Utrecht University

Veterinary Medicine

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Molecular Genetic Techniques Explained

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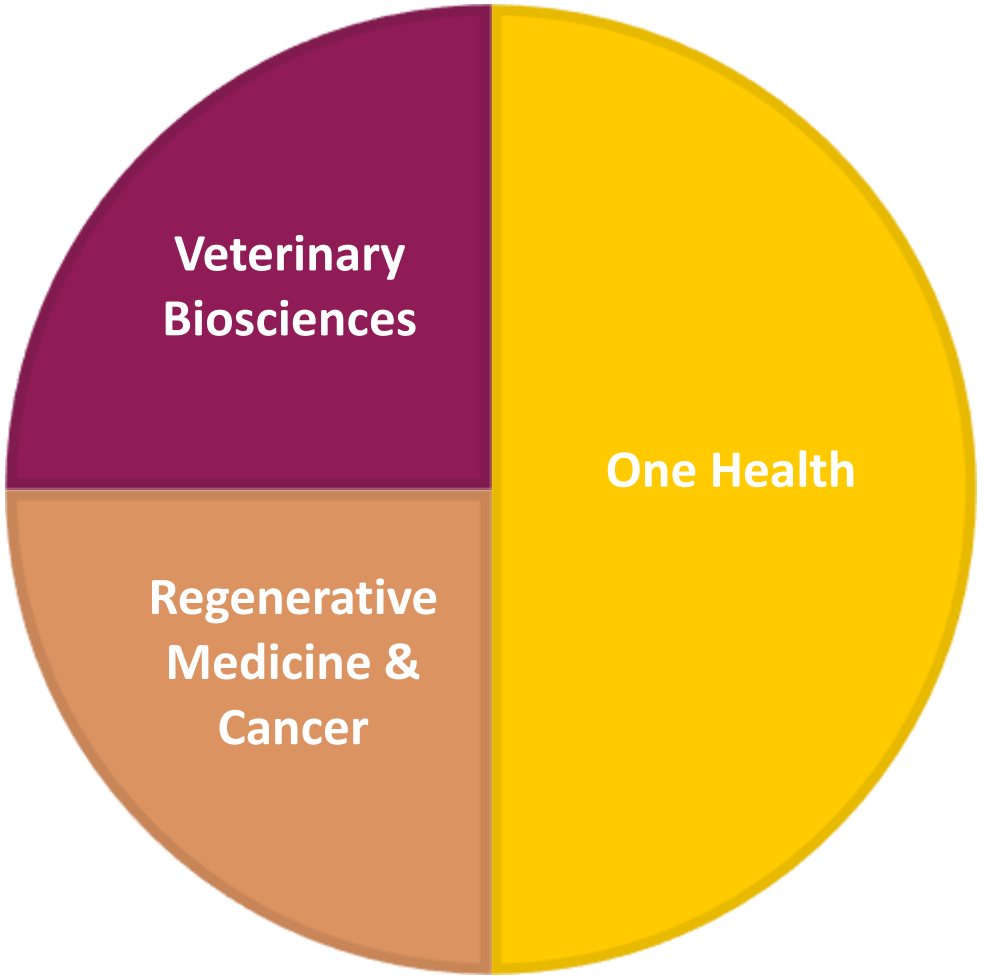
Research themes



Understanding and suppressing cancer

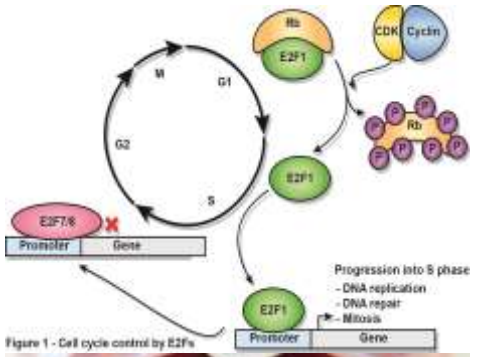
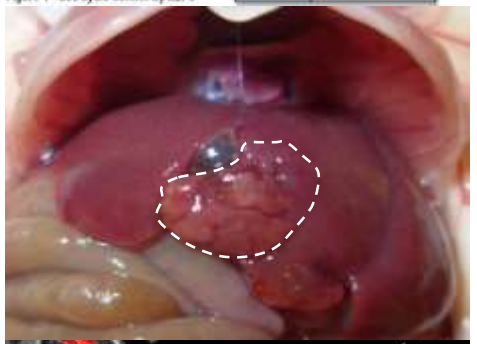
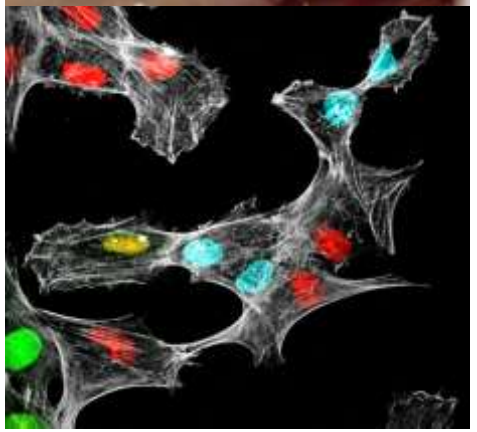


Figure 1 - Cell cycle control by E2Fs





Utrecht University umcu

dmpc
dutch molecular pathology center

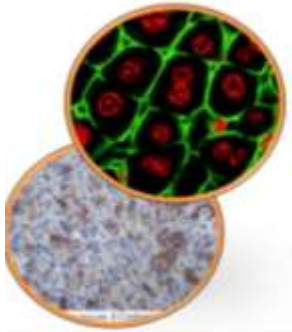
Building an interactive bridge between scientist and pathologist

Full laboratory animal necropsy service

training in mouse necropsy techniques:

In vitro model
Organoids

Histologic analysis
Organoids
Zebrafish
Embryos and placentas
Rodent tissues



Additional staining
Immunohistochemistry,
Immunofluorescence,
Histochemistry

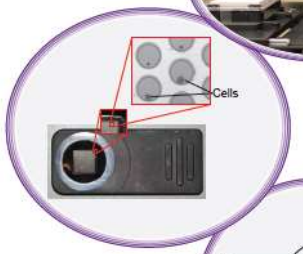
Molecular analysis
RNA, DNA, FACS analysis

Comprehensive Study Support
Optimizing your study design
Minimize animal usage
Quantitative and qualitative analysis
Interpretation and recommendations

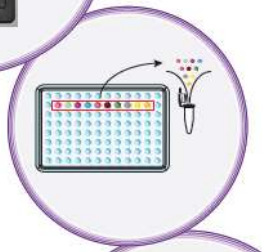
Single Cell Analysis Center Utrecht

Sample preparation
Tissue dissocation
into single cells

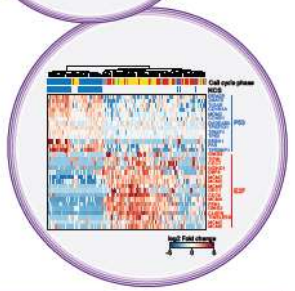
Cell capturing technology
Isolate, image, and
punch cells of interest



Barcoded NGS libraries
SMARTer, CEL-Seq2, WGA
RNA of DNA sequencing



Bioinformatic analysis
Quality control
Cell identification
Images & NGS data
Statistics



Research Facility Service
Optimizing study design
Sample preparation by experienced operators
Comprehensive basic bioinformatic analysis
Interpretation and recommendations

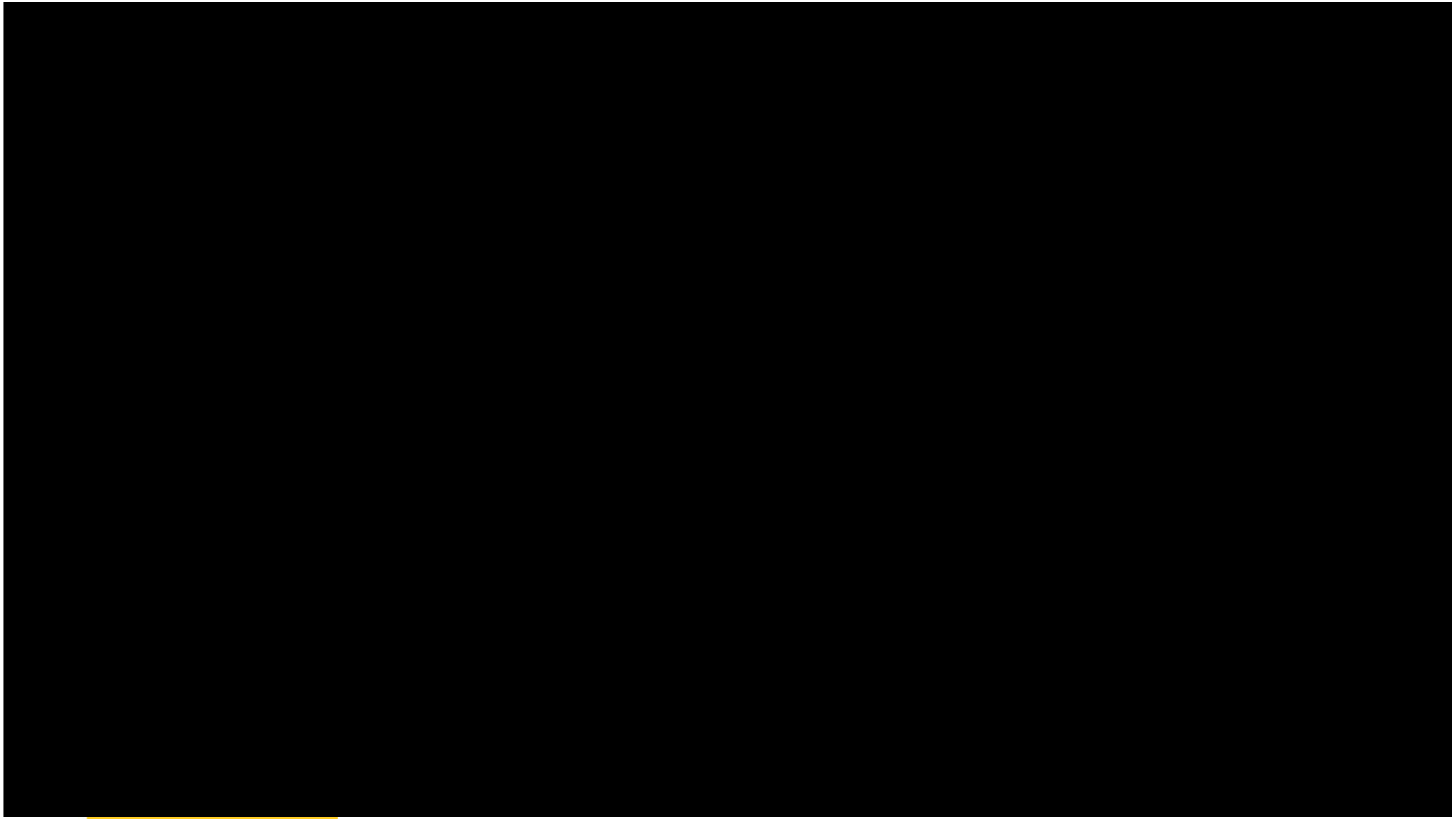
Content

- Introduction Molecular Pathology
- Data analysis on interactive scientific case studies
- Molecular genetic techniques
- Find mistakes in scientific abstract

Why gaining knowledge about Molecular Pathology ?

- Identifying cause of disease
- Understanding the pathogenesis
- Prognosing survival duration of patients
- Building interactive bridge between pathologist & scientist
- Discovering new diseases and novel therapy strategies
- Identifying new functions of genes
- Passing successfully the ECVP exam

From DNA to protein



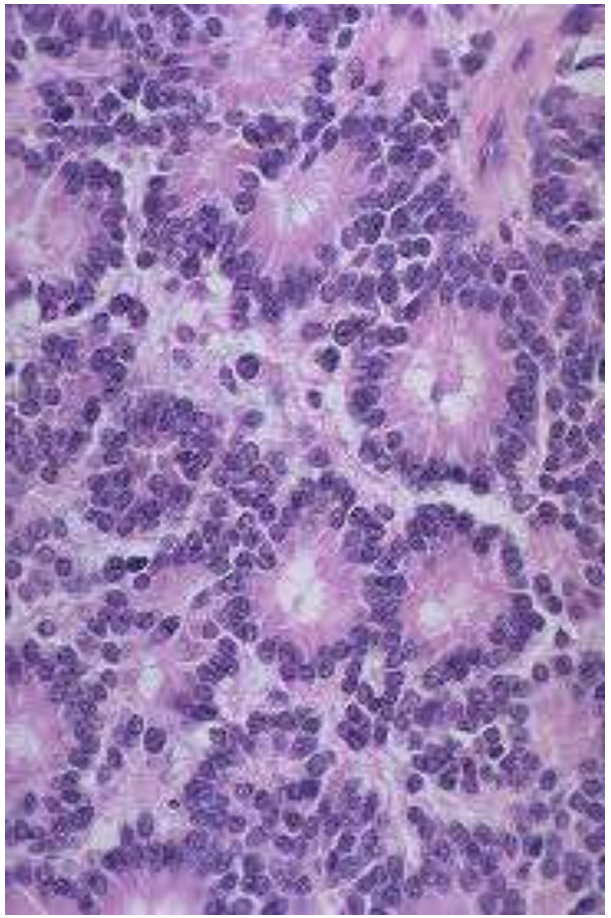
https://www.youtube.com/watch?v=rdF3mnyS1p0

ECVP exam questions: molecular genetic techniques

- What do you measure by the technique X?
- Briefly explain the principle of technique X
- Explain the differences between two techniques
- Explain the Crispr/Cas9 approach to make a knockout mouse
- Name two special stains to support your diagnosis
- Name three molecular techniques to confirm your etiological diagnosis
- Briefly outline the steps in the workflow of a NGS experiment

Retinoblastoma

1986



Flexner Wintersteiner rosettes

Cell cycle control by Rb-E2F pathway

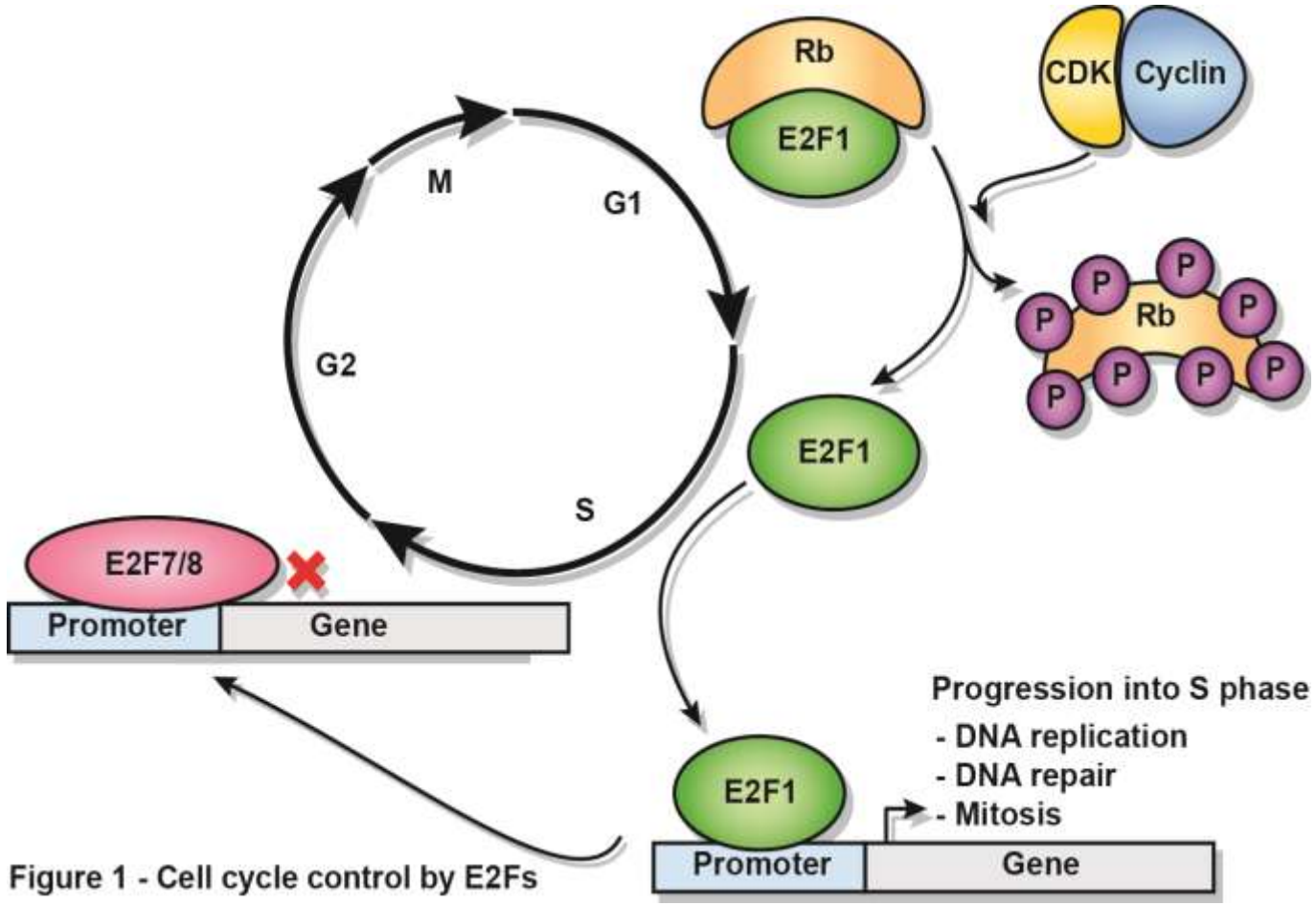
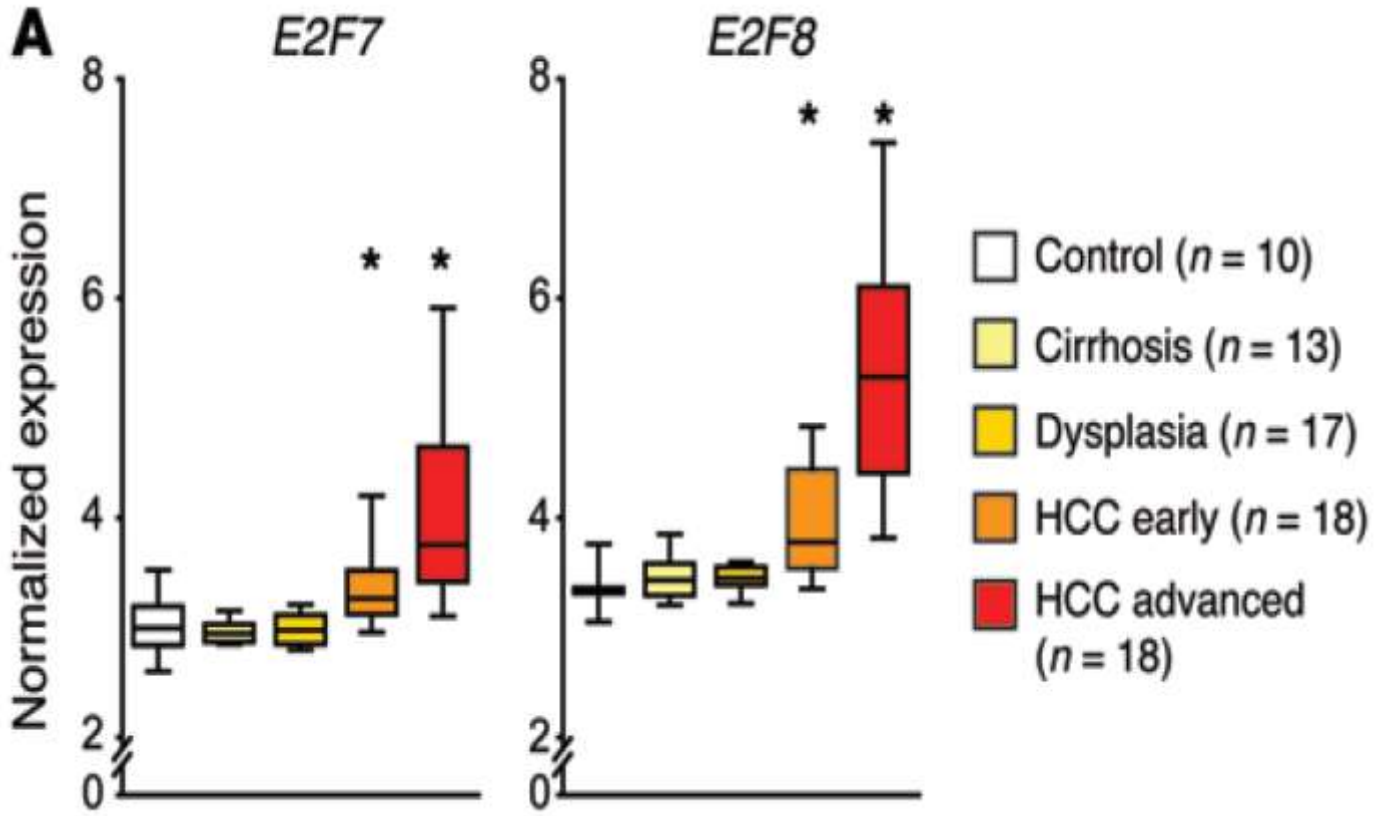


Figure 1 - Cell cycle control by E2Fs

What do you measure by microarray analysis?



Box plots showing the expression levels of *E2F7* and *E2F8* from patients with normal or diseased livers derived from Affymterix Microarrays.

Explain the principle of microarray analysis

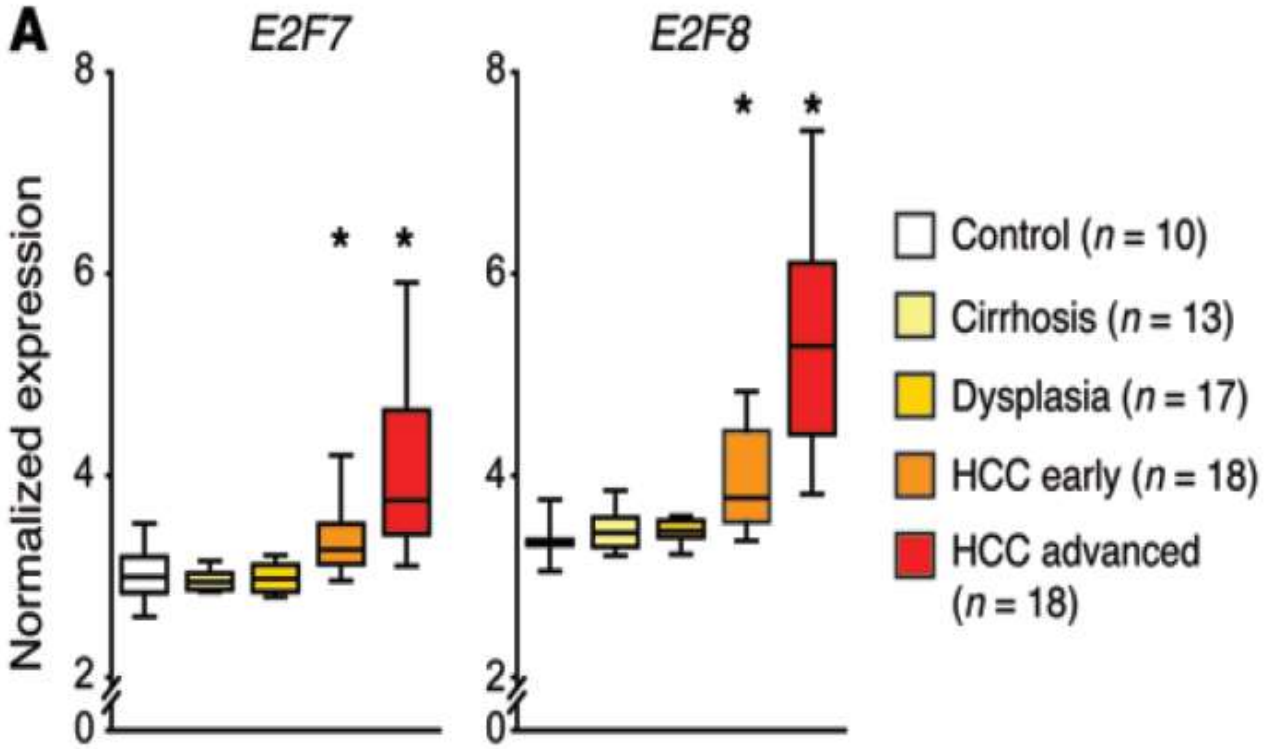
https://www.youtube.com/watch?v=yzBVOCwRanI

Name another technique to measure global RNA expression

Describe the data and provide an overall interpretation of the findings

Suggest a hypothesis about the role of E2F7 and E2F8 in liver cancer

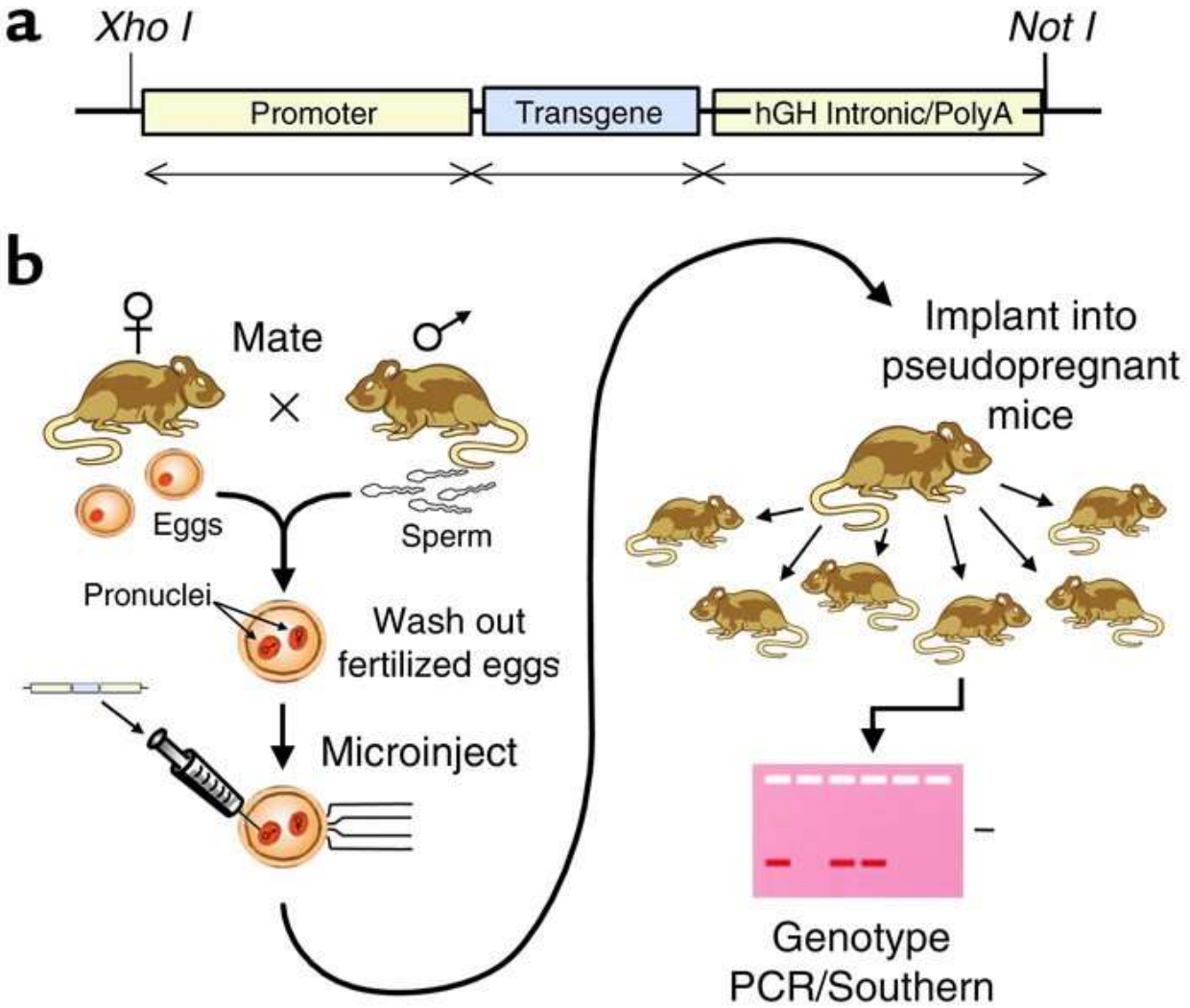
Provide one experiment and techniques where you could test your hypothesis



Box plots showing the mRNA levels of *E2F7* and *E2F8* from patients with normal or diseased livers derived from Affymterix Microarrays.

J Clin Invest. 2016 Aug 1;126(8):2955-69

Explain the principle of generating a transgenic mouse overexpressing a protein



Elias et al. J Clin Invest. 2003;111(3):291-297

Explain the principle of IHC

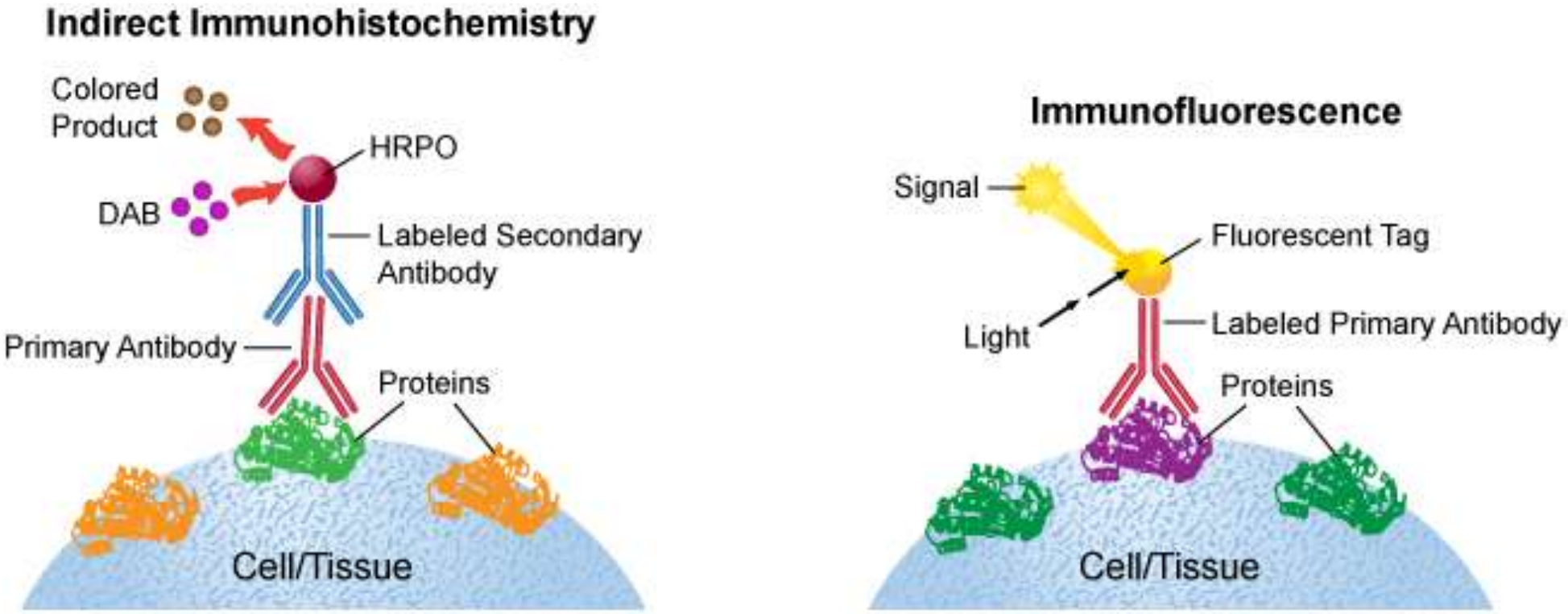
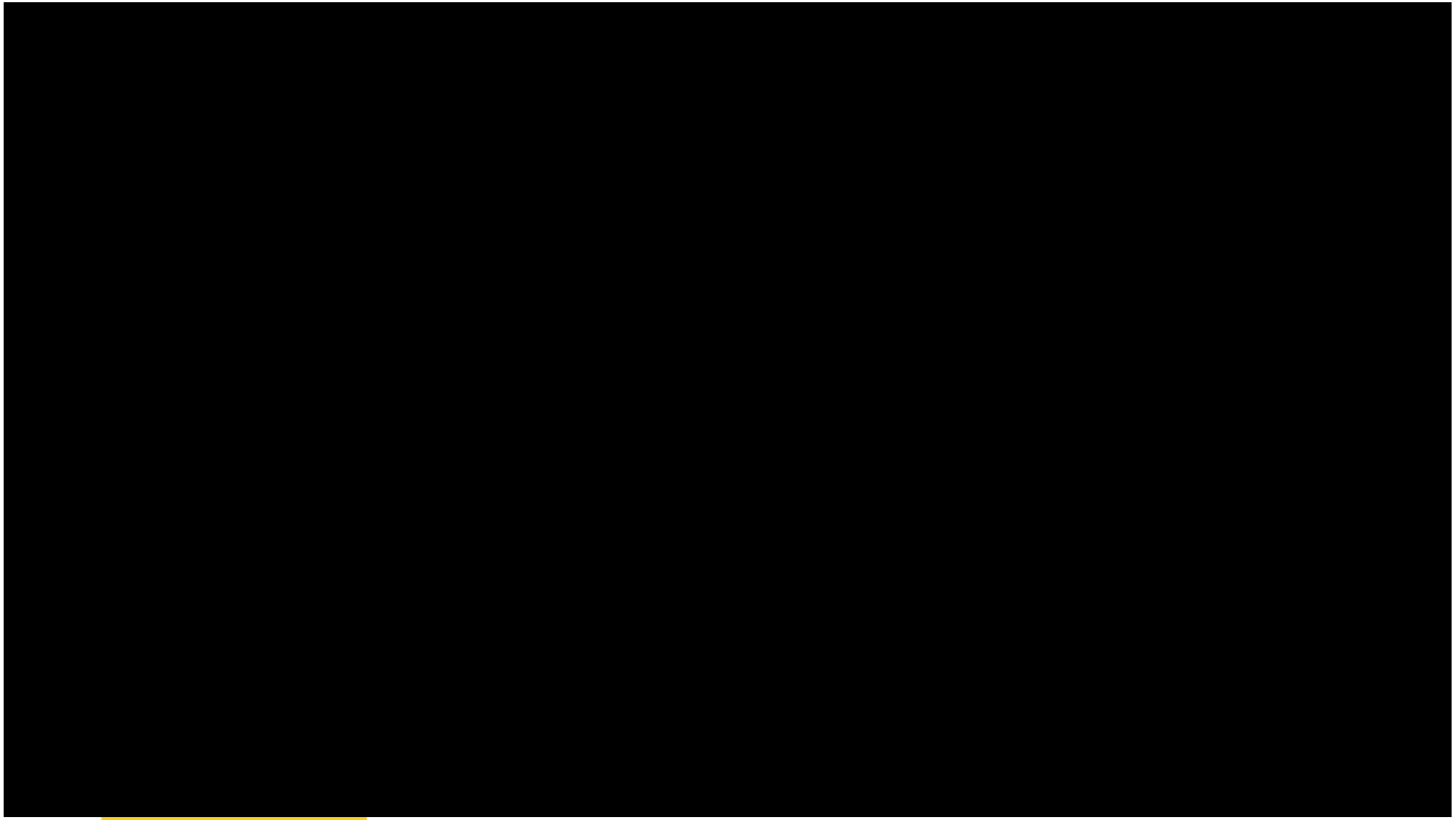


Diagram 1: *Illustration of Indirect Immunohistochemistry and Immunofluorescence methods.*

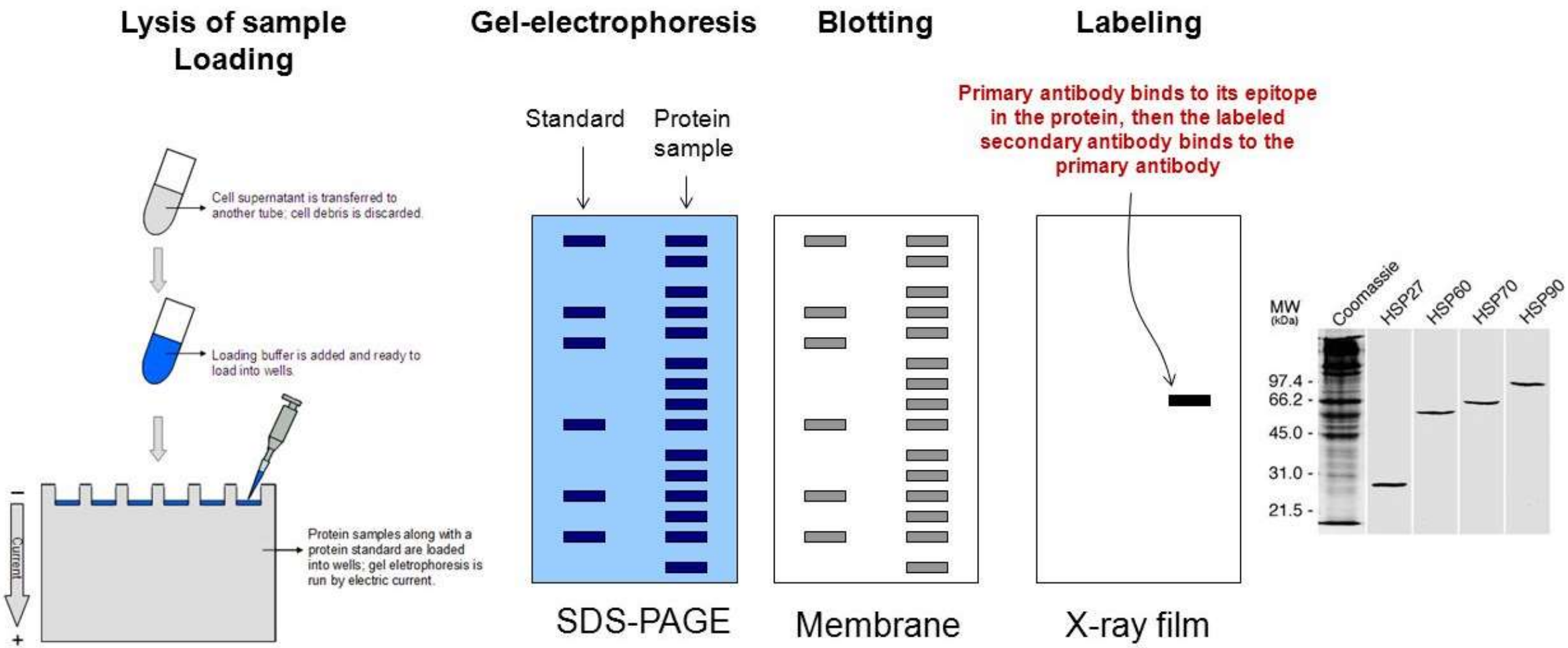
https://lookfordiagnosis.com/mesh_info.php?term=Immunohistochemistry&lang=1

Principles of ELISA



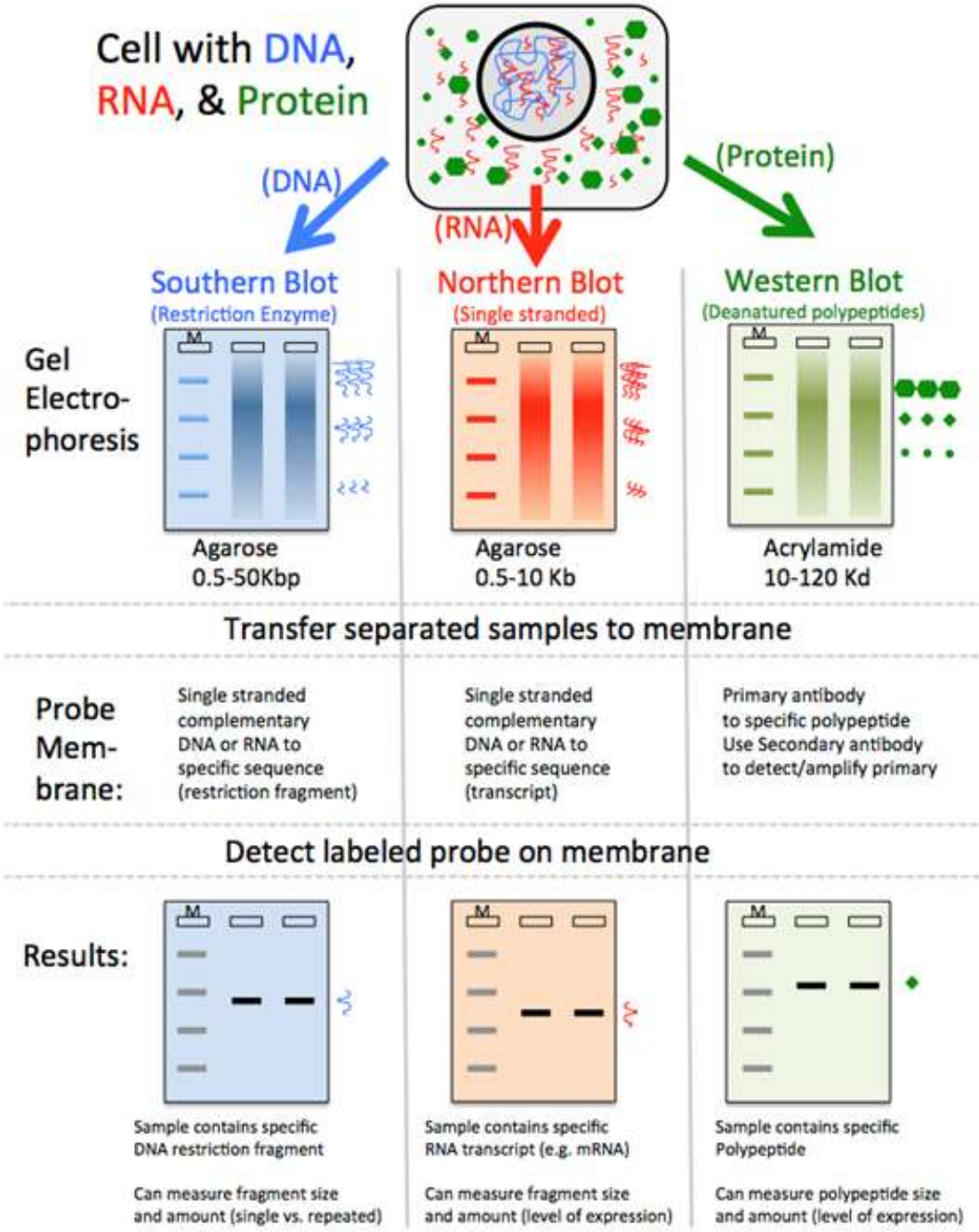
https://www.youtube.com/watch?v=lUWpWKVcmc4

Principle of Western blotting



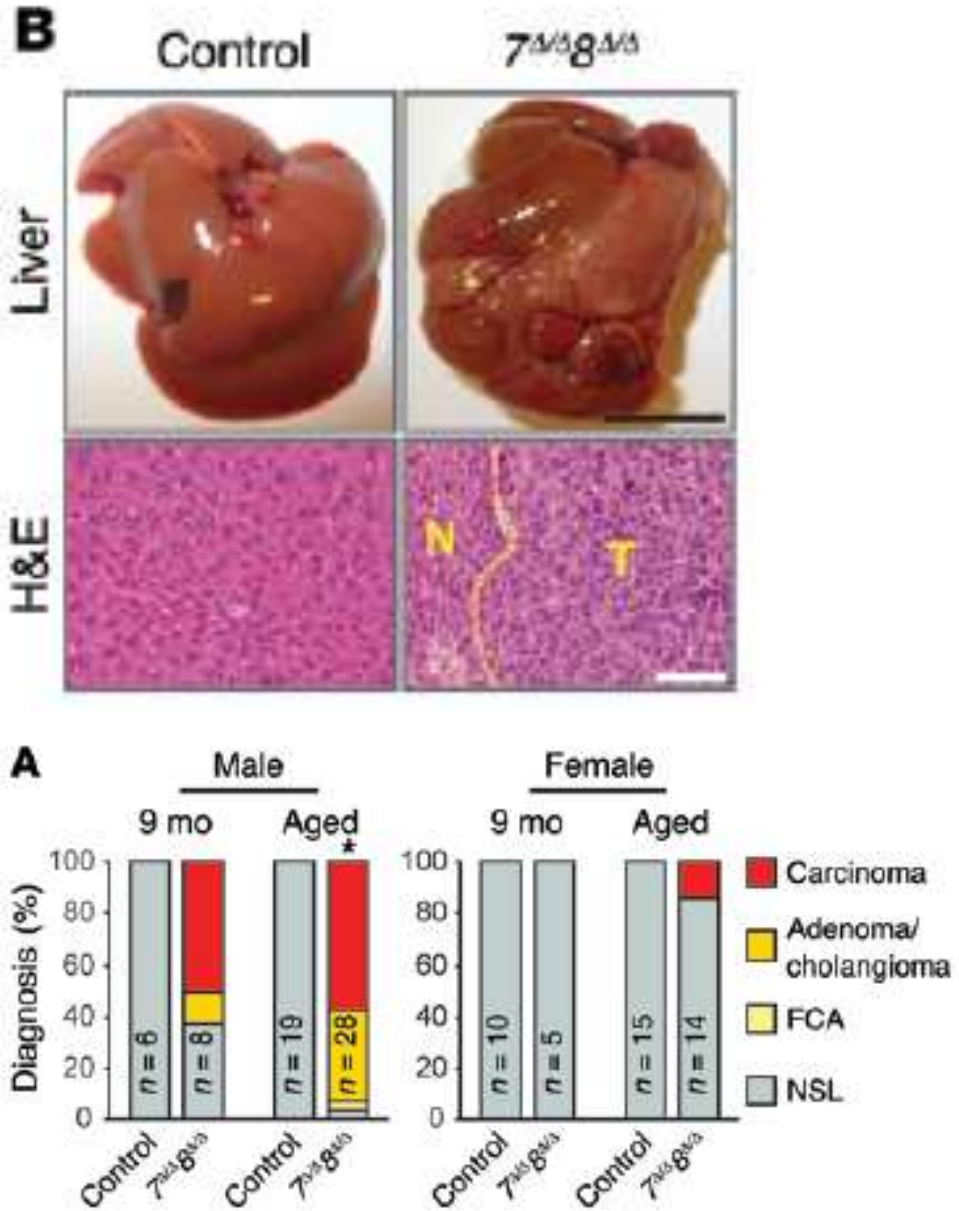
https://slideplayer.com/slide/8665767/

Southern, Northern and Western blotting



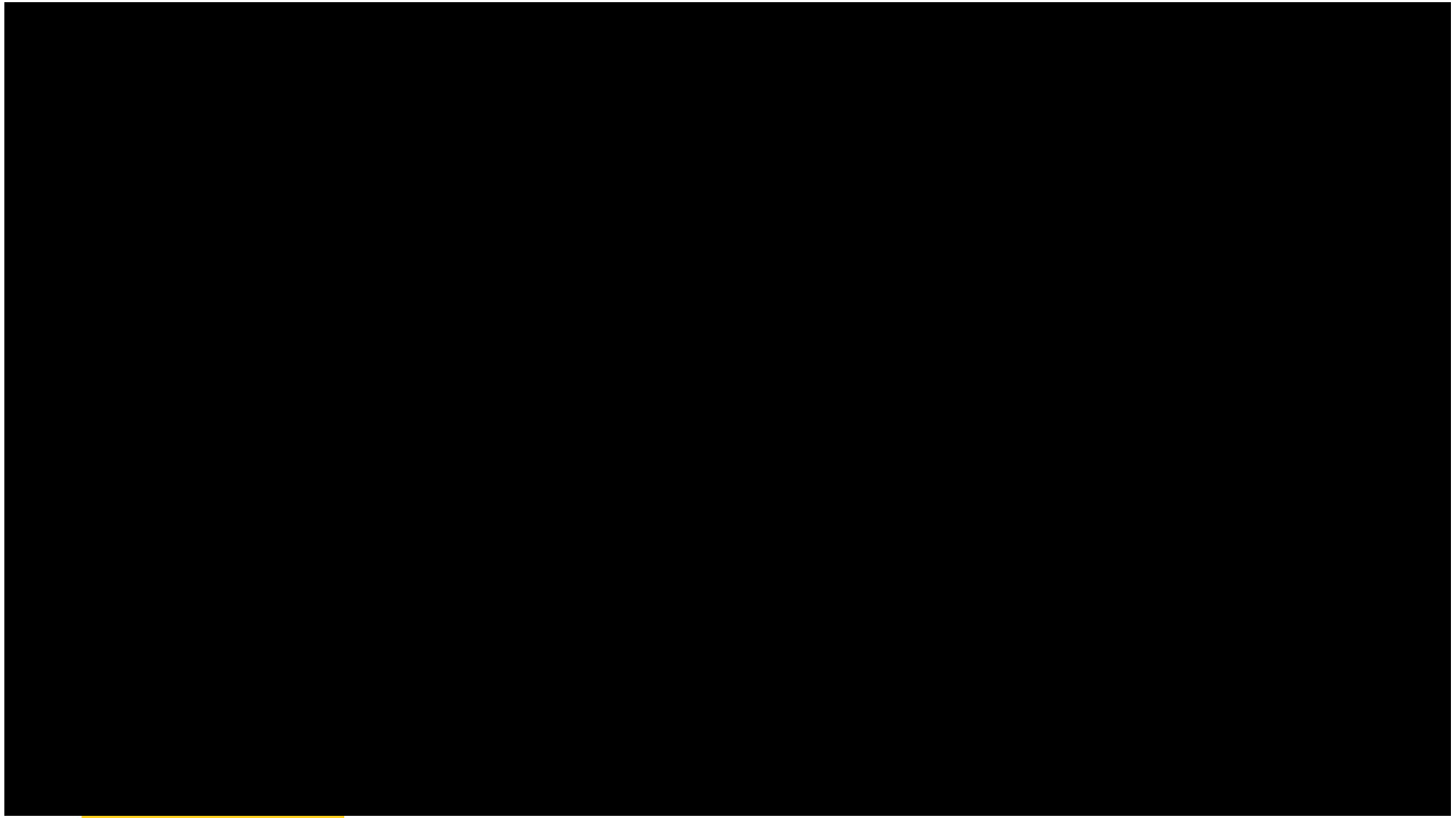
What would you expect when you knockout E2F7/8 in the mice concerning liver cancer?

Which techniques can be used to generate knockout mice?



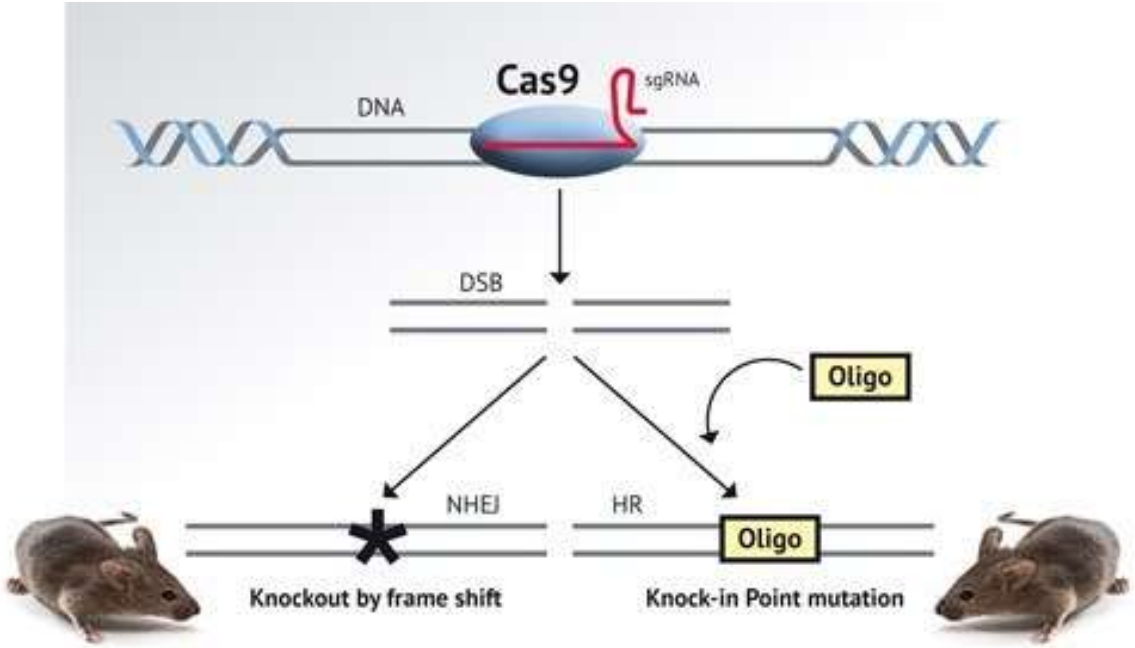
J Clin Invest. 2016 Aug 1;126(8):2955-69

Explain the Crispr/Cas9 approach to make a knockout mouse

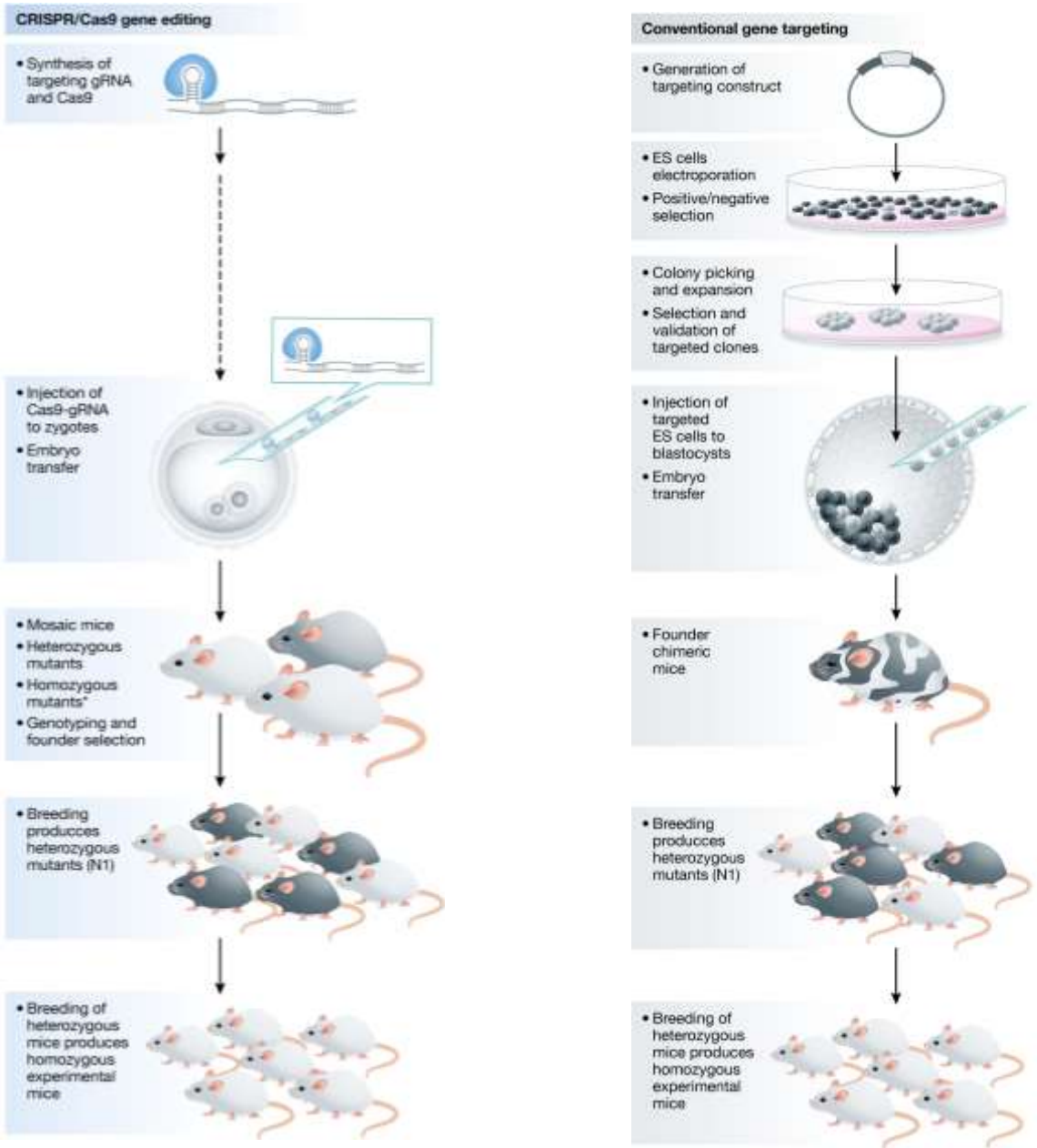


https://www.youtube.com/watch?v=4YKFw2KZA5o

Principle of gene editing in mice

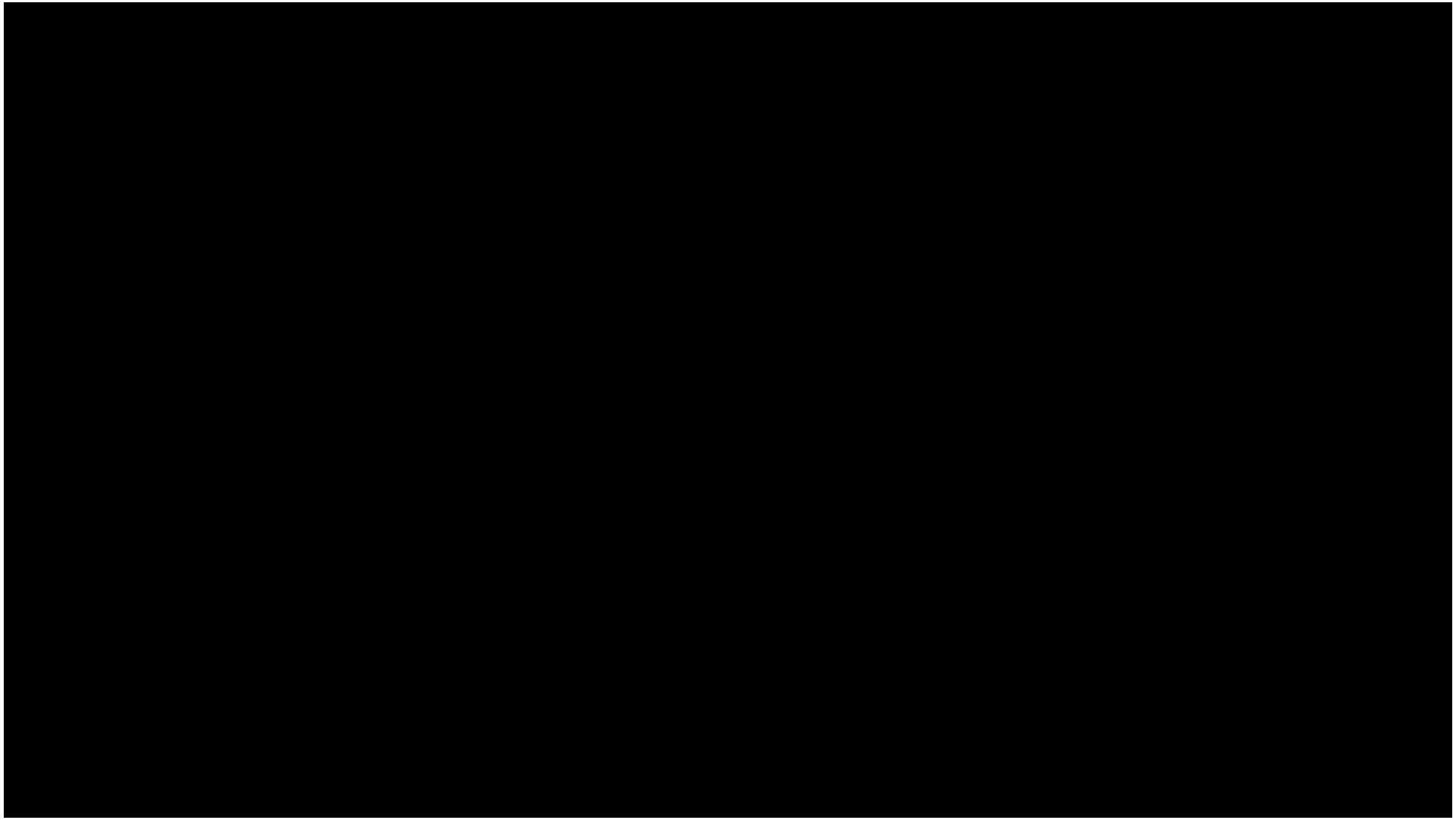


https://cmmt.ubc.ca/facilities-services/mouse-animal-production/maps-services/crispr-cas9/



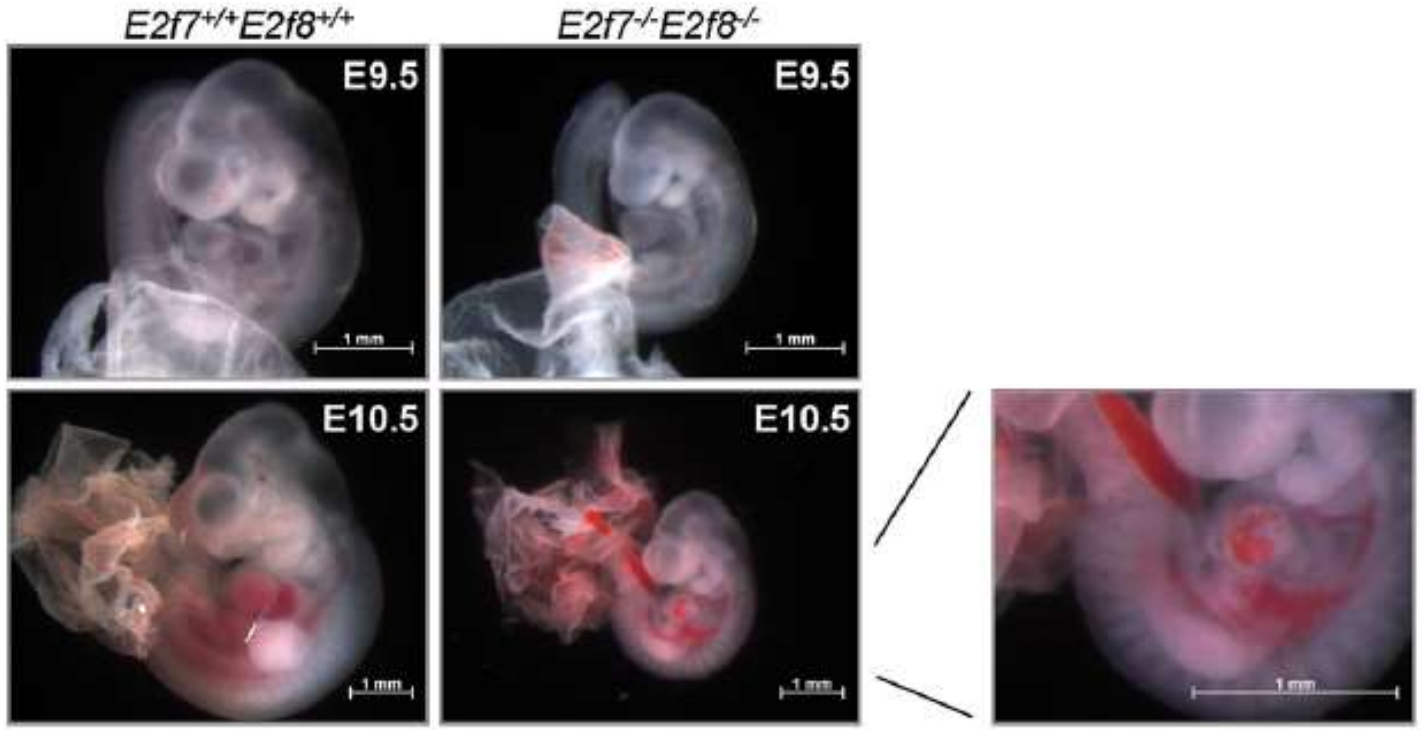
EMBO Rep, Volume: 18, Issue: 2, Pages: 187-193,

Explain the principles of the siRNA technology for knocking down gene expression



https://www.youtube.com/watch?v=cK-OGB1_ELE

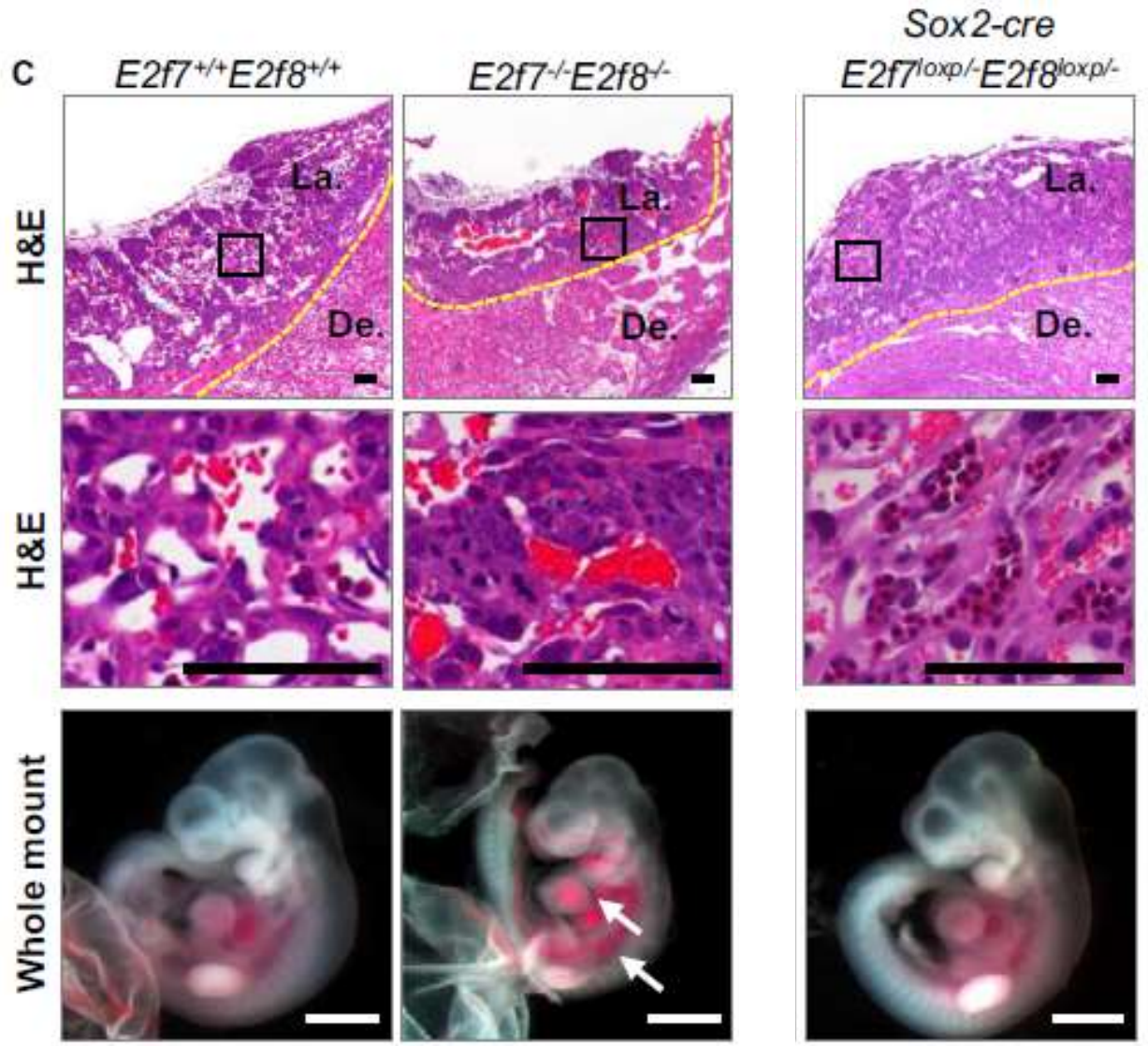
What if the ko mice show embryonic lethality?



What could be the cause of death?

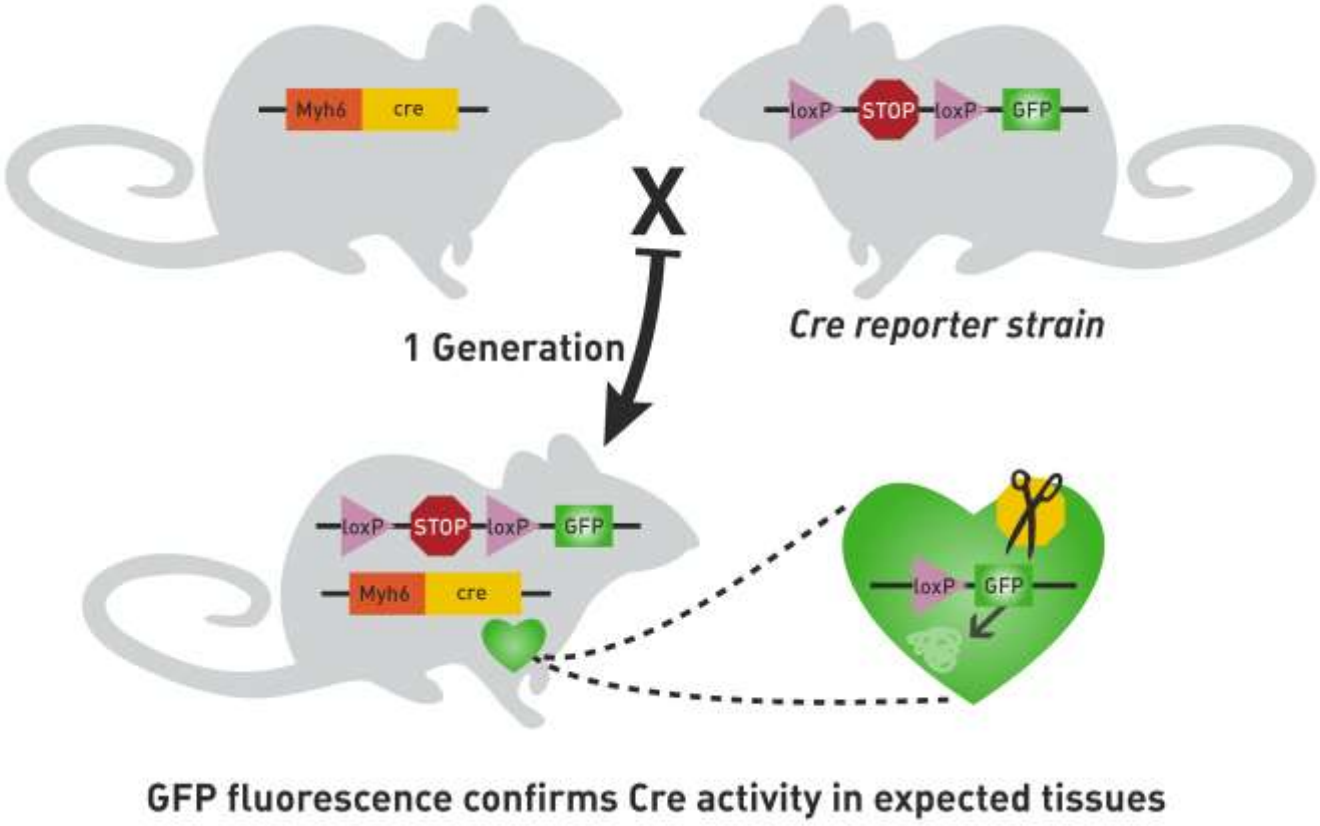
Dev Cell. 2012 ;22(4):849-62

How could you prove that the placenta is responsible for fetal death



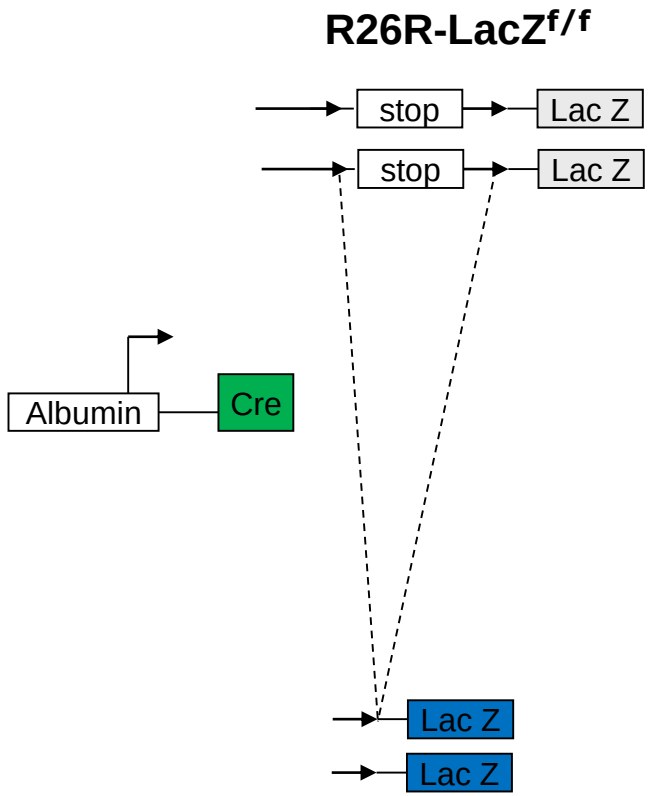
Dev Cell. 2012 ;22(4):849-62

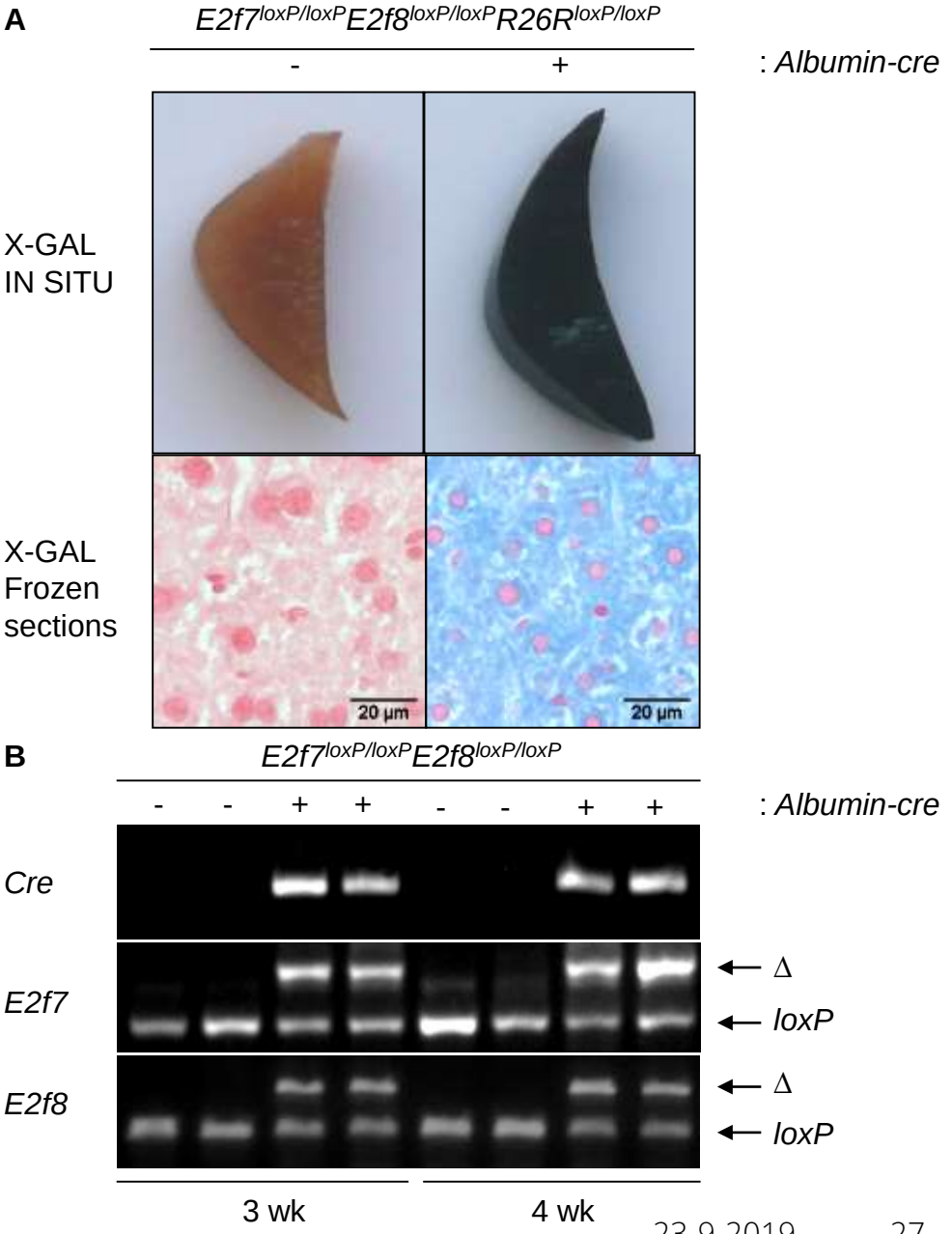
Explain the principle of generating conditional ko mice



https://www.jax.org/news-and-insights/jax-blog/2011/october/cre-lox-breeding-for-dummies-part-ii

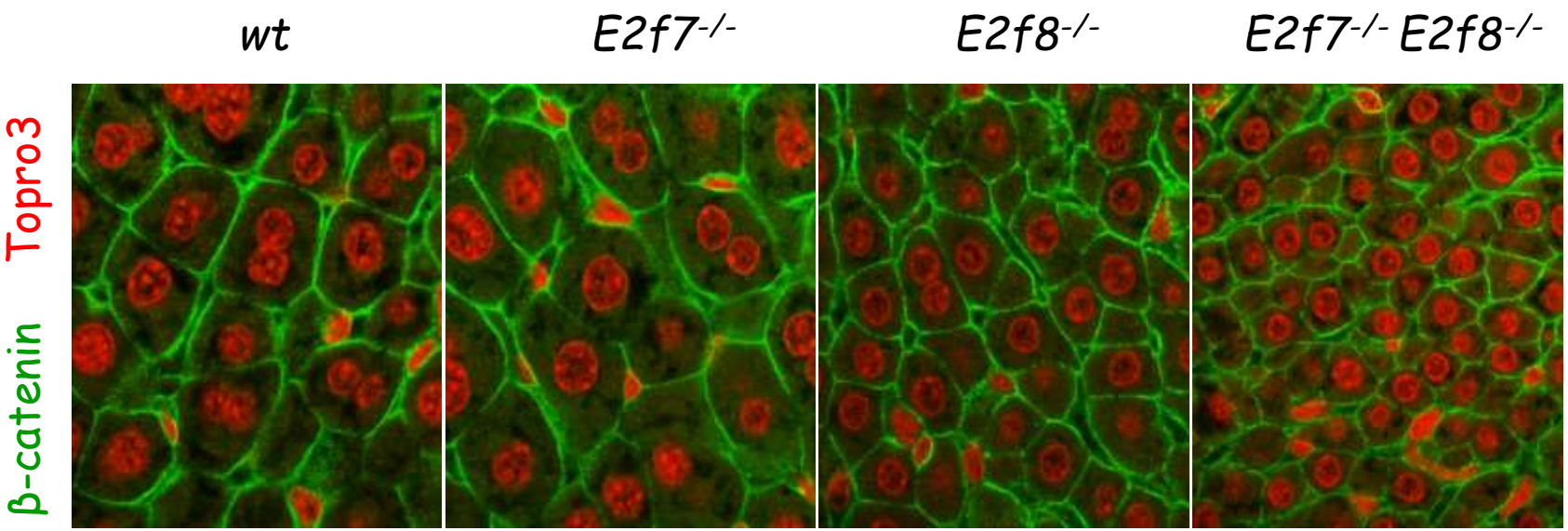
Liver specific deletion of E2F7/8





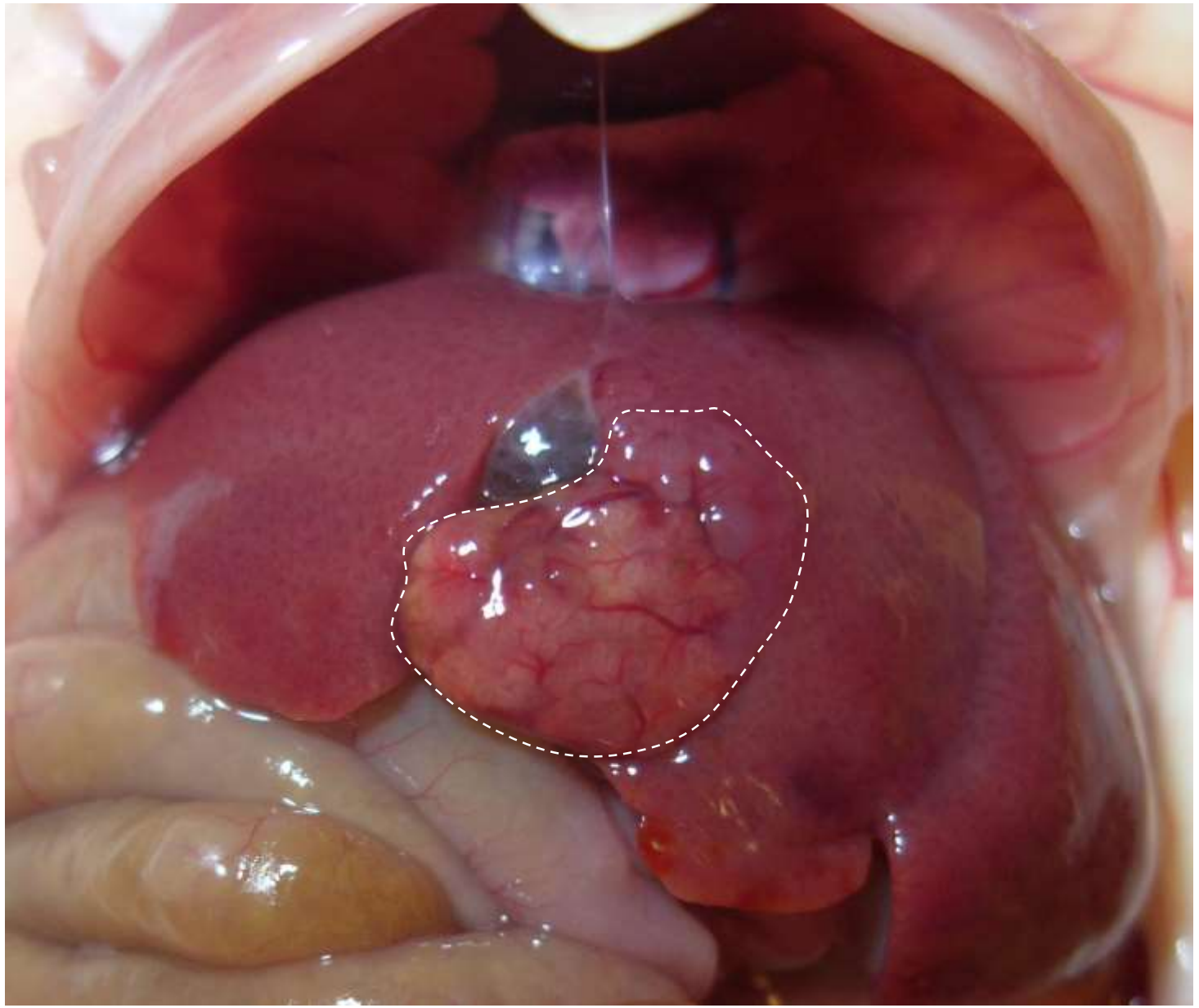
Pandit et al. Nature Cell Biology 2012

E2F7/8 is essential liver cell binucleation



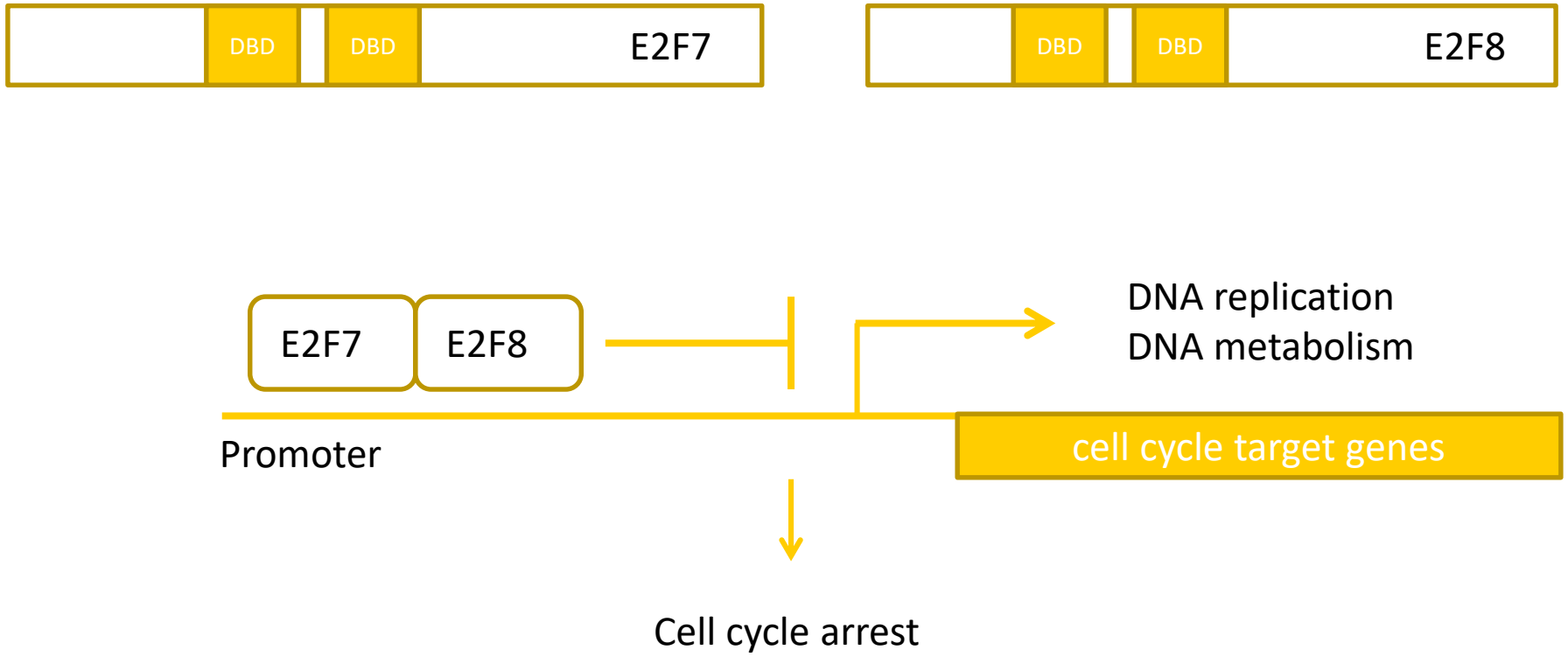
Pandit et al. Nature Cell Biology 2012

Inactivation of E2F7/8 results in liver tumors

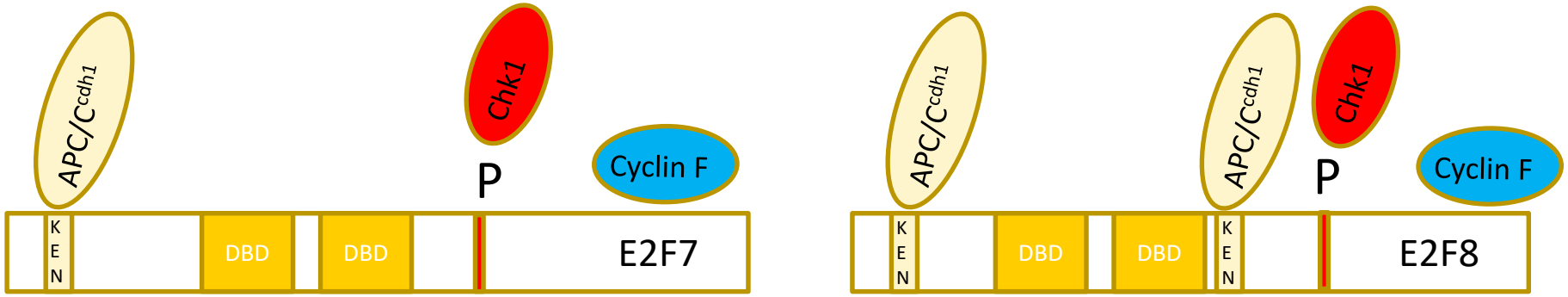


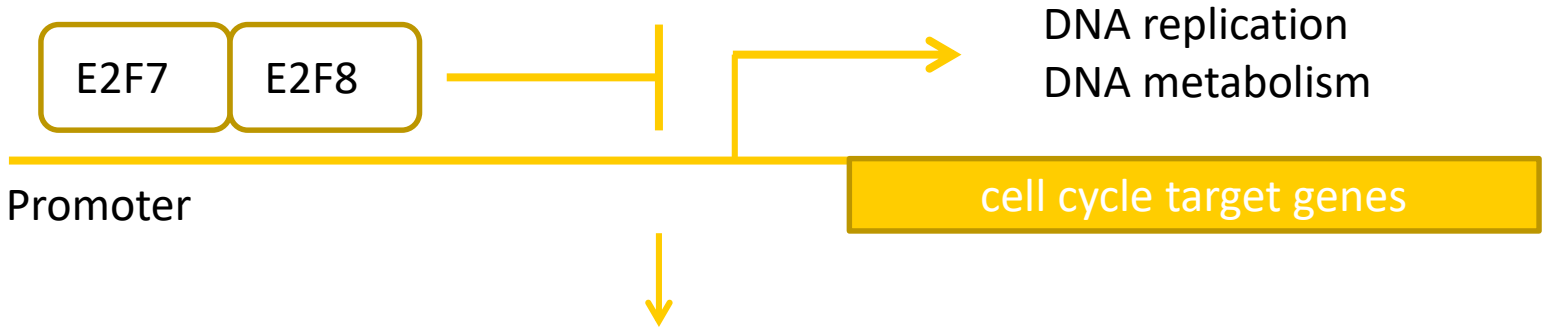
Pandit et al. Nature Cell Biology 2012

E2F7/8 are transcriptional repressors of cell cycle genes



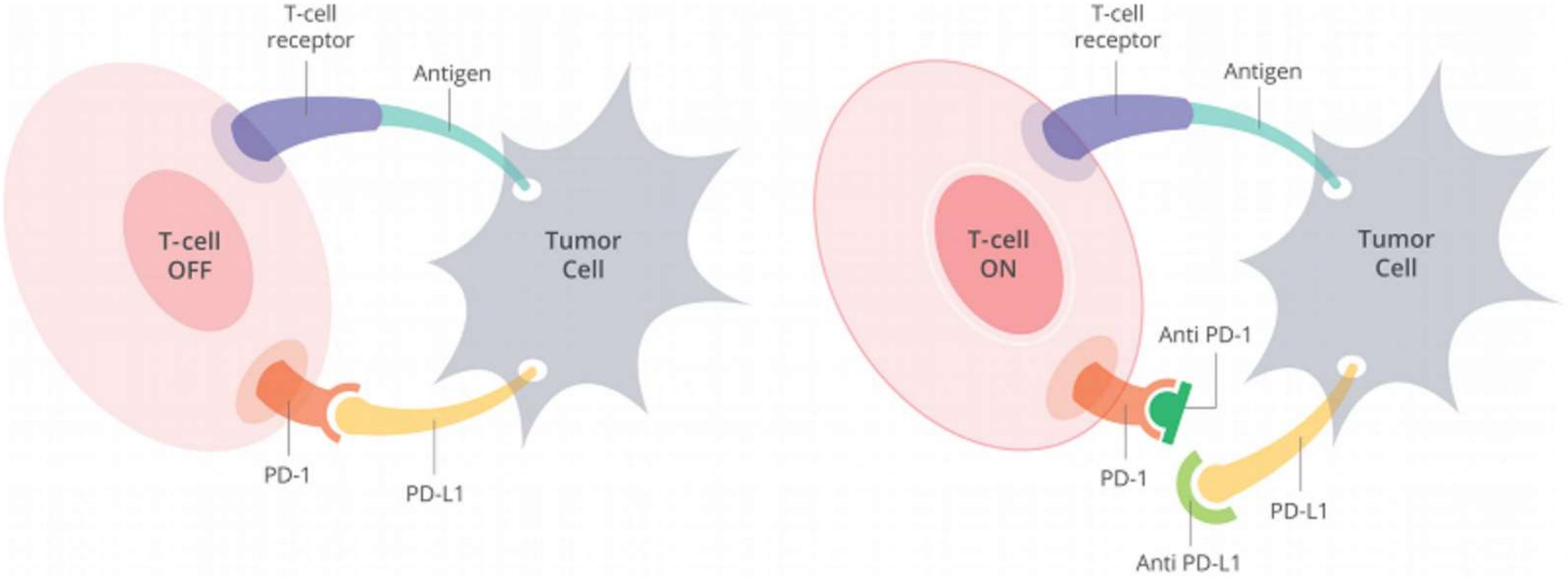
Targets to regulate E2F7/8





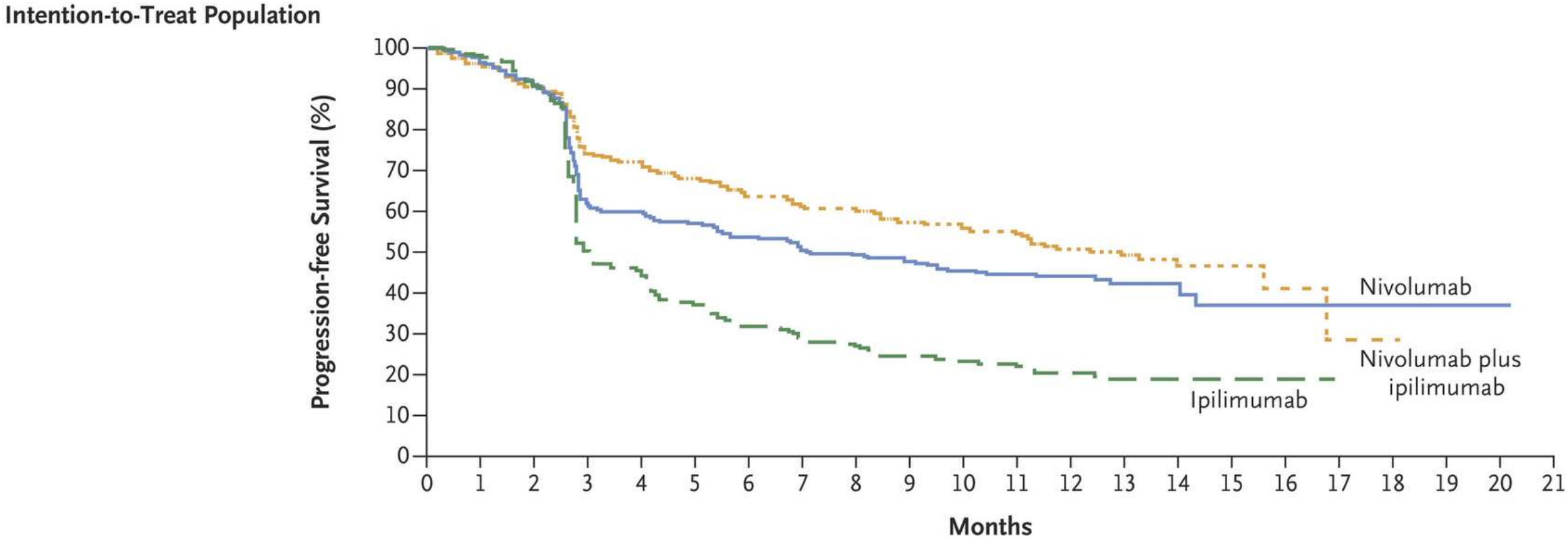
Boosting the immune system to fight cancer

- Tumor cells can express PD-L1 to inactivate attacking cytotoxic T-cells
- Therapeutic antibody against PD-L1 blocks this defense to unleash T-cell response



http://blogs.shu.edu/cancer/2016/11/09/how-pd-1-abrogates-the-anti-tumor-immune-response/

Which patients benefit from PD-1 inhibition (nivolumab)?

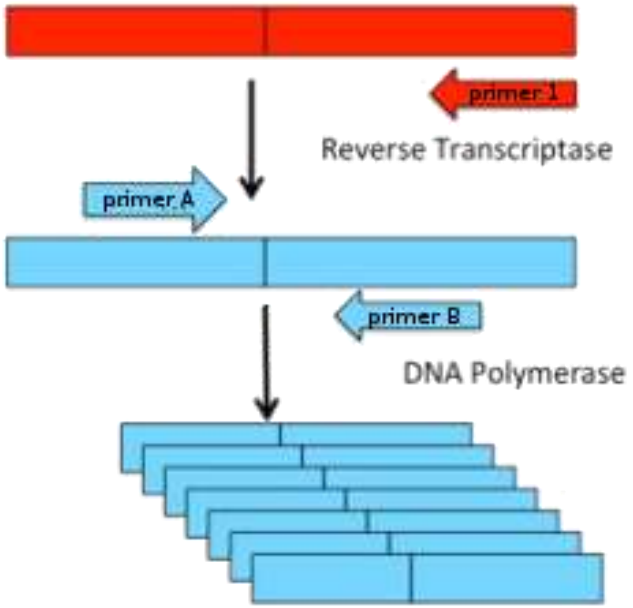


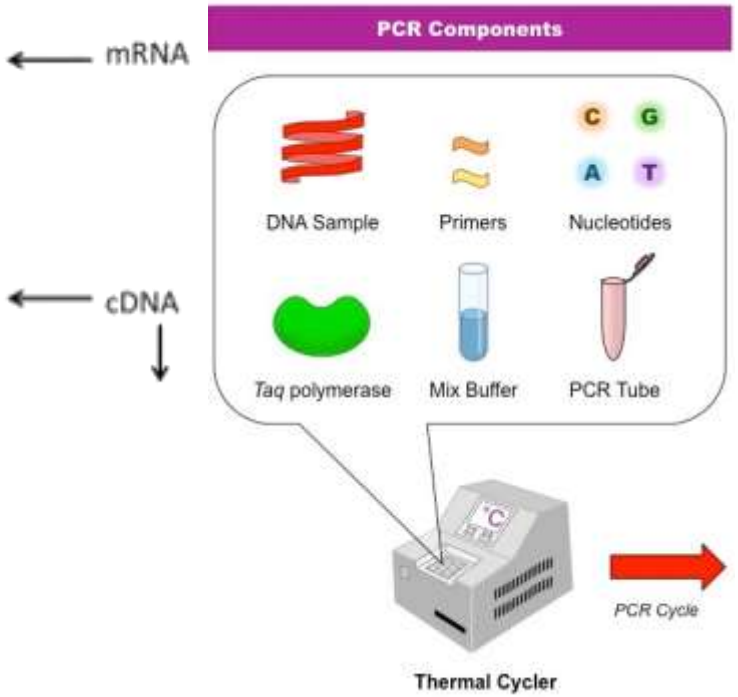
No. at Risk																						
Nivolumab	316	292	271	177	170	160	147	136	132	124	106	86	50	38	14	9	6	2	1	1	1	0
Nivolumab plus ipilimumab	314	293	275	219	208	191	173	164	163	151	137	116	65	54	18	11	7	2	1	0	0	0
Ipilimumab	315	285	265	137	118	95	77	68	63	54	47	42	24	17	7	4	3	0	0	0	0	0

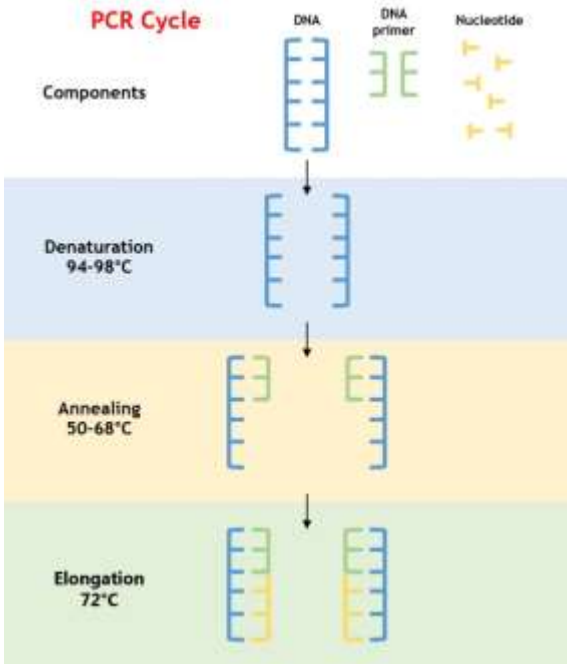
Larkin et al. N Engl J Med. 2015 Jul 2;373(1):23-34

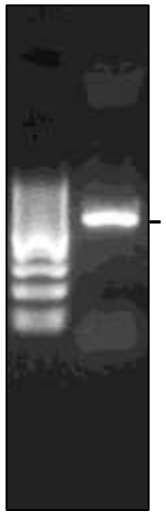
Molecular biology basics: quantifying DNA or mRNA

- Polymerase chain reaction (PCR) to amplify DNA.
- mRNA (unstable!) is first reverse-transcribed into cDNA.



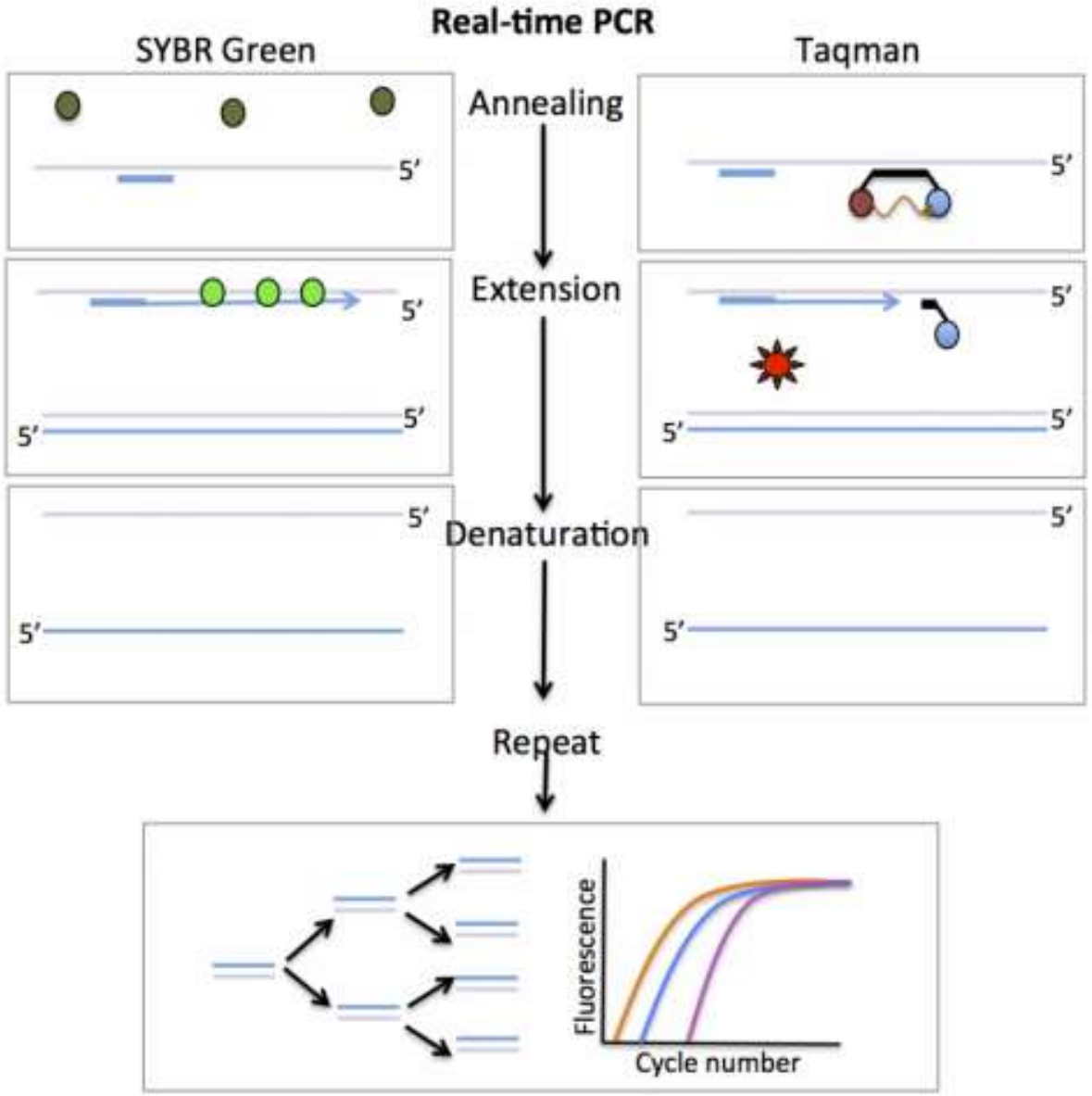






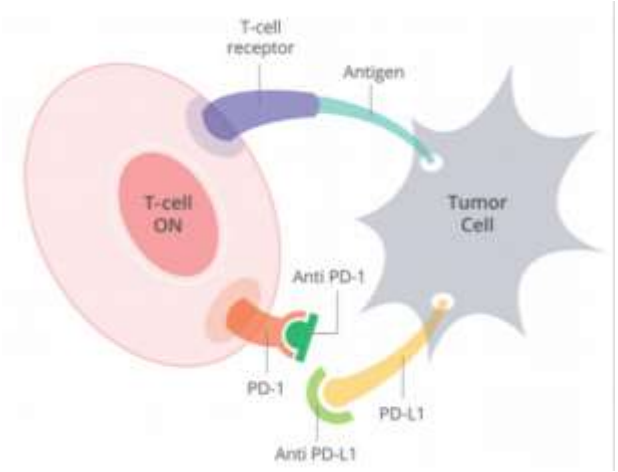
PCR product

Quantify biomarker mRNA; real-time PCR on cDNA



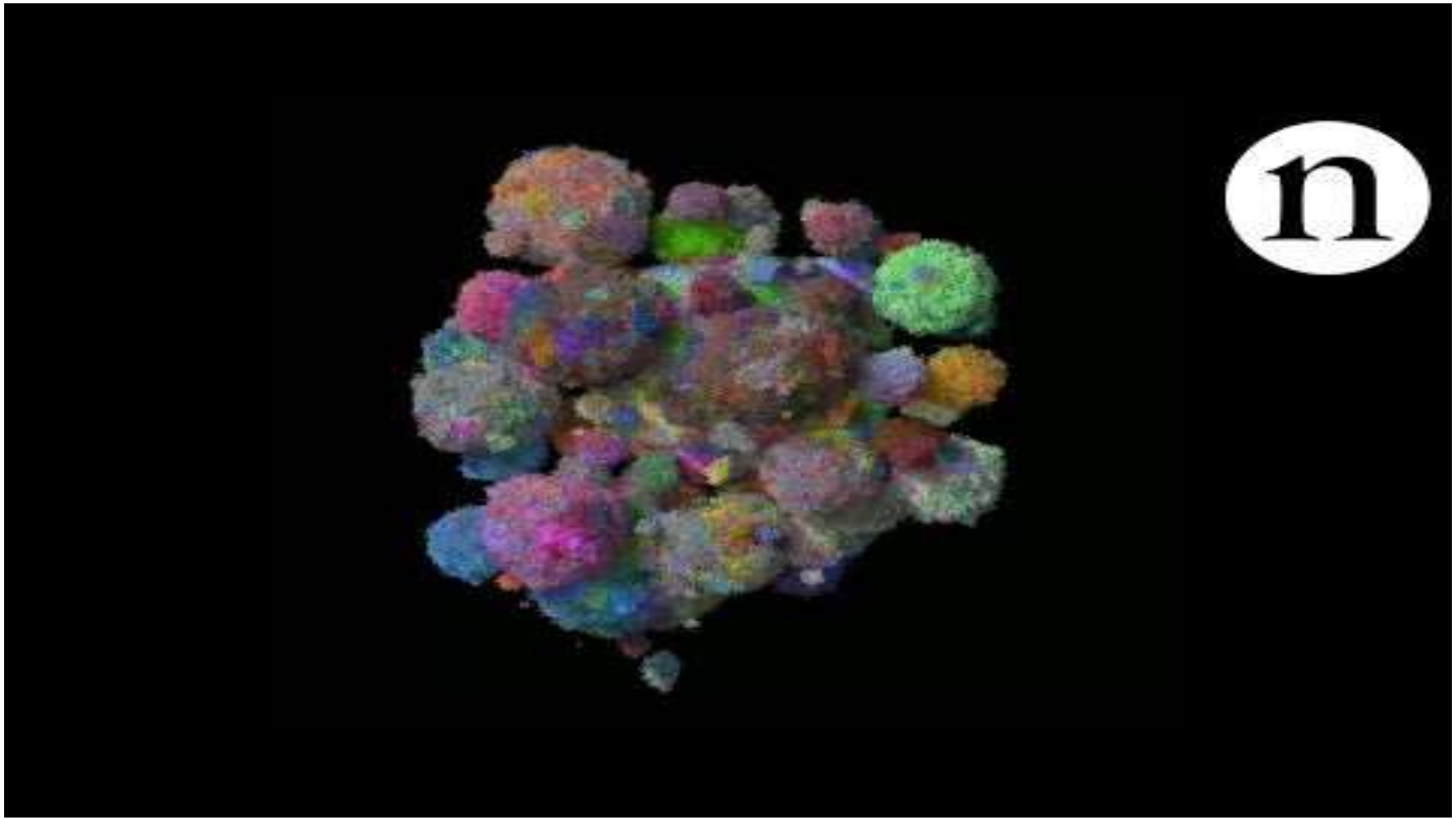
Questions:

- Which sample has the highest expression of biomarker X? Orange, blue or purple?
- If this is a qPCR on PD-L1 in 3 tumor samples: which tumor do you predict to respond best to immunotherapy?



The real world is more complex: tumors are heterogeneous

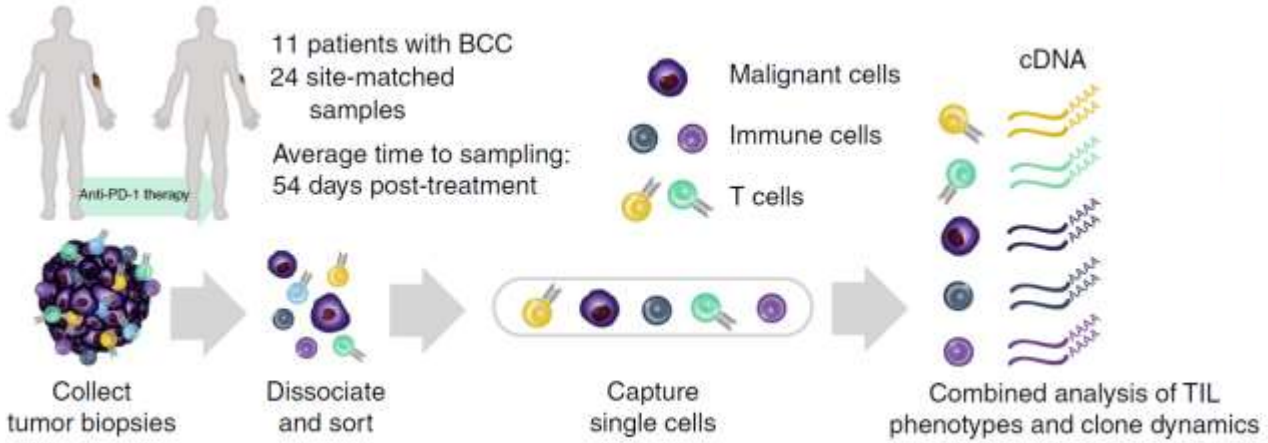
Overall PD-L1 expression turned out to be a poor predictor of treatment success

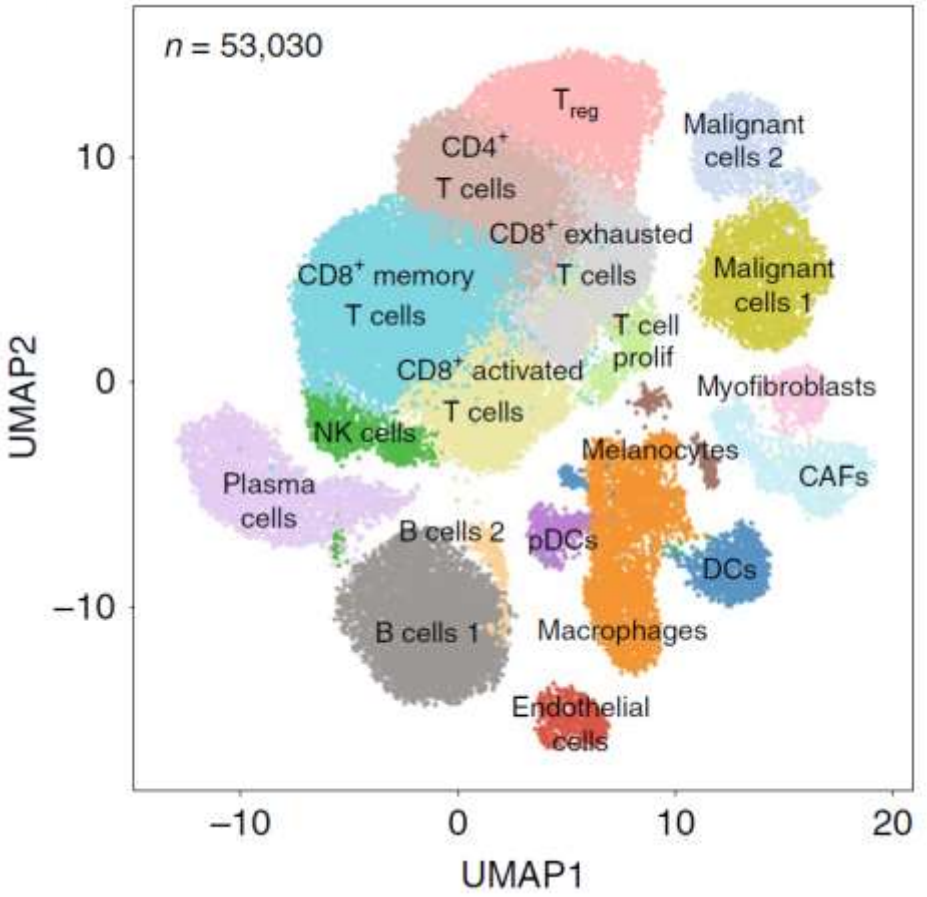


https://www.youtube.com/watch?v=lpytoIxRu0o

Single cell analysis

Question: can you briefly describe the data in the right plot? What does each dot stand for?

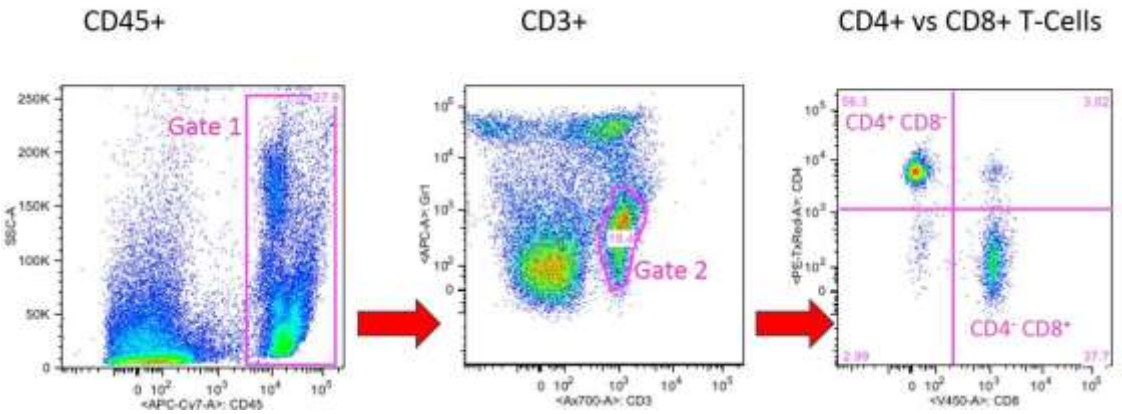


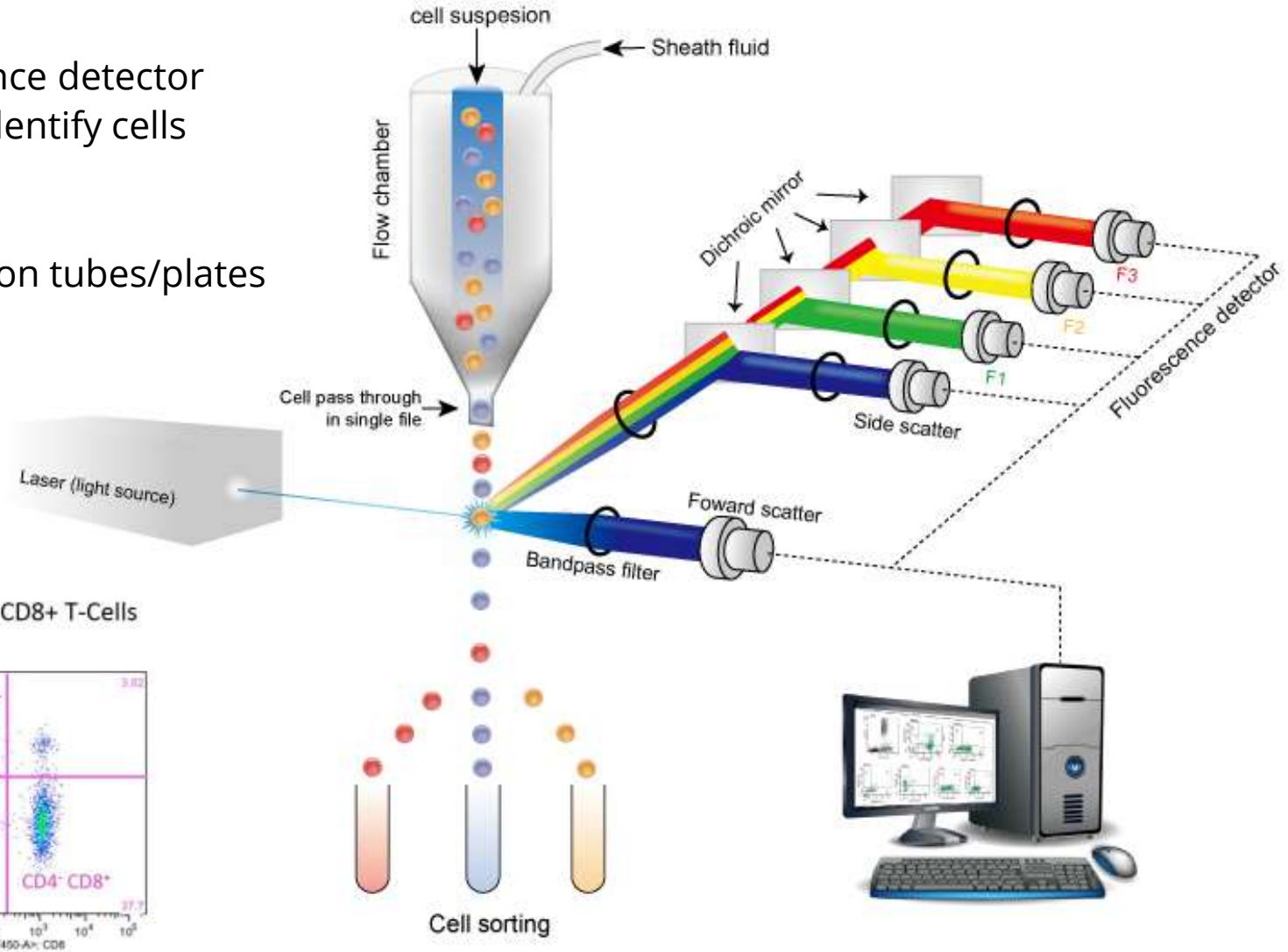


Yost et al. Nature Medicine 2019; 25: 1251-1259

Sorting single cells: flow cytometry

- Cell suspension passed through fluorescence detector
- Measure light scattering and intensity to identify cells
- Living/dead cells
- FACS - Flow Assisted Cell Sorting:
  (single) live cells can be sorted into collection tubes/plates for molecular assays



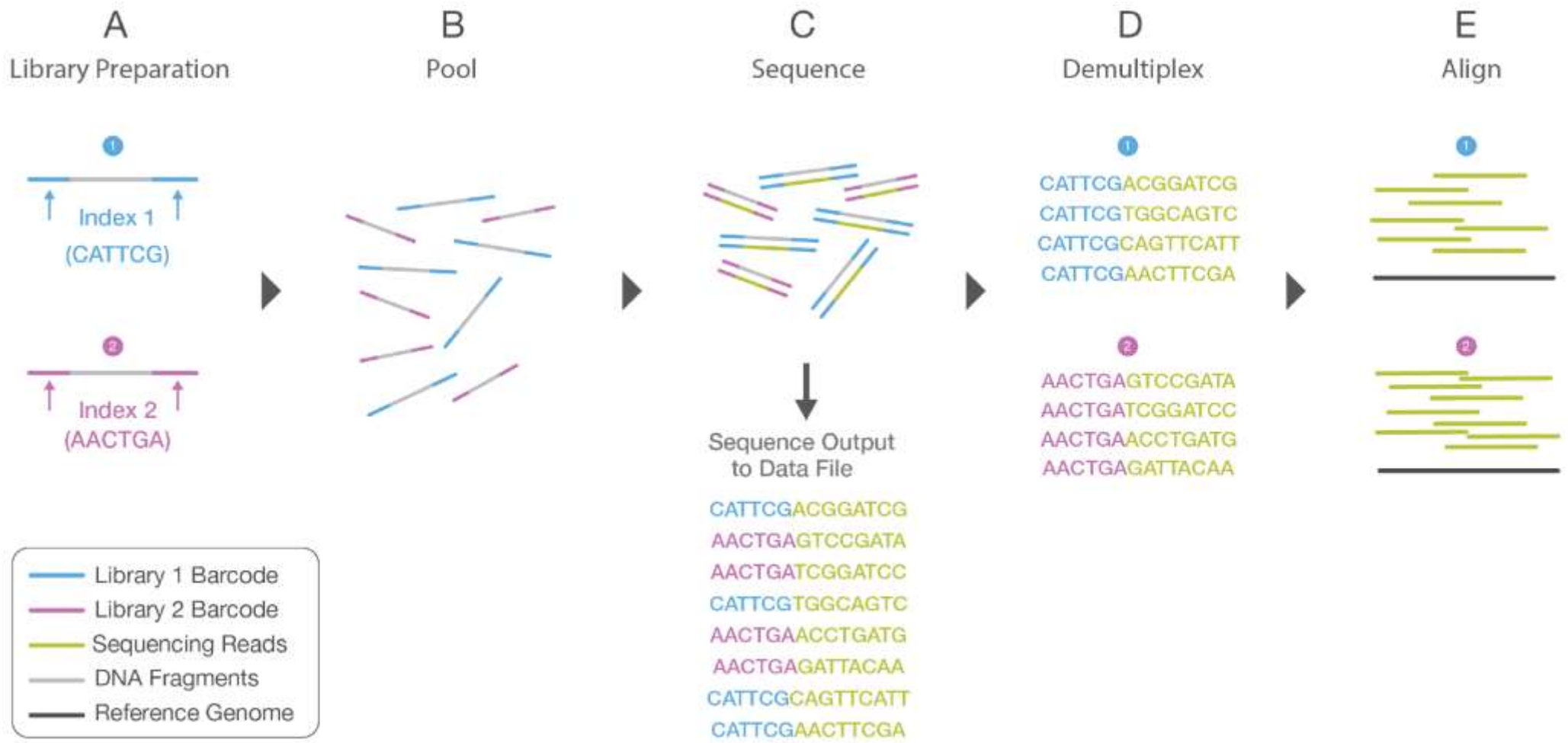


https://www.creative-diagnostics.com/flow-cytometry-guide.htm

https://www.antibodies-online.com/resources/18/1540/cd-marker-panel/

Technique: Next generation sequencing

Step A: reverse transcription (RNA) and amplification. Primers contain barcodes (Indexes)

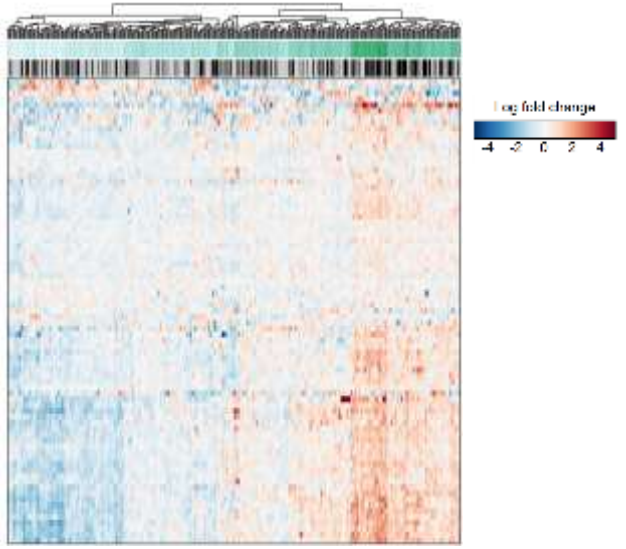


Technique: Next generation sequencing

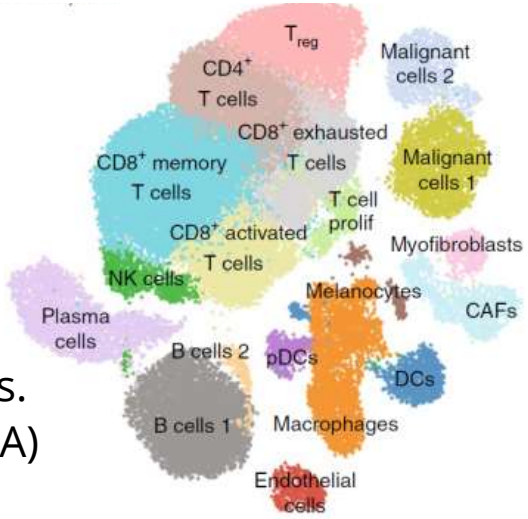
mRNA counts per cell:

	Cell 1	Cell 2	Cell 3	Cell 4	Cell …
Gene 1	0	87	12	3	…
Gene 2	13	2	0	15	…
Gene 3	321	250	130	40	…
Gene 4	4	0	7	8	…
Gene 5	0	0	0	14	…
Gene ….	…	…	…	…	…

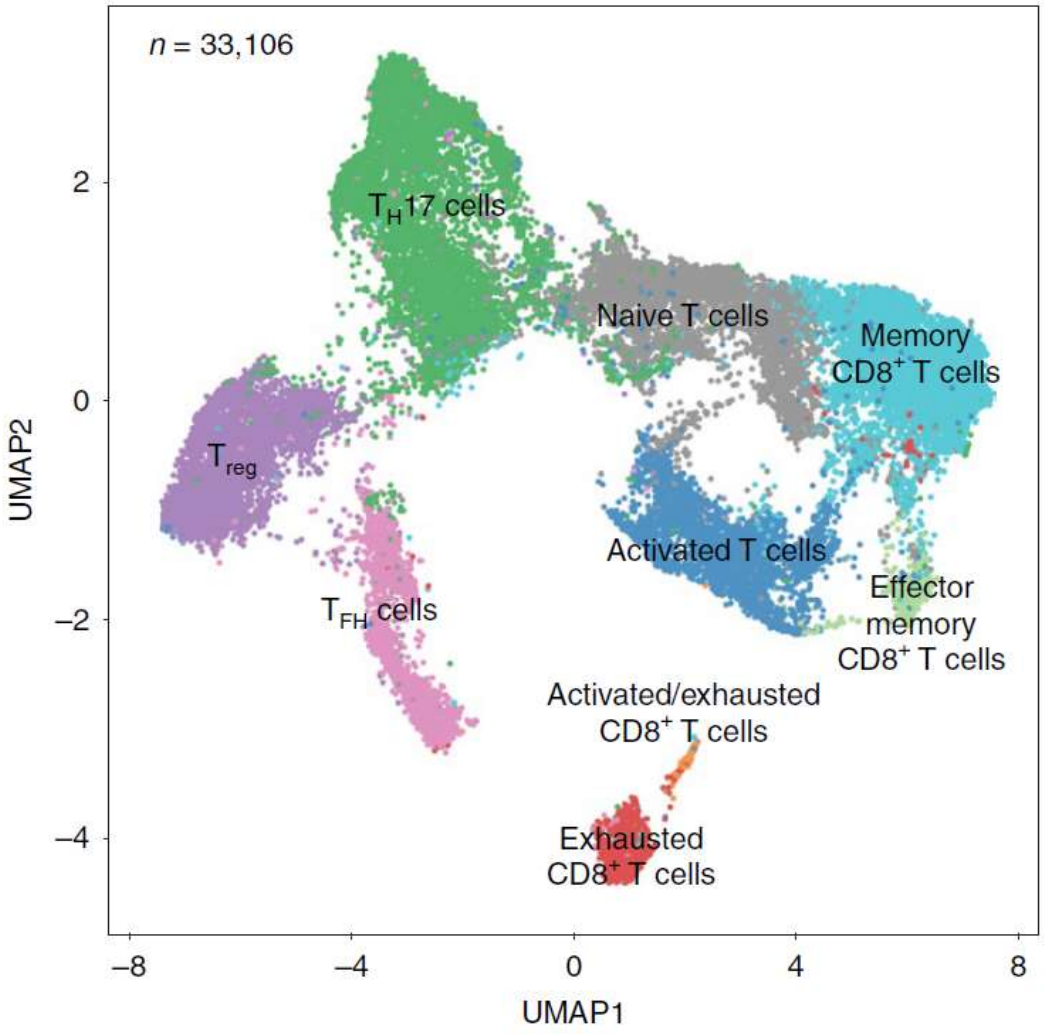
Expression heatmaps; clusters



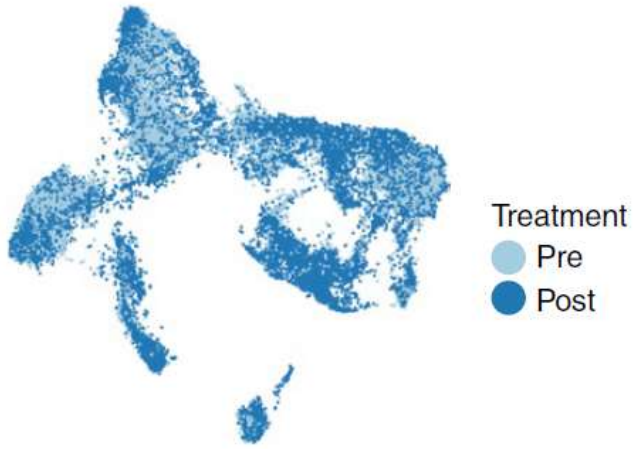
Data dimensionality reduction algoritms; identify subgroups of cells.
- Principal Components Analysis (PCA)
- tSNE
- Diffusion maps



Single cell RNA sequencing: analyzing T-cells



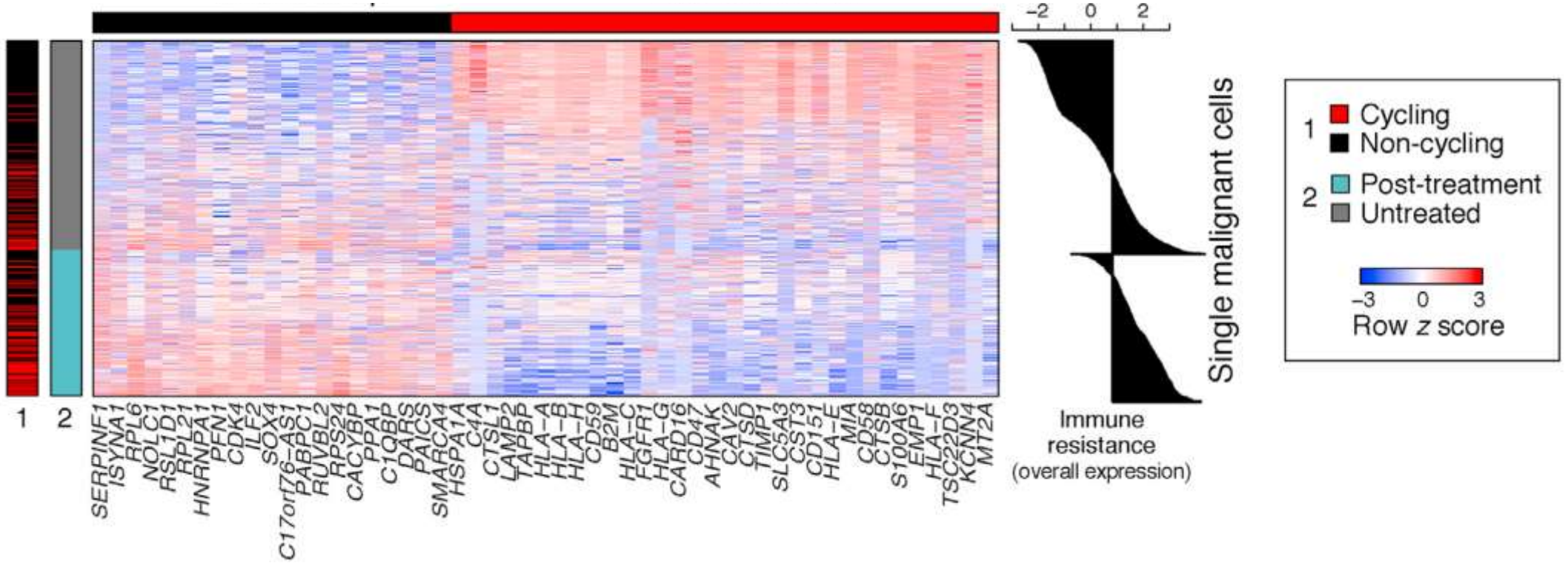
Question: Suggest how this technology can be used to analyze the effects of immune-checkpoint (PD1 blockade) in cancer



Yost et al. Nature Medicine 2019; 25: 1251-1259

Single cell RNA sequencing: analyzing cancer cell heterogeneity

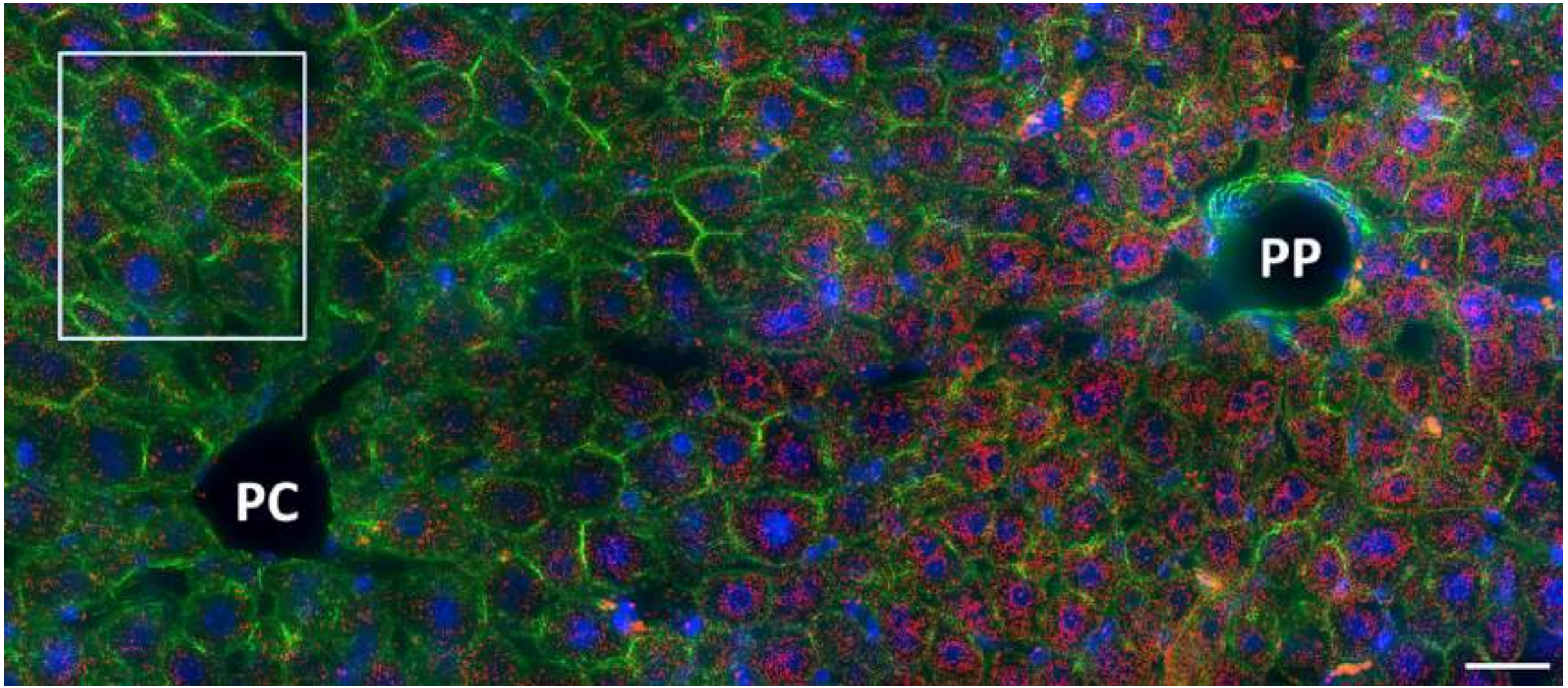
Subclones of tumor cells express genes involved in immune-resistance



Question: what essential information for a pathologist is lacking here? Suggest a complementary technique

Jerby-Arnon et al. Cell 2018; 175: 984-997

Spatial information - single cell in situ mRNA expression

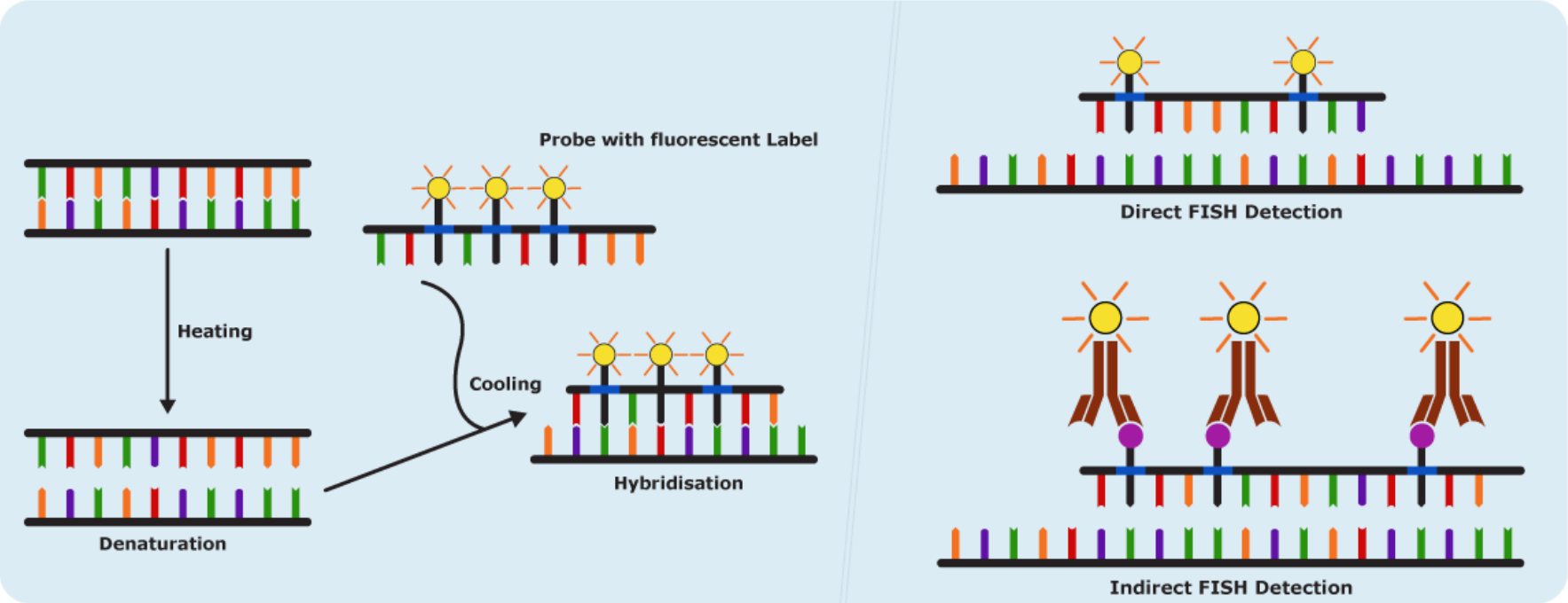


Blue: DNA (DAPI) Green: Actin (phalloidin) Red: Pck1 mRNA molecules

Halpern et al. Molecular Cell 2015

fluorescence in-situ hybridization (FISH)

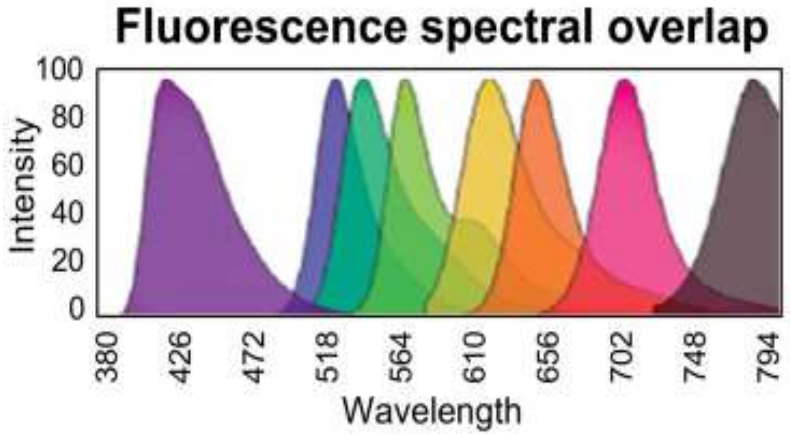
- Count RNA or DNA molecules on tissue section
- Fluorescent-labeled probe complementary to DNA/RNA of interest

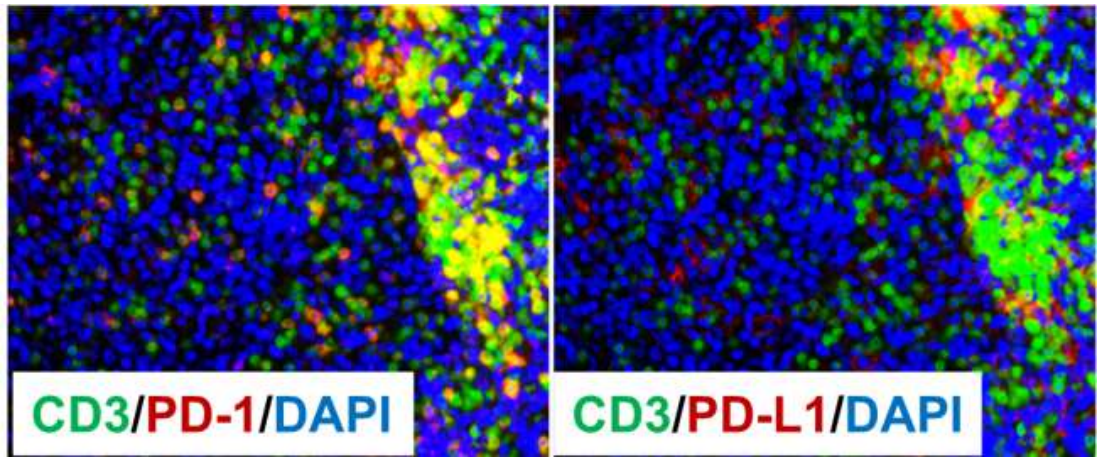


mRNA can serve as biomarker, but proteins eventually do the work

Classic method: immunofluorescence staining or immunohistochemistry
Fluorescence: only a handful of colors (i.e.) proteins can be measured

Question: how can the modern pathologist overcome this limitation?





Li et al. Modern Pathology 2019; 32: 741–754

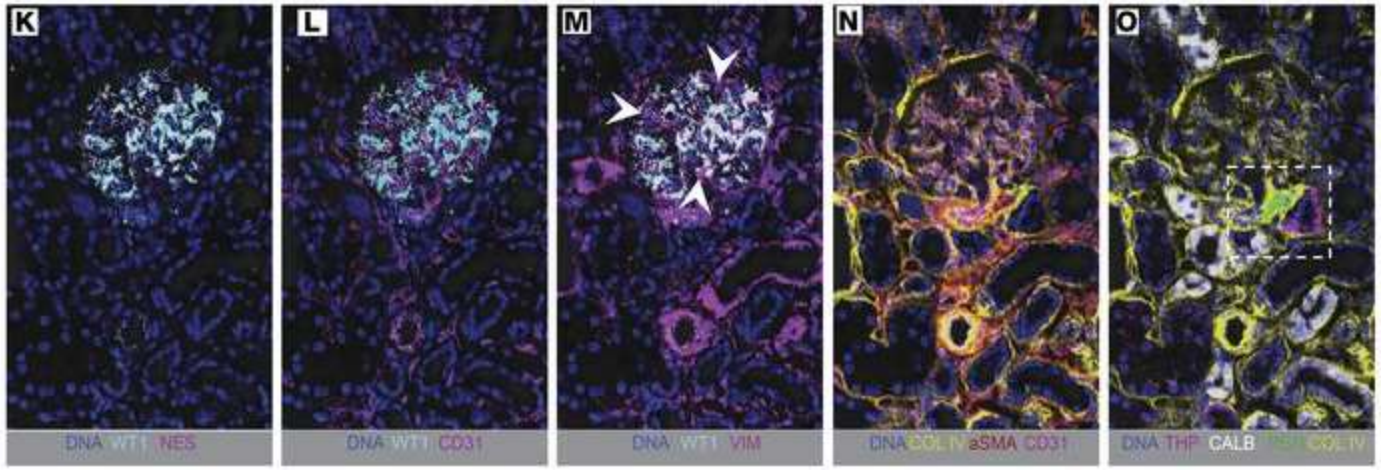
mRNA can serve as biomarker, but proteins eventually do the work

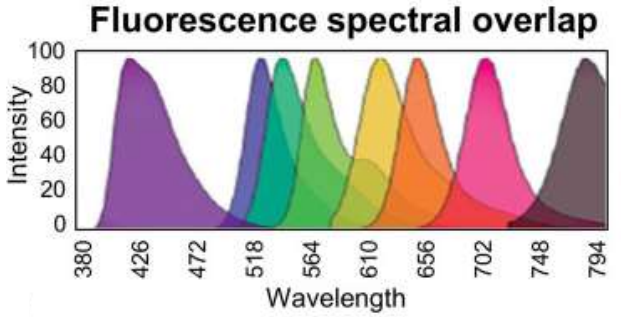
https://www.youtube.com/watch?v=1G6jTGAfgUc

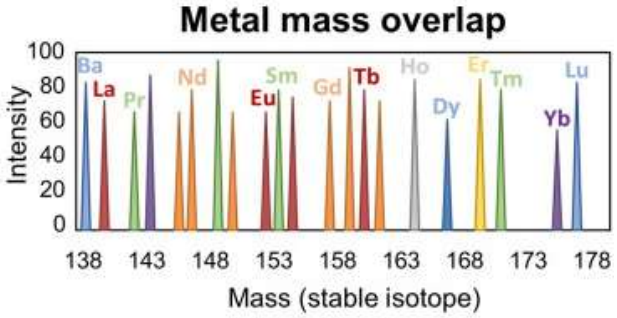
Mass spectrometry to measure proteins directly, or indirectly

- Proteome: mass spec on tissue biopsy

- Spatially resolved: imaging mass cytometry
  - label antibodies with metals instead of fluorescent proteins.
  - Mass cytometry: ~40 proteins per cell



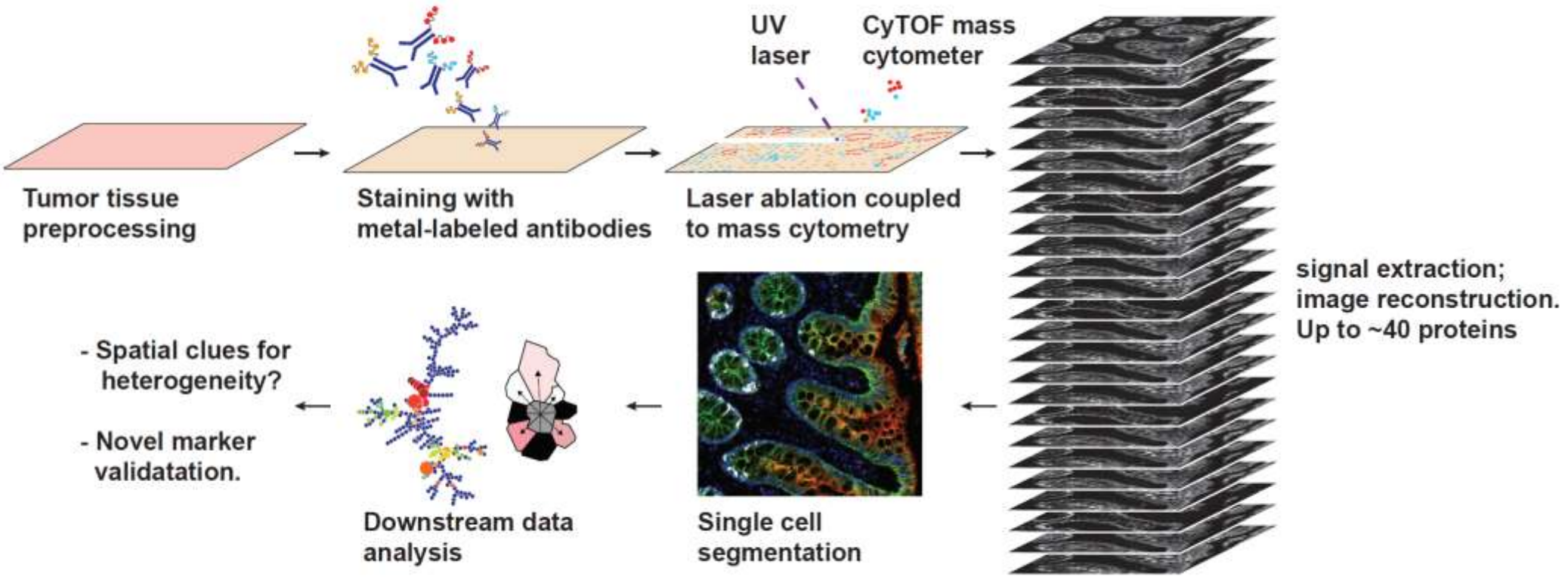




https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/mass-cytometry

Technique: Imaging mass cytometry

- Label (phospho-) proteins with metal istope-tagged antibodies
- Vaporize 1x1 um2 regions; measure amount of isotope per region with mass spec
- ~20-40 antibodies per experiment



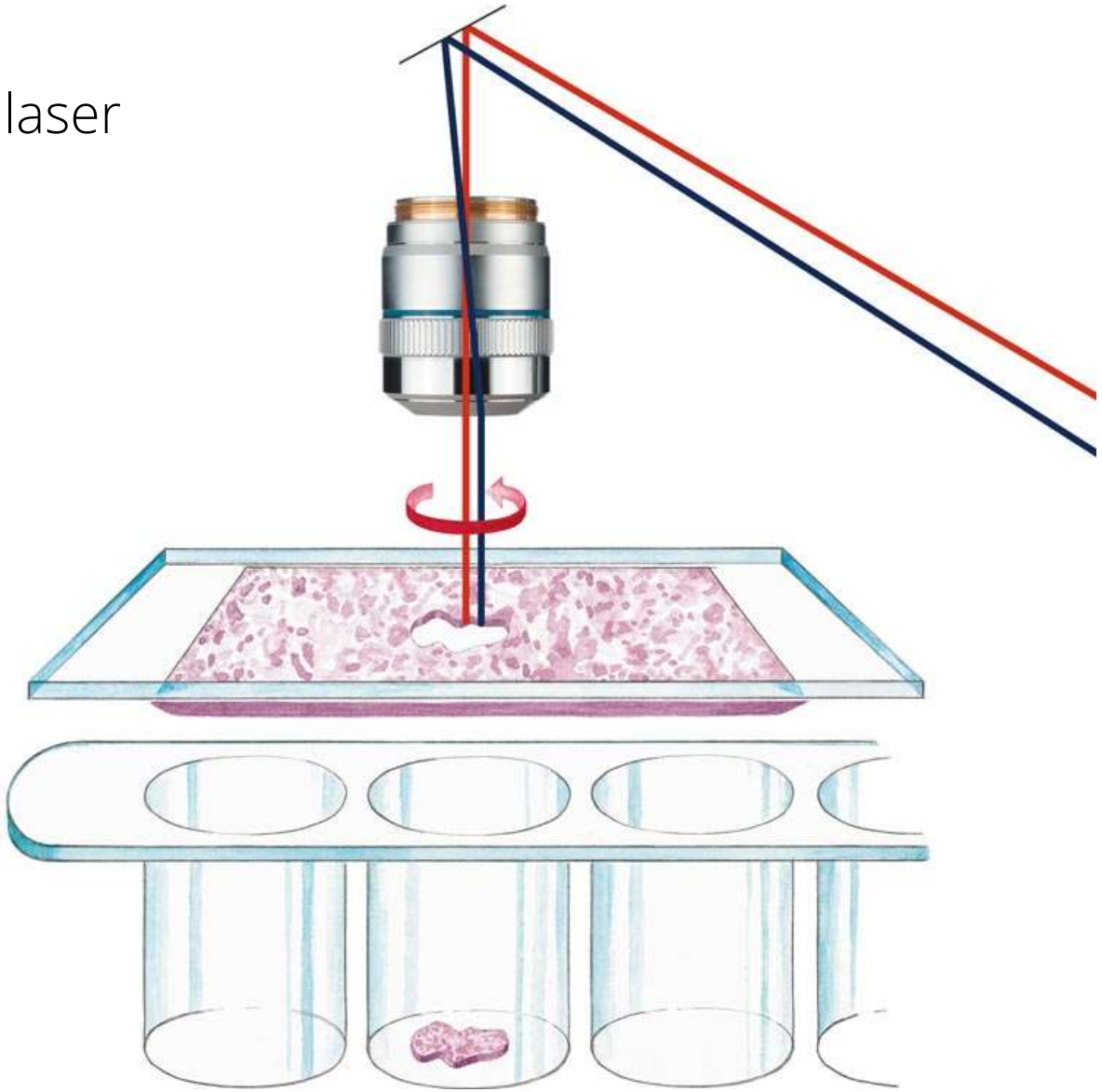
Studying biomarkers and cell-cell interactions

- Studying a marker in a tissue homogenate often not enough.
- Single cell techniques
- Multiple proteins, mRNA markers at the same time.

Question: your starting material is often a frozen section or a formalin-fixed, paraffin-embedded section.
Can you study novel biomarker panel x in archived patient samples?
If yes: how?

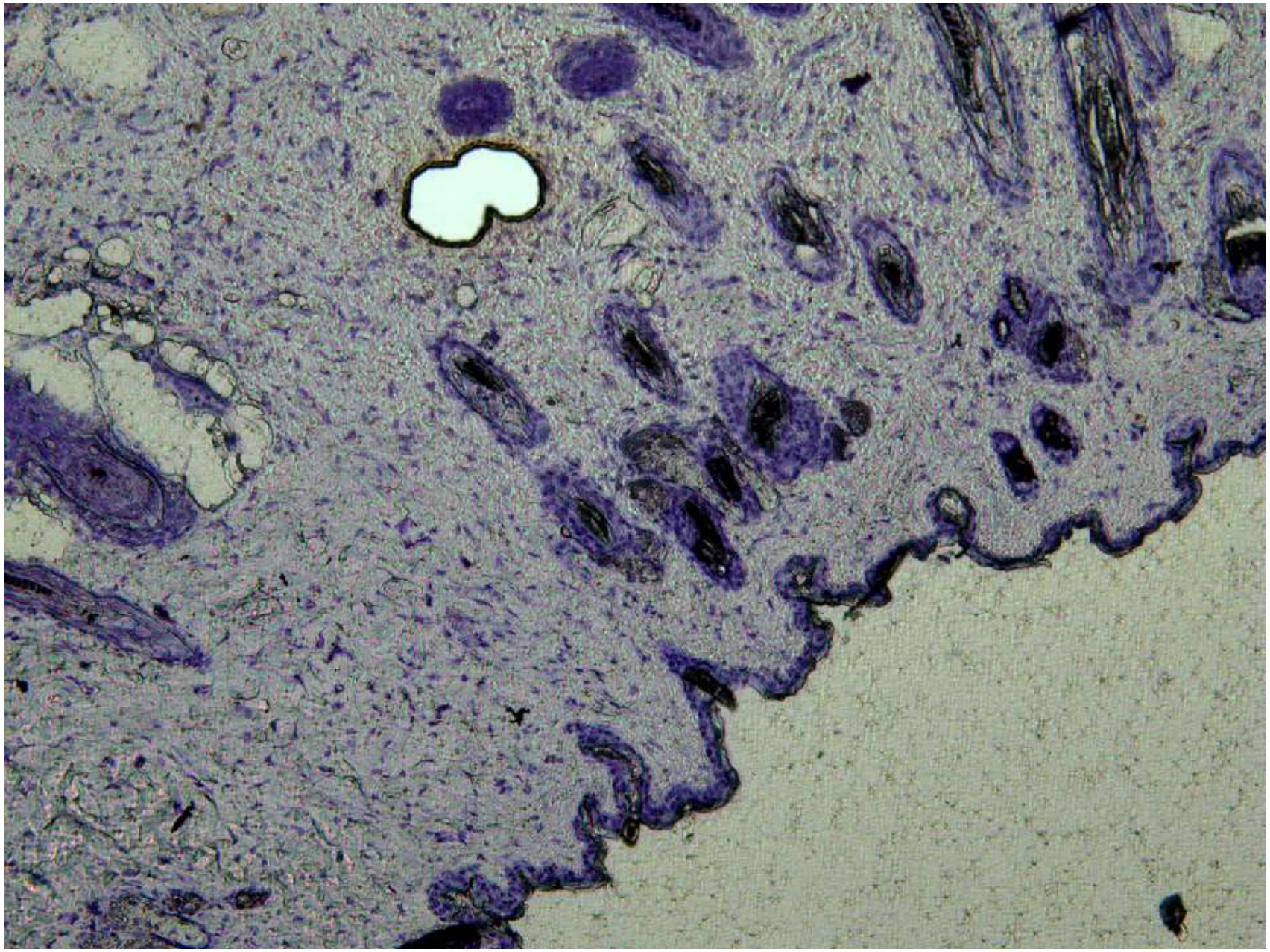
Laser microdissection

- Cut through histology section with laser
- Collect tissues of interest
- DNA / RNA / protein analysis
- High magnification: single cell
- Throughput: 96-well plates



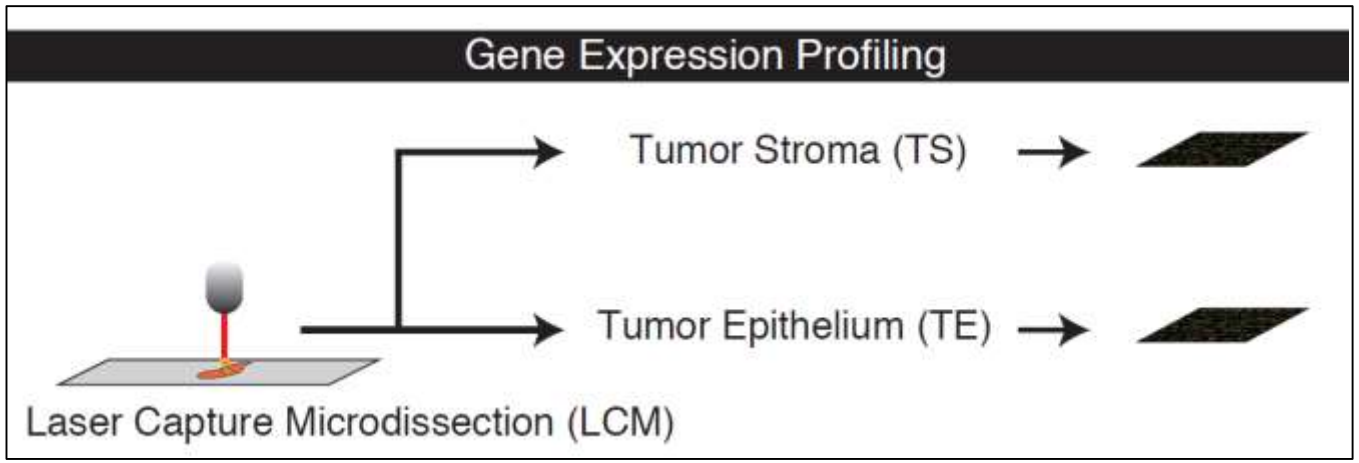
Leica Microsystems.com

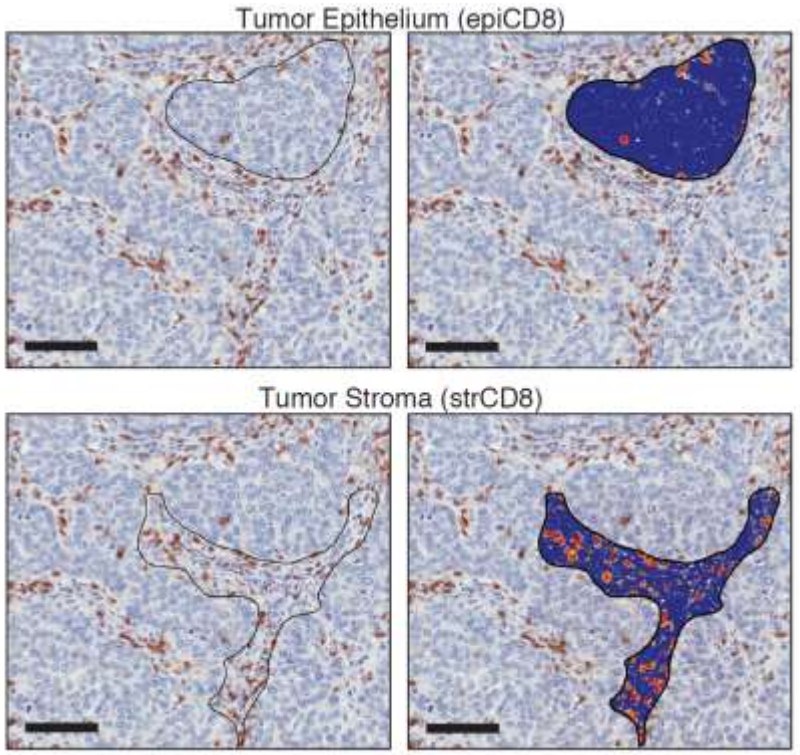
Laser microdissection



Laser microdissection

Question: Formulate a hypothesis how stroma versus tumor gene expression analysis could be used for biomarker discovery.





Summary: the molecular pathologist's toolbox

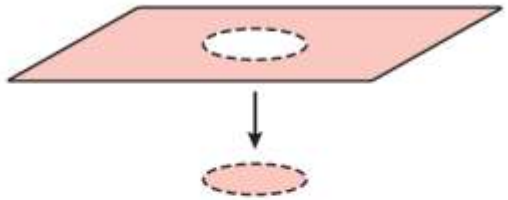
Section
(Frozen or FFPE)

Imaging

DNA
- FISH (in-situ hybridization)

RNA expression
- FISH (in-situ hybridization)

Protein expression
- IHC (1-2 proteins)
- IF staining (1-5 proteins)
- Imaging Mass Cytometry (<40 proteins)



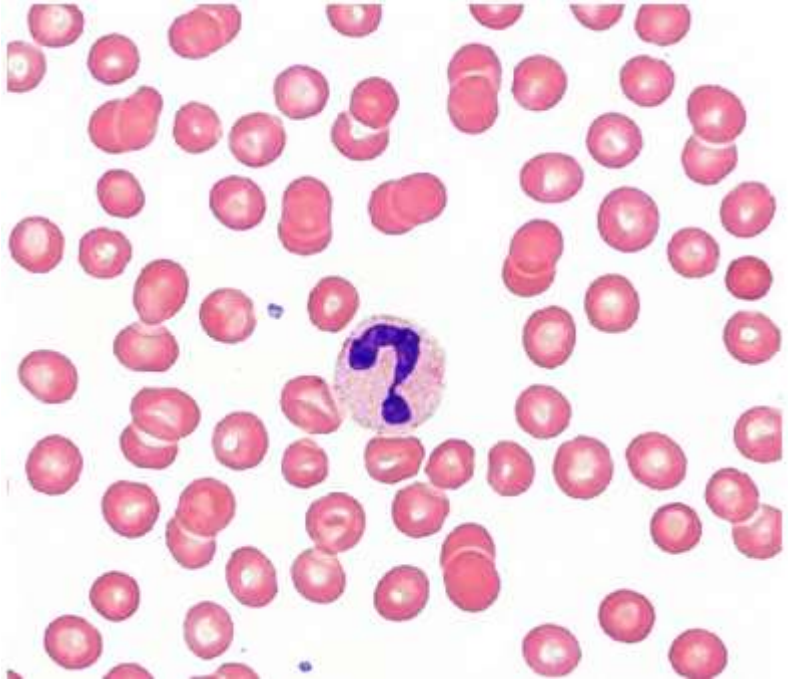
Molecular biology

DNA
- PCR
- DNA-sequencing (>1 cell; genome)

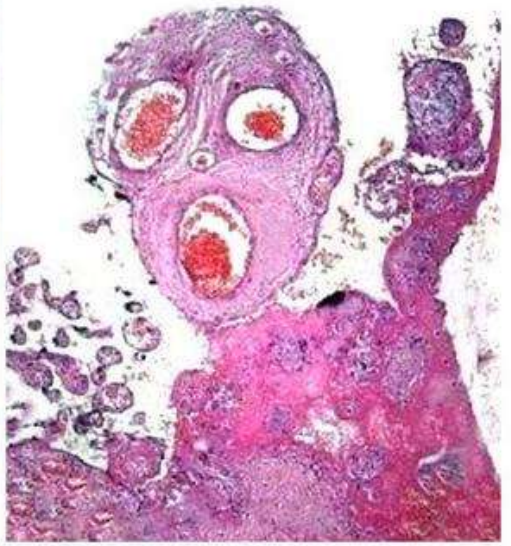
RNA expression
- qPCR (real-time PCR)
- RNA-sequencing (>1 cell; transcriptome)

Protein expression
- mass spec (1000s of cells; proteome)
- Western blot

RNA:
1. Deparaffinize
2. Digest protein (prot. K)
3. Isolate nucleic acids
4. Remove DNA (DNAse)
5. Wash and elute
6. RT and amplification



Histology's 'Placental villi'



Scientific abstract

- Invitation to review a scientific manuscript
- Before you accept or decline the invitation you review the abstract
- Identify mistakes in the abstract and briefly explain why they are wrong
- Mock exam