

Adult neural stem cells from the following brain regions have been well-characterized:

- A. Hippocampus
- B. Retina
- C. Hypothalamus
- D. Subventricular zone

During embryonic development of the brain in mammals, which of the following is (are) true ?

- A. Radial glial cells appear first and then give rise to neuroepithelial cells
- B. Neural stem cells make neurons, astrocytes and microglial cells
- C. The last neuronal layer of the cerebral cortex that is formed is the one closest to the ventricle
- D. Neural stem cells from different brain regions not produce the exact same progeny

Tools in stem cell biology

What you will hear about today

1. How to investigate stem cell differentiation:

- In vitro
- In vivo

1. Pluripotent stem cells as a tool for:

- the study of early embryogenesis
- modeling human diseases

I. Tools to investigate stem cell differentiation

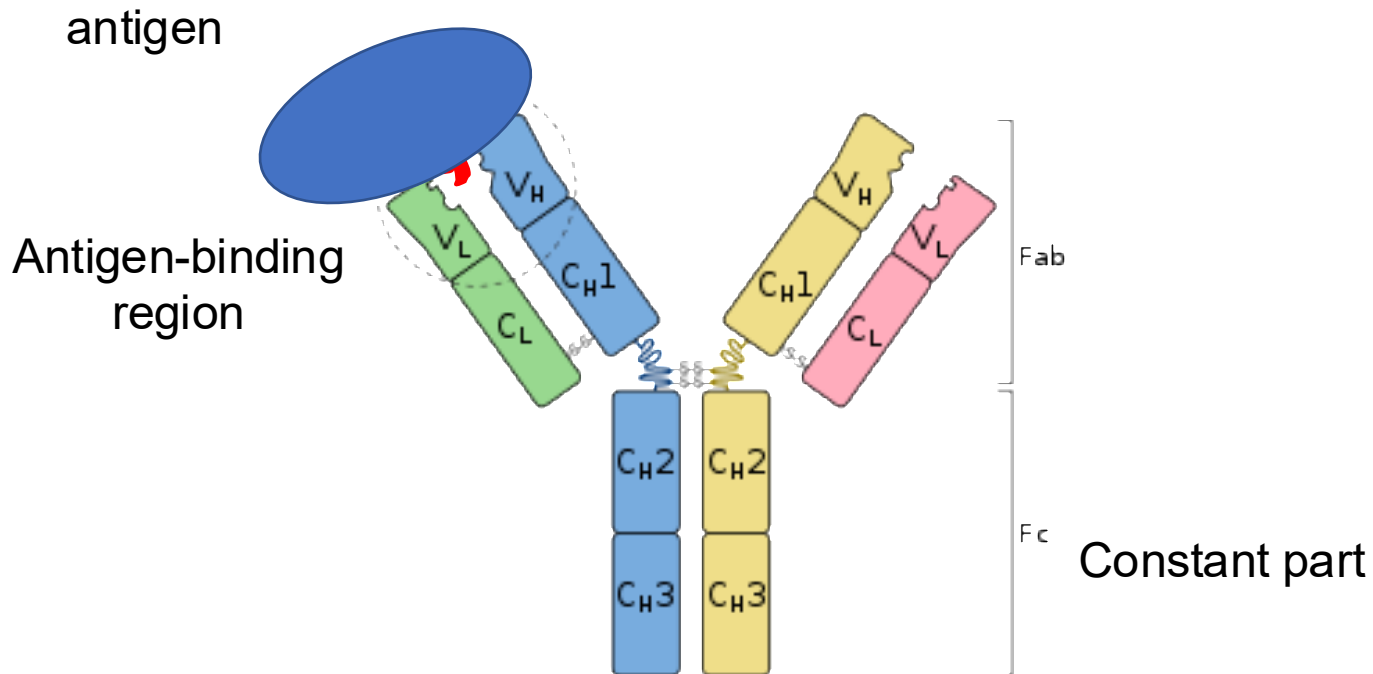
Tools to investigate stem cell differentiation

1. Immunofluorescence
2. RNA **F**luorescence **I**n **S**itu **H**ybridisation (RNA FISH)
3. Promoter-reporter constructs
4. Reporter-based lineage tracing
5. Sequencing-based lineage tracing

1. Immunofluorescence

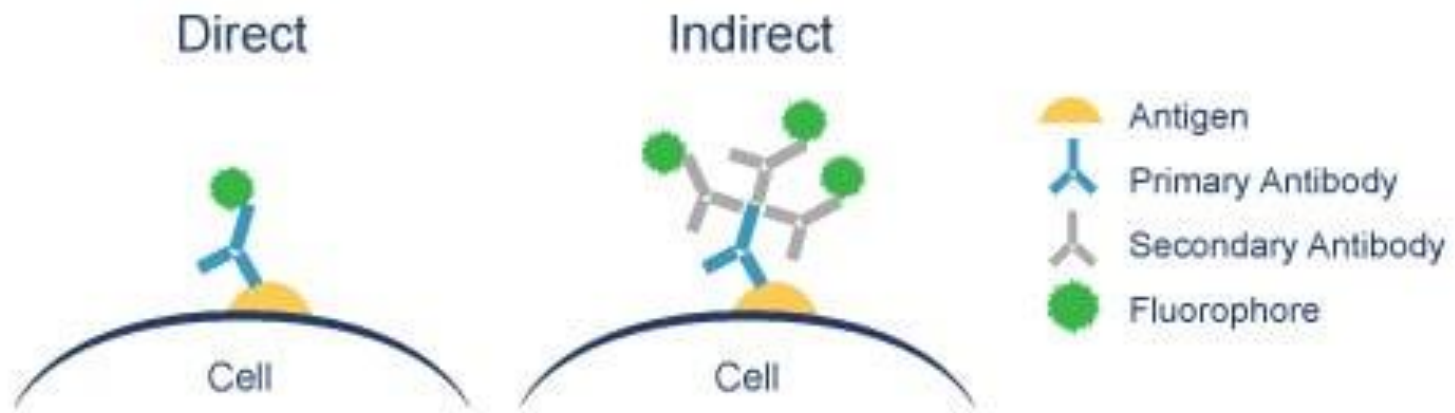
Immunofluorescence - principle

- Immunofluorescence uses antibodies to specifically stain proteins
- Antibodies are glycoproteins produced naturally by our immune cells (B-cells) in response to foreign proteins (for example from viruses, bacteria etc.)
- Each antibody binds **specifically** to an **antigen**.

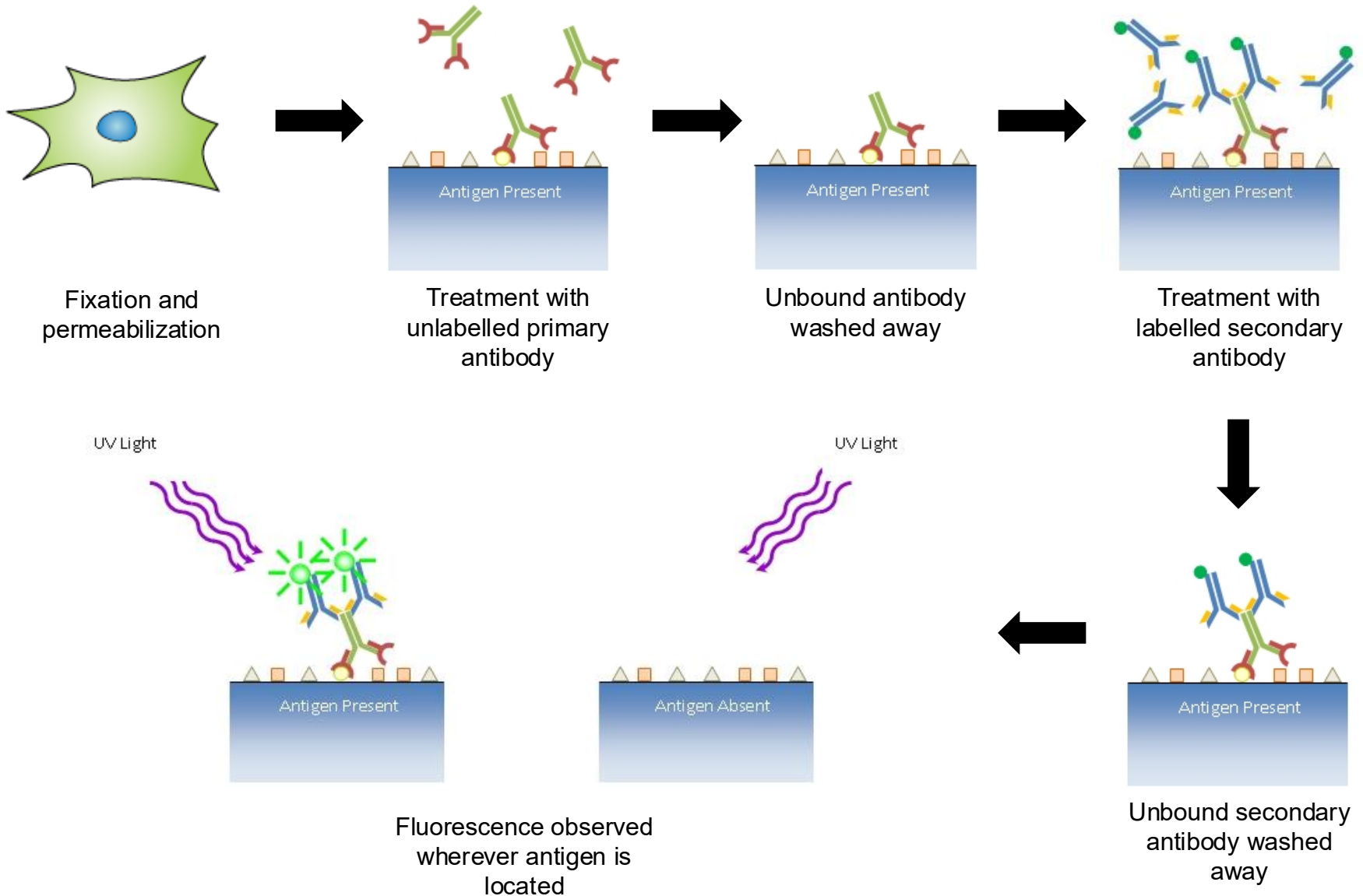


How does immunofluorescence work?

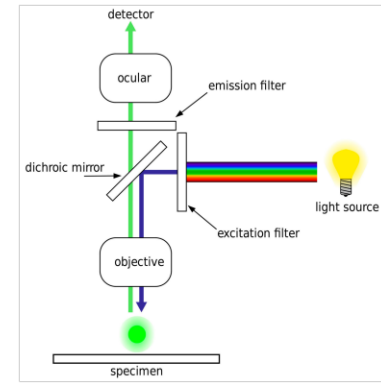
Antibody binding is detected using fluorophores in a one or two step reaction



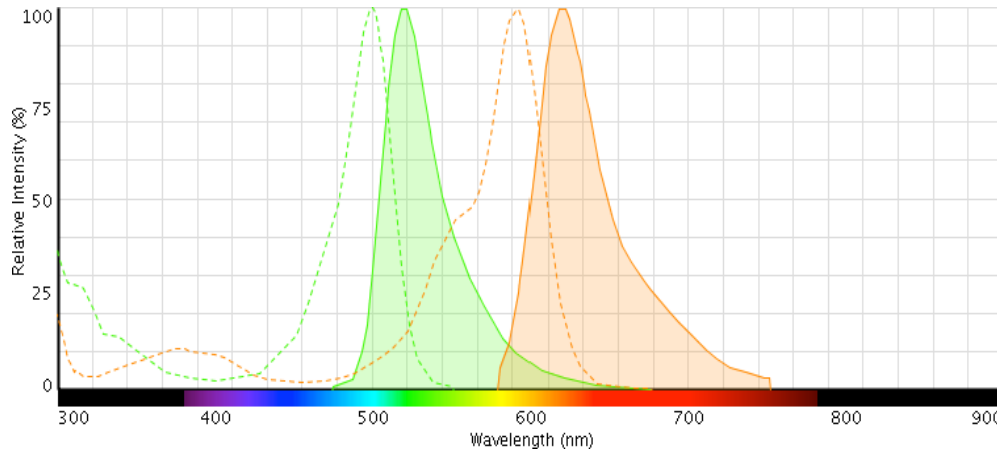
How does immunofluorescence work?



Is it possible to image several fluorophores at the same time?



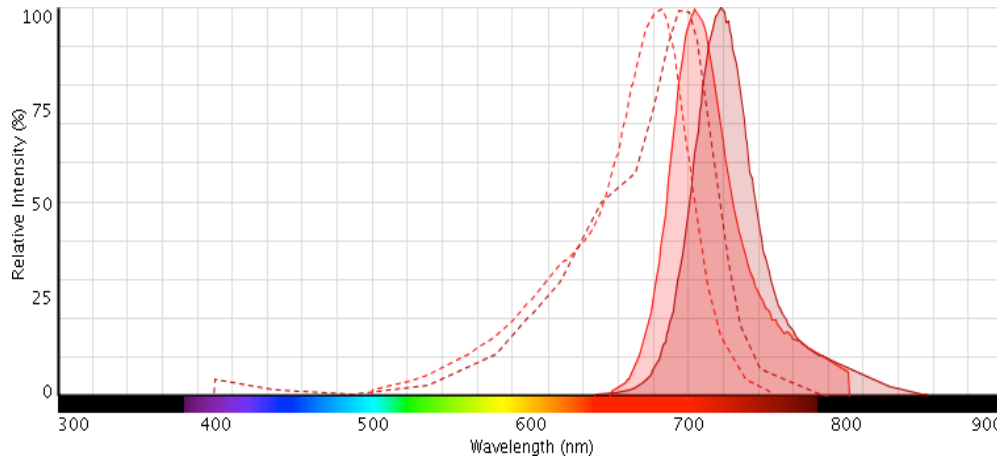
Spectra of the fluorophores **Alexa 488** and **Alexa 594**



→ Yes, if the spectra are not overlapping too much

 Excitation spectrum
 Emission spectrum

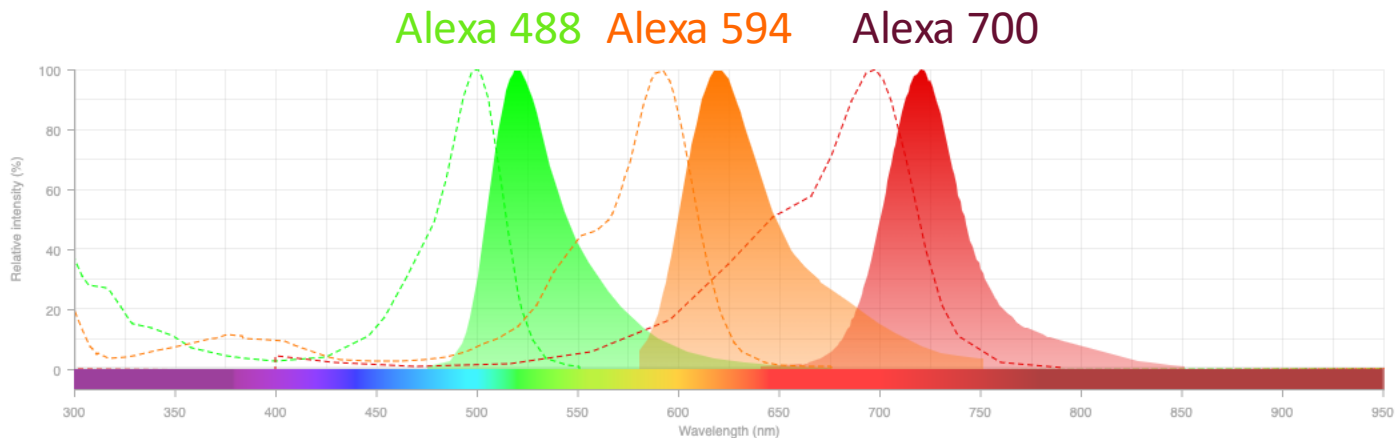
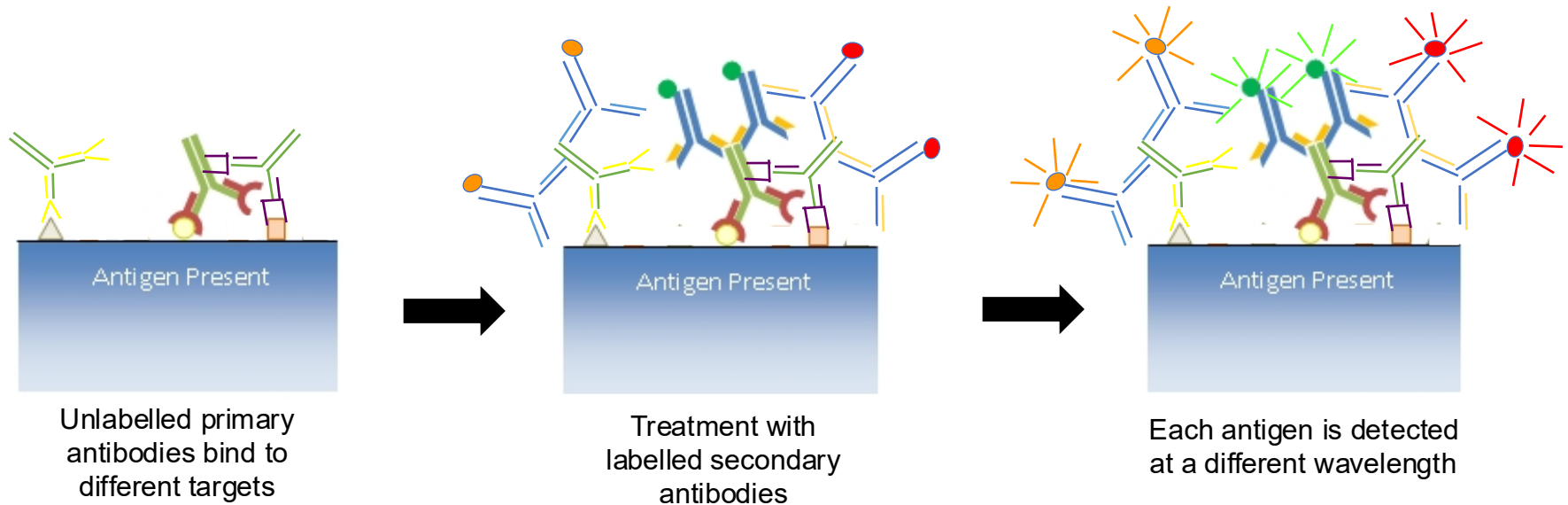
Spectra of the fluorophores **Alexa 680** and **Alexa 700**



→ No if the spectra are overlapping too much

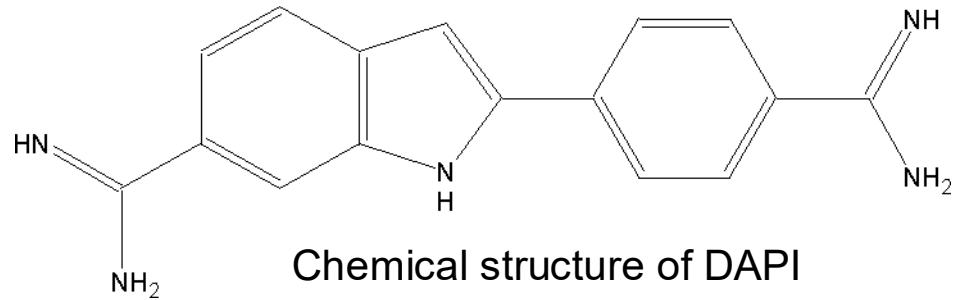
Immunofluorescence with several antibodies

- It is possible to use multiple antibodies until all filters of the microscope are used (often 4-5 channels)

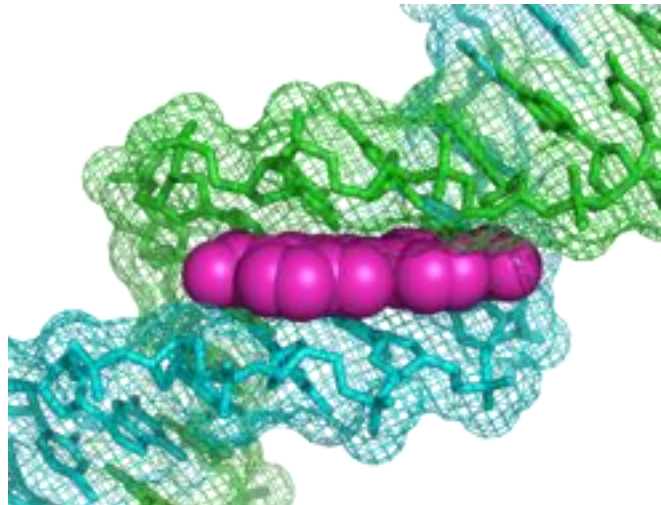


How to stain the cell nucleus?

- By using a chemical, for example 4',6-diamidino-2-phenylindole (**DAPI**) that binds to DNA
- It emits in the **blue** spectrum, and can be used in combination with green-emitting and red-emitting dyes

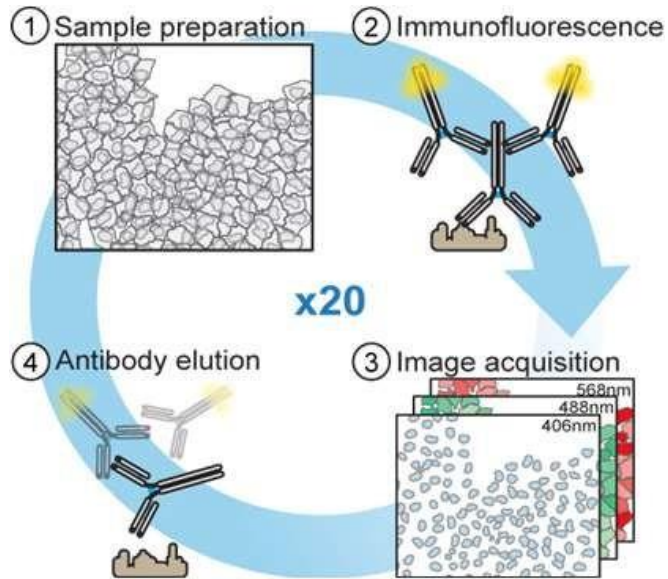


Model showing DAPI (pink)
bound to DNA

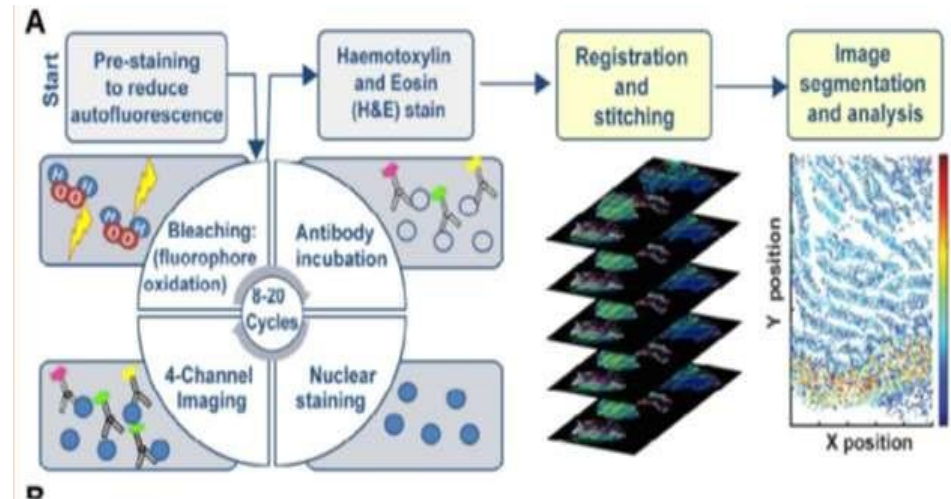


Multiplexing through cyclic immunofluorescence

- Different cyclic immunofluorescence protocols have been published
- Antibodies are eluted/bleached after imaging to allow repeat staining

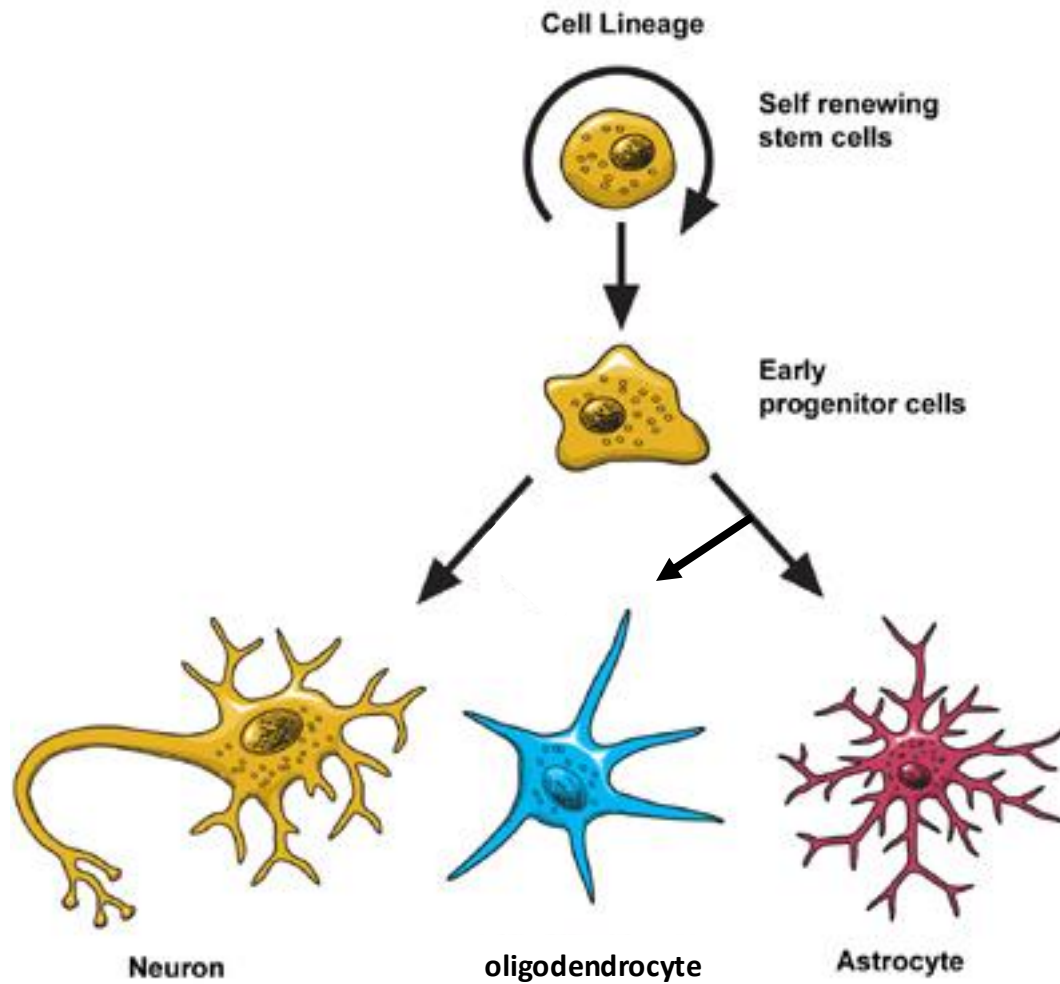


Gut et al., Science 2018



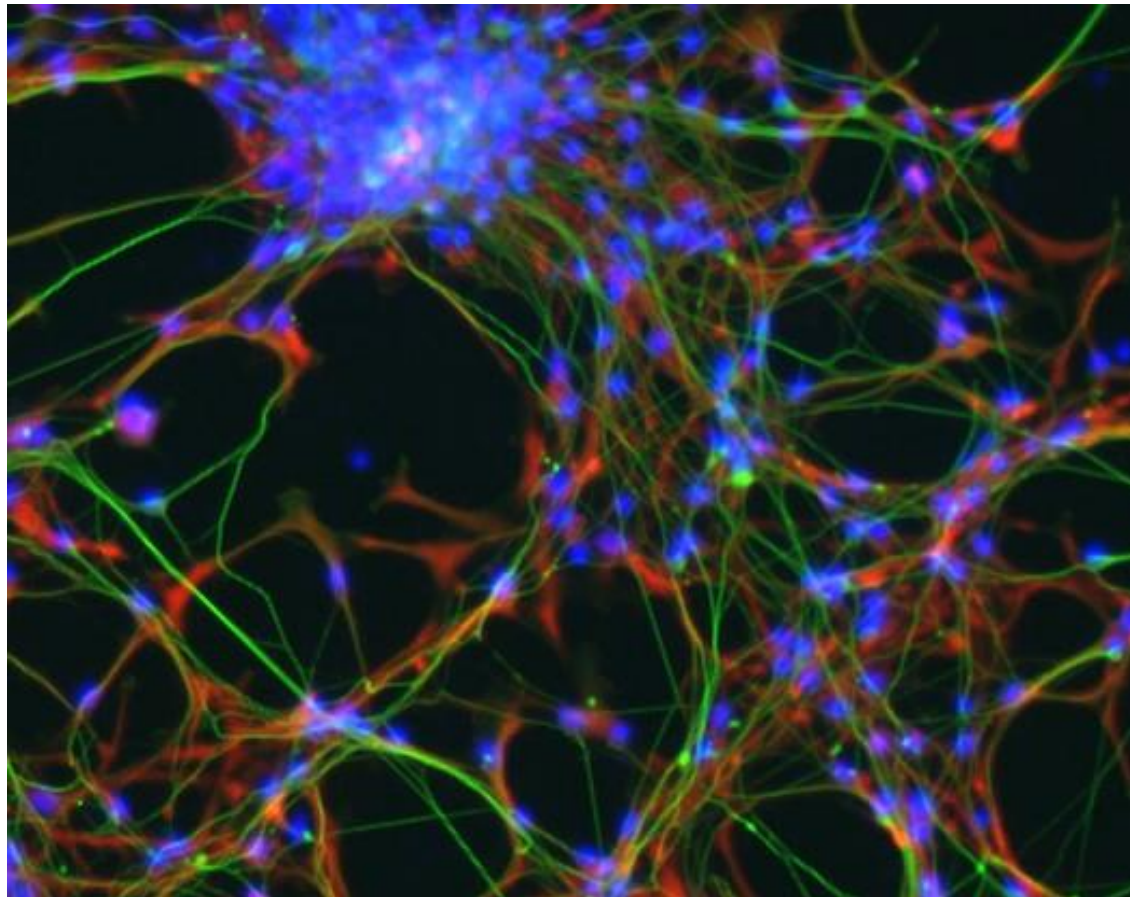
Jia-Ren Lin et al., eLife 2018

Application: in vitro differentiation towards neurons



Application: in vitro differentiation of ESCs towards neurons

In vitro differentiation of embryonic stem cells towards neurons
Immunofluorescence against Nestin (expressed in neuronal progenitors) and β 3-tubulin
(expressed in neurons) and DAPI staining



Nucleus:
DAPI stain

Neuronal
progenitors:
Nestin

Neurons: β 3-
tubulin

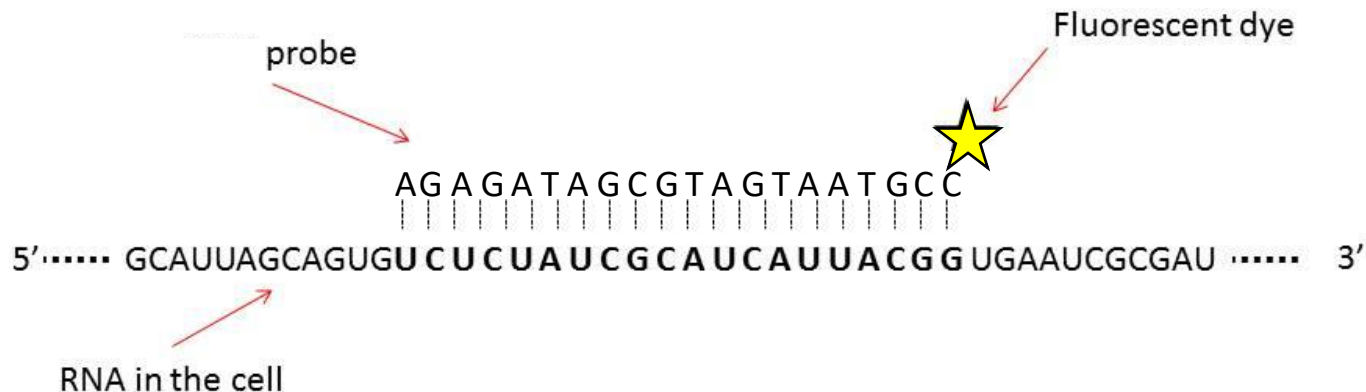
David Suter, unpublished

2. RNA FISH

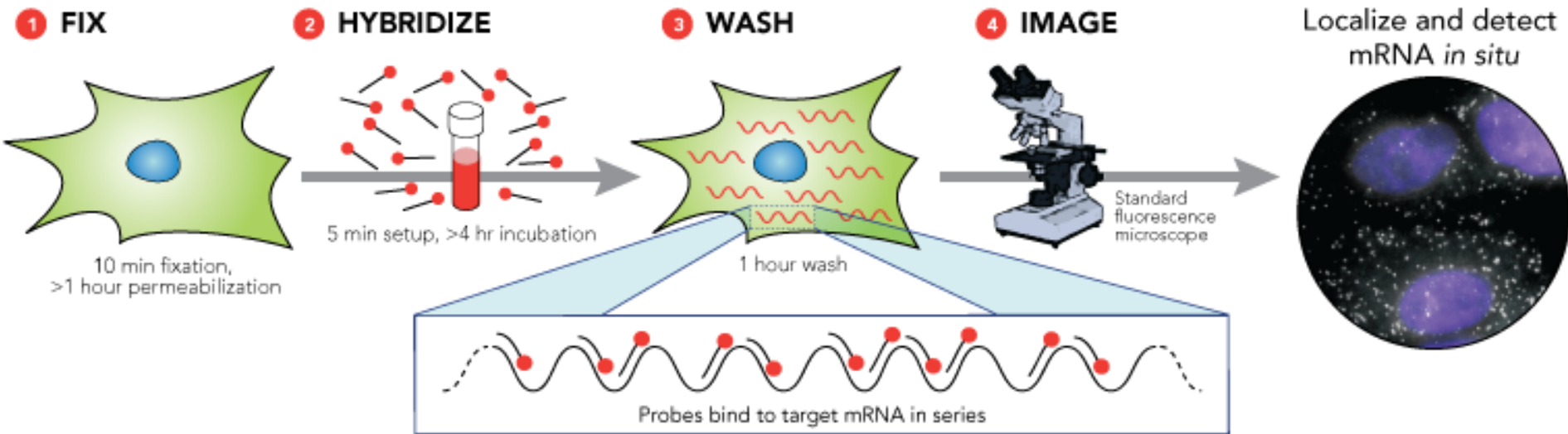
RNA fluorescence in situ hybridization (RNA FISH)

In RNA FISH, **several small single stranded DNA molecules** complementary to the same mRNA molecule and marked with fluorescent molecules (the **probes**) are hybridized (= bound by base complementarity) to their target mRNA

→ Allows to **localize** and **count** mRNA molecules



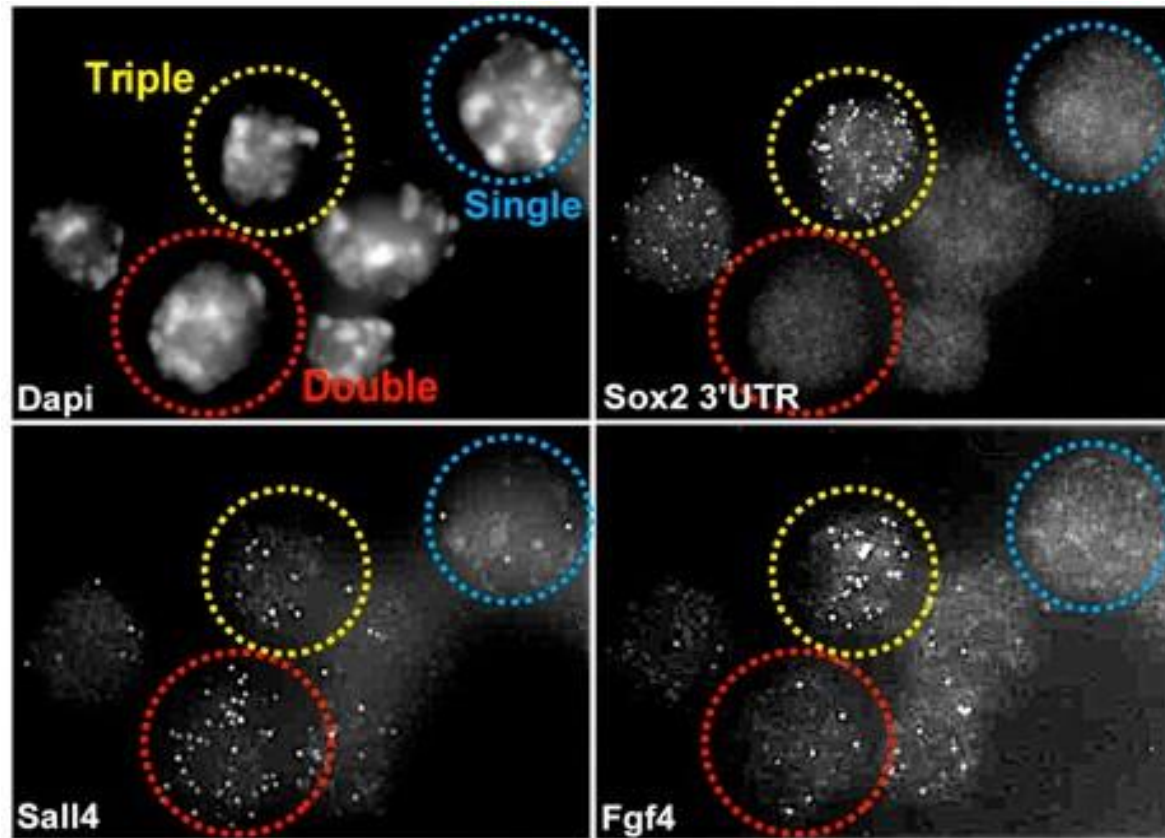
RNA FISH – experimental setup



Stellaris® RNA FISH

RNA FISH allows to localize and count mRNA molecules

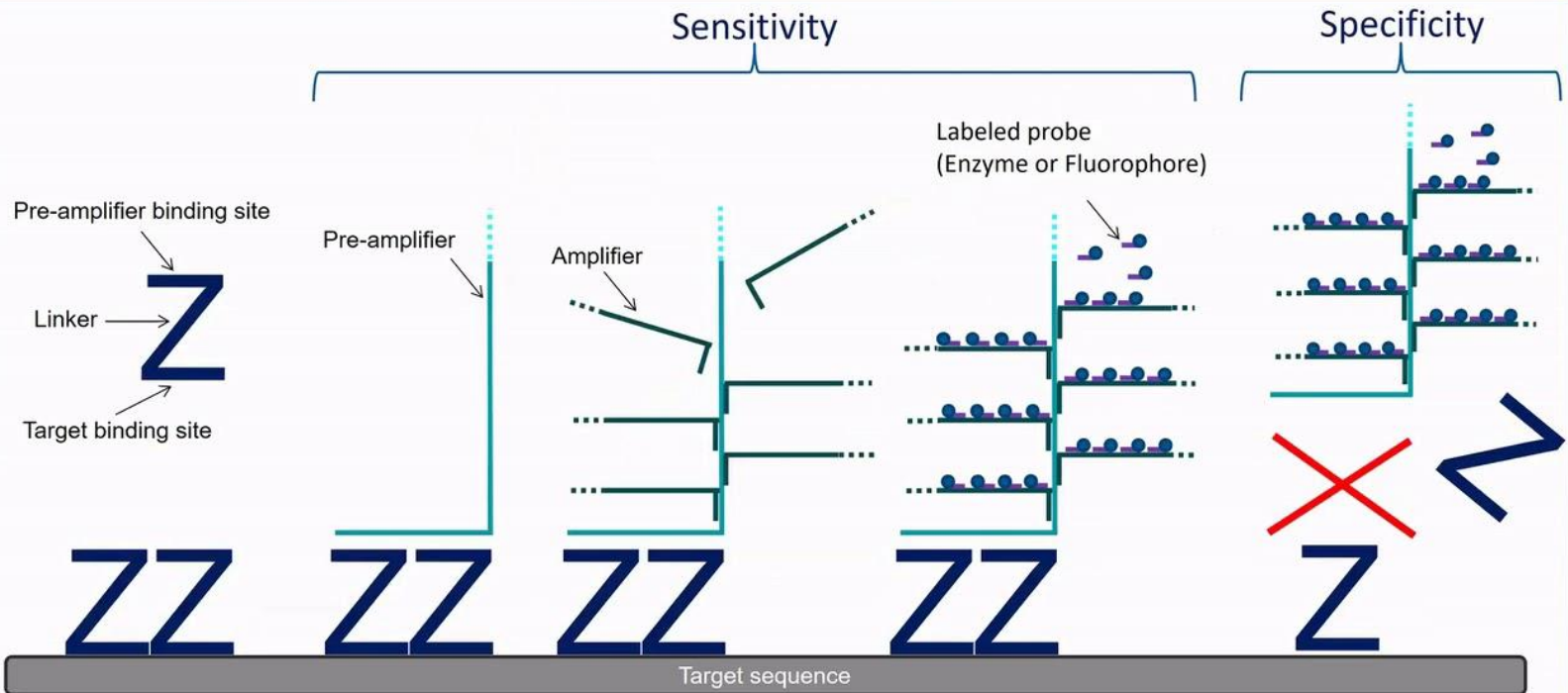
Each dot represents a single mRNA molecule
→ Allows to count the number of each mRNA



Buganim et al., Cell 2012

Different signal amplification strategies exist

RNAscope® Signal Amplification - highly sensitive & specific



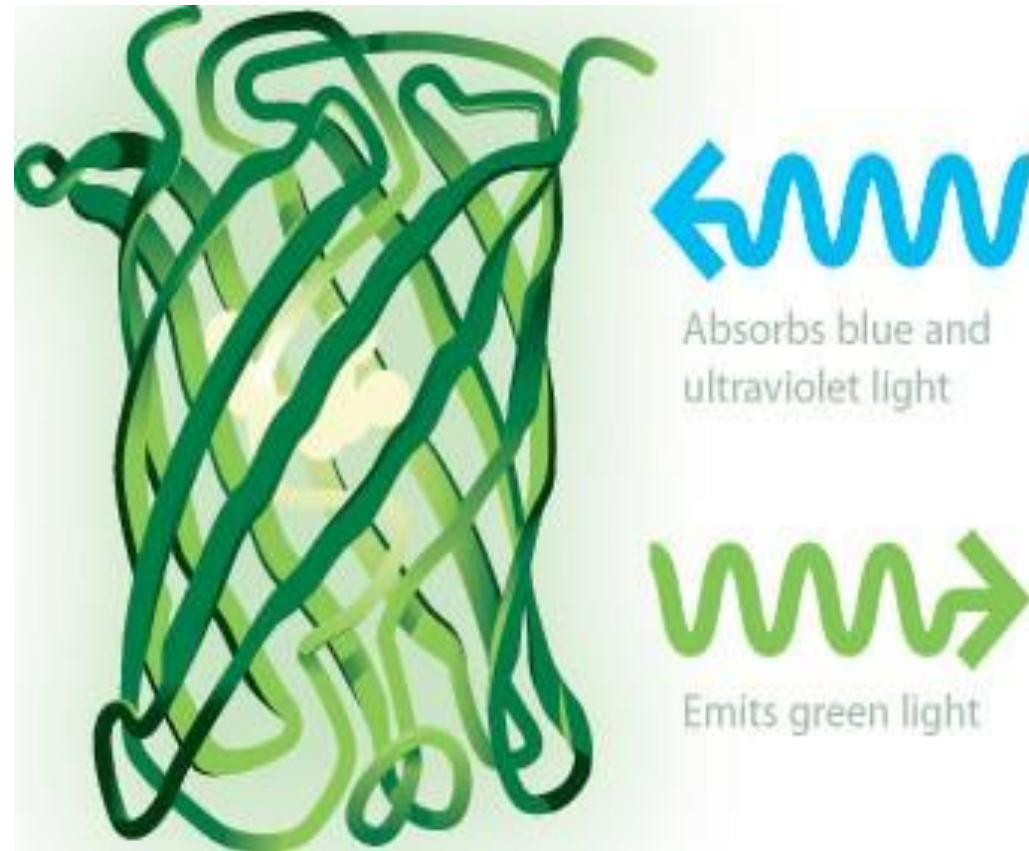
3. Promoter-reporter constructs

How to mark living cells: fluorescent proteins

GFP (Green Fluorescent Protein)



Aequorea victoria



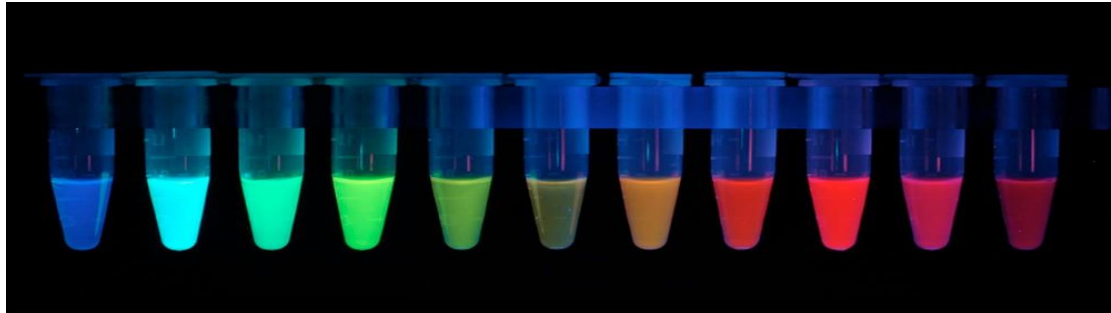
Fluorescent proteins have a low degree of toxicity

Mice can be engineered to express GFP

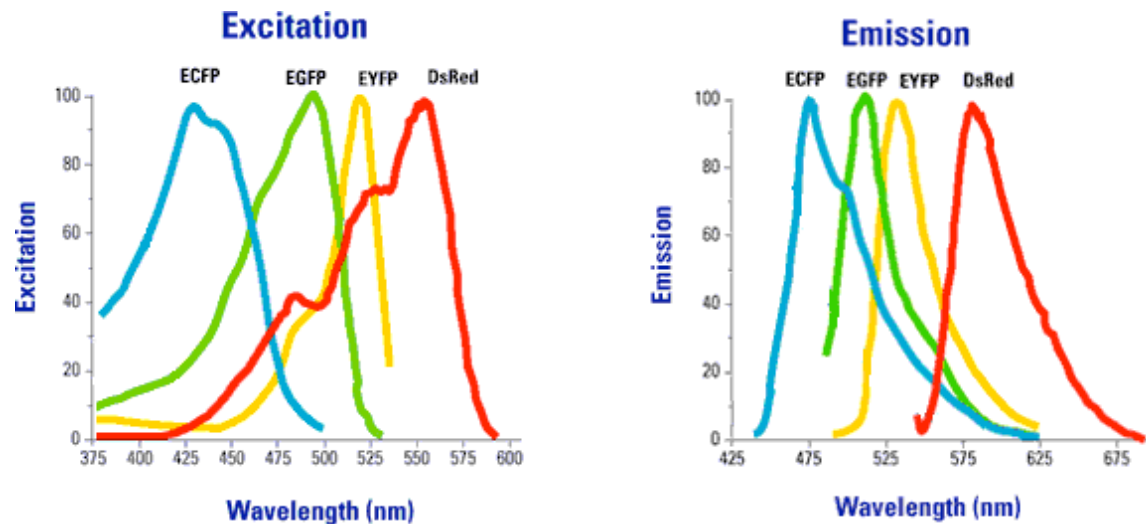


A colorful palette

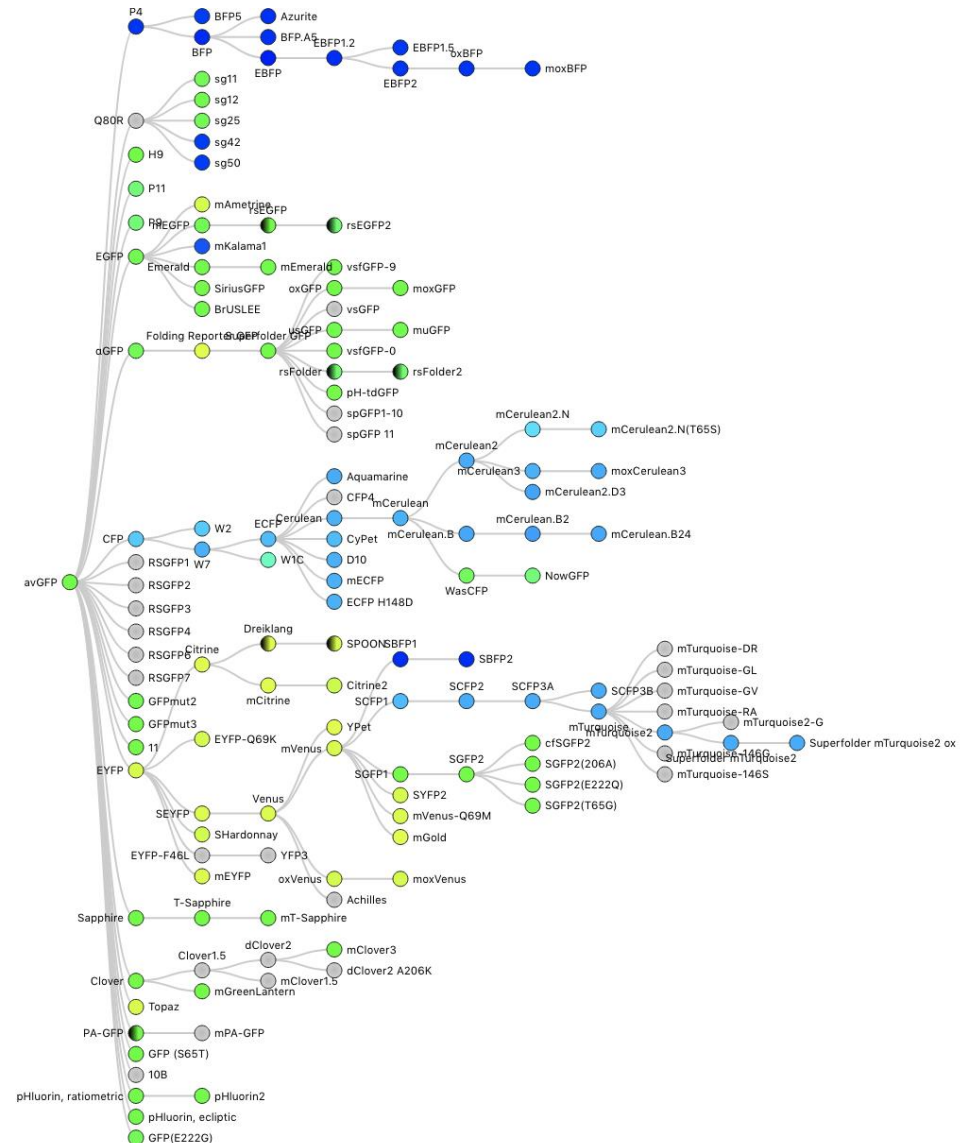
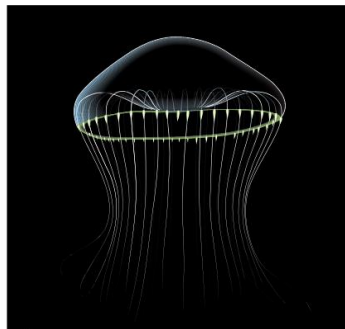
- New fluorescent proteins have been developed through mutations in the GFP gene



- Different fluorescent proteins have different excitation and emission spectra



The “evolution tree” of GFP



→ The fluorescent protein database (<https://www.fpbase.org/>): for info about spectra, properties, microscope set-ups...

Fluorescent proteins can be used as cell-specific reporters

Promoter active in neurons

GFP



Non-neuronal cell: no GFP expressed

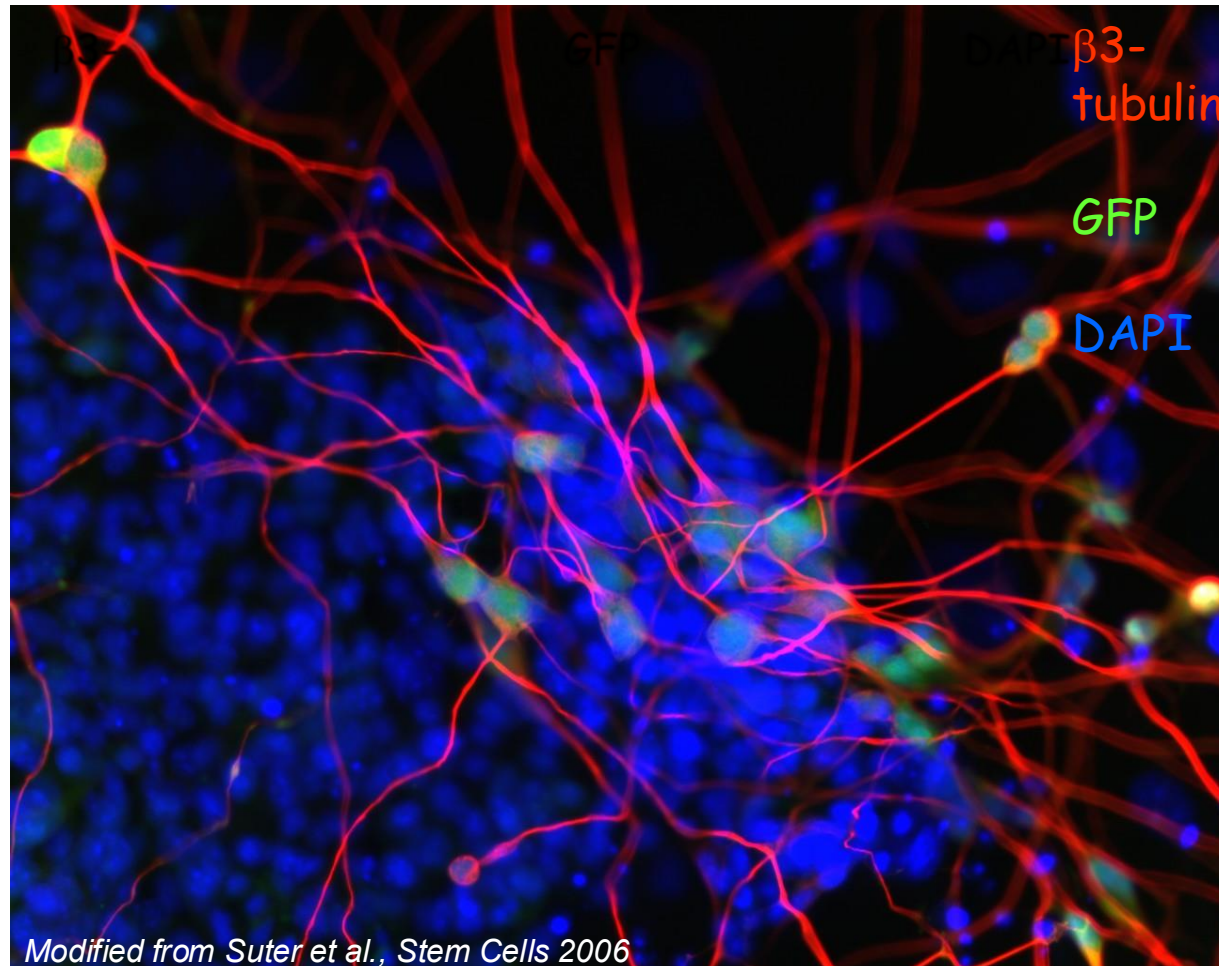


Neuron: GFP expressed



Application: tracking cellular differentiation with fluorescent proteins

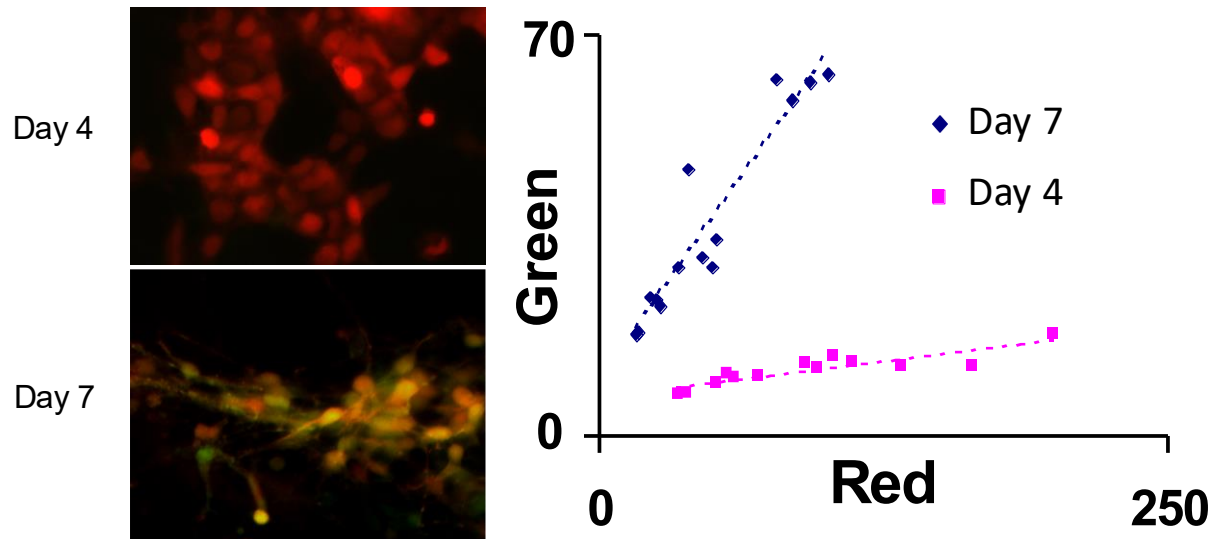
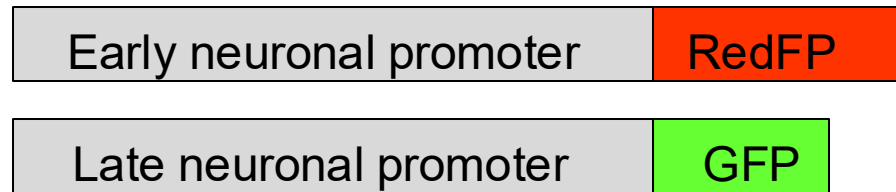
- ES cells carrying a neuronal promoter driving the expression of GFP are differentiated towards neurons.
- In addition: immunofluorescence against β 3-tubulin (expressed in neurons) and DAPI staining



Application: tracking cellular differentiation with fluorescent proteins

→ Promoter active in a specific cell type allows to follow differentiation by fluorescence imaging

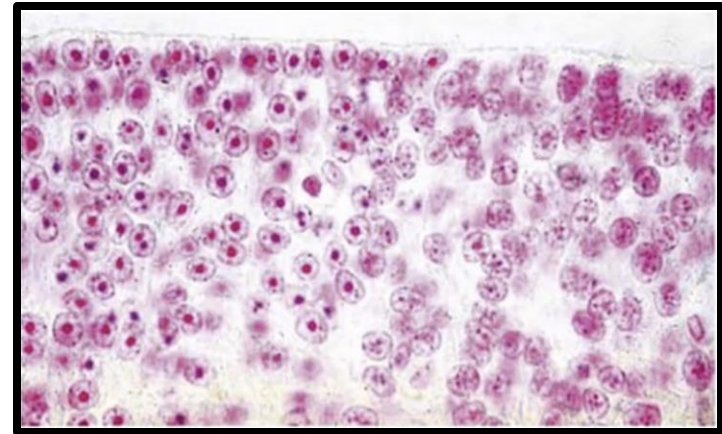
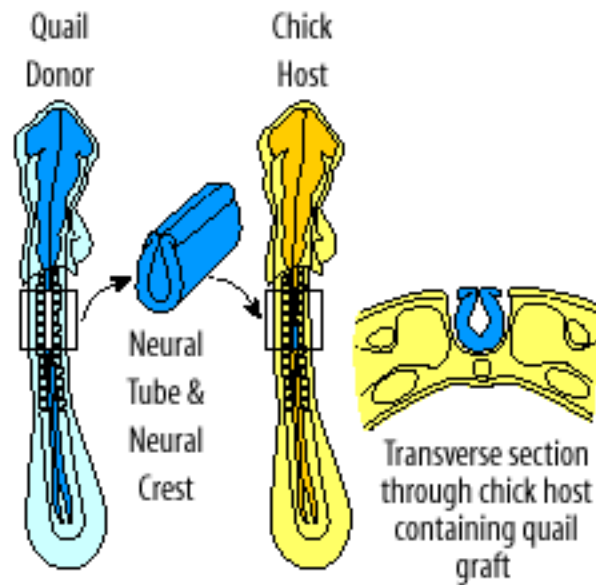
→ In vitro differentiation of embryonic stem cells towards neurons



Modified from Suter et al., Stem Cells 2006

Application: tracking cell fate after transplantation

Tracking cell fate after transplantation: seminal experiment by Nicole Le Douarin



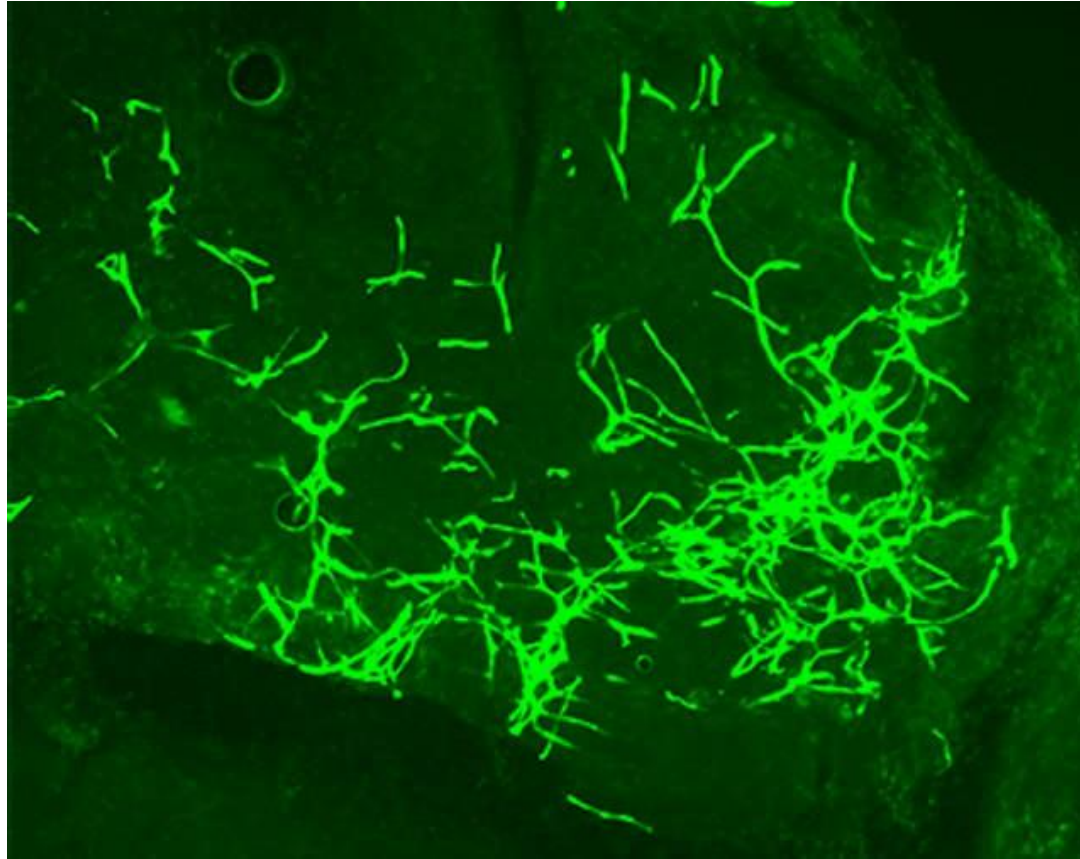
Key concept: quail cells have a prominent nucleolus that serves as a permanent marker

→ Can be discriminated from chick cells

Application: tracking cell fate after transplantation

Cells from a GFP mouse can be isolated and transplanted into a non-GFP mouse

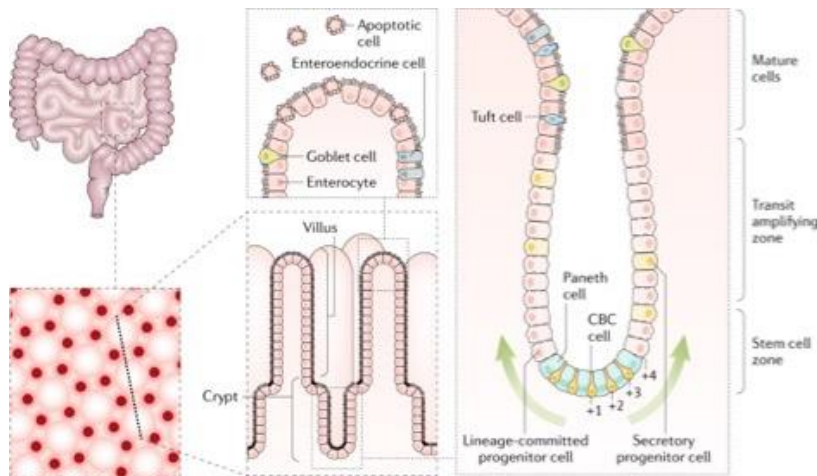
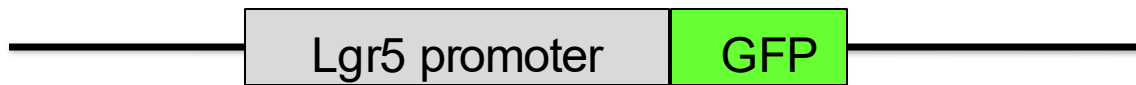
→ This allows to track the fate of the transplanted cells



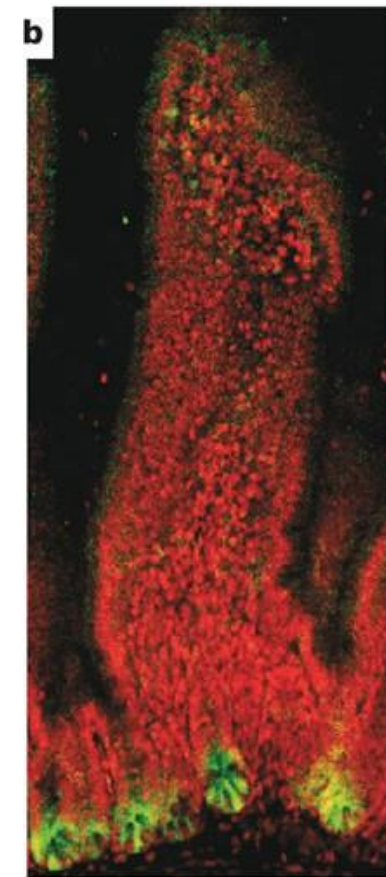
Example: transplantation of vascular endothelial stem cells
→ The resulting vessels can be directly seen by GFP
fluorescence in the recipient mouse

Application: In vivo labeling of stem cells

Intestinal stem cells can be labeled in vivo by controlling GFP expression by an endogenous gene (*Lgr5*) that is active specifically in these cells



Gehart & Clevers, Nature 2018

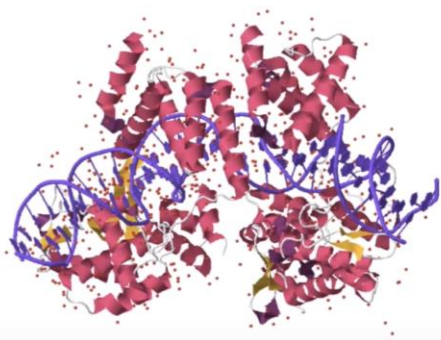


Barker et al., Nature 2007

4. Reporter-based lineage tracing

The Cre-Lox recombination system

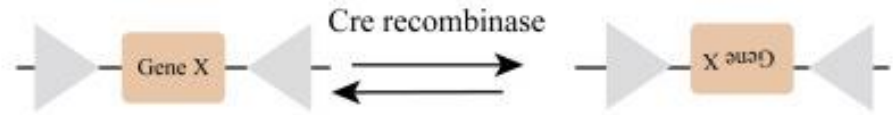
Is it possible to stably label endogenous stem cells and follow their fate ?



LoxP

5' -ATAACTTCGTATA-GCATA CAT-TATACGAAGTAT-3'
3' -TATTGAAGCATAT-CGTATGTA-ATATGCTTCAATA-5'

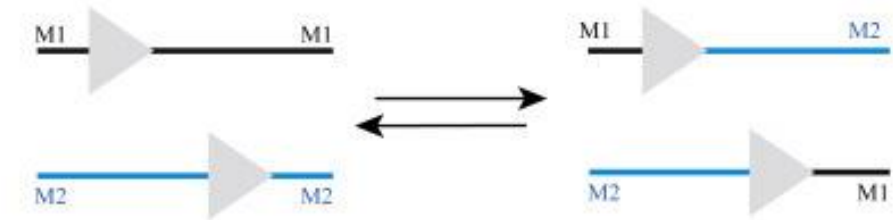
Inversion



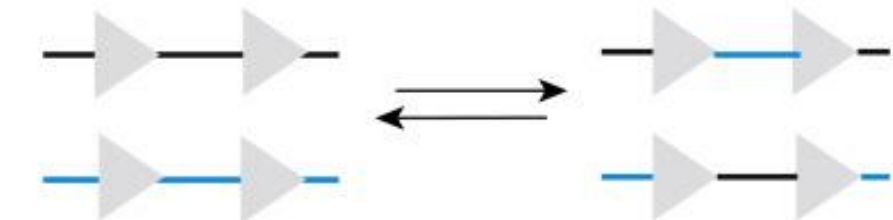
Deletion/Insertion



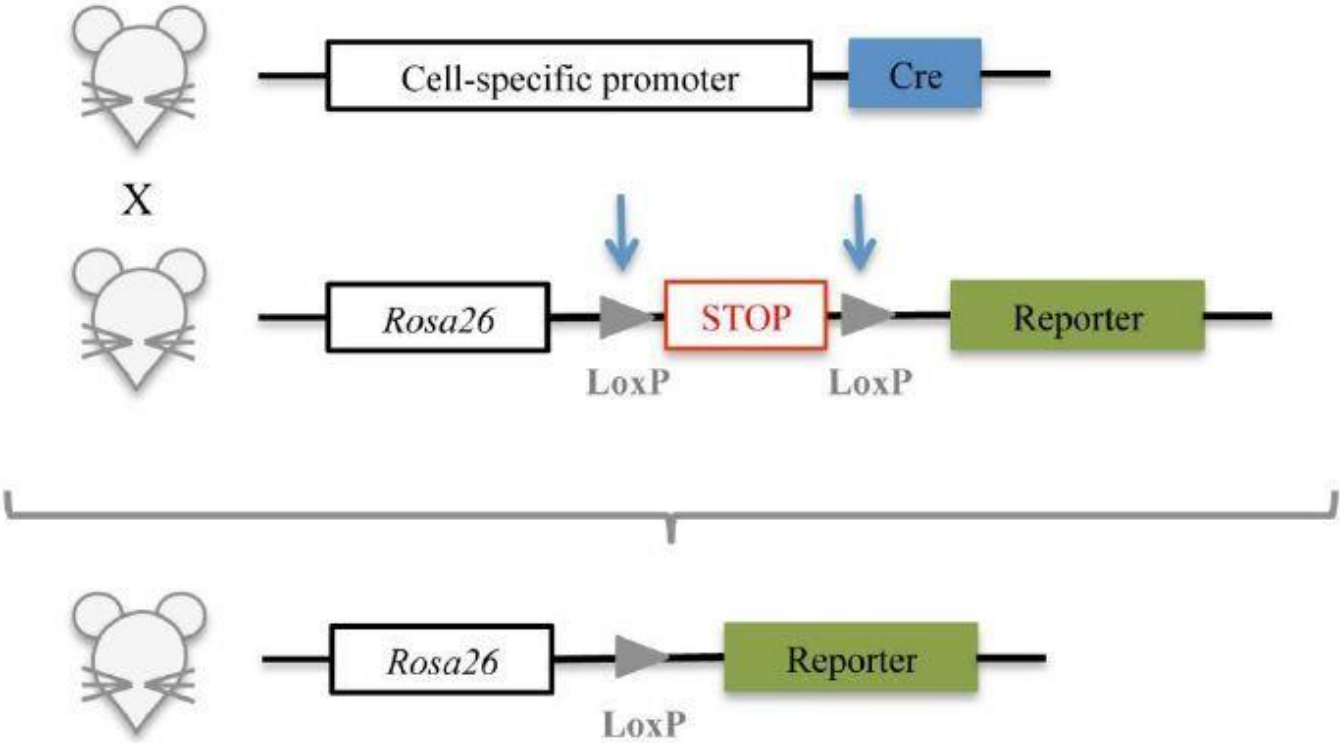
Translocation



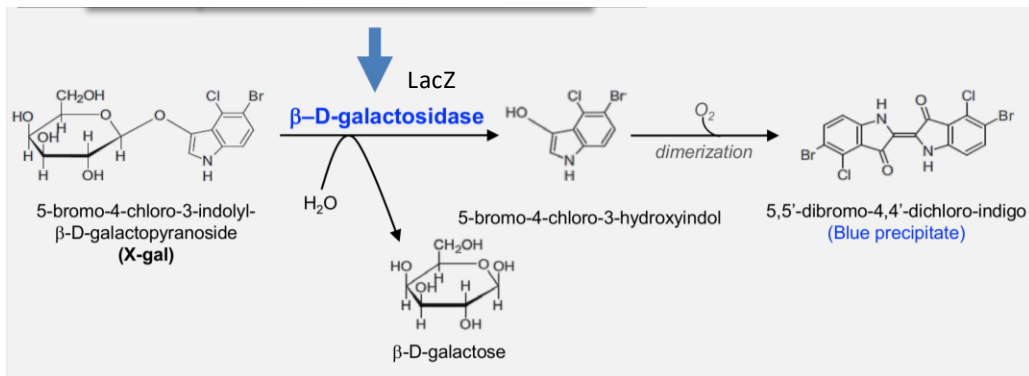
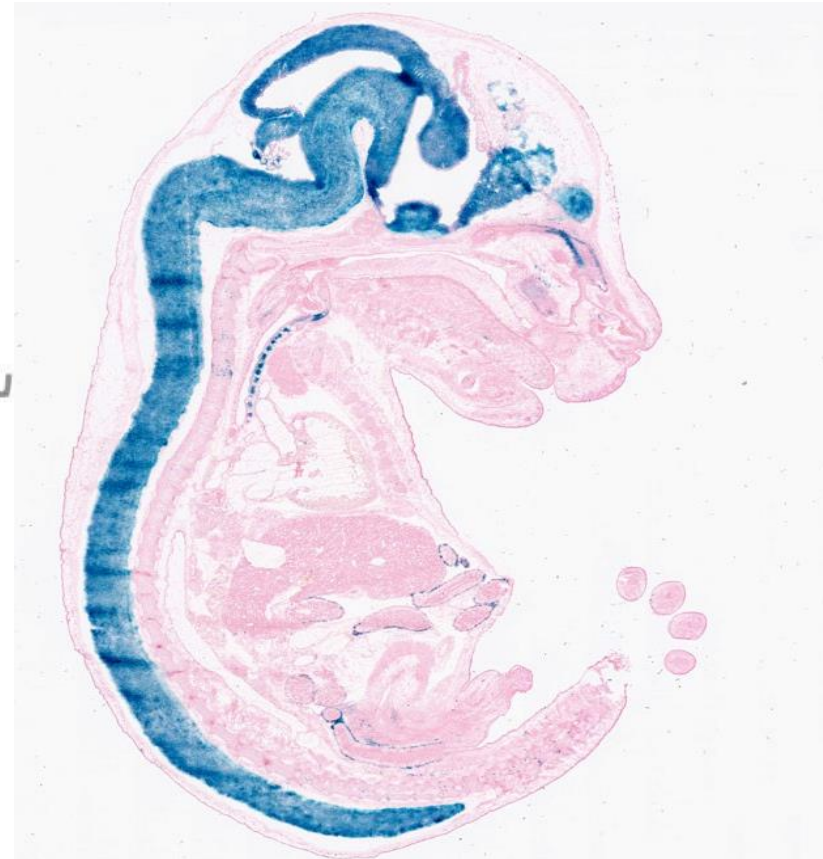
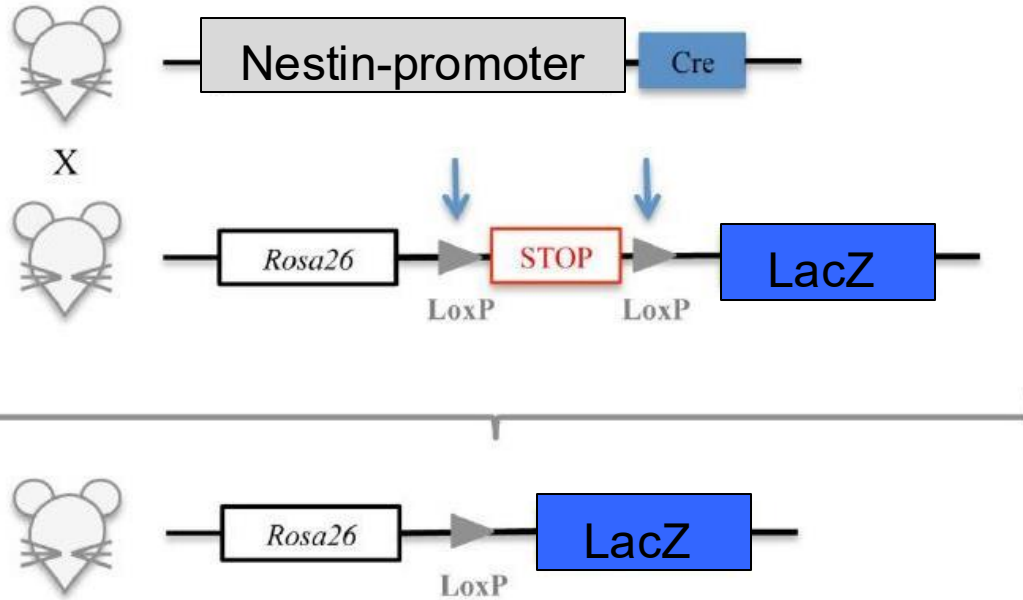
Cassette exchange



Cre-Lox system can be used to label progeny of specific cells



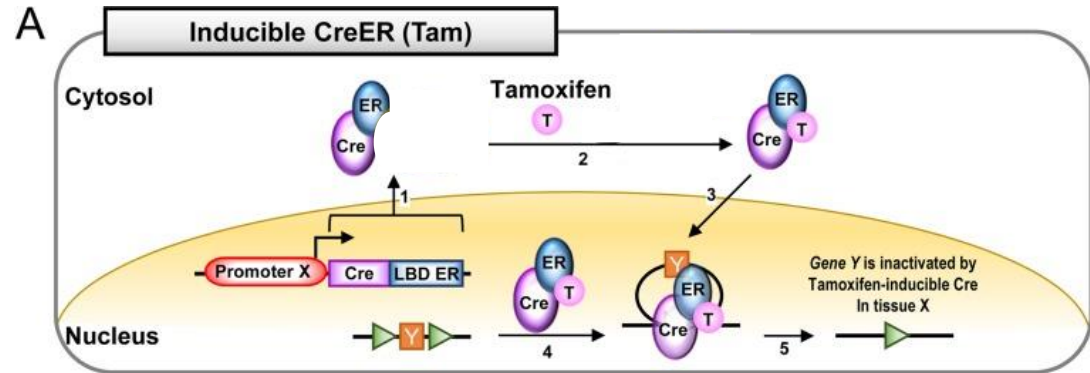
Cre-Lox system can be used to label progeny of specific cells



Inducible Cre-Lox recombination

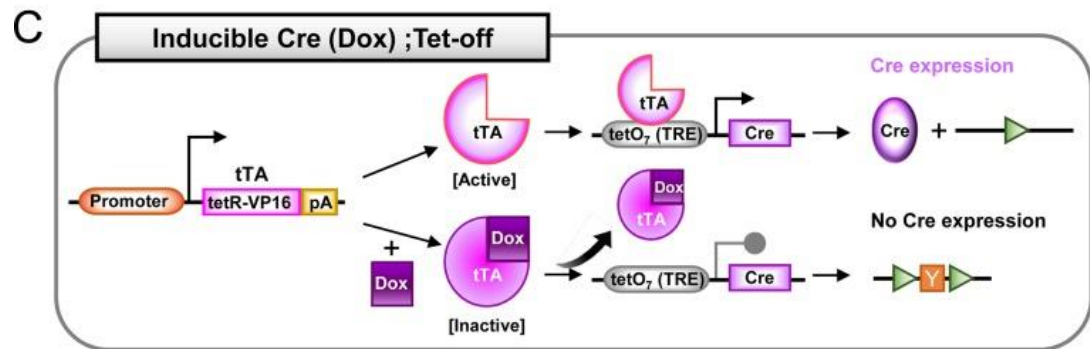
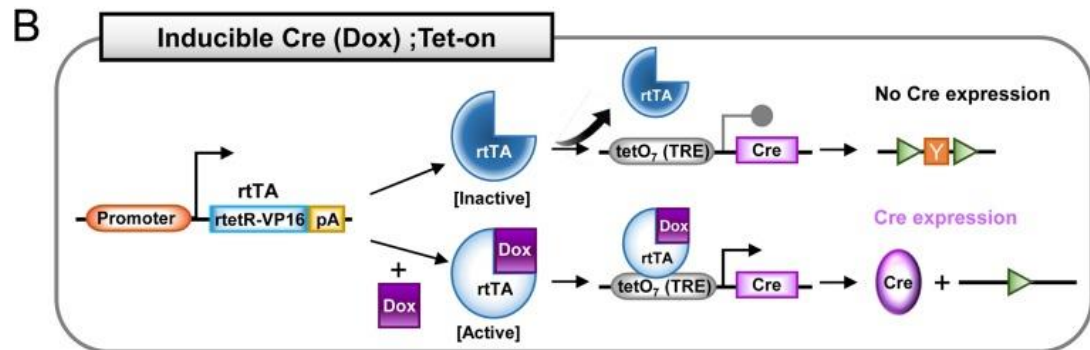
Tamoxifen (Tam) - mediated:

- Cre fused to a modified estrogen receptor (CreER) cannot enter the nucleus
- Tamoxifen binding to the ER induces nuclear translocation of Cre



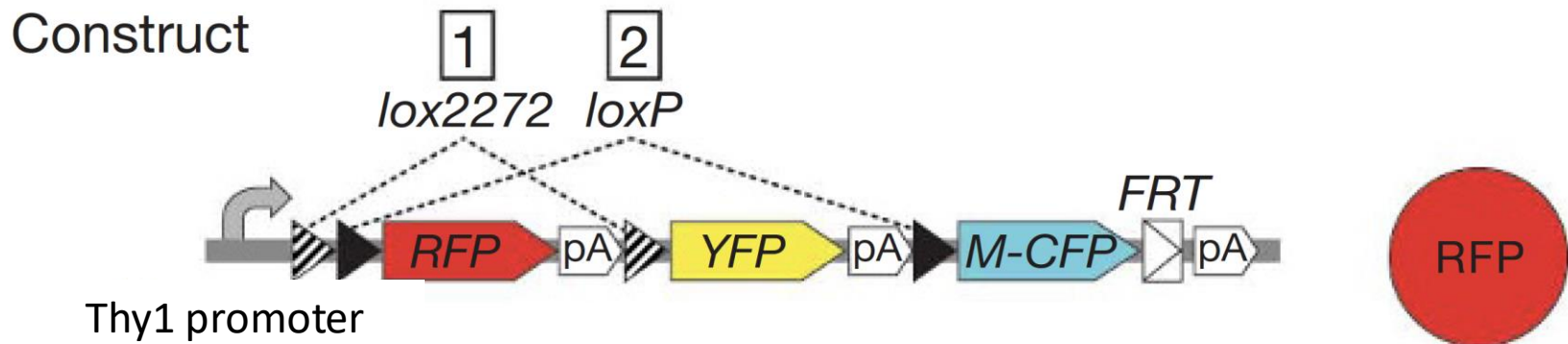
Doxycycline (Dox) - mediated:

- Cre expression is driven by a tetO promoter
- (B) In presence of Dox, rtTA induces Cre expression
- (C) In absence of Dox, tTA induces Cre expression
- (C) In presence of Dox, Cre expression is inhibited



Multicolor lineage tracing: the brainbow mouse

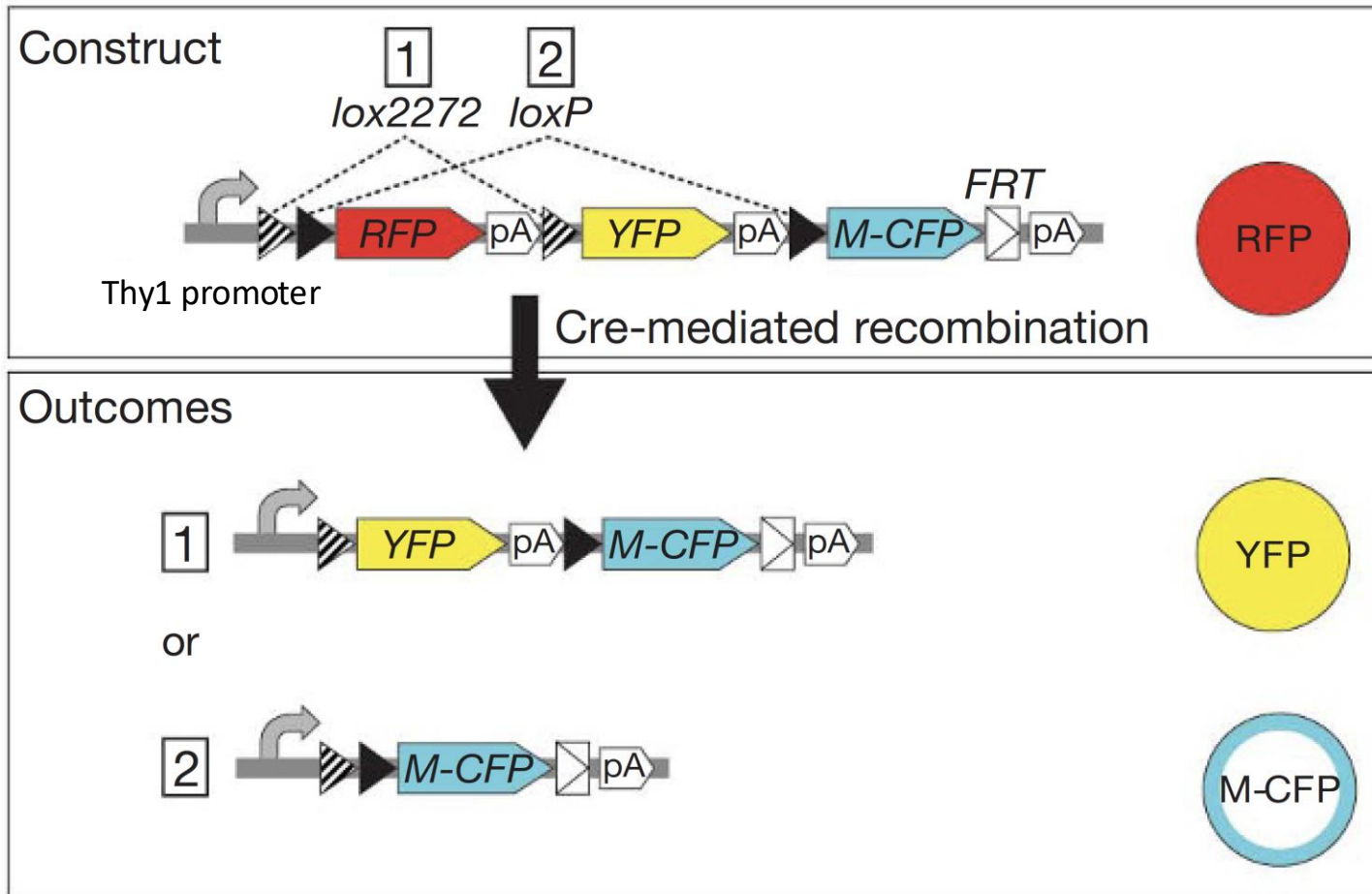
- A **DNA sequence coding for several fluorescent proteins** is inserted into the mouse genome.
- These proteins can only be expressed in the brain (neuron specific promoter)
- Upon induction, the Cre recombinase **randomly permutes parts of this sequence** so that only one of the fluorescent proteins is expressed



Multicolor lineage tracing: the brainbow mouse

Each permutation results in expression of a single fluorescent protein

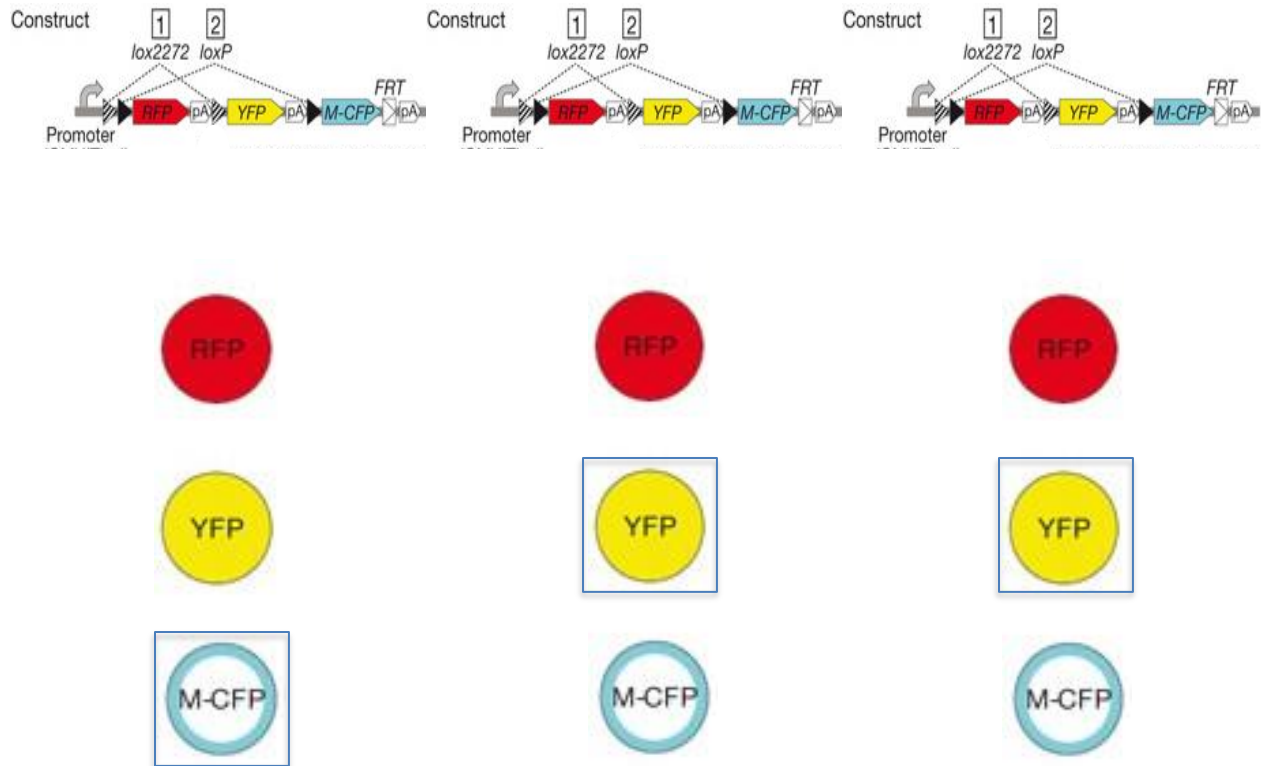
a *Brainbow-1.0*



Multicolor lineage tracing: the brainbow mouse

If the DNA sequence is present in 3 copies:

→ 3 different fluorescent proteins are expressed in each cell!

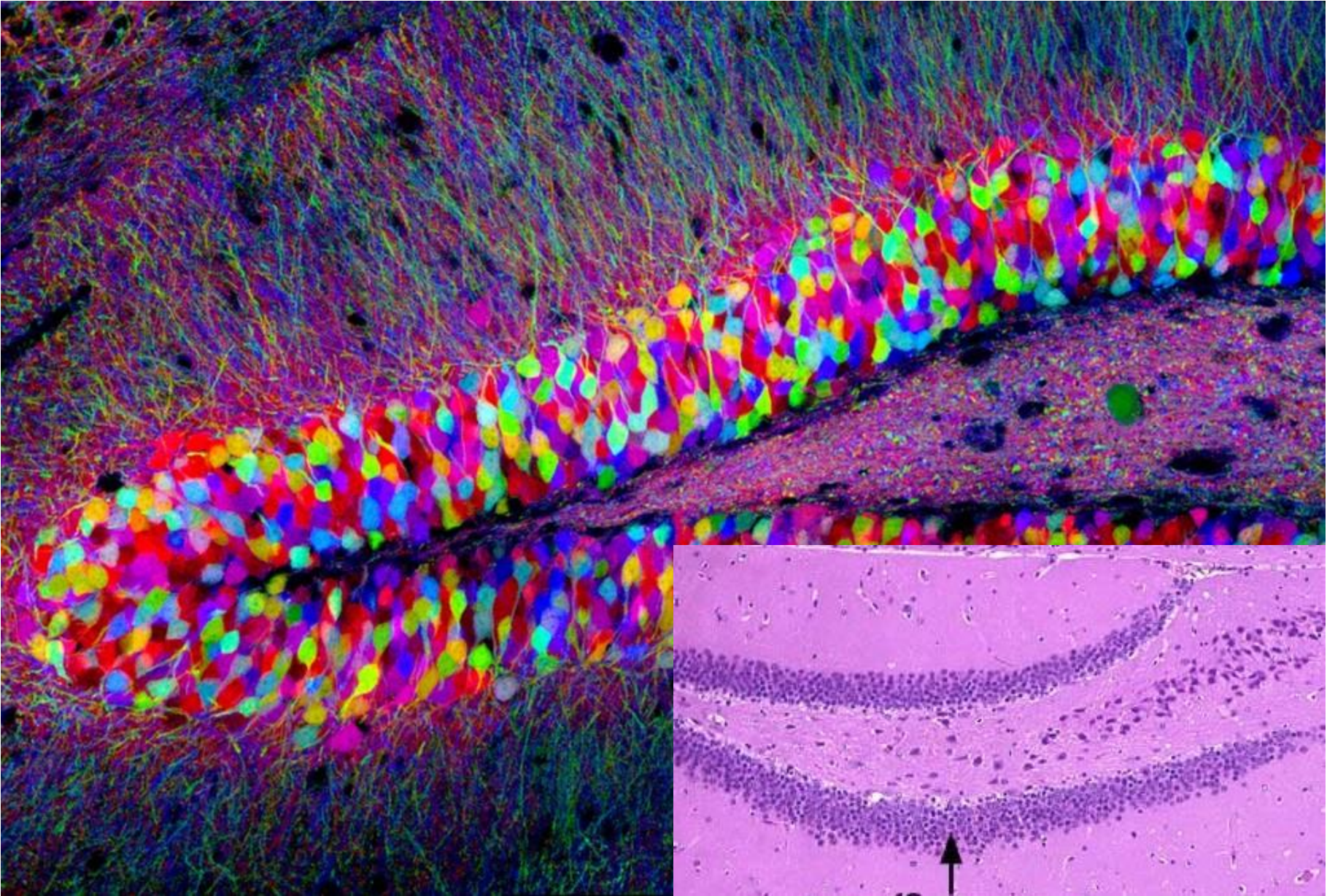


a XFP combinations

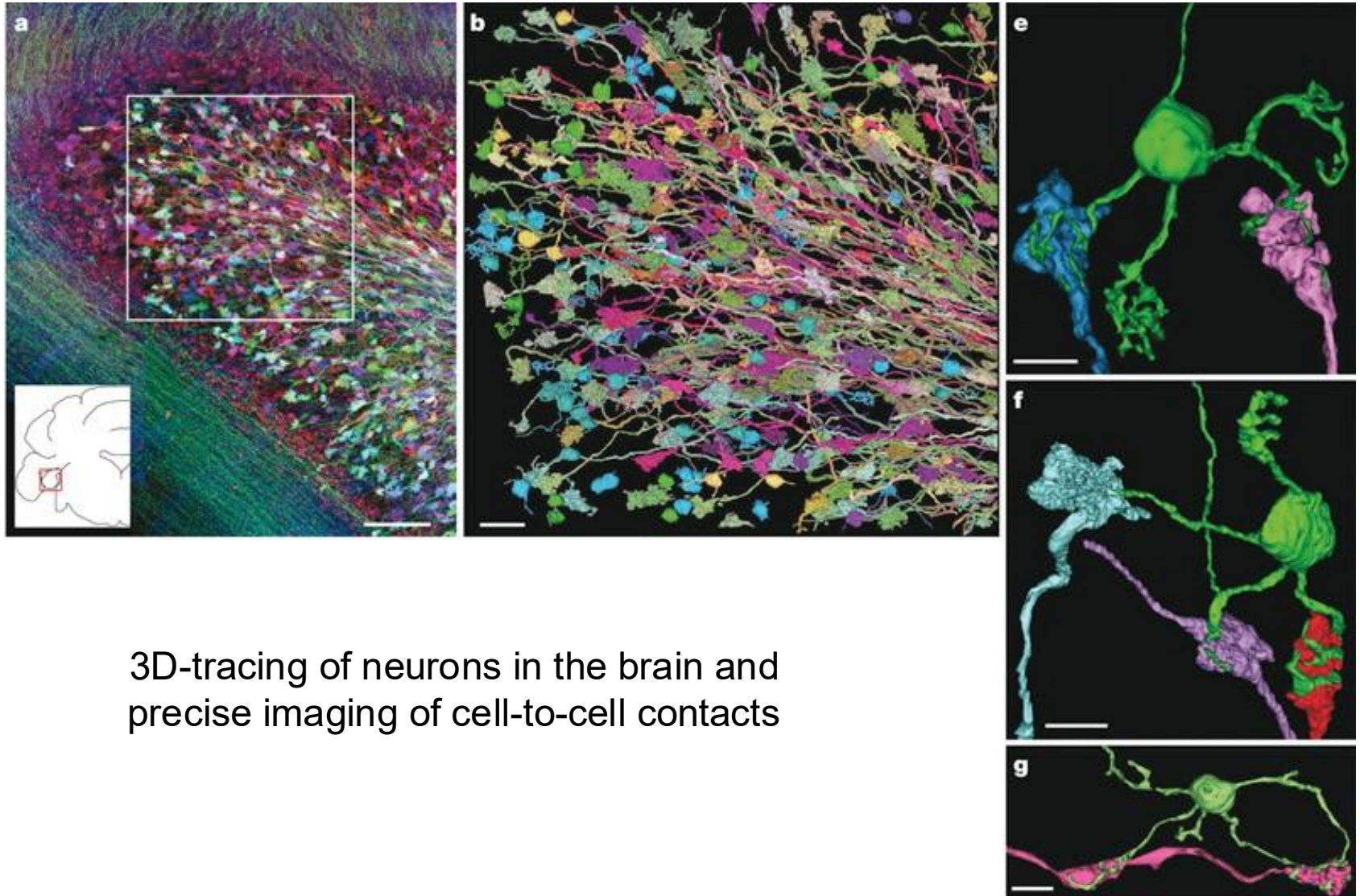
Outcome for each copy Resulting colour

1	2	3	Resulting colour
C	C	C	Blue
C	C	Y	Light blue
C	Y	Y	Blue-green
Y	Y	Y	Green
Y	Y	R	Light green
Y	R	R	Orange
R	R	R	Red
R	R	C	Magenta
R	C	C	Purple
R	C	Y	Grey

Neurons of the brainbow mouse

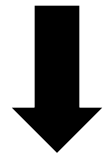
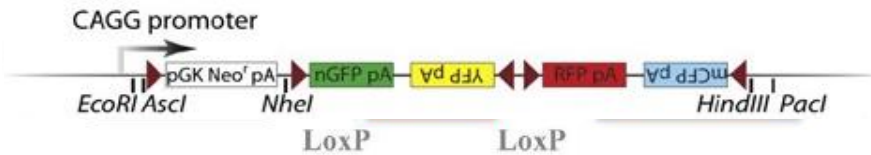
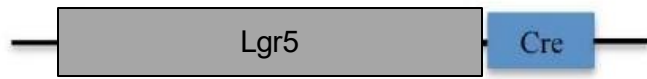


Neurons of the brainbow mouse



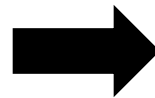
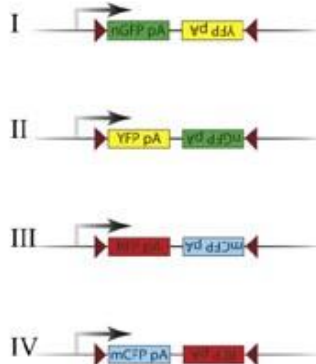
3D-tracing of neurons in the brain and
precise imaging of cell-to-cell contacts

Multicolor lineage tracing: the confetti mouse

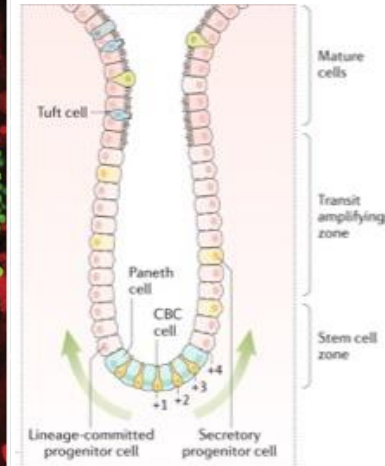
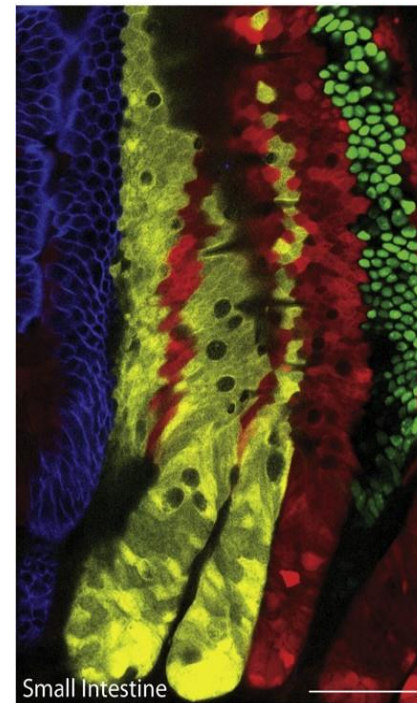


Cre recombination

Outcomes



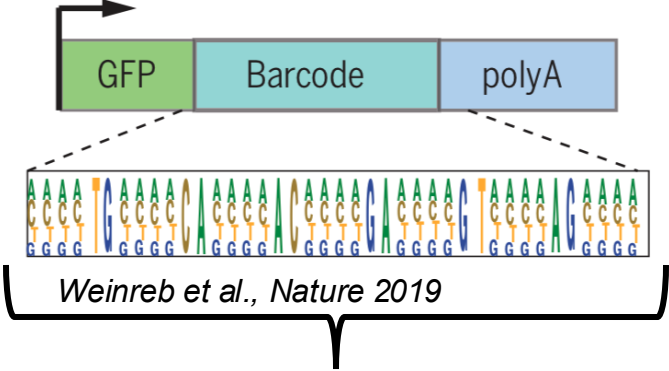
The progeny of each Lgr5-expressing single cell is marked with a different color



5. Sequencing-based lineage tracing

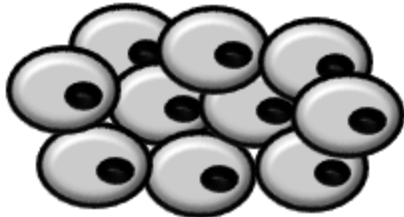
The principle of cellular barcoding

Labelling cells with nucleic acid sequences



Lentiviral library

Cell population of interest



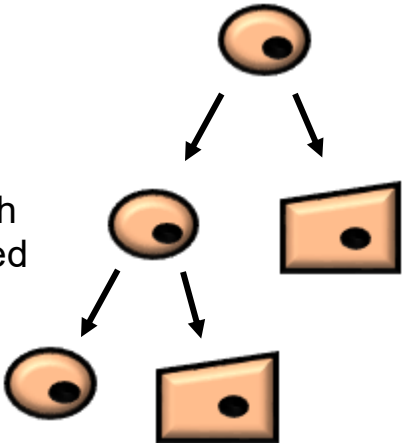
Selection of barcoded cells (FACS/antibiotics)



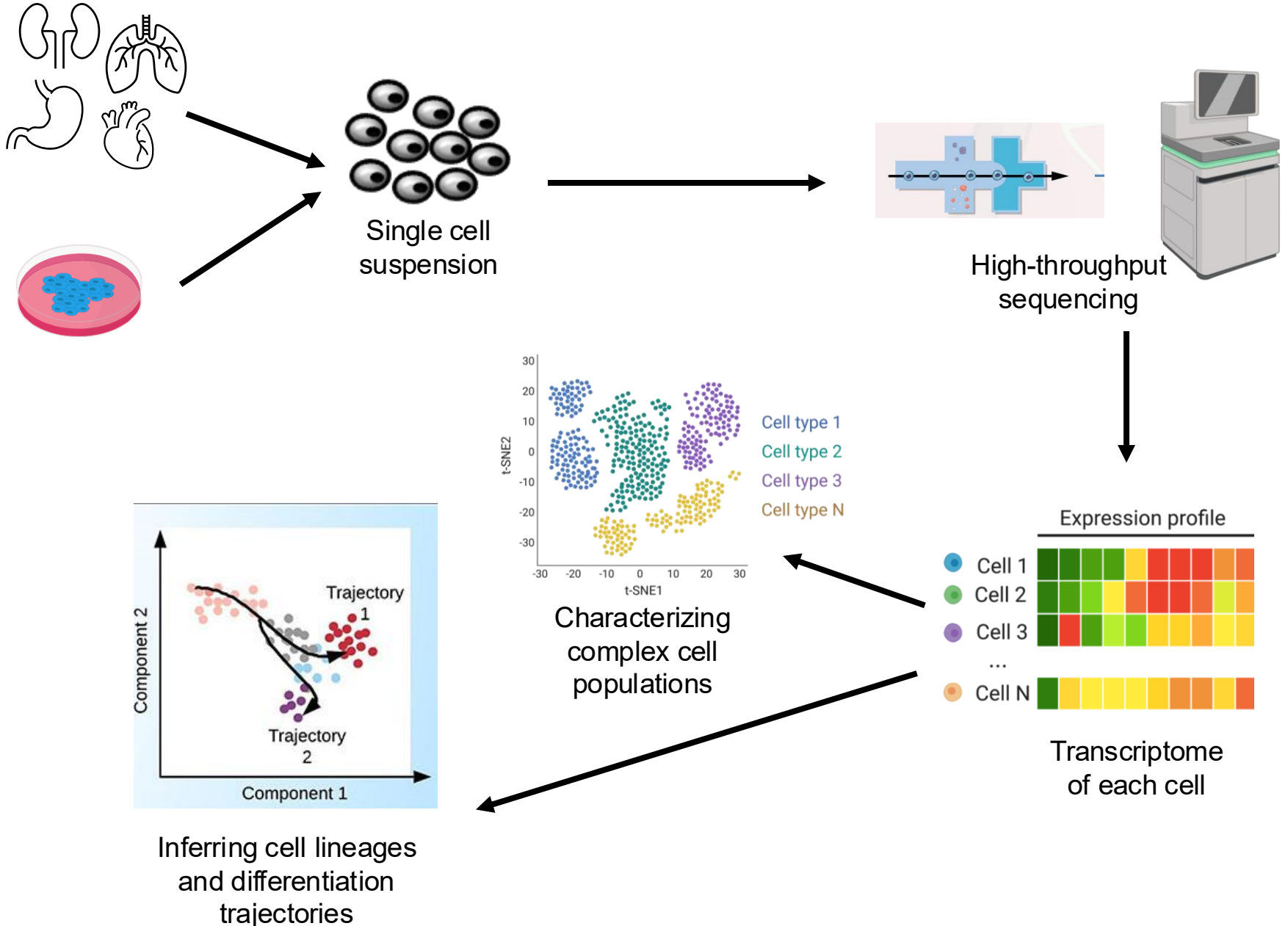
Differentiation



The unique barcode of each cell is transmitted to its progeny



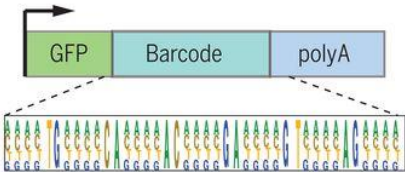
Single cell analysis (scRNA-Seq)



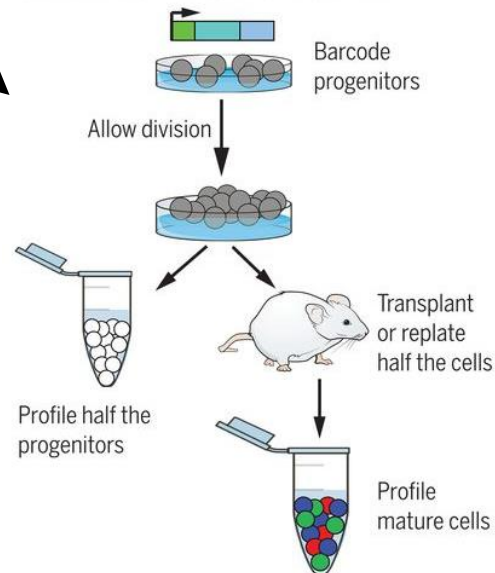
Application: Cellular barcoding to trace HSC differentiation

Lineage and RNA recovery (LARRY)

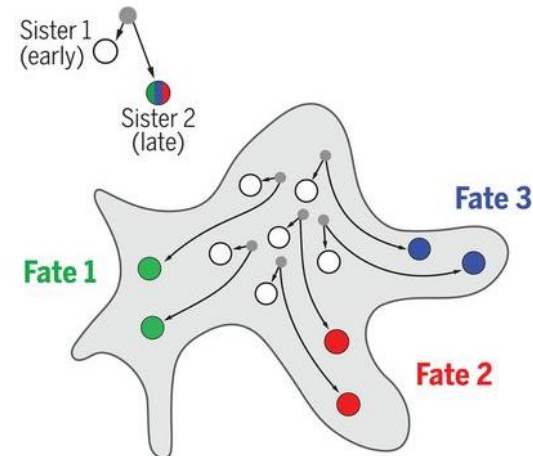
Lineage tracing + single-cell RNA seq



Clonal barcoding experiment

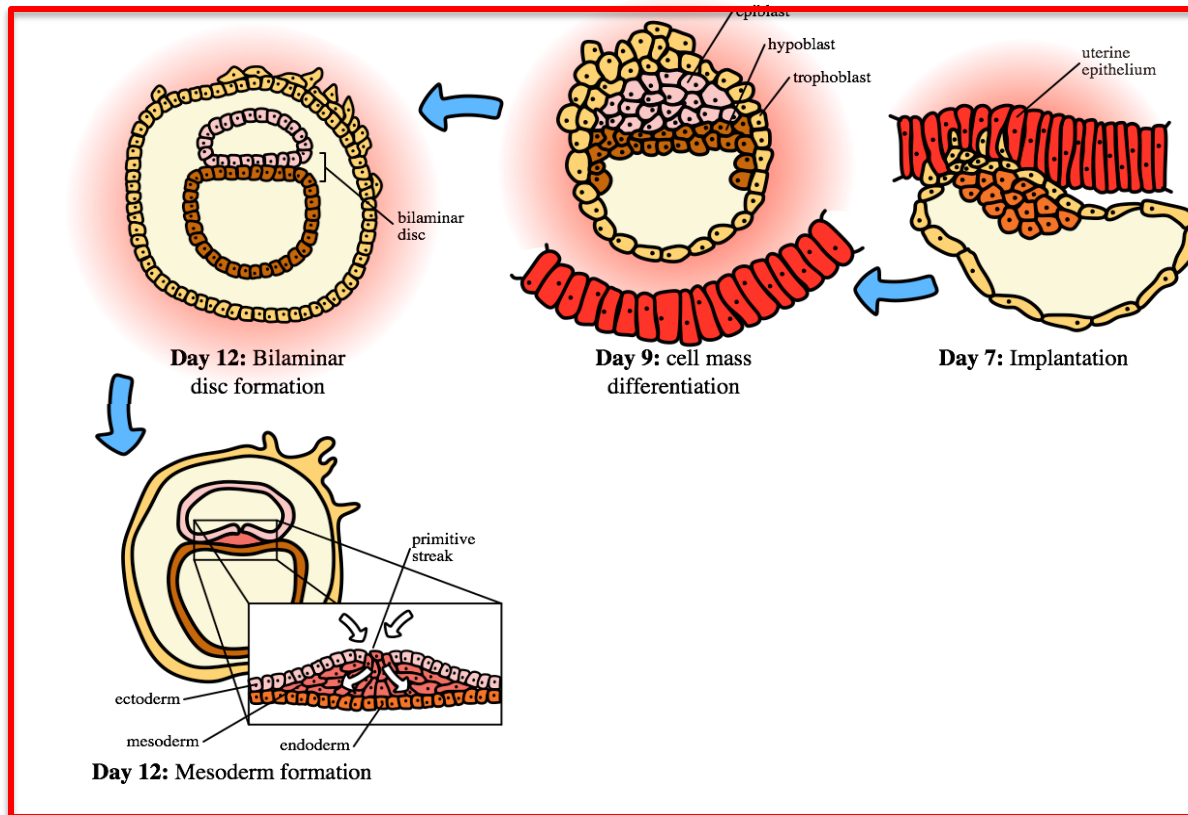
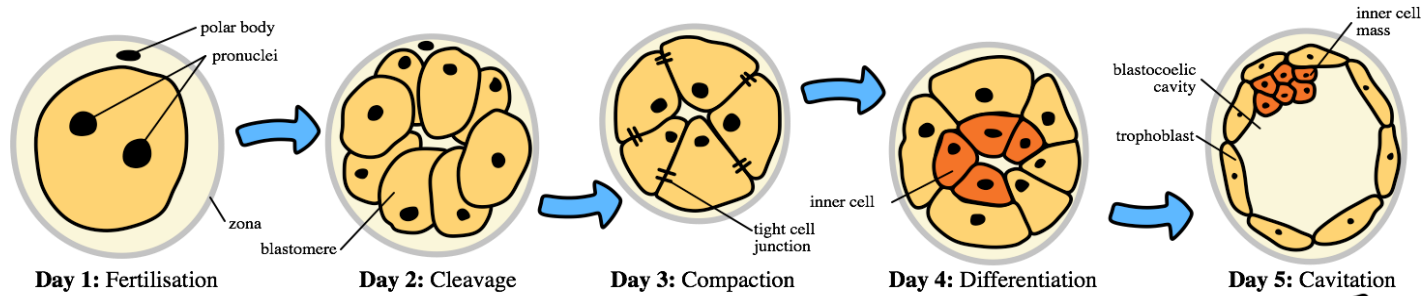


Map of progenitor fate



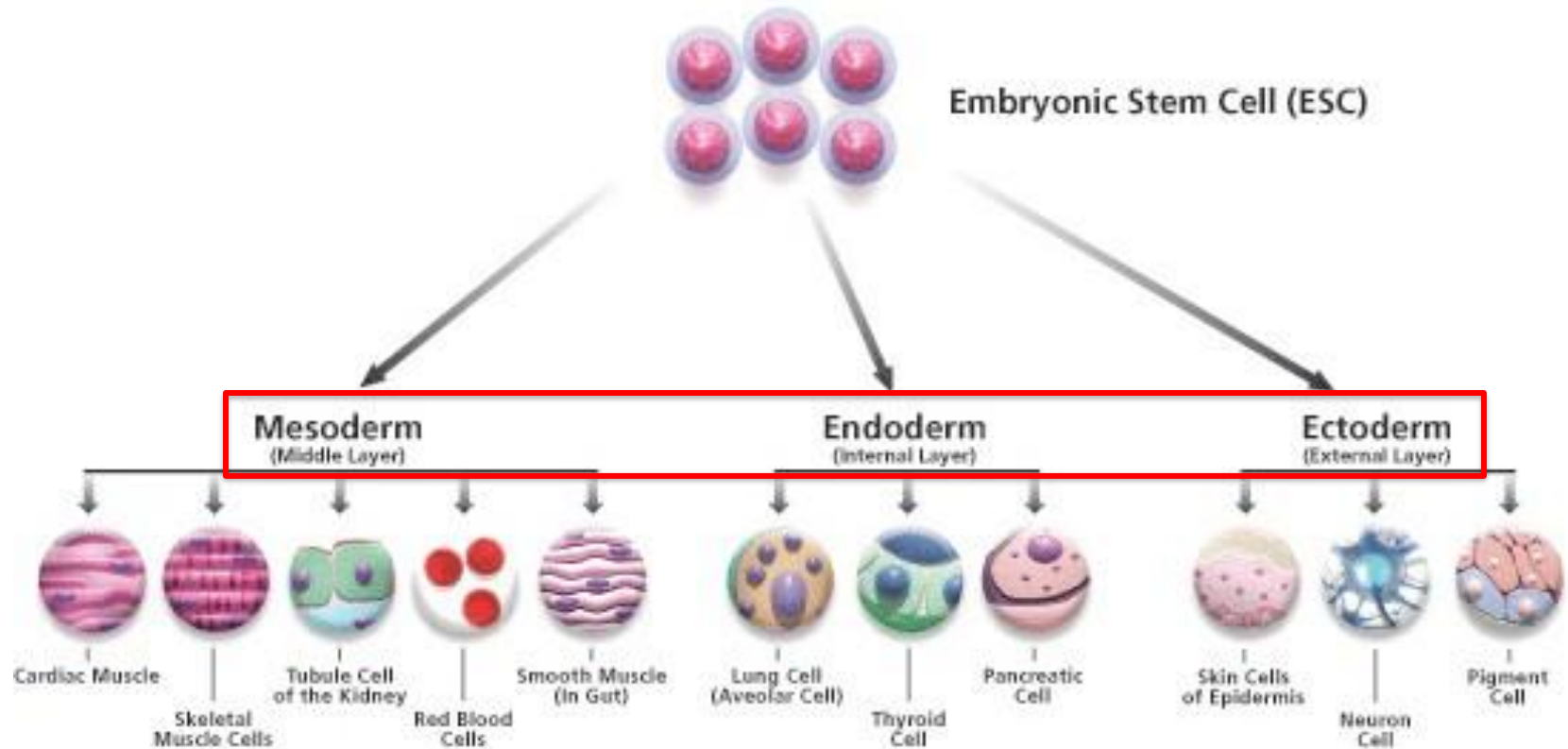
II. Using stem cells to model early embryogenesis

How can we study early embryogenesis?



Very difficult to study in vivo !

How can we study early embryogenesis?



Timing of embryogenesis..

3 weeks

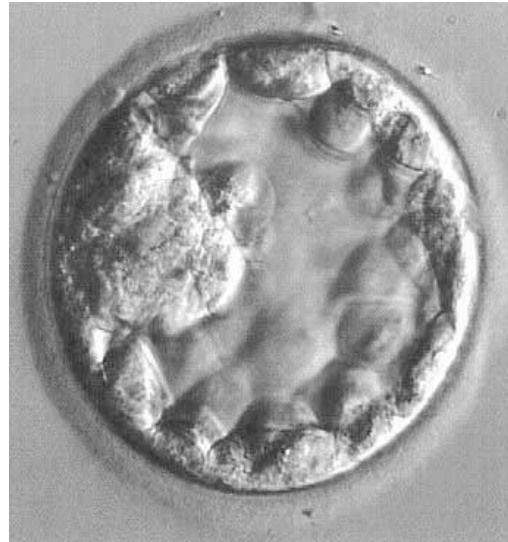


40 weeks



..parallels ES differentiation timing

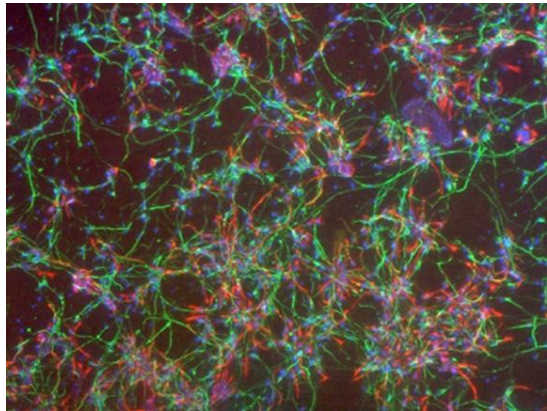
5 days



3 weeks

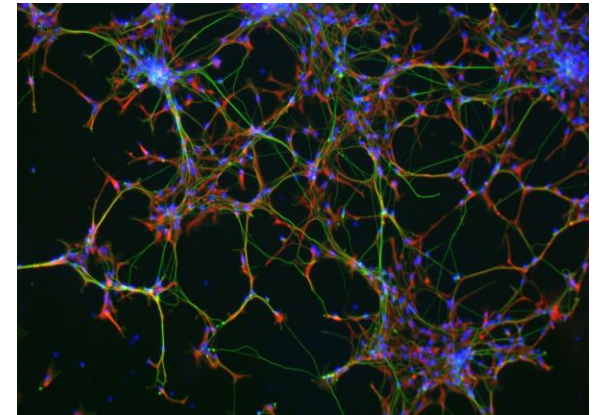


Mouse ES cell-derived neurons



www.csc.mrc.ac.uk

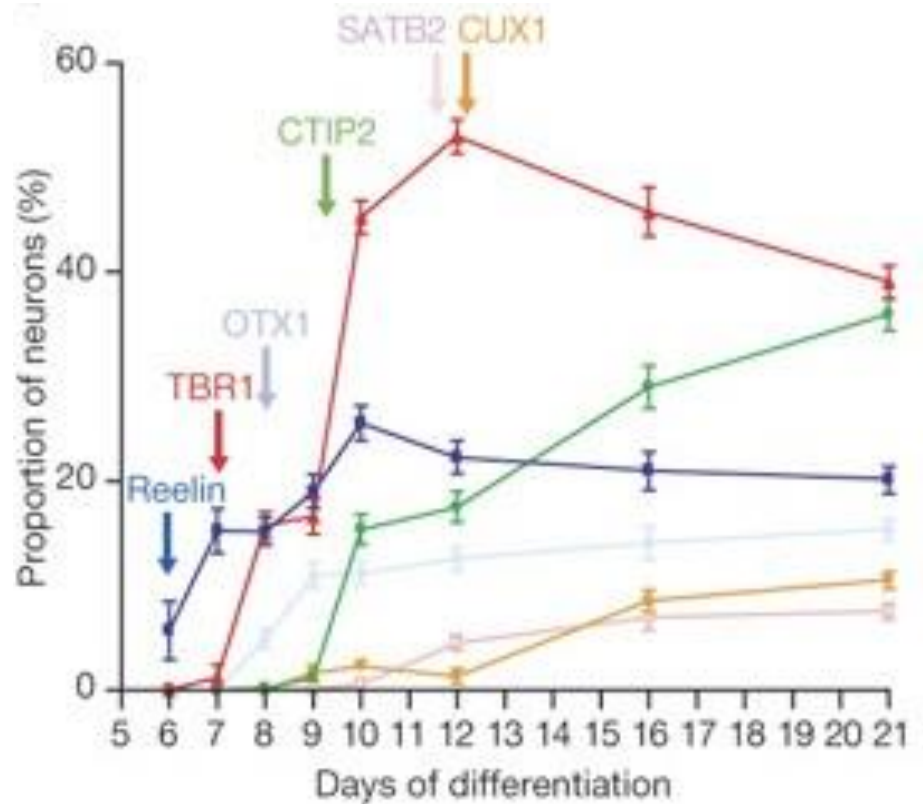
Human ES cell-derived neurons



Suter et al., unpublished

How can we study early embryogenesis?

Layers	Markers	Birthdate
Cajal-Retzius neurons	Reelin, calretinin, p73, TBR1	E10.5–11.5
Upper layers	SATB2, CUX1	E13.5–16.5
Deep layers	CTIP2, SOX5, OTX1, ER81, TBR1, TLE4, FOXP2	E11.5–14.5
Subplate	TBR1, calretinin, reelin	E10.5–13.5

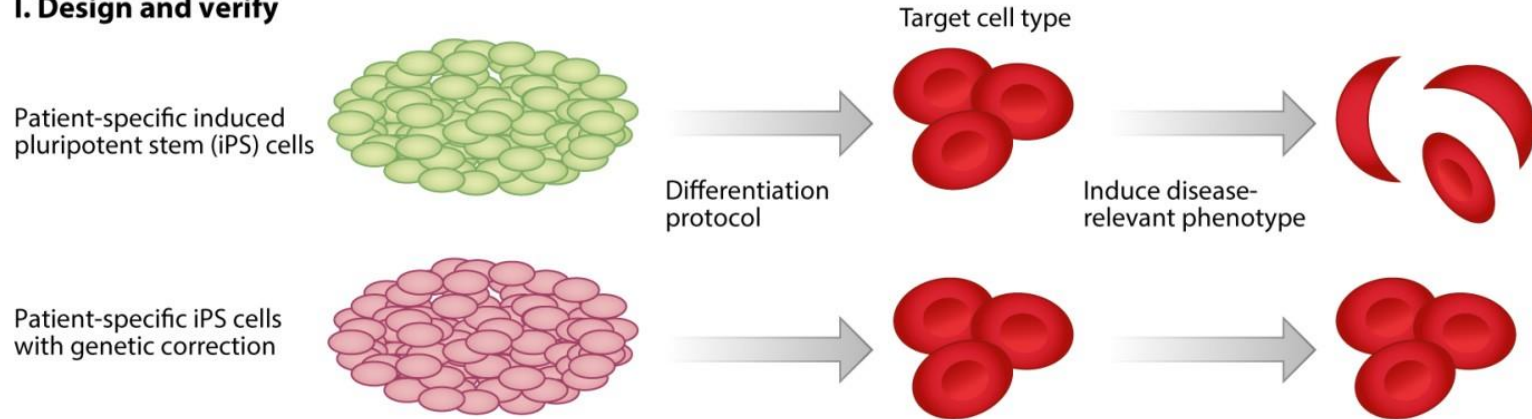


III. Using stem cells to model disease

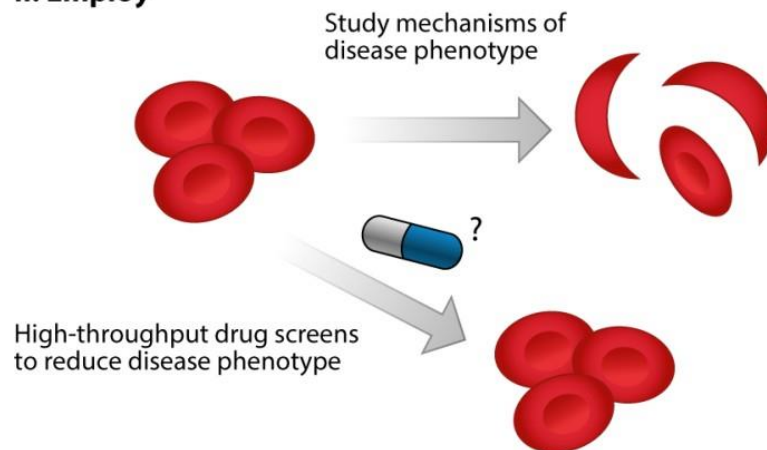
Using pluripotent stem cells to model diseases

Steps of disease modeling

I. Design and verify



II. Employ



Issues with patient-derived cells

- Patients differ from each other not only in their diseases but at millions of genomic loci
- Each iPS cell line from the same patient behaves differently

Genome editing to create disease-specific in vitro models

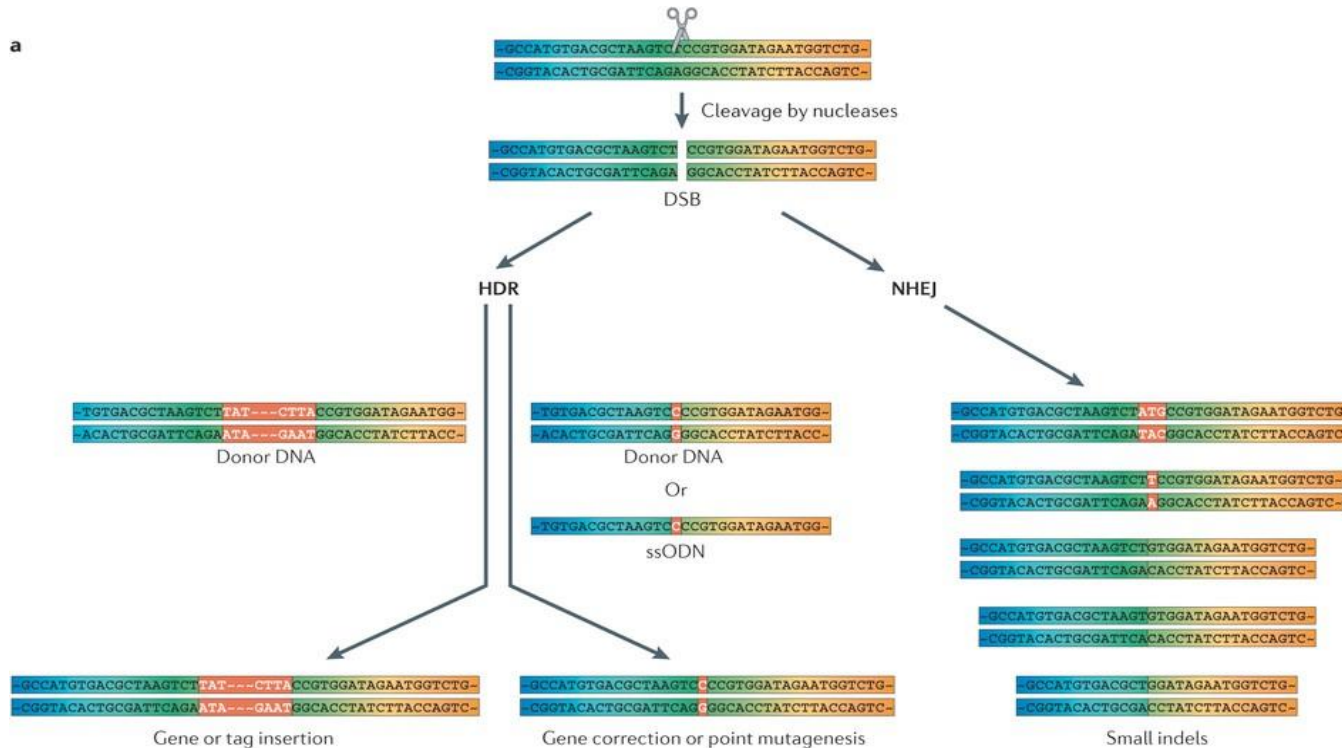


- Starting from a given pluripotent cell line that is modified **specifically** at the disease locus allows a much cleaner model
- How to introduce specific mutations in the genome of pluripotent cells ?

Current genome editing technologies

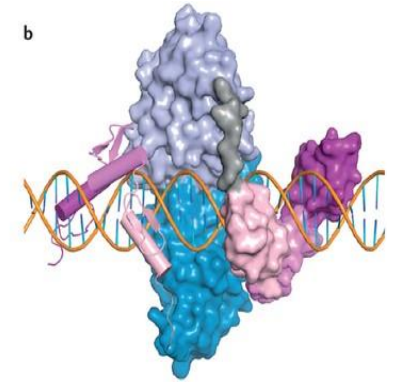
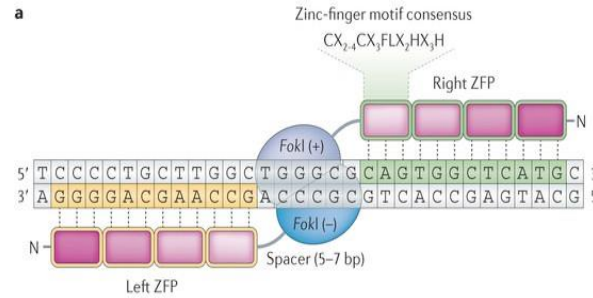
Principle: cleavage of a specific genomic sequence by nucleases

→ The break will be repaired either by non-homologous end-joining (NHEJ) or by homology-directed recombination (HDR)



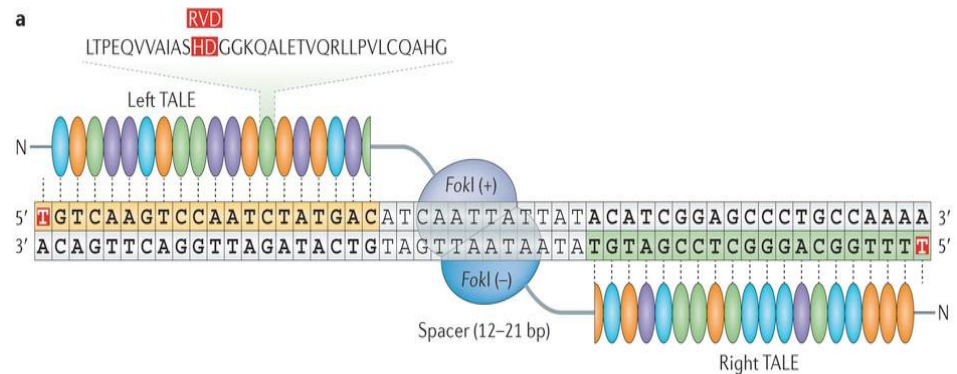
Three different generations of nucleases

1. Zinc-finger nucleases: specific aa motifs recognize specific triplets of base pairs

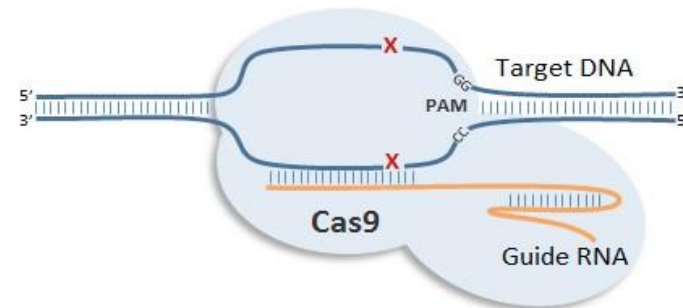


Nature Reviews | Genetics

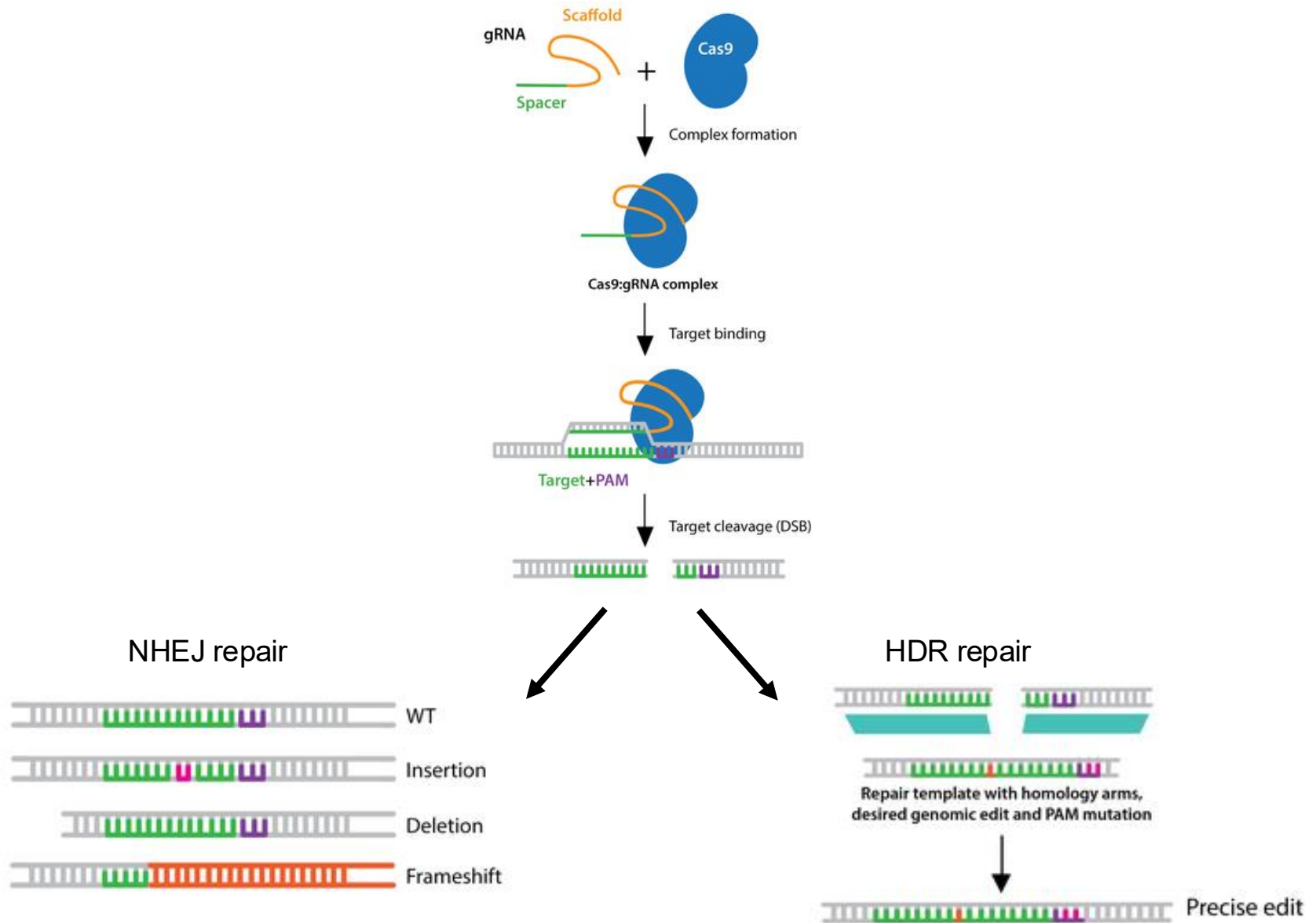
2. Transcription activator-like effector nucleases (TALEN)



3. CRISPR-Cas9 nucleases

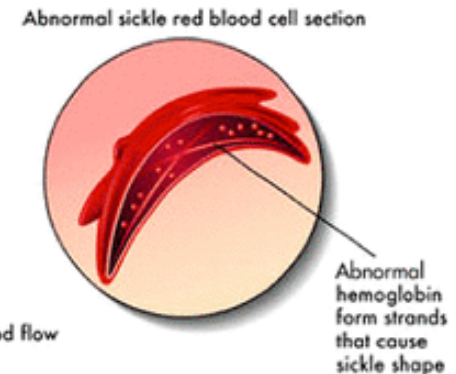
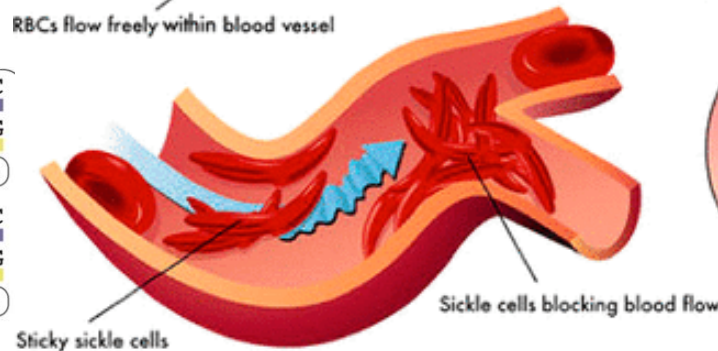
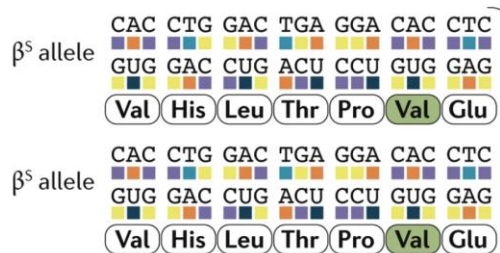
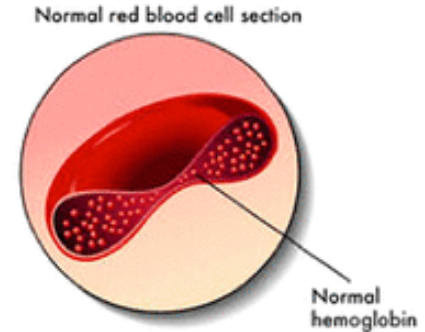
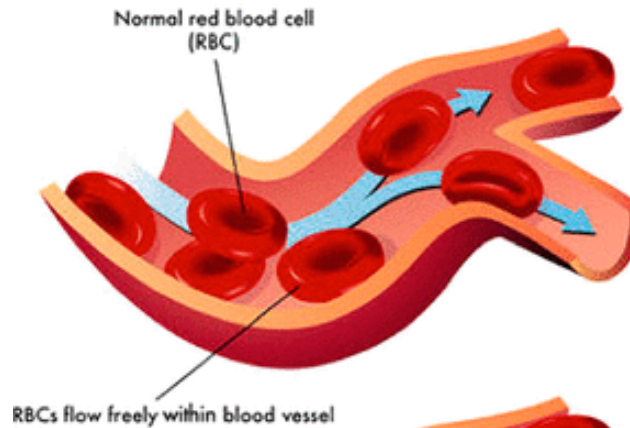
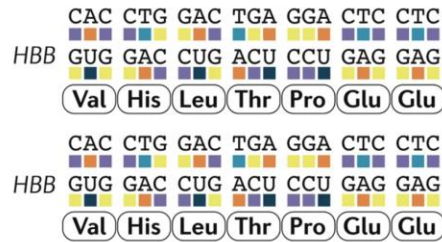


CRISPR-Cas9 nuclease system



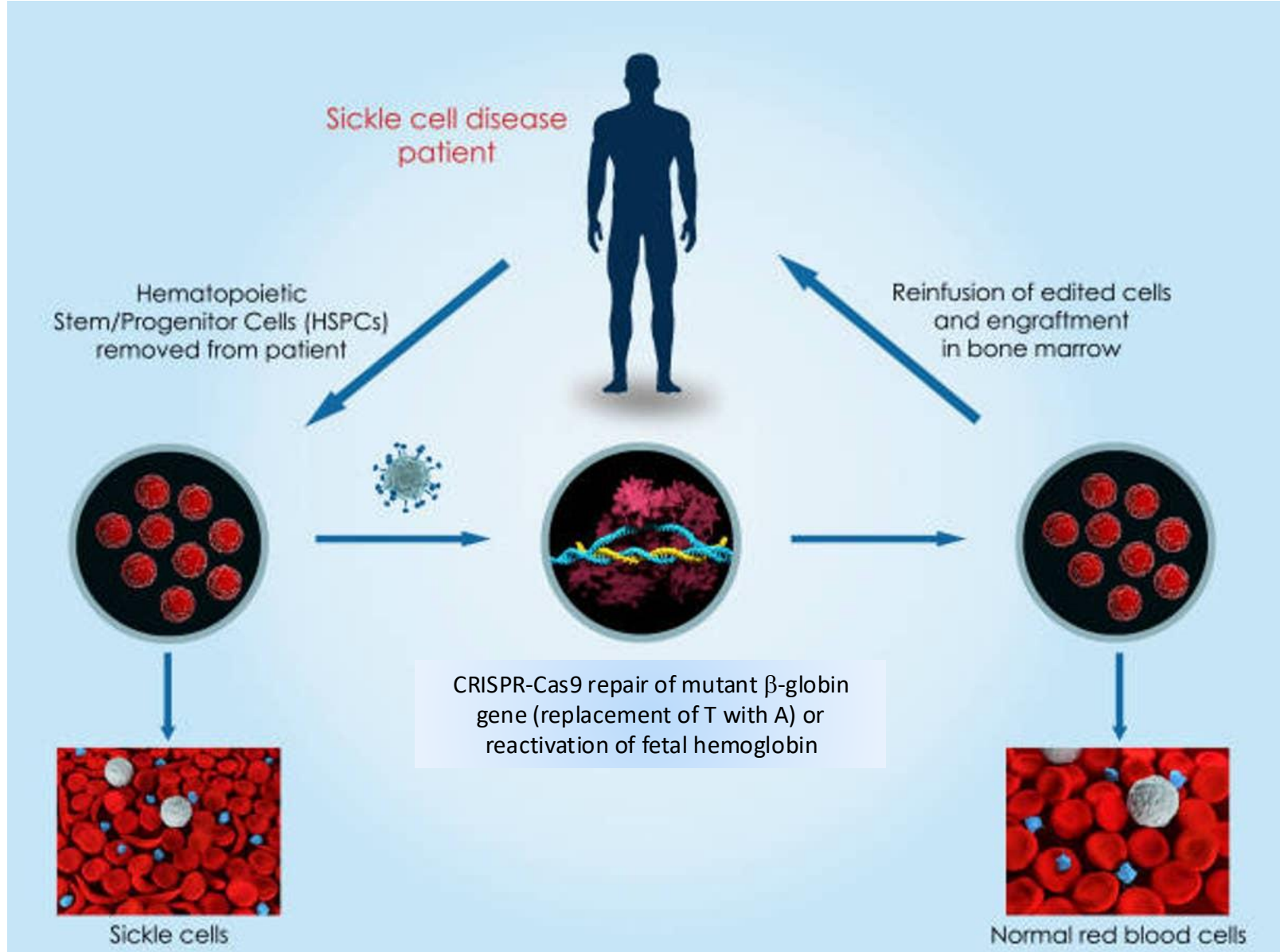
CRISPR-Cas9 based gene therapy for sickle cell anemia

Sickle-Cell Anemia

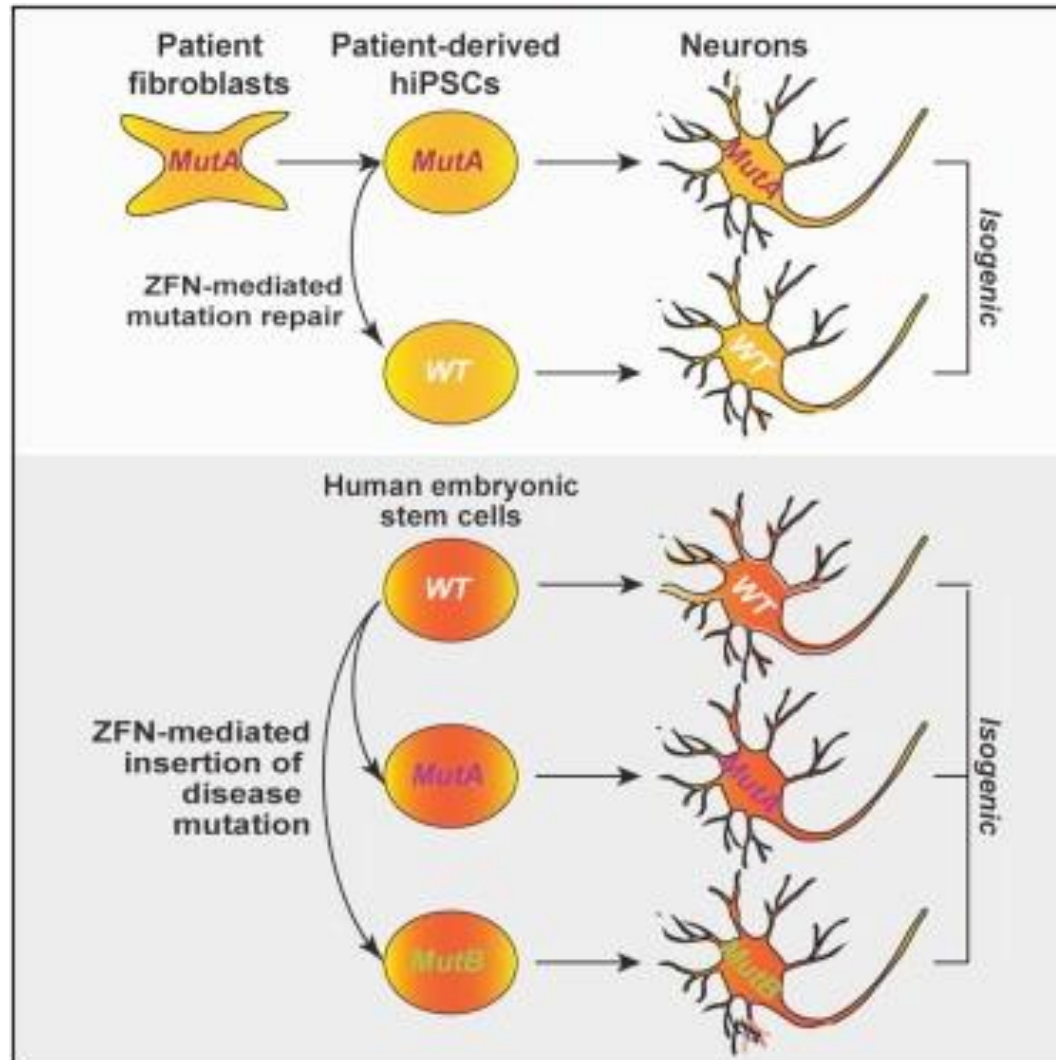


Ortiz et al., 2018

CRISPR-Cas9 based gene therapy for sickle cell anemia

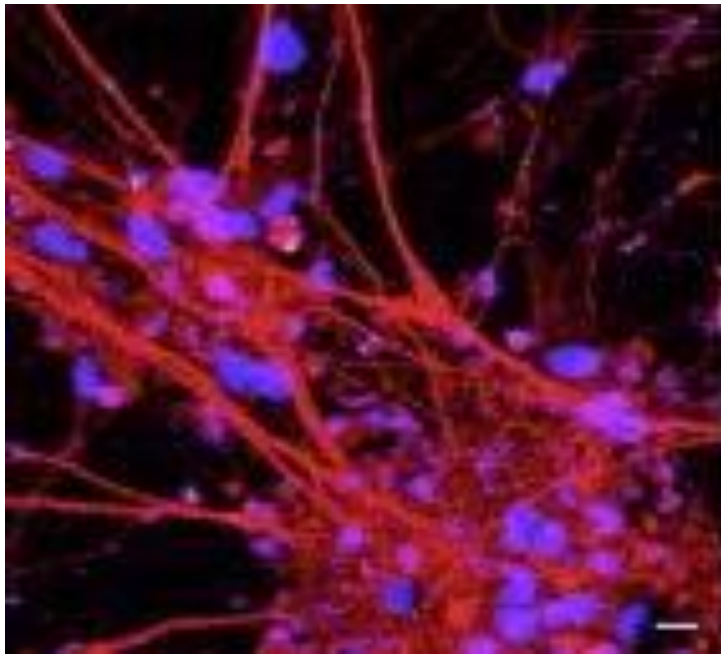


Genome editing model of Parkinson's disease

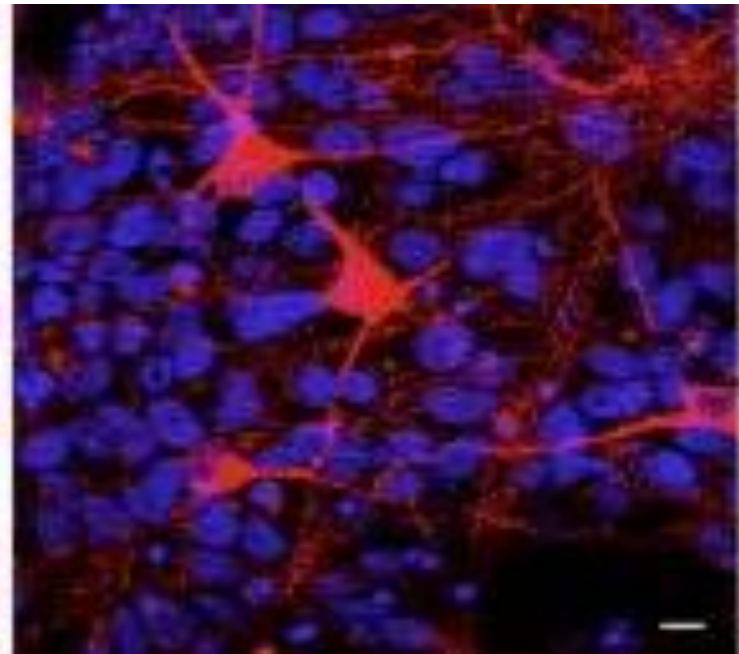


Genome editing model of Parkinson's disease

Corrected point mutation



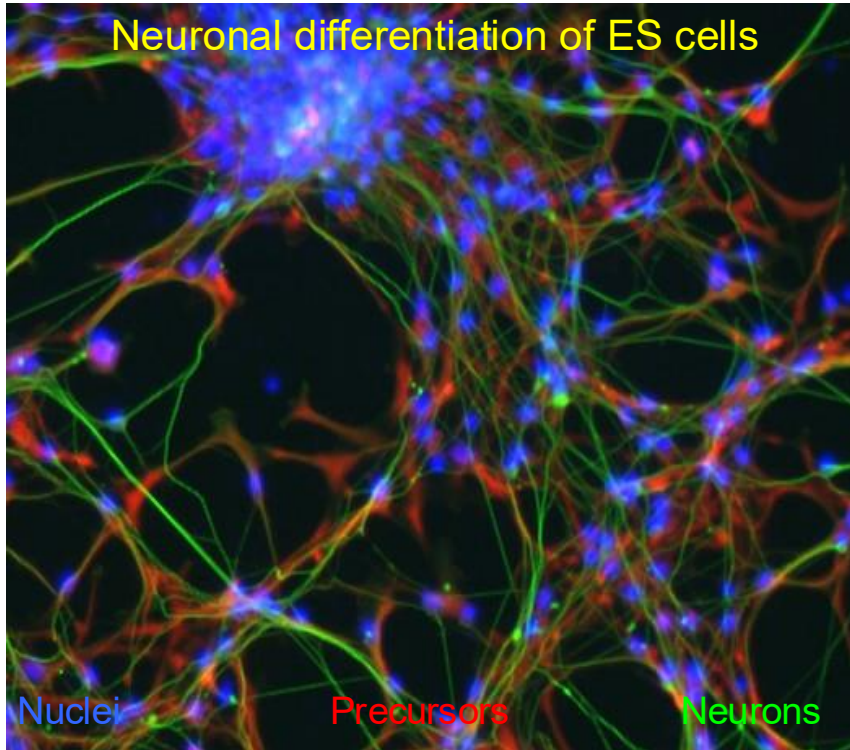
Point mutation causing Parkinson's disease



TH+ neurons
DAPI

Limitations of in vitro differentiation of ES cells to model diseases

ES cell differentiation in a dish is “messy”

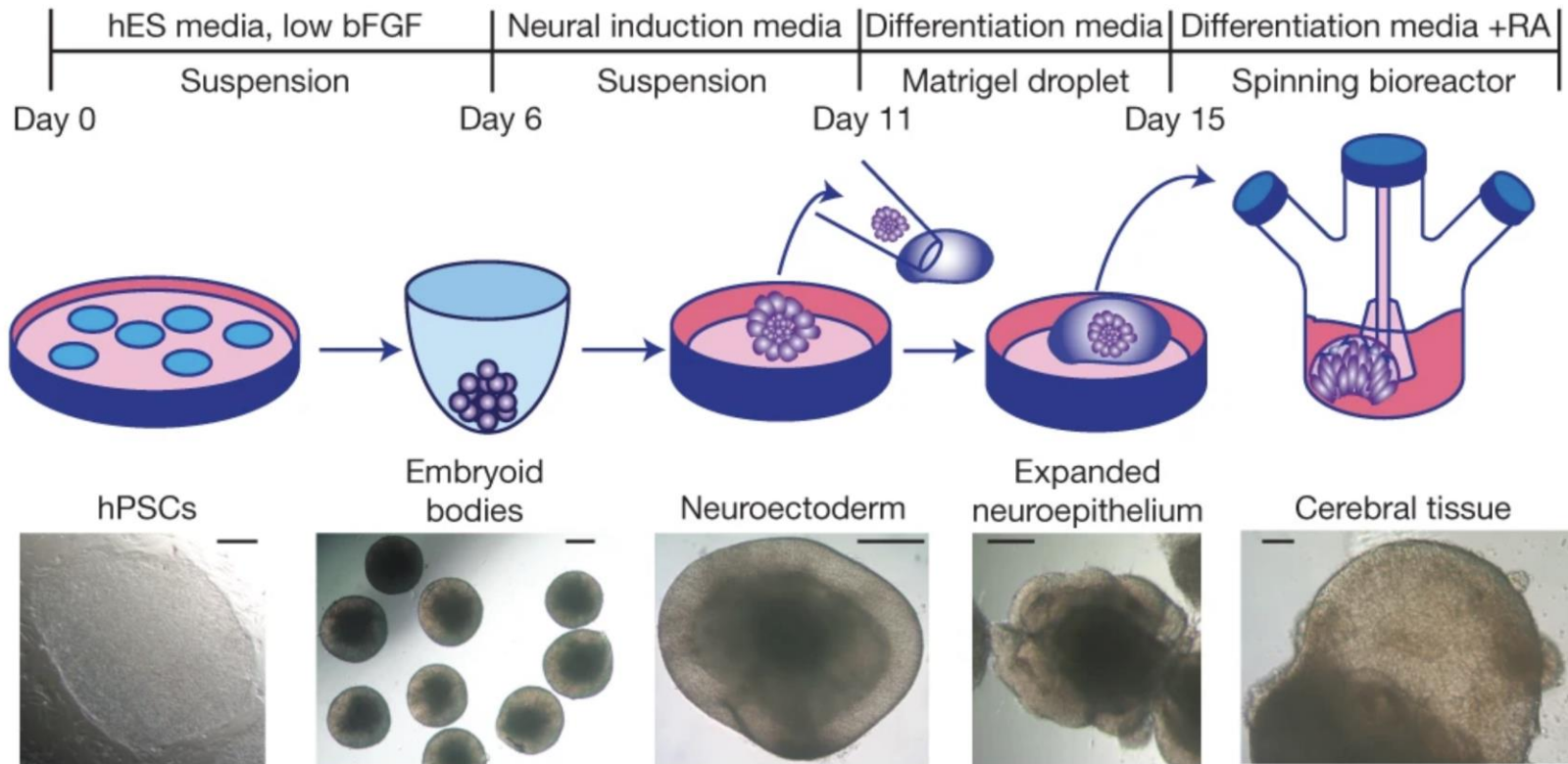


David Suter, unpublished



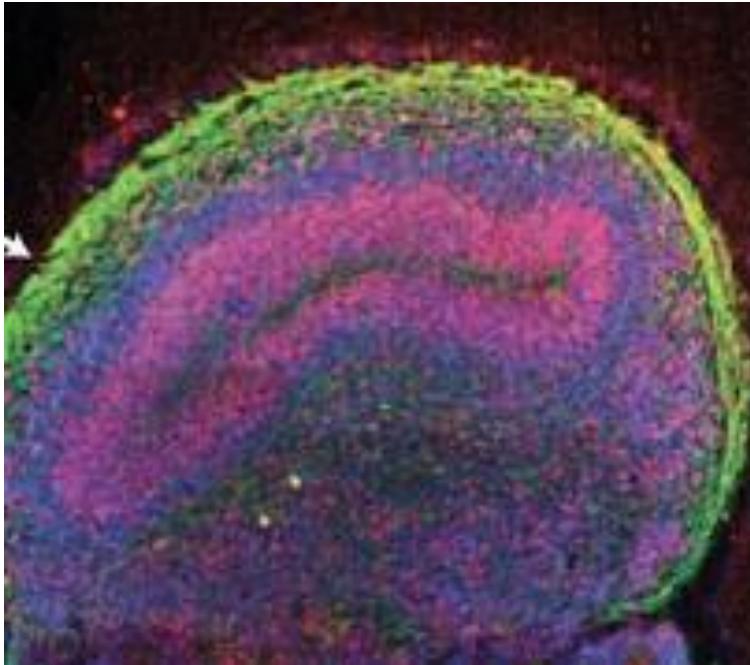
→ Stem cells can be differentiated as 3D structures to form organ-like structures (“organoids”)

“Minibrains” generated from human ES cells

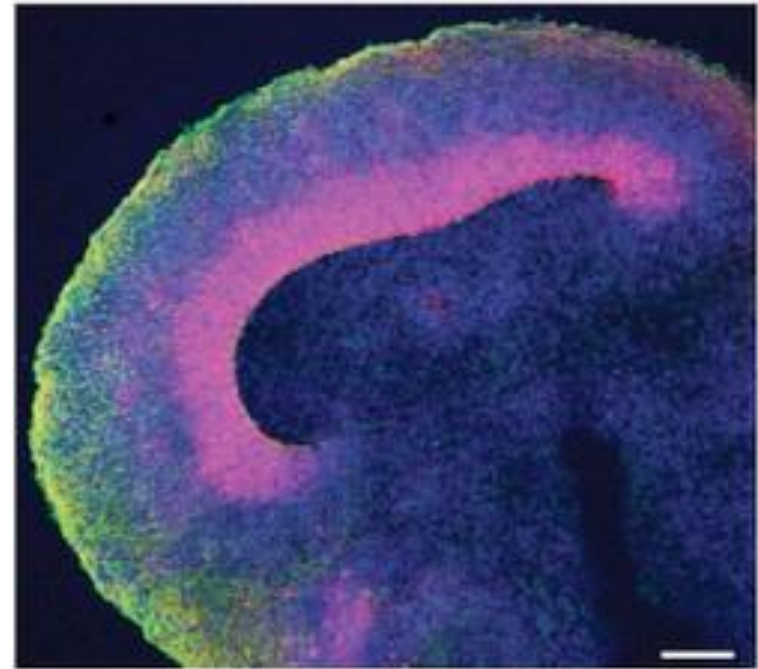


“Minibrains” generated from human ES cells

Human ES cell-derived minibrain



Human fetal brain



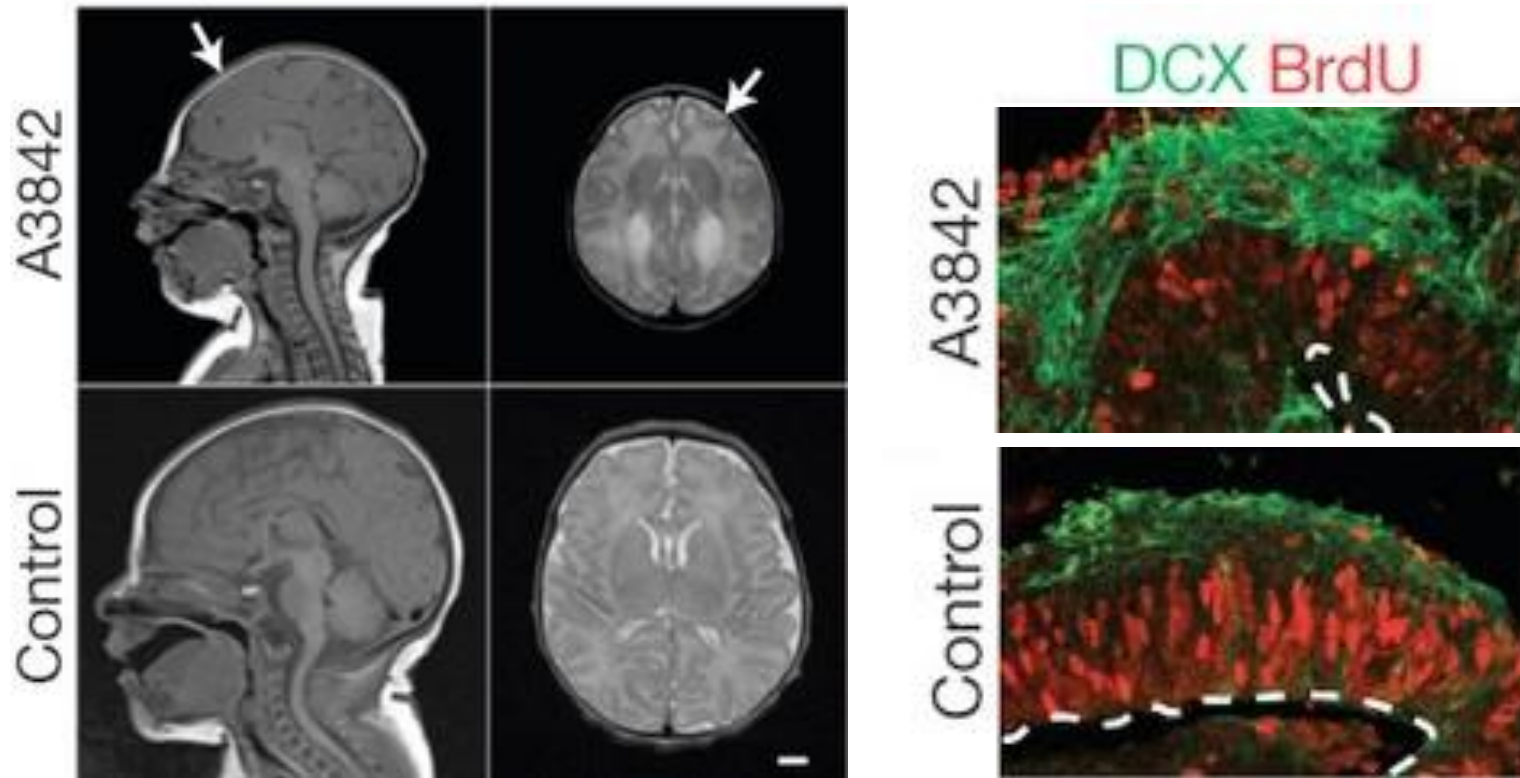
Red: neuronal progenitors

Green: mature neurons

Blue: cell nuclei

“Minibrains” to model human disease

Human iPS cells generated from a patient with a mutation in the CD5RAP2 protein



In vitro intestine from intestinal stem cells

