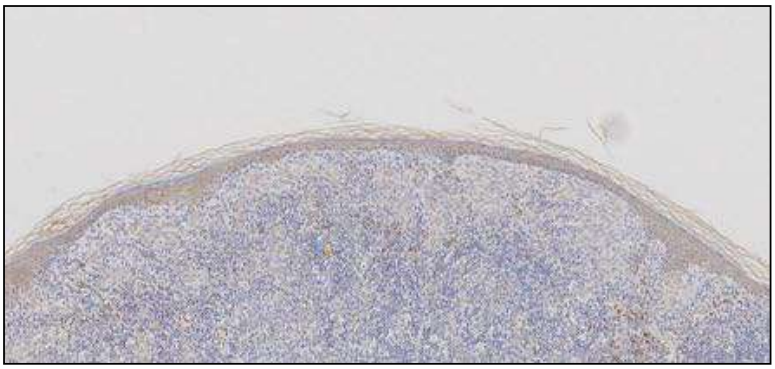
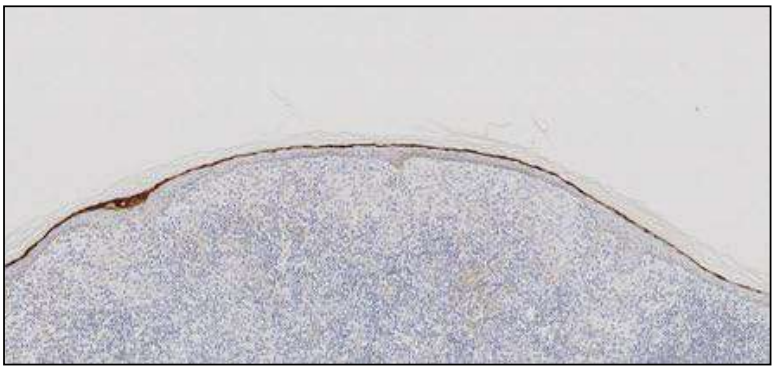
The AMLo Biomarker – Low Risk

AMBRA1

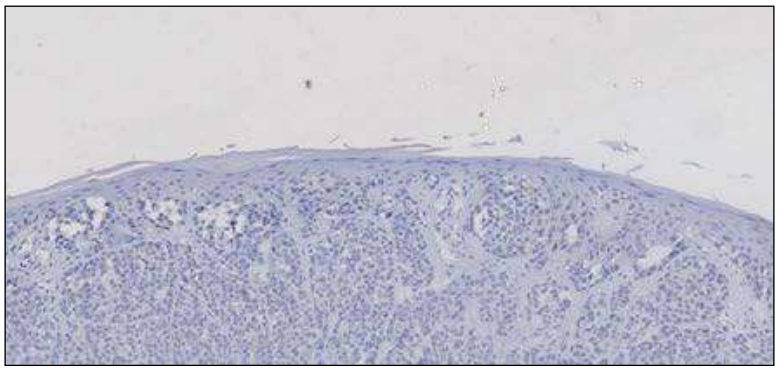


Loricrin

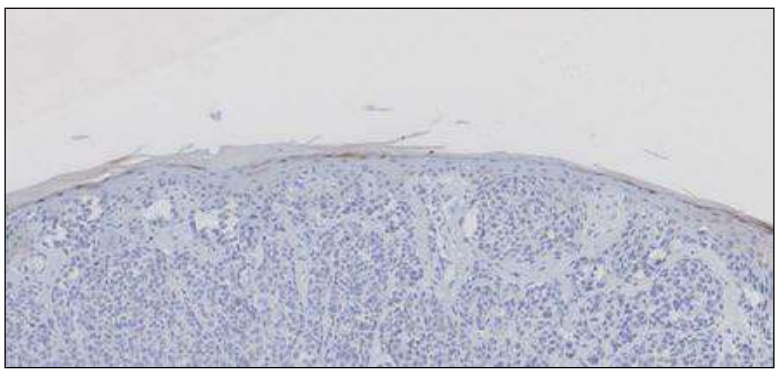


The AMLo Biomarker – High Risk

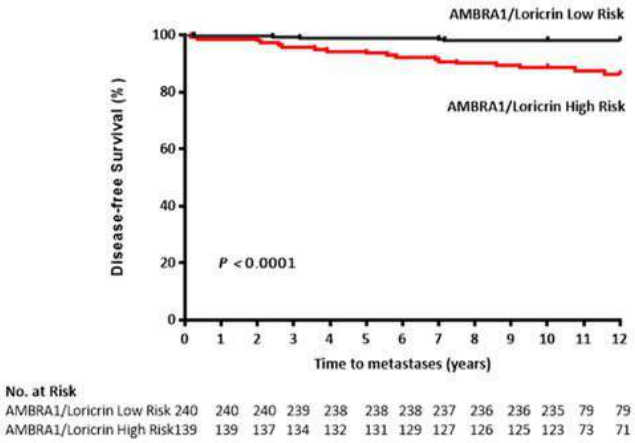
AMBRA1



Loricrin



The AMLo Biomarker – Combined Cohort



Variable	Univariate Hazard Ratio (95% CI)	P value	Multivariate Hazard Ratio (95% CI)	P value
AMBRA1/Loricrin	3.61 (1.79-7.29)	0.00033	3.18 (1.56-6.48)	0.00142
Breslow Depth	5.63 (2.52-12.6)	<0.0001	4.87 (2.06-11.5)	0.0003
Age	0.99 (0.96-1.01)	0.35	0.98 (0.96-1)	0.084
Gender	1.66 (0.73-3.76)	0.23	1.67 (0.74-3.8)	0.22

Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
78.3%	66.0%	12.9%	97.9%

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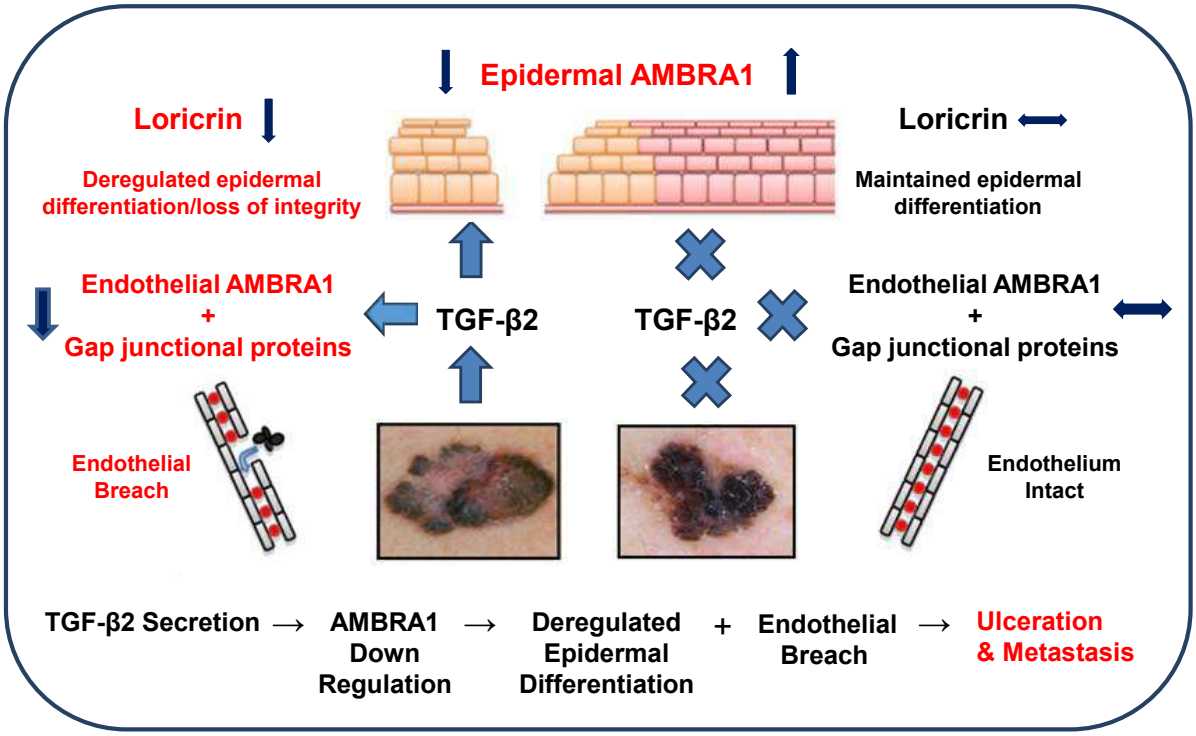
How does TGFB2 influence metastasis



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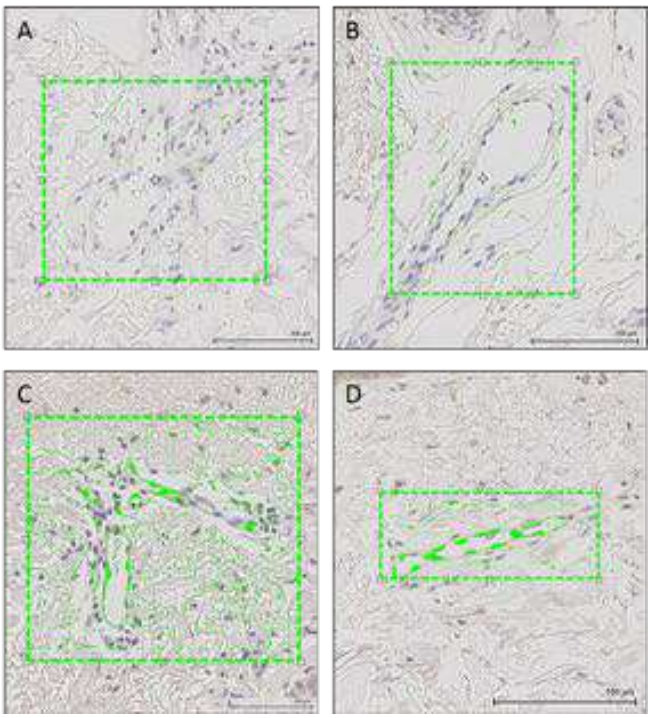
The AMLo Biomarker – Endothelial involvement



What next?

Previous Funding Streams

Melanoma Focus Group - £100 000 Patient Impact Programme

iCURE Programme – £40 000

Innovate UK - £210 000

Current Funding

NIHR Health Technology Assessment – Evidence based assessment of AJCC I melanoma
£215 000

NIHR i4i – Development of AMLo Biomarker
£1.2 million

NICE AdviseMe Prize winners 2018

AMLo Biosciences Ltd

AMLo BIOSCIENCES

beyond your diagnosis

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www.amlo-biosciences.com

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The future

Academic

- Further AJCC I/II cohorts from Buffalo, Tauranga, Barcelona, Kyoto
- Links with Brisbane, Berlin, Turin, Brazil, Sweden, Russia
- Prospective trials for SLNB stratification and WLE margins
- Finalise role of AMLo in melanoma management
- Drug development work

Commercial

- CE Marking of clinical grade antibody
- NICE Guidelines
- CLEA labs in US

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Acknowledgements

Dermatological Sciences, Newcastle University

Prof Penny Lovat

Dr Marialena Anagnostou
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Dr Batoul Nasr
Dr Diana Tang
Dr Ashleigh McConnell
Dr Marie Labus

Histopathology, The James Cook University Hospital, Middlesbrough

Dr Ed Carling
Mrs Alison Greenwood
Dr Graeme Watson

Cancer Research UK, London Research Institute

Mr Stuart Horswell

Biomarkers in Melanoma
Practical Session

Dr Ashleigh McConnell, Professor Penny Lovat & Dr Rob Ellis

Newcastle University
AMLo Biosciences Ltd.

Nov 2018

Outline

- **Immunohistochemistry**
- **The AMLo Biomarker**
- **Practical Examples: Your turn to be the pathologist**
- **Statistical Analysis**

Immunohistochemistry

- Visualises distribution and amount of antigens or proteins (Nucleic acids/lipids/carbohydrates) in tissue sections
- Uses a specific antigen-antibody reaction tagged with a visible label
- Doesn't destroy tissue architecture, assess expression of antigen in the microenvironment
- Can be preformed on formalin fixed paraffin embedded (FFPE) and frozen tissue, smears, cytospins
- Label antibodies with a chromogen that produces a visible colour, fluorochrome, radioactive element, colloidal gold

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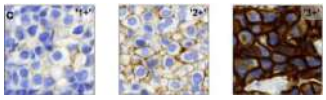
Pathology Lab IHC Workflow

HER2 Scoring



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Immunohistochemistry Protocol:
Tissue Fixation

FFPE

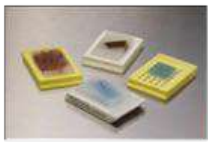
Tissue Fixation, embedding & sectioning

Frozen

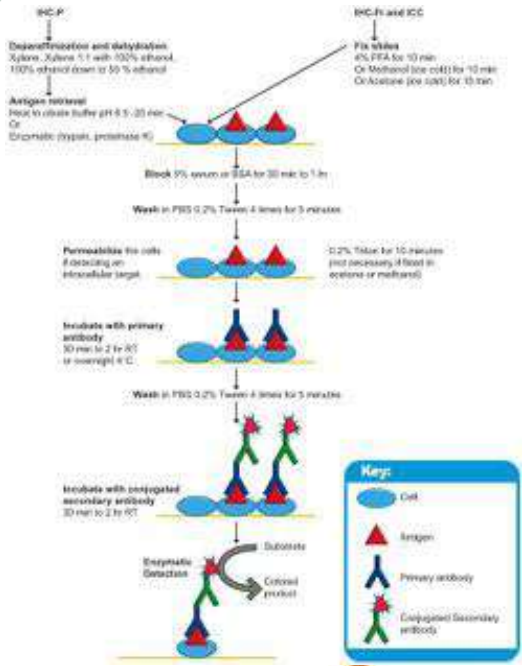
OCT & Sectioning

Tissue Handling and Fixation FFPE

- Fix to prevent autolysis, bacterial decomposition, preserve structure and integrity for processing
- Fixation induces artefact and masks epitopes
- 10% formalin 24 hours room temperature



- Frozen tissue embedded in cryoprotective embedding medium OCT, stored at -80°C



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Immunohistochemistry Protocol:
Antigen retrieval

FFPE

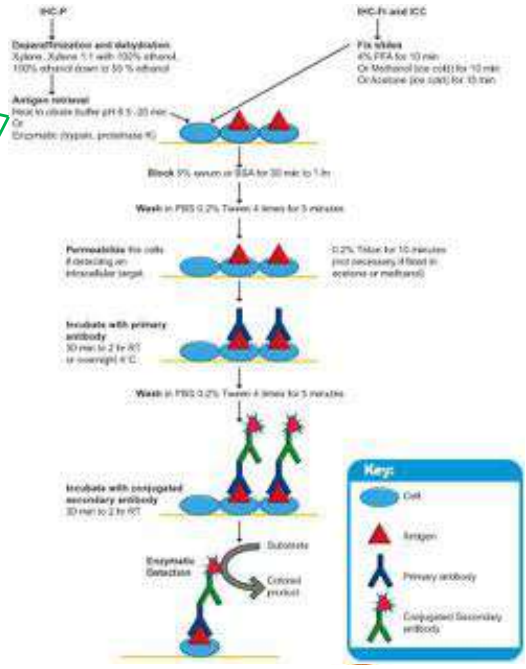
Tissue Fixation, embedding & sectioning

Frozen

OCT & Sectioning

Antigen Retrieval

- Antigen/epitopes of interest can be masked by fixation or 'over fixation'.
- Common buffers: Sodium citrate pH6, Tris/EDTA pH9
- Various methods of applying heat, microwave, pressure cooker, water baths



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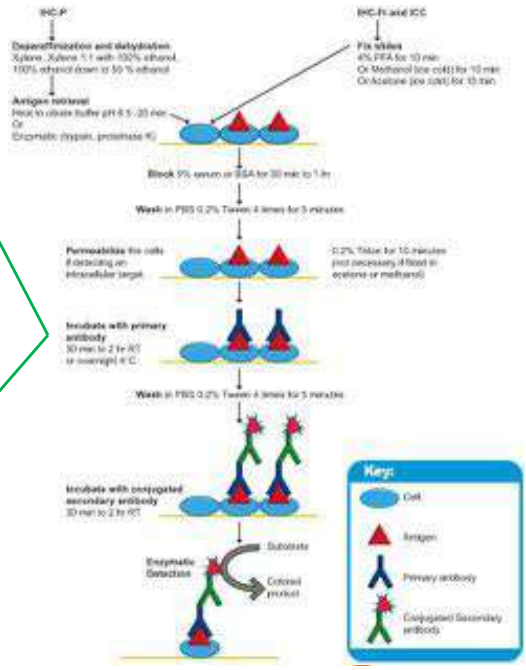
Immunohistochemistry Protocol:
Primary antibody binding to selected antigen

FFPE

Tissue Fixation, embedding & sectioning

Frozen

OCT & Sectioning



Primary Antibody

- Do your research
- Optimise the antibody dilution and incubation times
- Include a 'null primary' – no primary antibody slide
- Good positive and negative controls, FFPE cell pellets over expressing protein, or KD
- Good antibody, protein highly expressed
- Poor antibody, low expression in tissue

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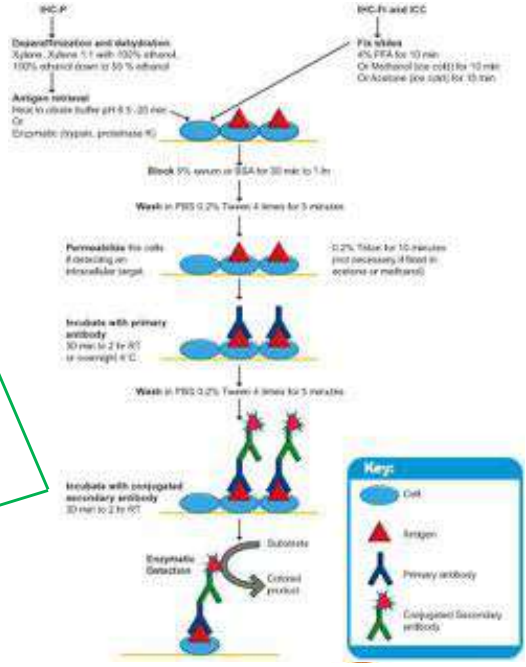
Immunohistochemistry Protocol:
primary antibody binding detection and visualization

FFPE

Tissue Fixation, embedding & sectioning

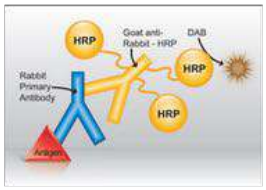
Frozen

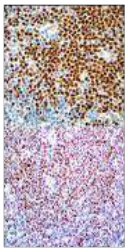
OCT & Sectioning



Detection System

- Peroxidase (Horse radish peroxidase HRP) most common.

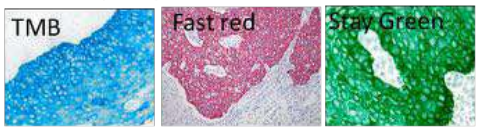




DAB

AEC

- Streptavadin
- Alkaline phosphatase



- Counterstain with Hematoxylin

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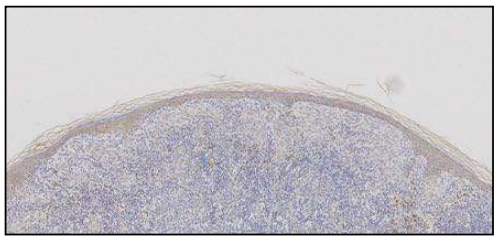
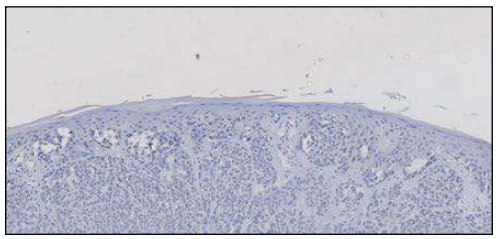
The AMBLor Biomarker - Practical

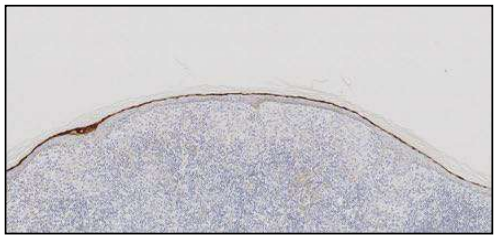
AMBRA1 maintained



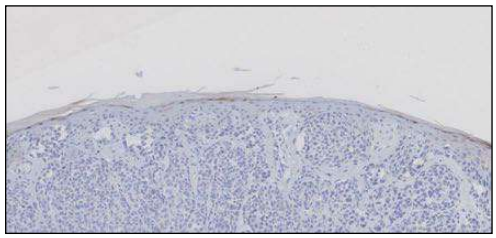
AMBRA1 lost



Loricrin maintained



Loricrin lost



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The AMBLor Biomarker - Scoring

	Maintained	Decreased/Lost
AMBRA1	1	0
Loricrin	1	0

	High Risk	Low Risk
AMBLor Score	1 or 2	0

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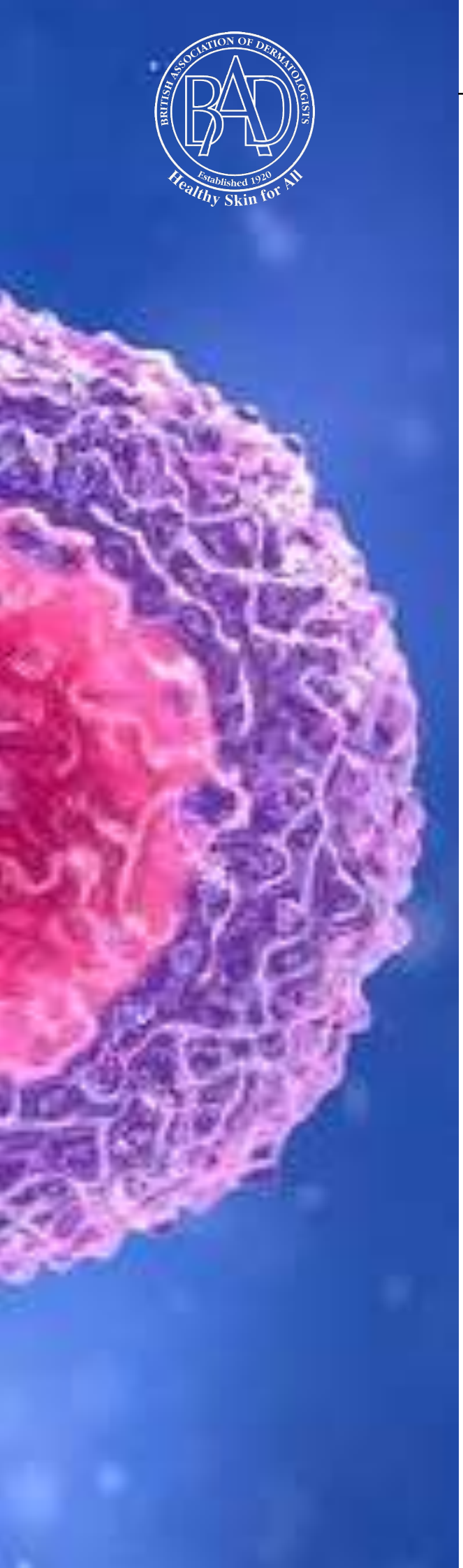


"CYTOMETRY": HOW TO BECOME A CELL DETECTIVE

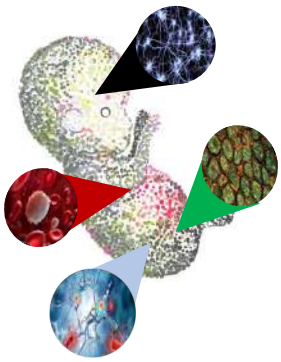
Andrew Filby

"Cytometry": How to become a cell detective

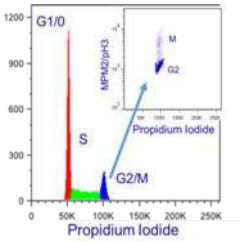
Dr Andrew Filby
Director of Newcastle Cytometry Platform and Single Cell Unit
Dermatology: Research techniques course
Newcastle: 13/11/2018

Heterogeneity: The biggest challenge to ALL cellular research

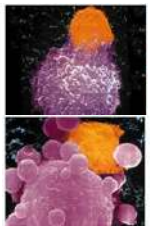
1. Different (stable) cell types



2. Transition states (temporal)

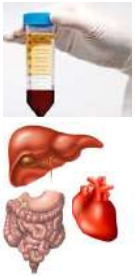


3. Functional states



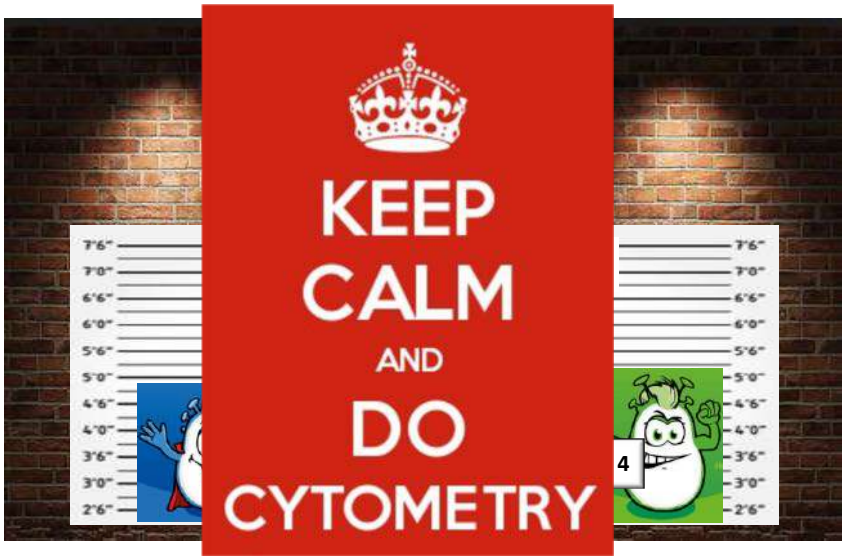
Cells doing a "job" such as killing others

4. Location



- **Establishing cellular identity is a fundamental question to understanding health and disease**

How do we begin to prove Cellular Identity?



The talk in 3 words…

Cytometry
Cytometer
Cytometrist

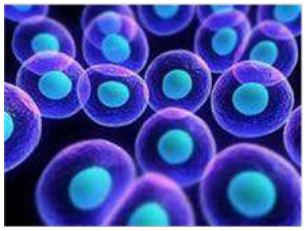
Cytometry

Greek = "Kytos"
"hollow basket"
Relates to CELL

Greek = "Metria"
"Process of Measuring"

Cytometry: We turn cells into numbers…..

Cell Biology

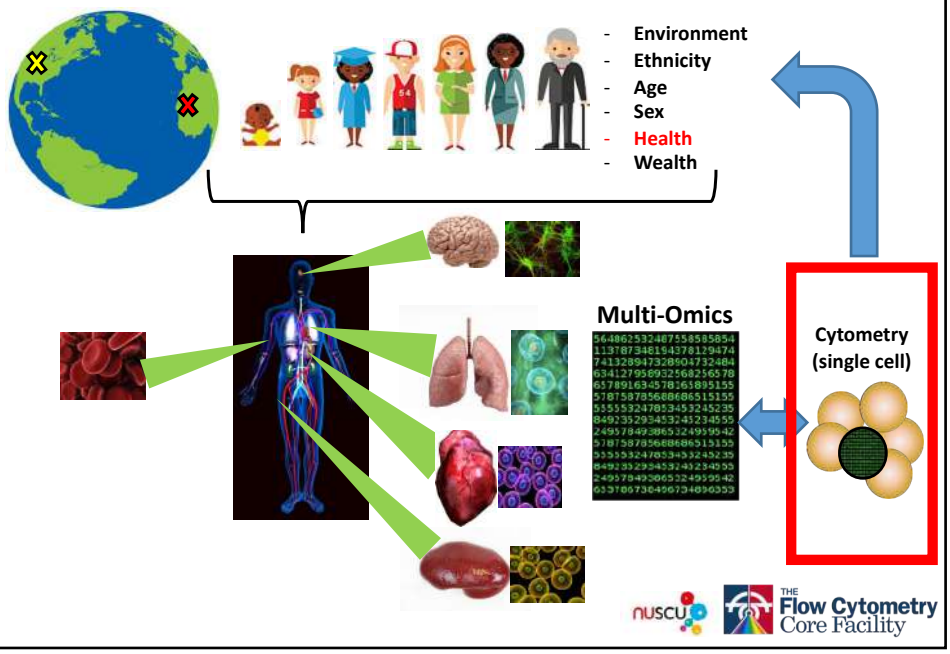


Numbers

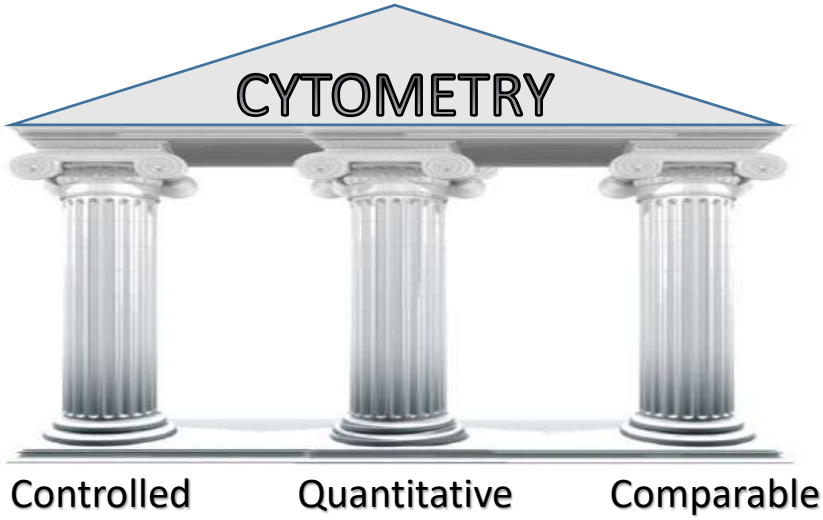
Questions we want to ask/answer with these numbers:

- What types of cells are there?
- How many are there?
- What do they do normally/abnormally?
- Can cell A become cell B?
- What role in development/disease?

Cytometry in the context of "BIG" data?



The 3 pillars and foundations of cytometry



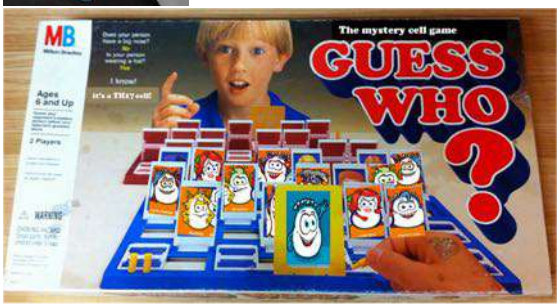
The talk in 3 words…

Cytometry
Cytometer
Cytometrist

A cytometrists asks as many informative questions to cells as we can

Daddy…what do you actually do?

Cytometrists are "cellular detectives"



- **Does it express CD4? Yes**
- **Does it express IL-4? No**
- **Does it express IL-17? Yes**
- **Does it express FoxP3? No**
- **Does it express RORγt? Yes**
- **Is it a TH17 cell?**
- **Yes!**

nuscu THE Flow Cytometry Core Facility

The Newcastle Cellular detectives..

David McDonald

Carly Foster

Satomi Miwa

Jack Wigham Andrew Fuller Gillian Hulme

Solving “crimes against biology” and bringing the perpetrators to justice

nuscu THE Flow Cytometry Core Facility

Welcome to the Newcastle “Cellular Detective Agency”

THE Flow Cytometry Core Facility

NICR: Herschel

iCFL

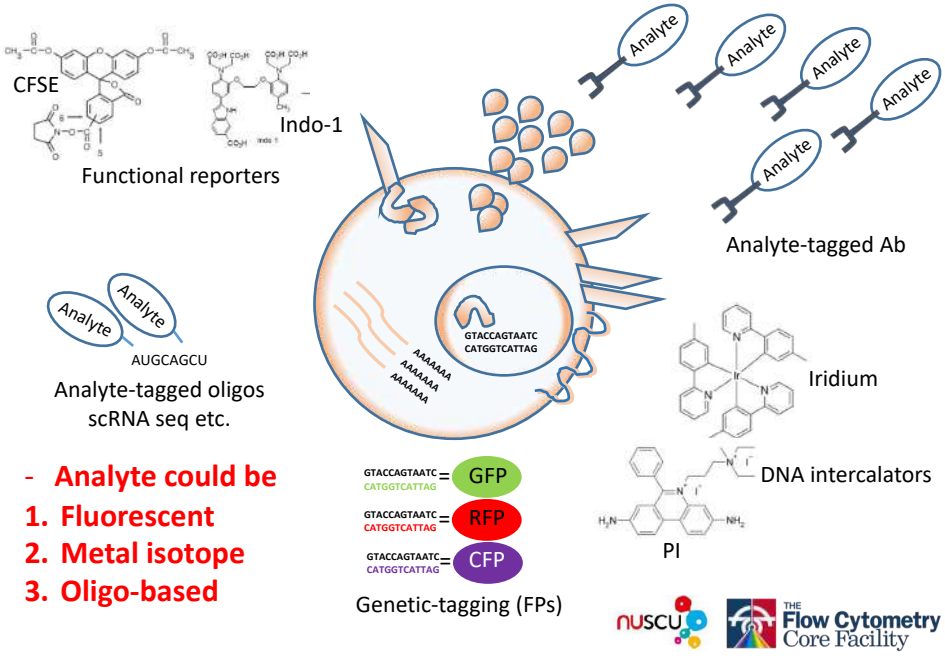
William Leech Medical School

Main HUB

NICR: POG

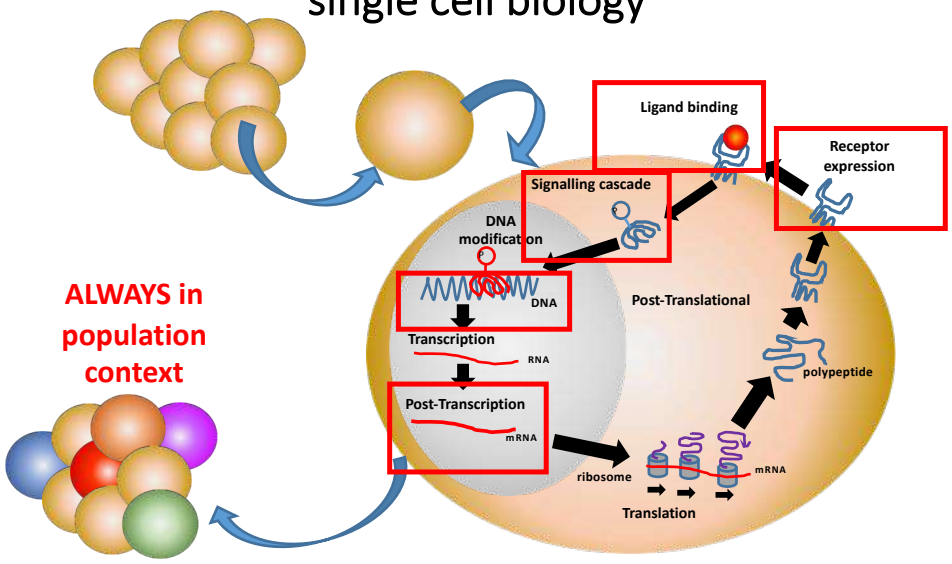
Meeting the cytometry needs of over 300 users across several different disciplines/institutes

How do we ask these questions? With labels



- **Analyte could be**
1. **Fluorescent**
2. **Metal isotope**
3. **Oligo-based**

We can ask our questions in many aspects of single cell biology



ALWAYS in population context

The talk in 3 words…

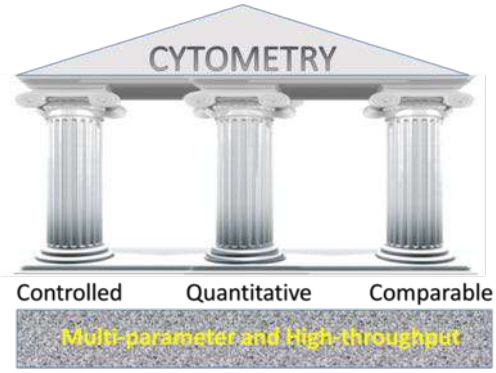
Cytometry
Cytometer
Cytometrist

So many cytometers and so many ways to do cytometry

NUSCU

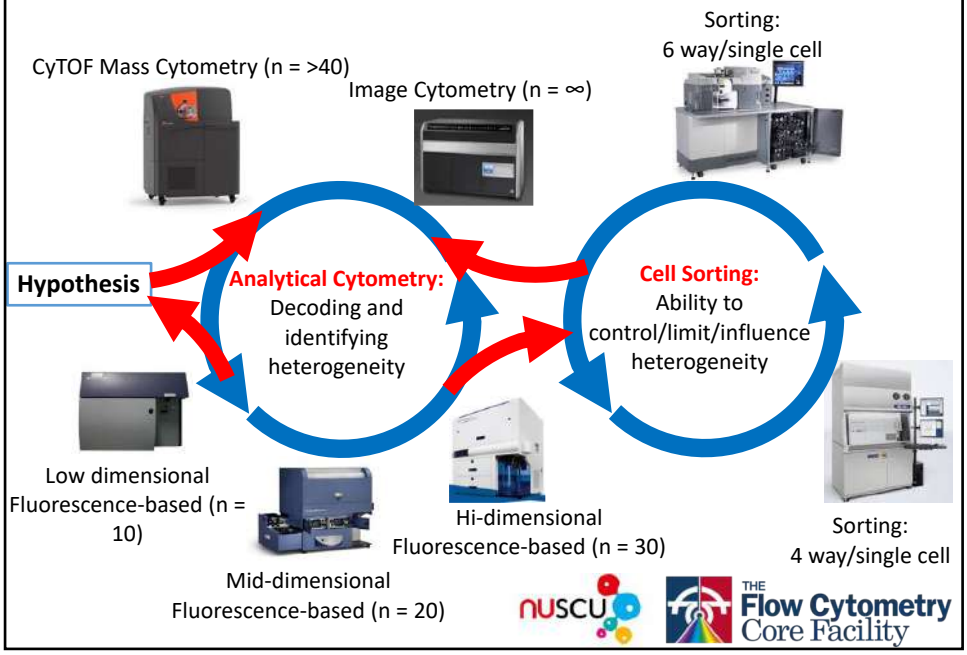
THE Flow Cytometry Core Facility

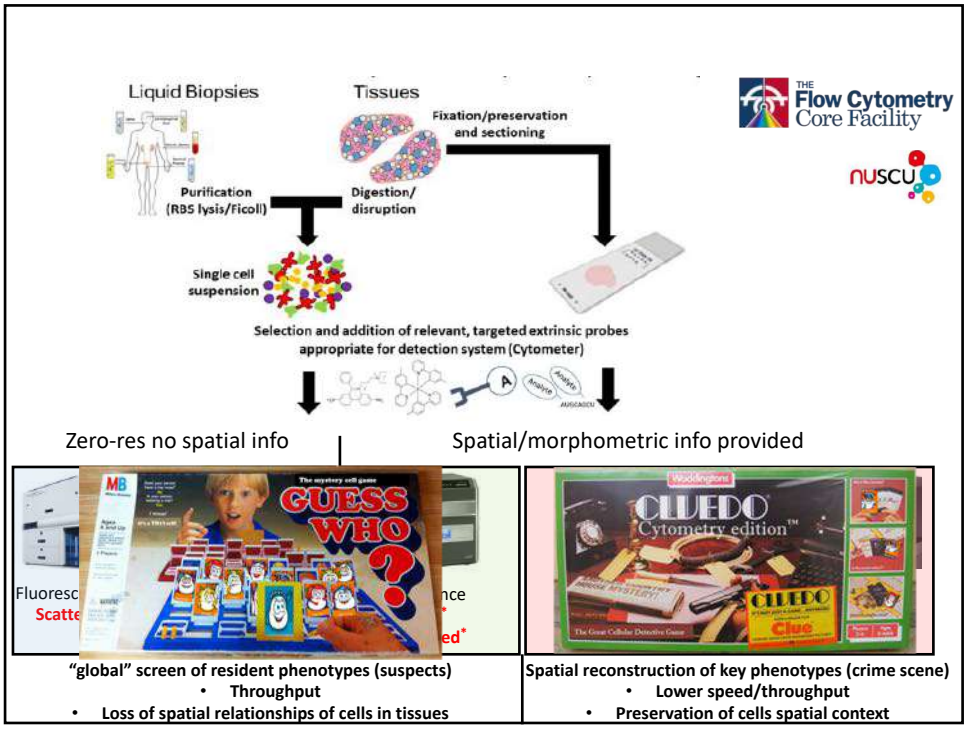
The 3 pillars and foundations of CYTOMETERS



A CYTOMETER generates and measures signals from single cells in a high-throughput, (semi) quantitative, multi-parameter fashion

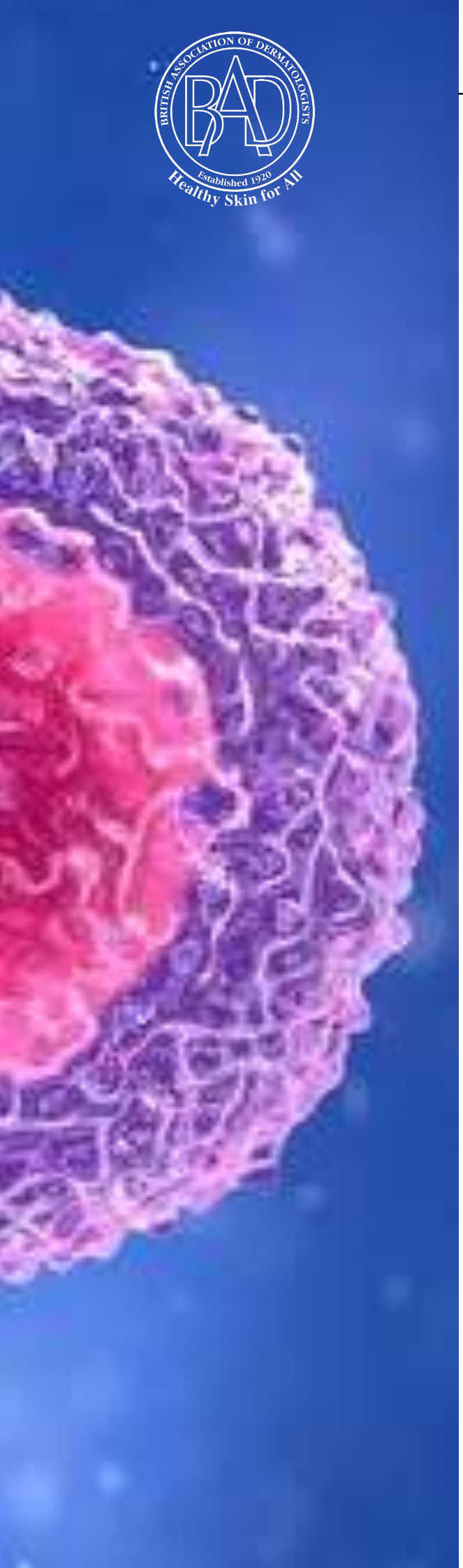
Know and master your cellular heterogeneity @ FCCF





Summary

- Heterogeneity pervades **ALL** cellular systems
- Cytometry helps us to **APPRECIATE** **and** **DECODE**
- We do this using specialised systems called **Cytometers**
- People who do this are **Cytometrists aka "Cell Detectives"**
- Cytometry is always single cell and usually multiparameter
- It is ALWAYS controlled and quantitative
- It allows us to measure and master cellular heterogeneity

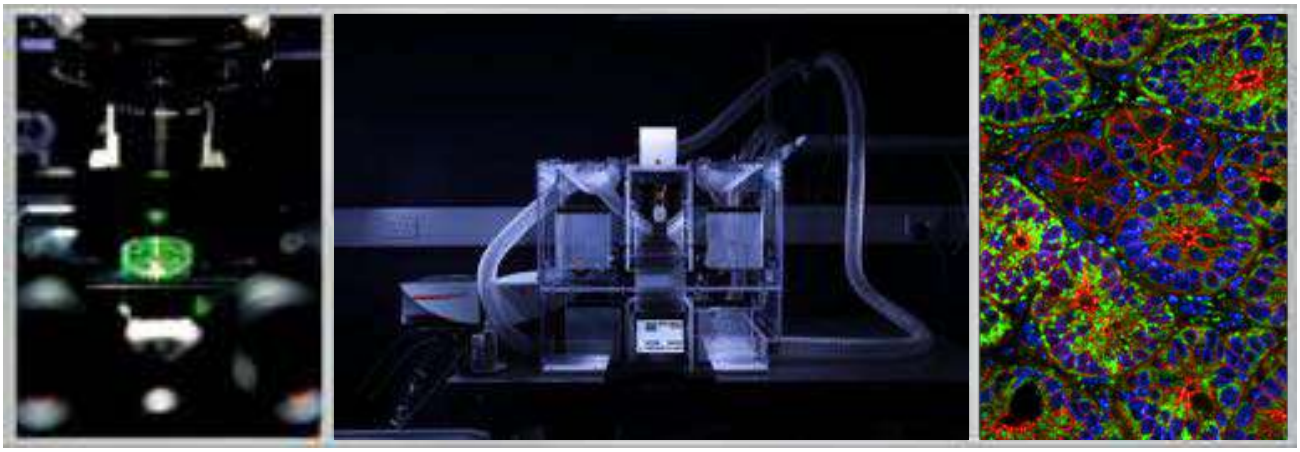


MULTI-MODAL MICROSCOPE BASED IMAGING: FROM THE ORGANELLE TO THE ORGAN

Alex Laude

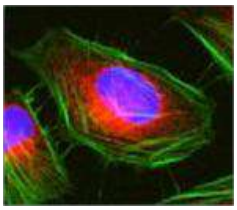
Multi-modal microscope based imaging: From the organelle to the organ

Alex Laude
The BioImaging Unit

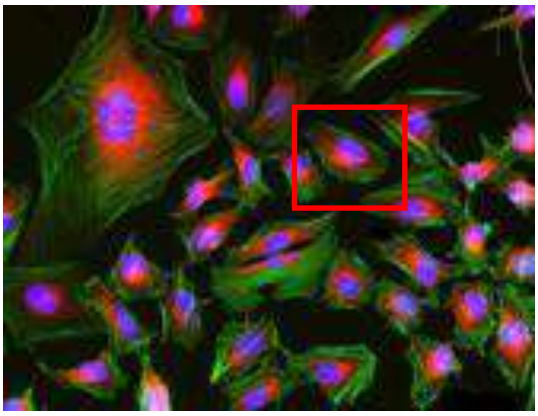


Bias……

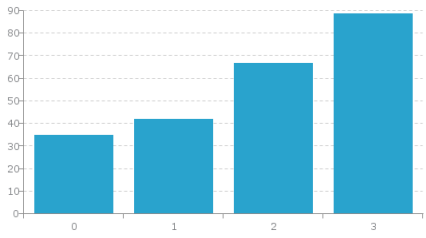
- Everybody publishes the best image…right?
  - They may only report what they want to see



- But is that image representative of the population?

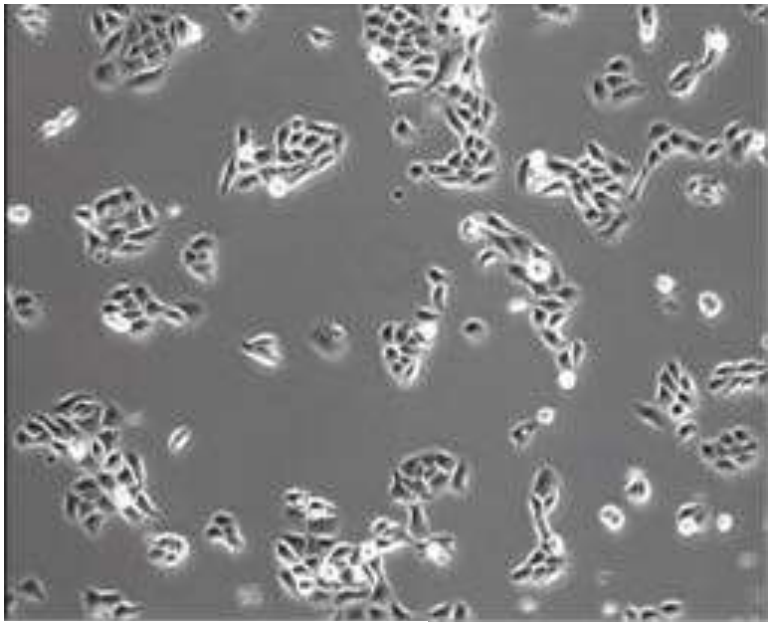


- There is a need to quantify our observations.



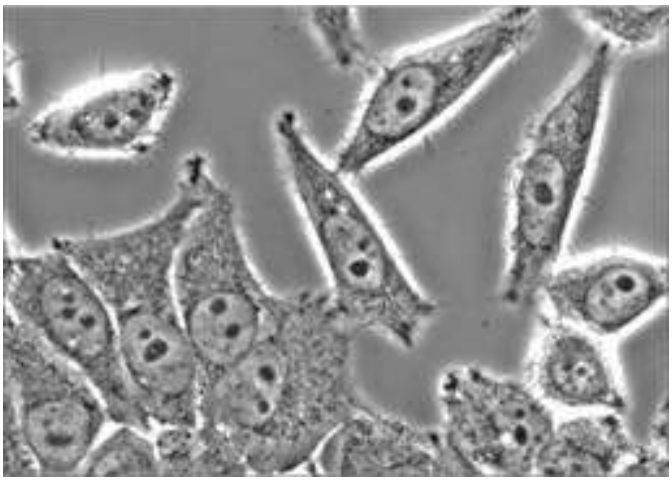
- But what features can we quantify and how do we go about it?

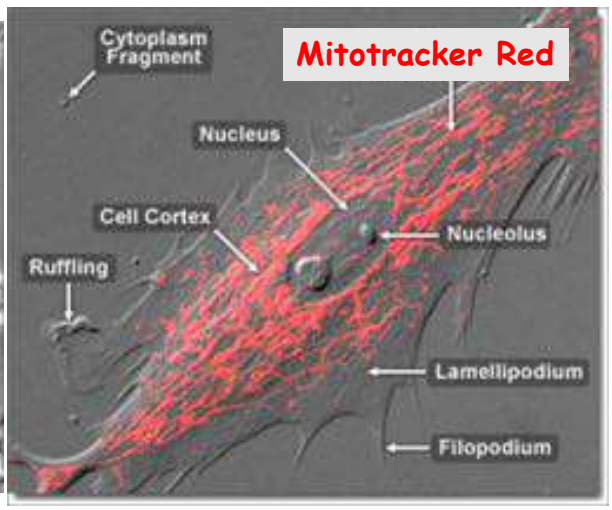
Contrast



HeLa cells (epithelial)
Phase contrast time lapse
(5min/frame, 18hrs)
Playback x 3600

Use of fluorescence – increase contrast





Brightfield imaging: limited ability to make out intracellular organelles
Impossible to identify individual proteins / processes
we need to make them standout from the background

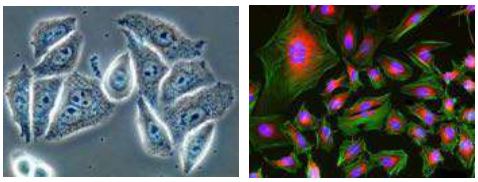
Typical research microscope

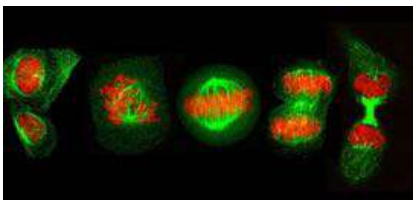
X-Y-Z-λ–position-*t* (6D)

Multi-dimensional, multi-modal imaging *at the sub-cellular level*

X-Y	X-Y-Z	X-Y-Z-λ	X-Y-Z-λ-position	X-Y-Z-λ–position-*t*
2D	3D	4D	5D	6D

- **Brightfield, phase and DIC microscopy**
- **Wide field fluorescence microscopy**
  - ***multi-parametric (x5) analysis***
- **Confocal & Multi-photon microscopy**
- **Live-Cell imaging**
  Fast dynamic processes (>10fps)
  Longer lasting (days / weeks)

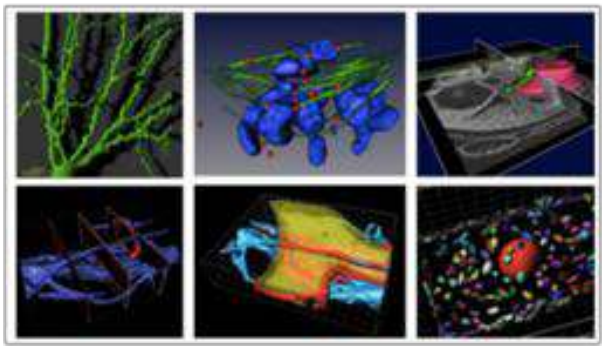




…..and analysis

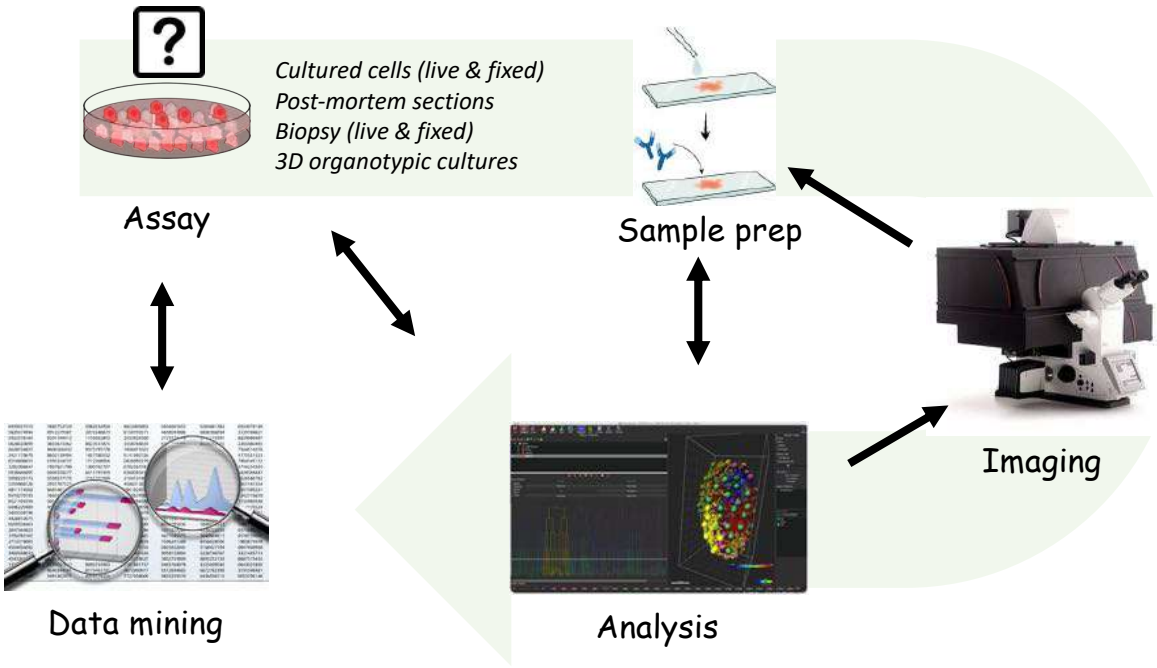
X-Y 2D | X-Y-Z 3D | X-Y-Z-λ 4D | X-Y-Z-λ-position 5D | X-Y-Z-λ–position-*t* 6D

- **Segmentation**
- **Intensity analysis**
- **Colocalisation**
- **2D & 3D tracking**
- **3D visualisation**
- **Deconvolution**

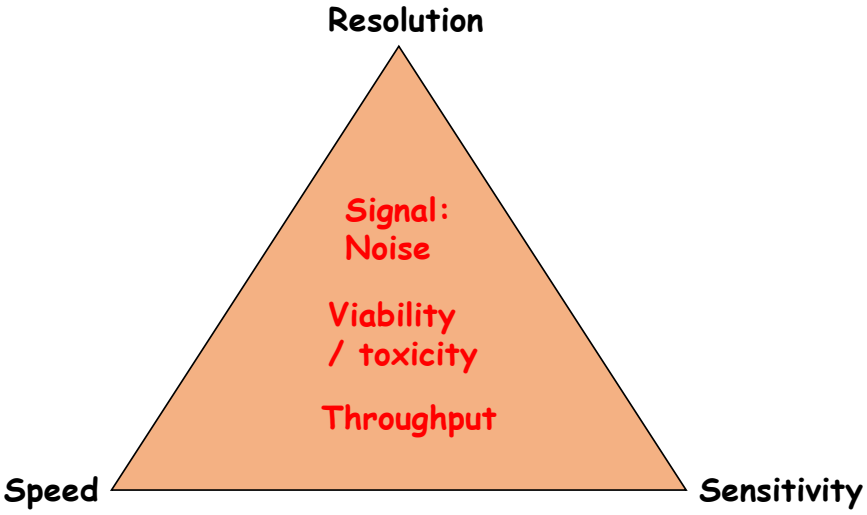


IMARIS® | Volocity | SVI | NIS-Elements | CellProfiler cell image analysis software | ImageJ

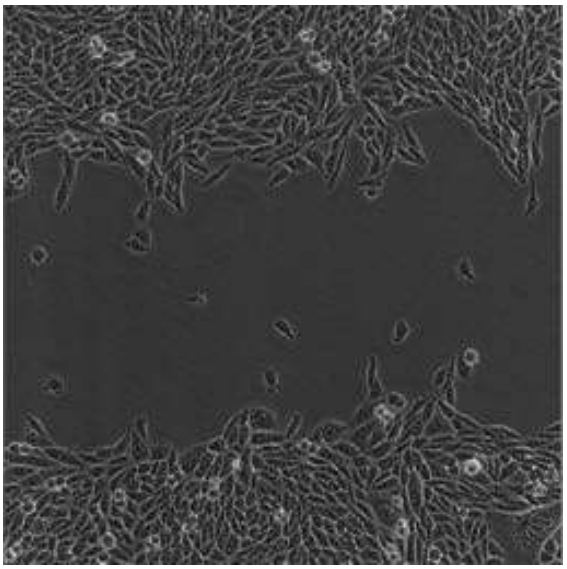
Imaging workflow

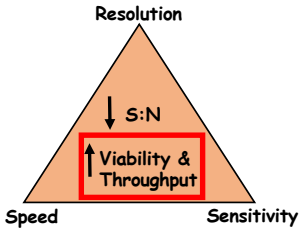


Choosing the right imaging modality: *The 'Triangle of Compromise'*



Choosing the right imaging modality: *brightfield time-lapse*

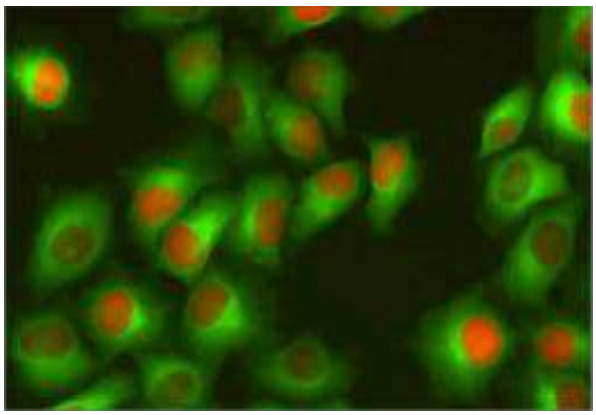




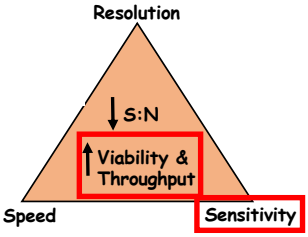
- **Cultured fibroblasts**
  - **Monitored using Nikon BioStation**

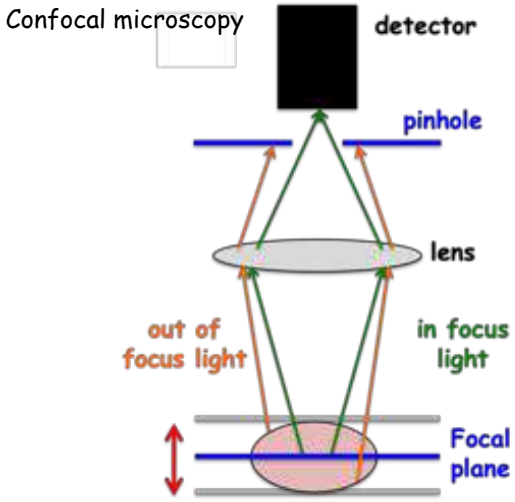
Capture @ 1fph
Playback x25k

Choosing the right imaging modality: *3D confocal time-lapse*



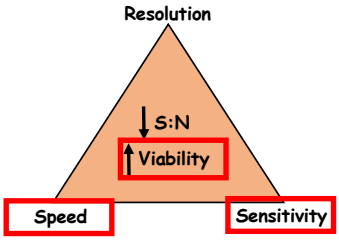
Capture @ 12fph
Playback x300

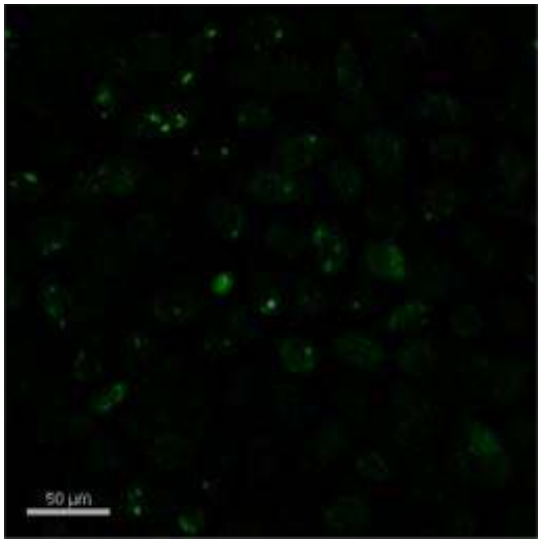




- **Cultured HeLa cells expressing GFP-tubulin & RFP-histone protein**
- **Multi-point acquisition over 18 hours**
- **Maximum intensity projection image formed from 7-confocal stacks**
  - **Several fields observed**

Choosing the right imaging modality: *resonant confocal or spinning disk*

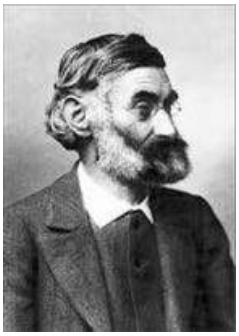




- **Skin cell monolayer loaded with Fluo Ca^{2+} indicator**
  - **Monolayer 'wounded' - intercellular Ca^{2+} signalling**

Capture @ 5fps
Playback x4

Super resolution imaging: *resolving structures*

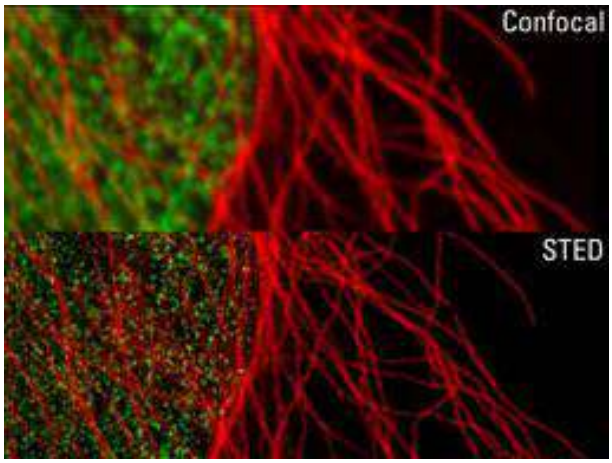


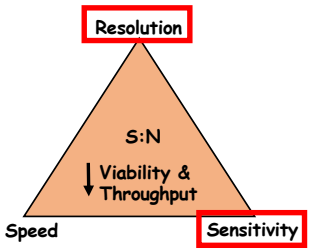
Ernst Abbe

$$d\ (\text{resolving power}) = \frac{\text{Wavelength of light}}{2 \times \text{Numerical aperture of lens}}$$

$$d_{GFP} = \frac{500\ nm}{2 \times 1.4} \qquad d = 179\ nm$$

Choosing the right imaging modality: **STED** super resolution

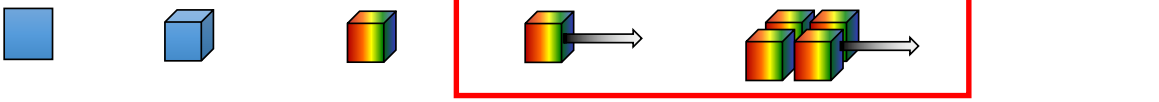




- **Leica SP8 STED super resolution**
  - **Up to 60 nm X-Y resolution**
  - **Up to 130 nm Z resolution**
  - **Fixed samples**
  - **White light laser – 200 excitation possibilities**
  - **Hybrid detectors – sensitive**
  - **Live-cell capability**

Multi-dimensional, multi-modal imaging *at the sub-cellular level*

X-Y	X-Y-Z	X-Y-Z-λ	X-Y-Z-λ-position	X-Y-Z-λ–position-*t*
2D	3D	4D	5D	6D

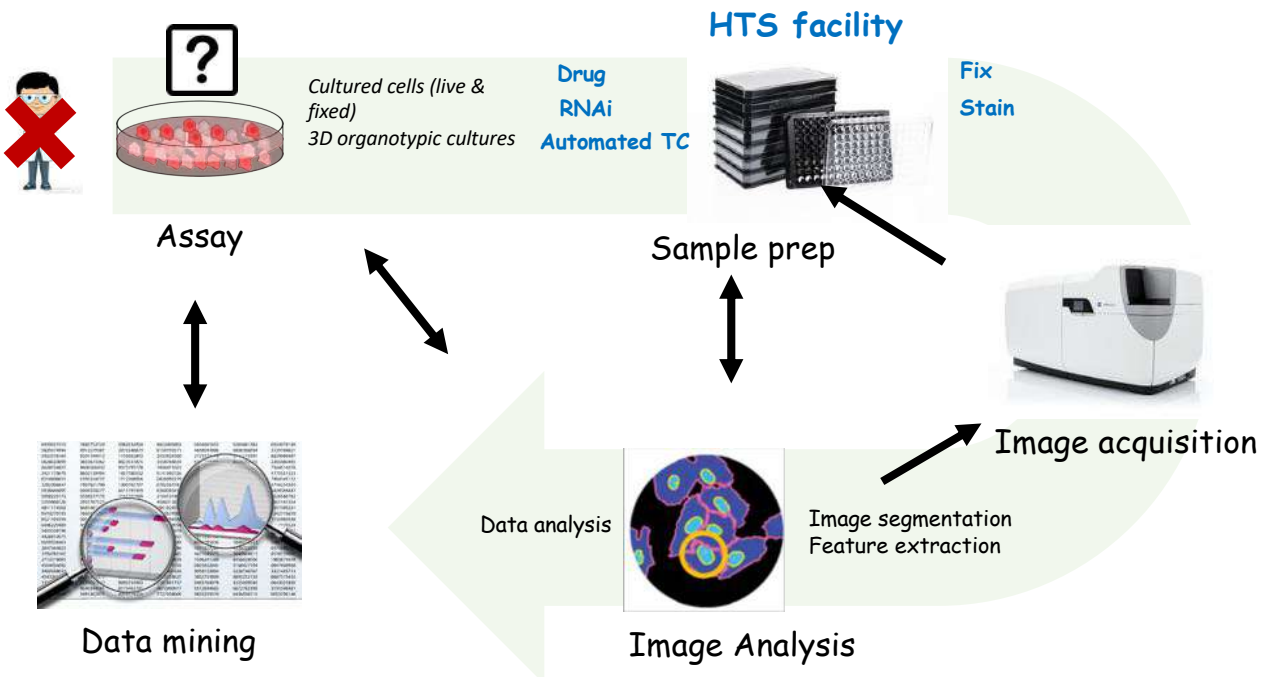


'Scalability'

- **Brightfield, phase and DIC microscopy**
- **Wide field fluorescence microscopy**
  - ***multi-parametric (x5) analysis***
- **Confocal microscopy**
- **Super resolution microscopy (live & fixed)**
- **Live-Cell imaging**
  Fast dynamic processes (>10fps)
  Longer lasting (days / weeks)
- **Automated high-content imaging / screening (live & fixed)**
  population data at the sub-cellular level

THE BioImaging Unit

High-content imaging / screening / analysis - typical workflow

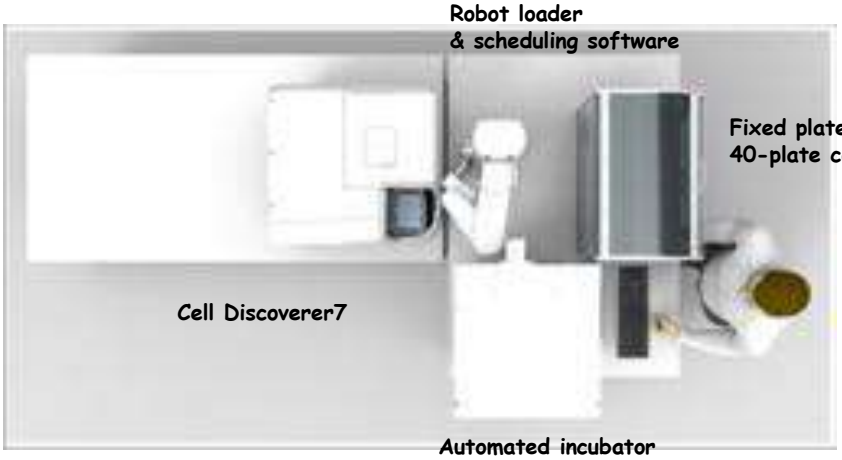


THE BioImaging Unit

Automated, live-cell microscope - Zeiss Cell Discoverer7

Zeiss Cell Discoverer7

Robot loader
& scheduling software



Fixed plate storage
40-plate capacity

Cell Discoverer7

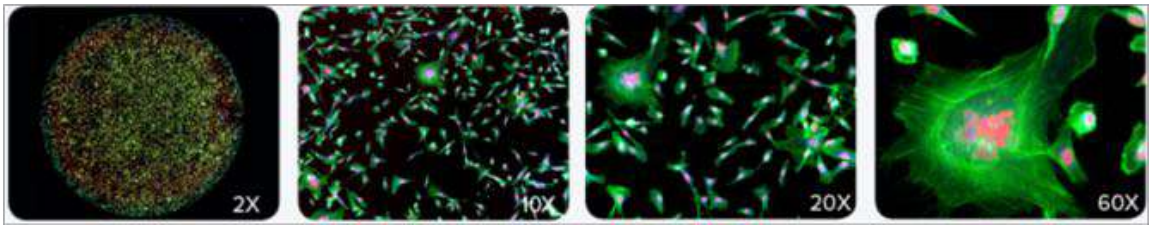
Automated incubator
20-plate capacity

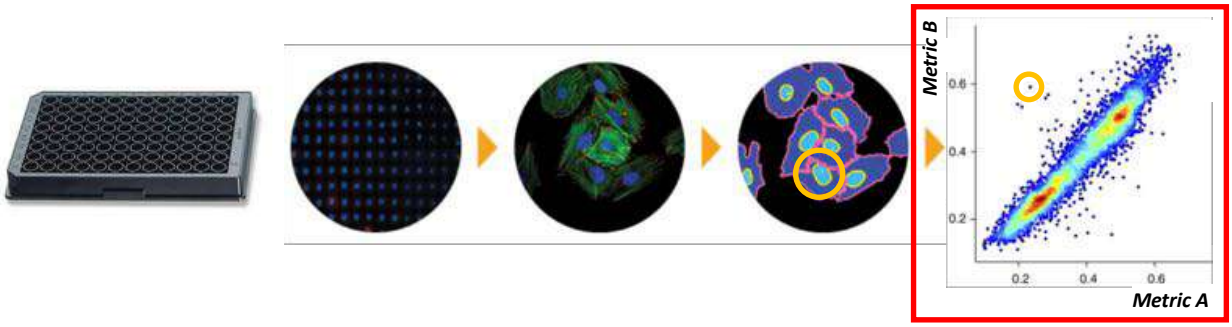
Wellcome funded

THE BioImaging Unit

High-Content / Throughput Imaging
Automated 'smart' multi-colour imaging & analysis

...one cell at a time





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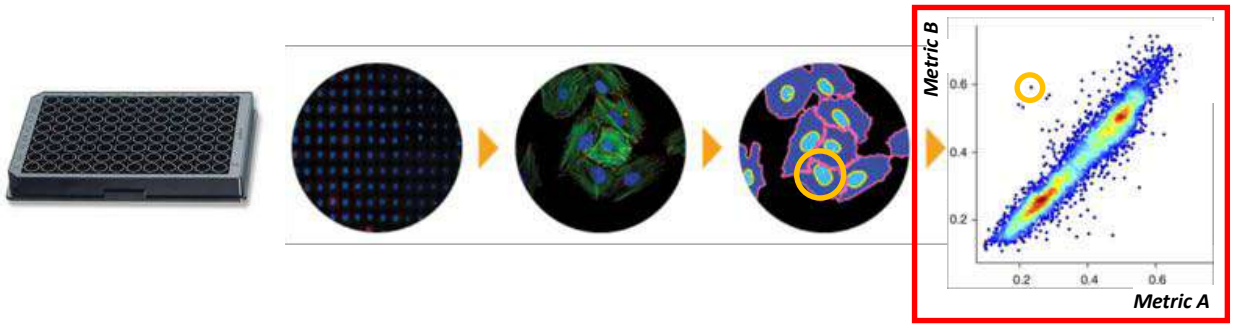
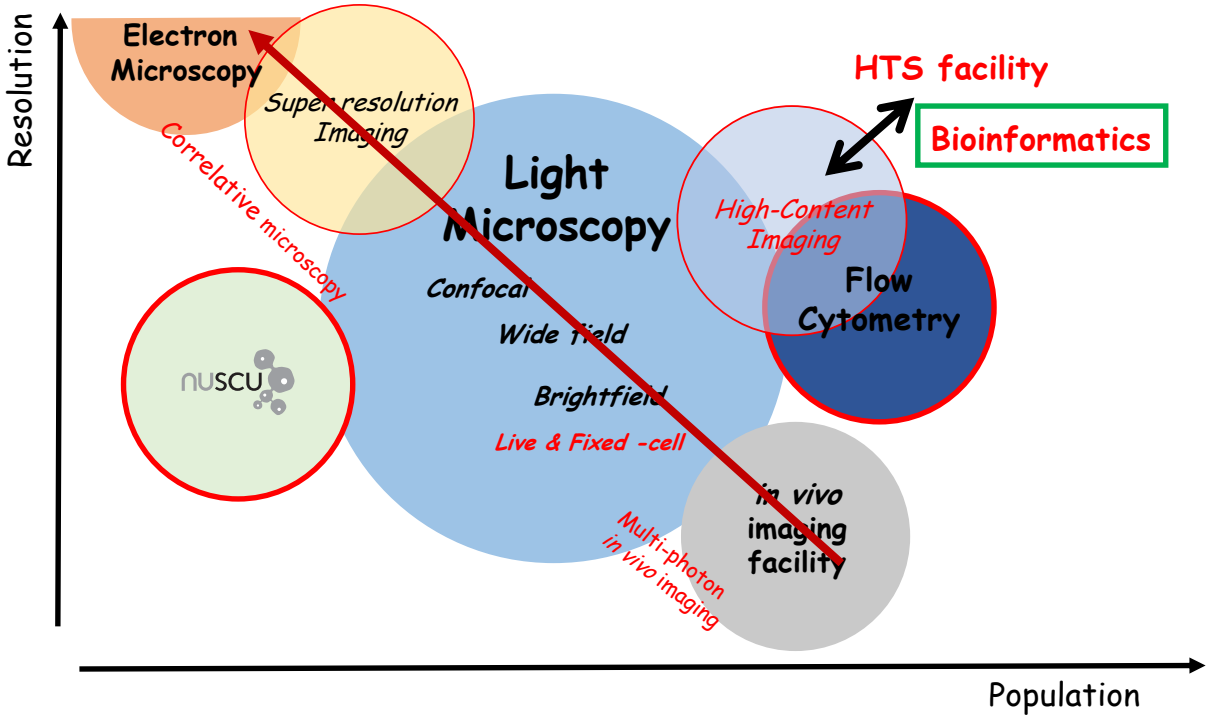
High-Content / Throughput Imaging
Data analysis

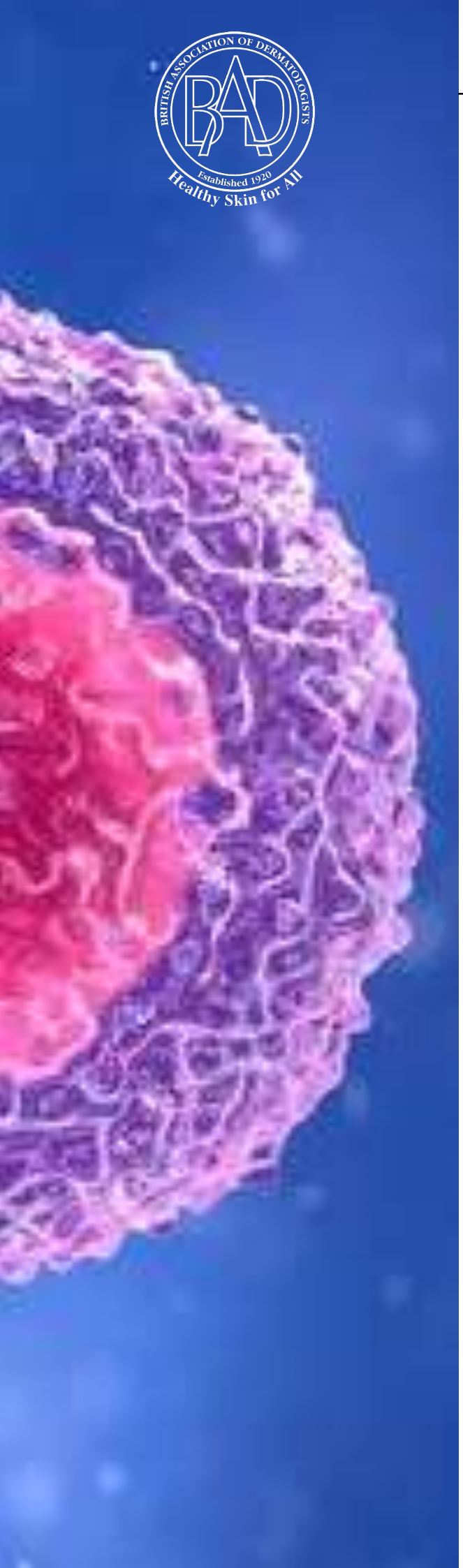


Statistical Analysis (pooling data from well and plates)

- Cell count (live / dead)
- Segmentation (requires cell surface / cytoplasm marker)
- Spot count (per cell)
- Intensity of protein (per cell or population)
- Morphology

The BioImaging network





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- Newcastle Hospital Healthcare Charity
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- Melanoma Focus
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- Biomedical Research Council
- AmLo Biosciences Ltd.
- Innovate UK
- 4ward North
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