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

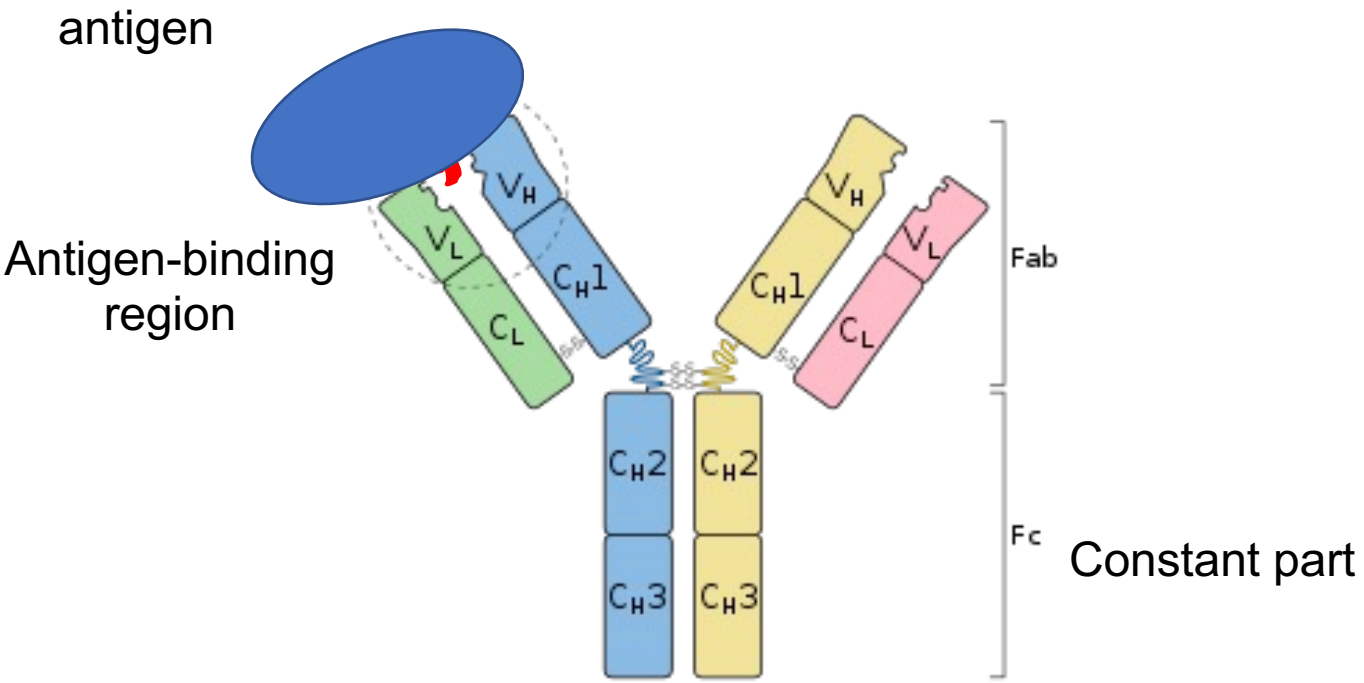
Tools to investigate stem cell differentiation

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

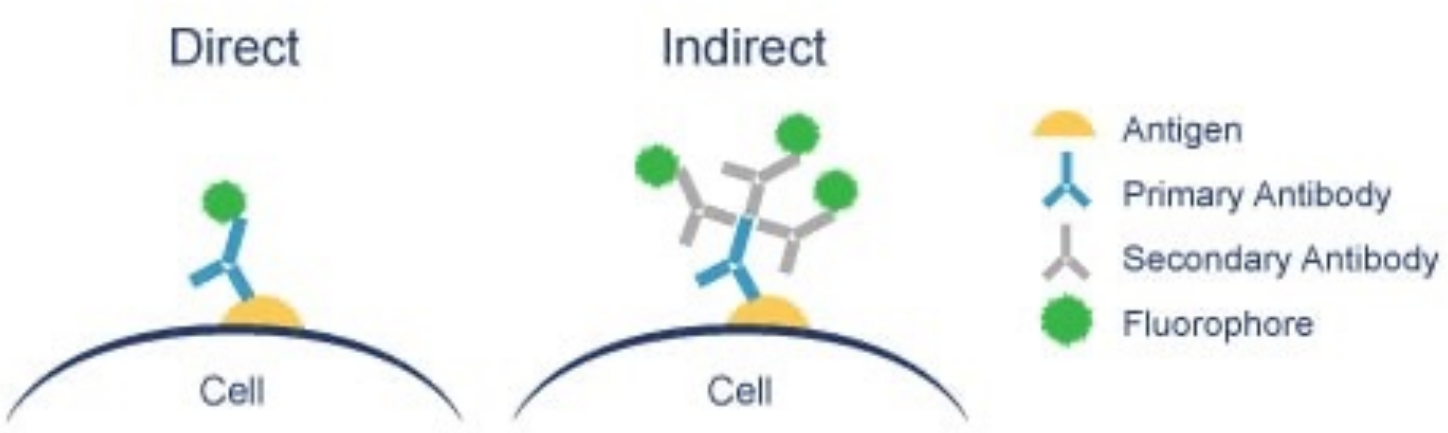
Immunofluorescence - principle

- 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**.

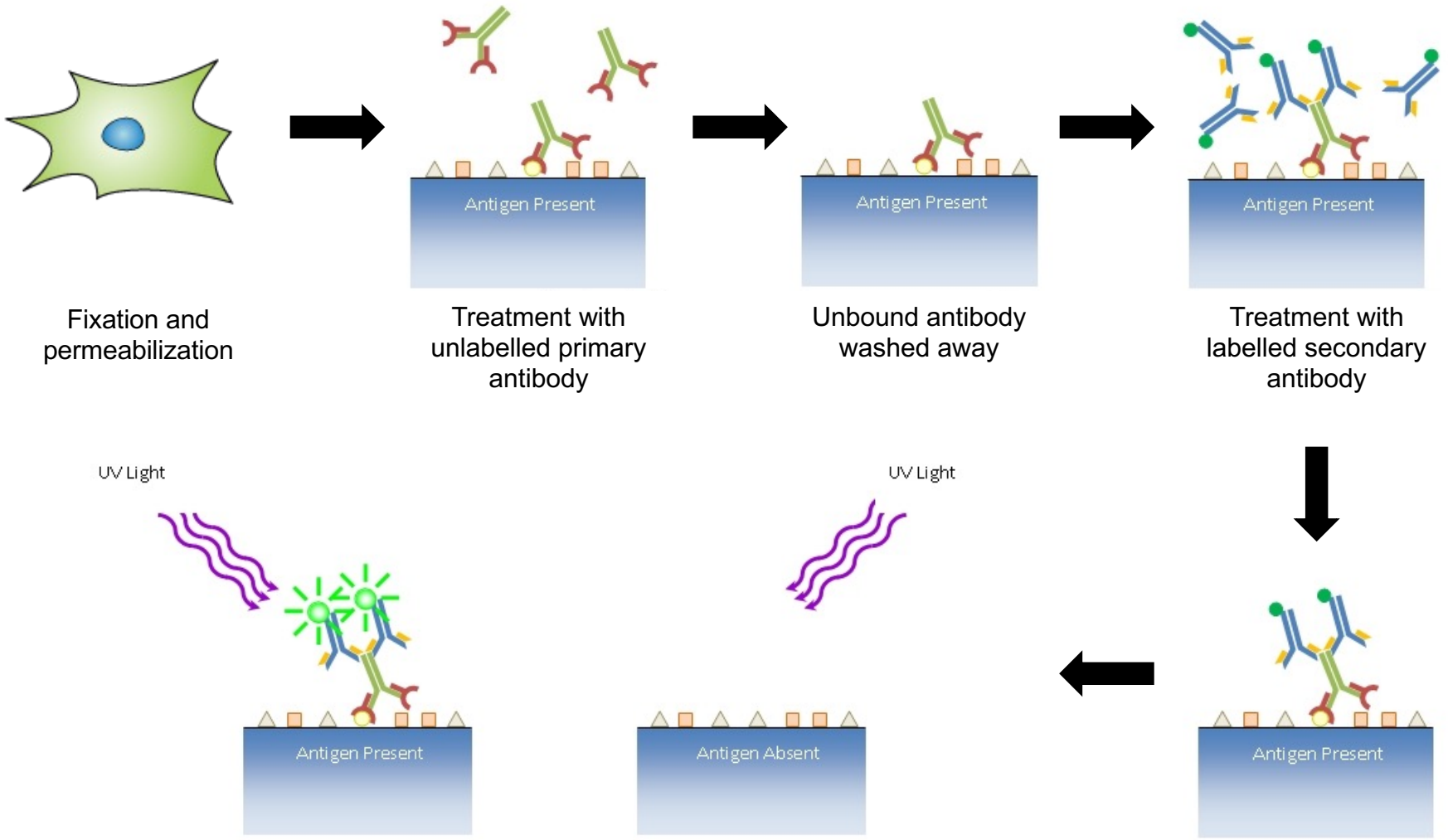


How does immunofluorescence work?

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



How does immunofluorescence work?



Fixation and permeabilization

Treatment with unlabelled primary antibody

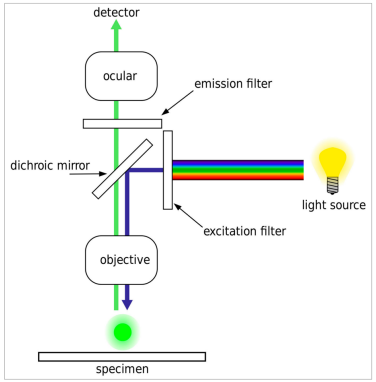
Unbound antibody washed away

Treatment with labelled secondary antibody

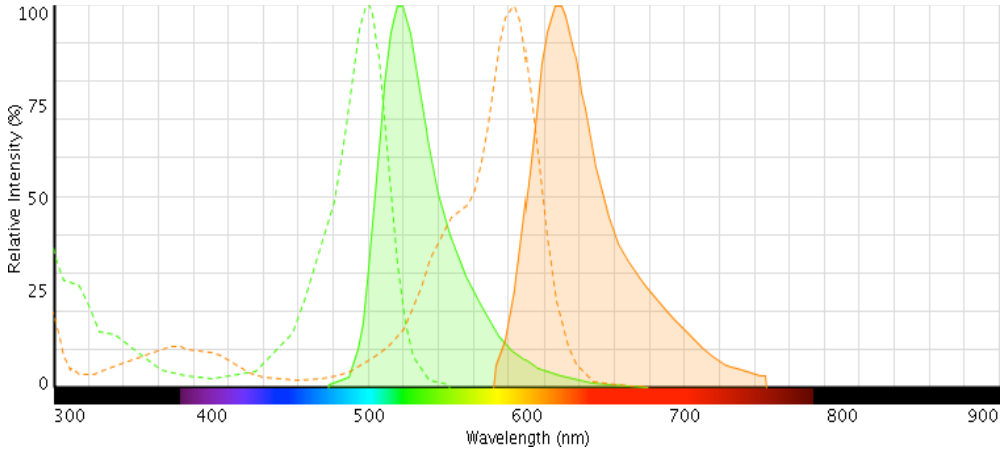
Unbound secondary antibody washed away

Fluorescence observed wherever antigen is located

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



Spectra of the fluorophores Alexa 488 and Alexa 594

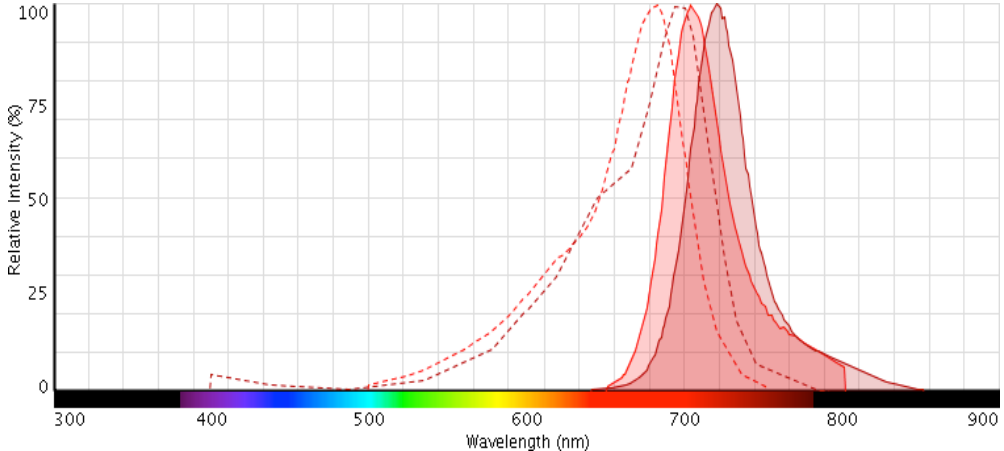


→ Yes, if the spectra are not overlapping too much

Excitation spectrum

Emission spectrum

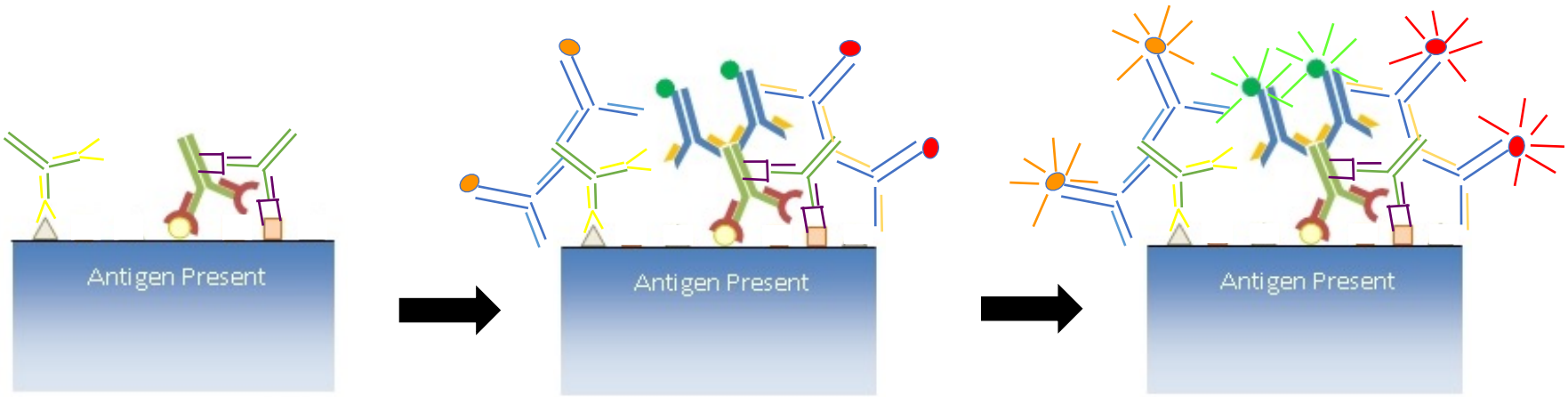
Spectra of the fluorophores Alexa 680 and Alexa 700



→ No if the spectra are overlapping too much

Immunofluorescence with several antibodies

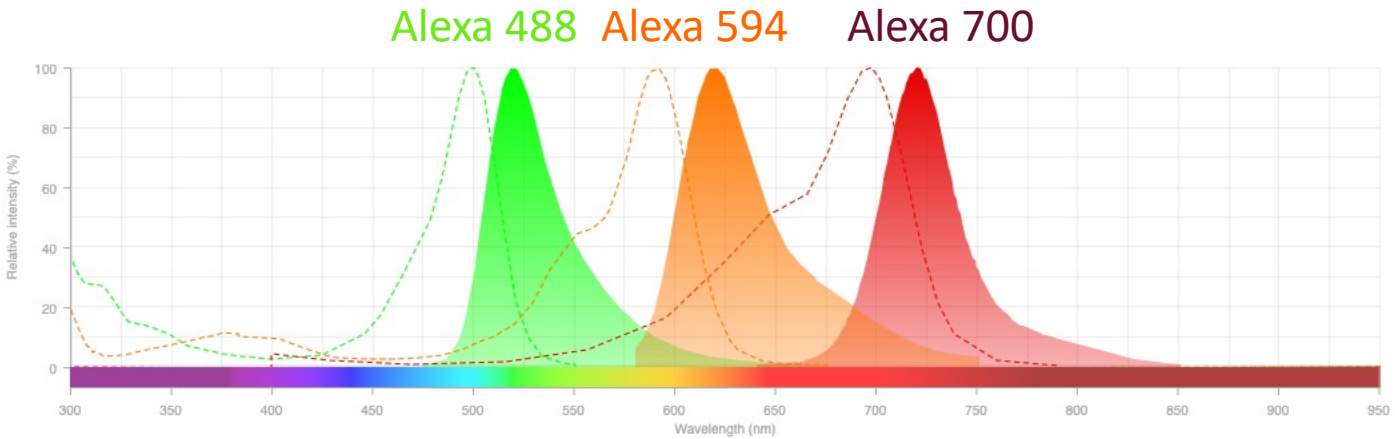
- It is possible to use multiple antibodies until all filters of the microscope are used (often 4-5 channels)



Unlabelled primary antibodies bind to different targets

Treatment with labelled secondary antibodies

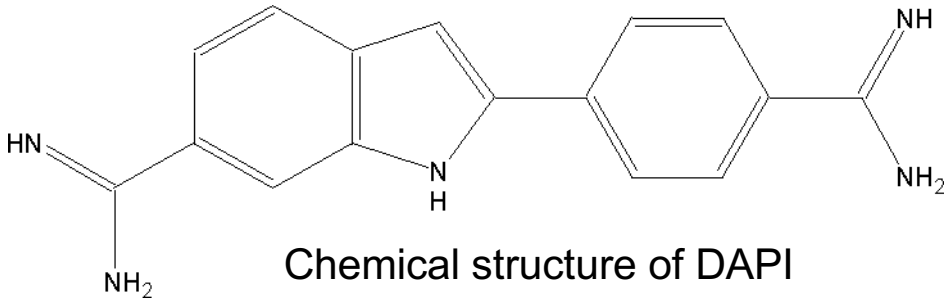
Each antigen is detected at a different wavelength



How to stain the cell nucleus?

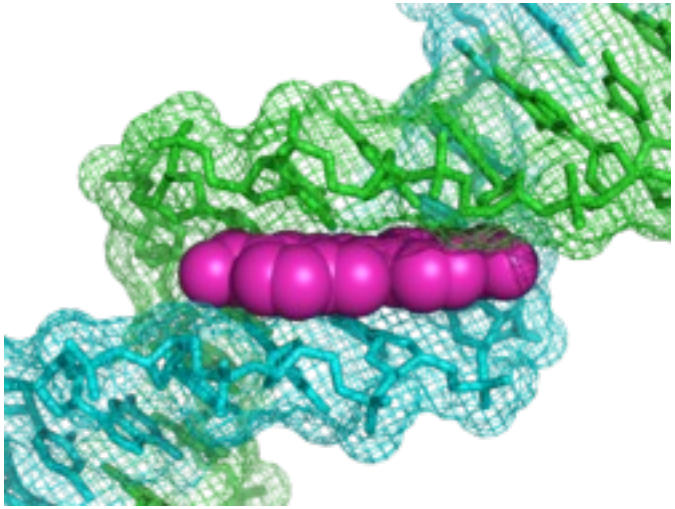
→ By using a chemical, for example 4',6-diamidino-2-phenylindole (**DAPI**) that binds to DNA

→ It emits in the **blue** spectrum, and can be used in combination with green-emitting and red-emitting dyes



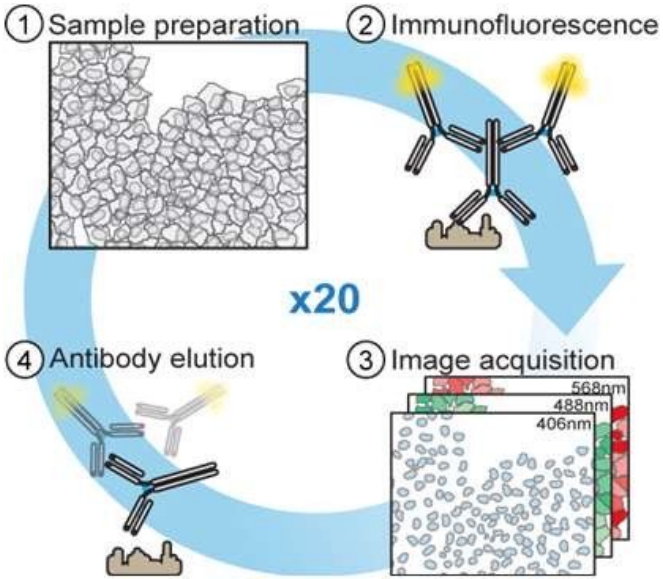
Chemical structure of DAPI

Model showing DAPI (pink) bound to DNA

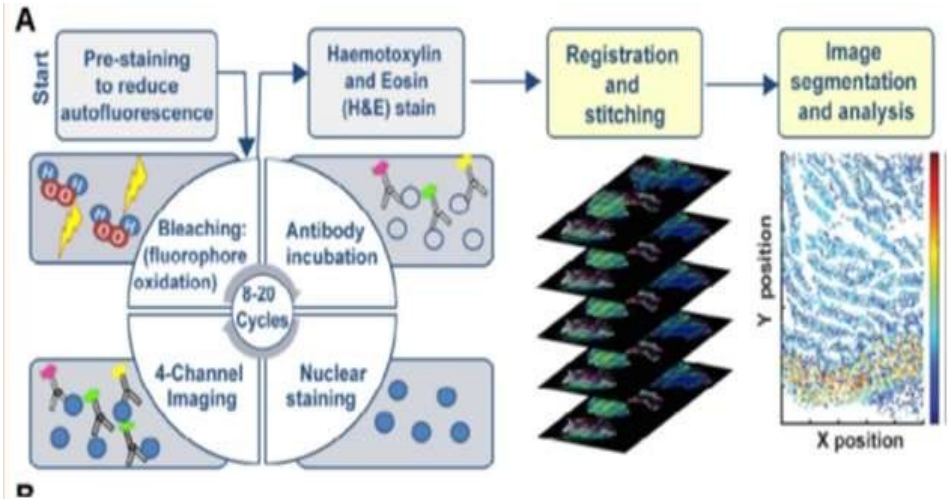


Multiplexing through cyclic immunofluorescence

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

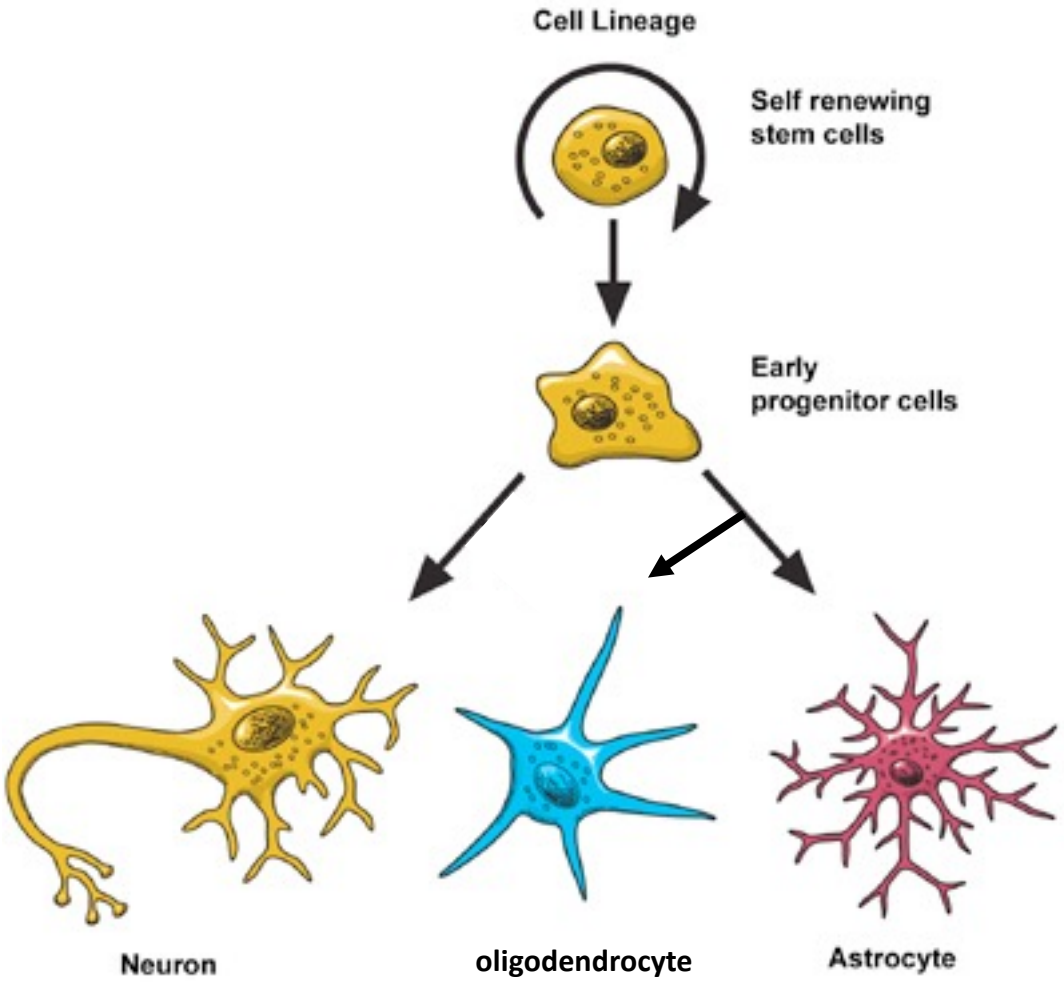


Gut et al., Science 2018



Jia-Ren Lin et al., eLife 2018

Application: in vitro differentiation towards neurons



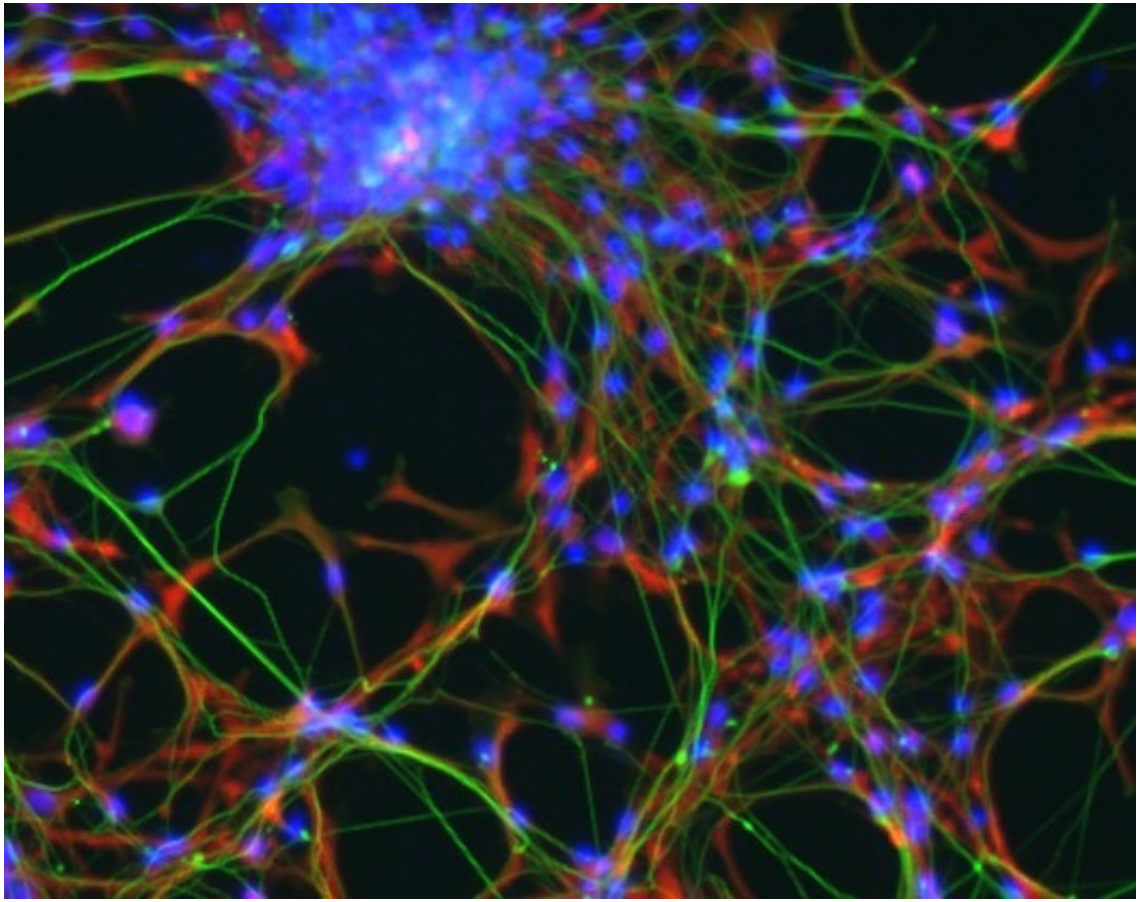
Application: in vitro differentiation of ESCs towards neurons

In vitro differentiation of embryonic stem cells towards neurons
Immunofluorescence against Nestin (expressed in neuronal progenitors) and β3-tubulin (expressed in neurons) and DAPI staining

Nucleus: DAPI stain

Neuronal progenitors: Nestin

Neurons: β3-tubulin



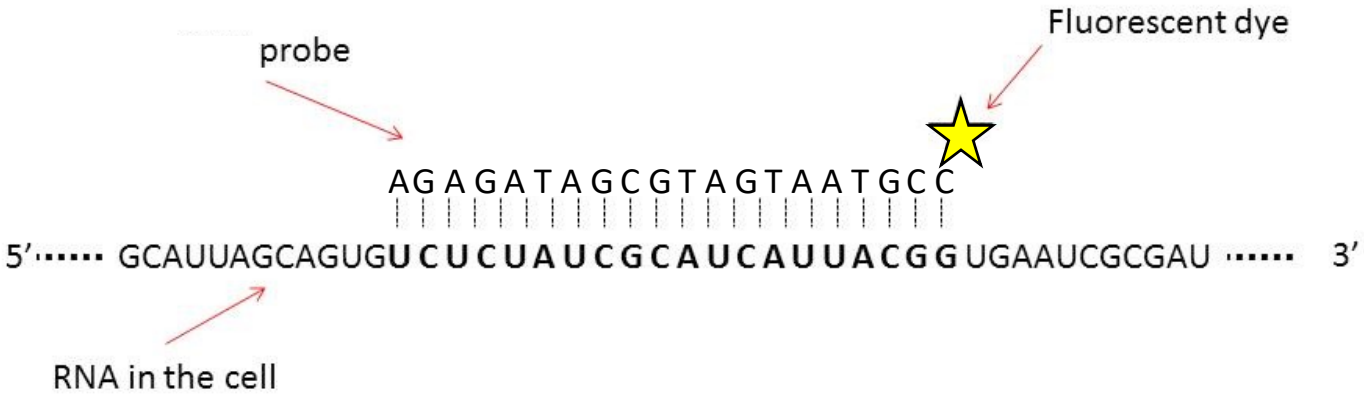
David Suter, unpublished

2. RNA FISH

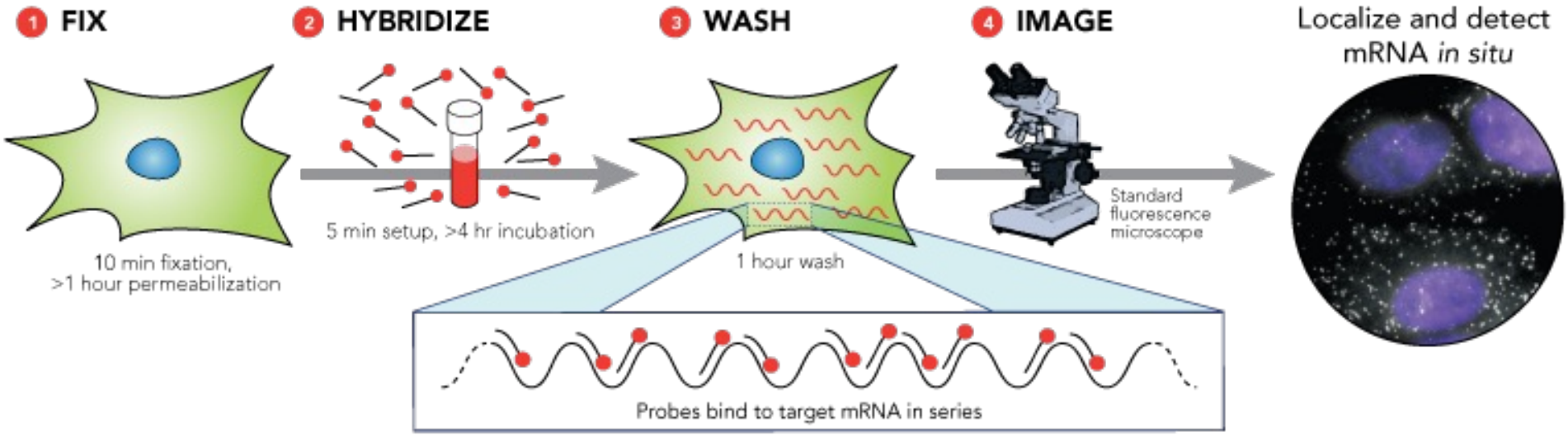
RNA fluorescence in situ hybridization (RNA FISH)

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



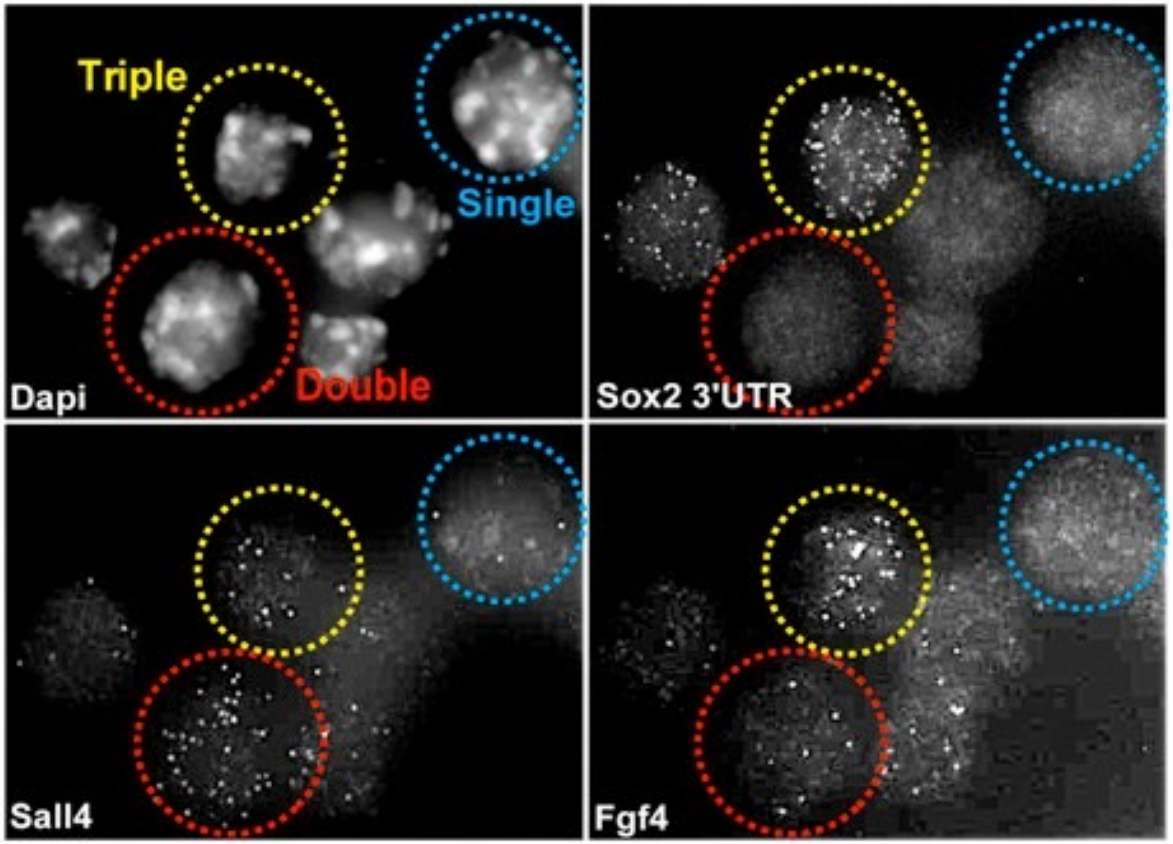
RNA FISH – experimental setup



Stellaris® RNA FISH

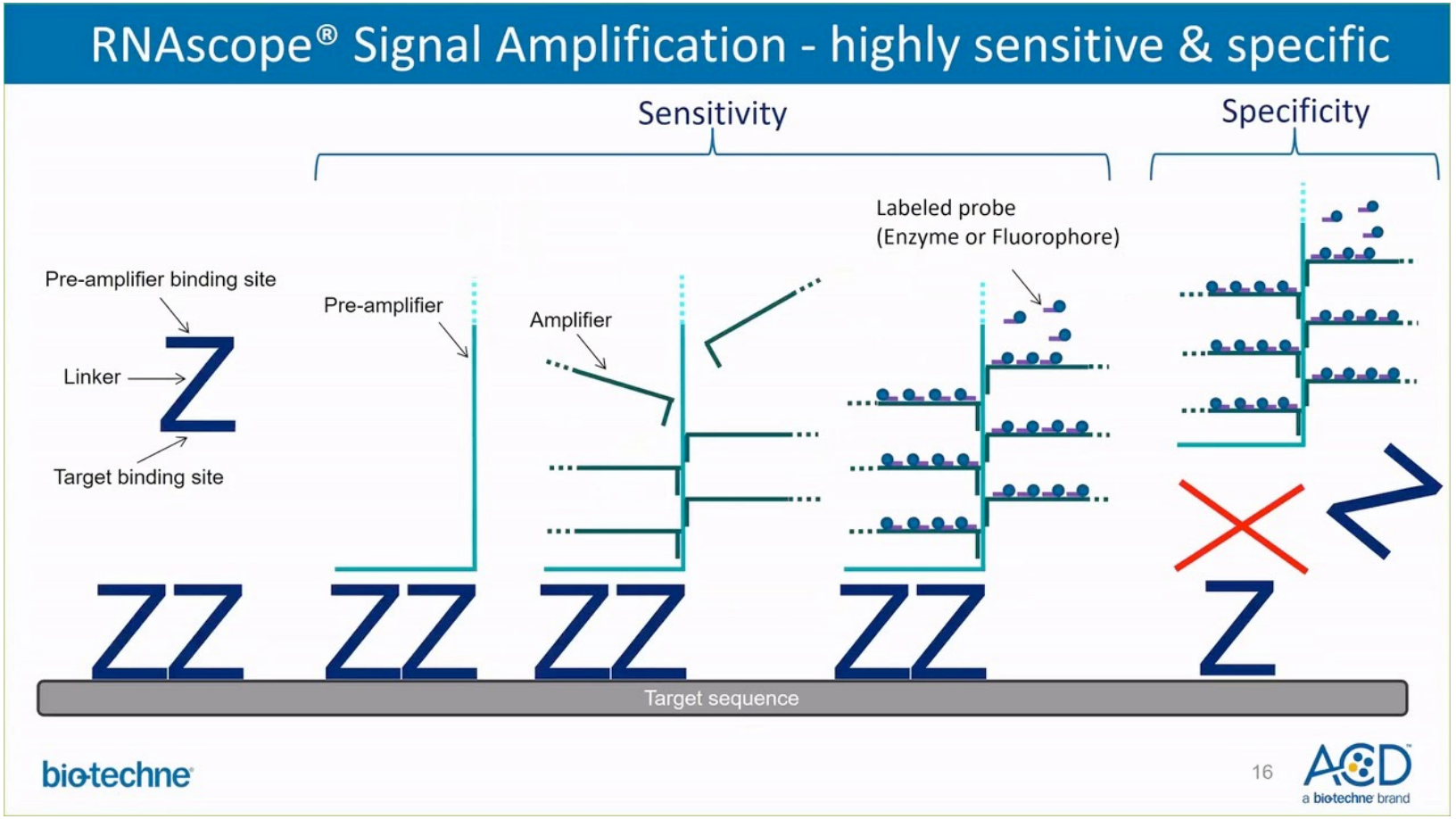
RNA FISH allows to localize and count mRNA molecules

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



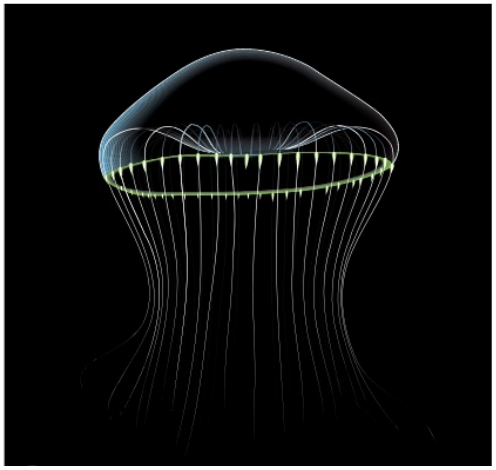
Buganim et al., Cell 2012

Different signal amplification strategies exist



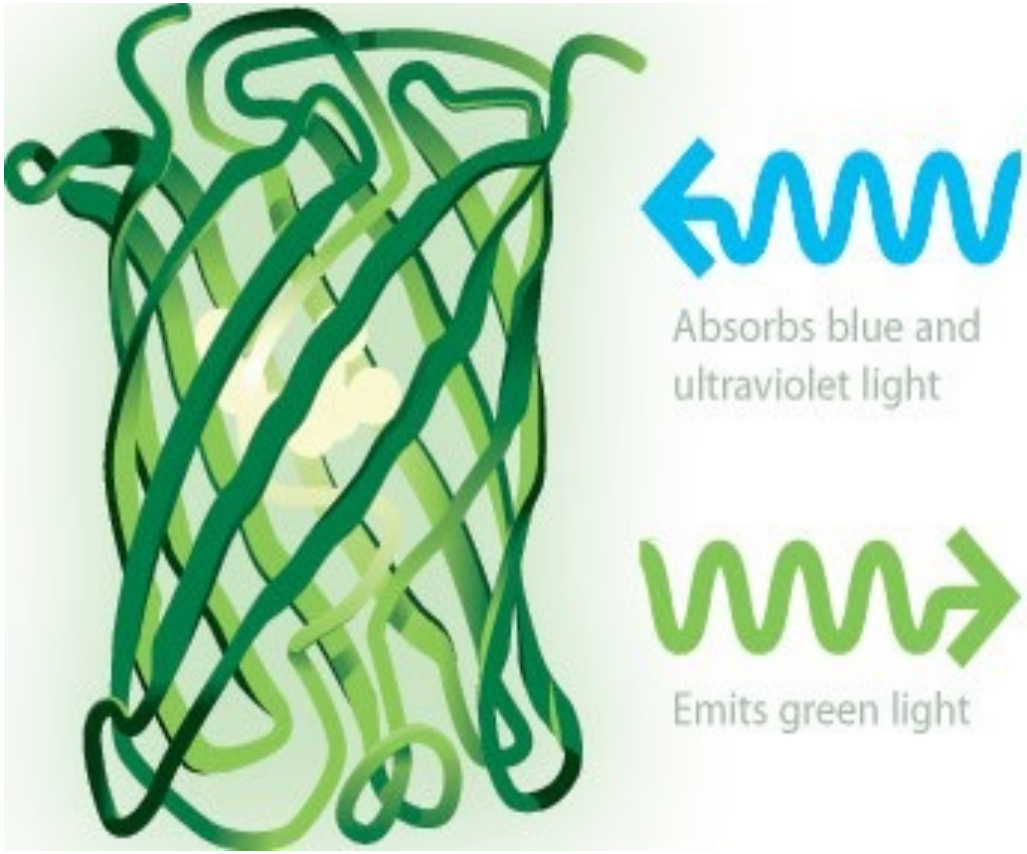
3. Promoter-reporter constructs

How to mark living cells: fluorescent proteins



Aequorea victoria

GFP (Green Fluorescent Protein)

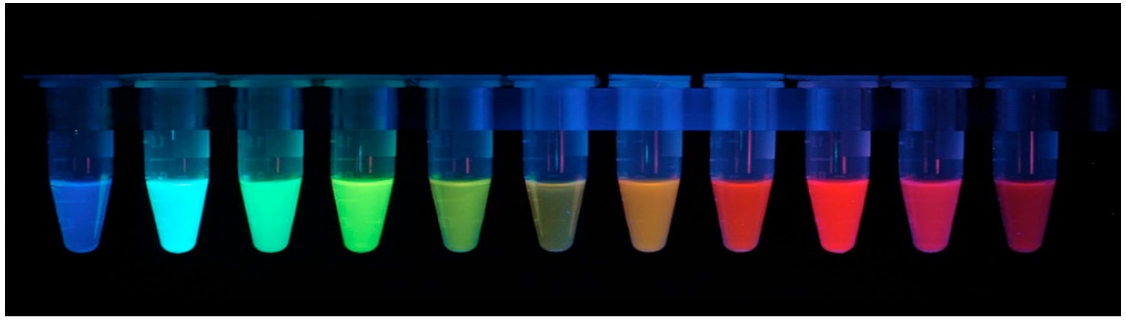


Fluorescent proteins have a low degree of toxicity

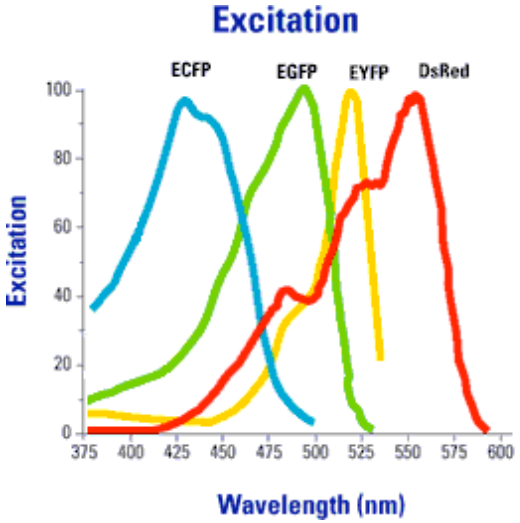
Mice can be engineered to express GFP

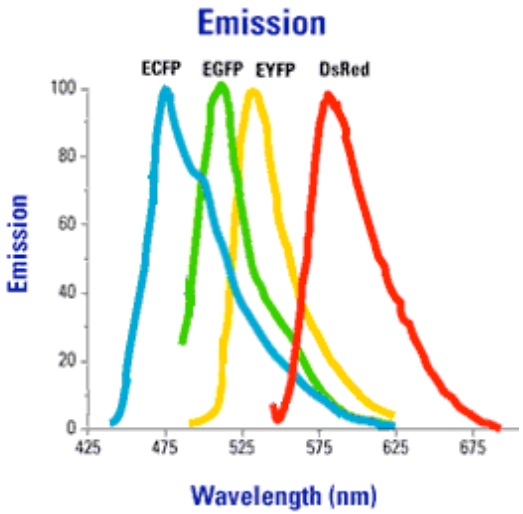
A colorful palette

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

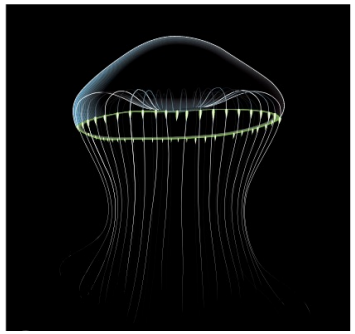


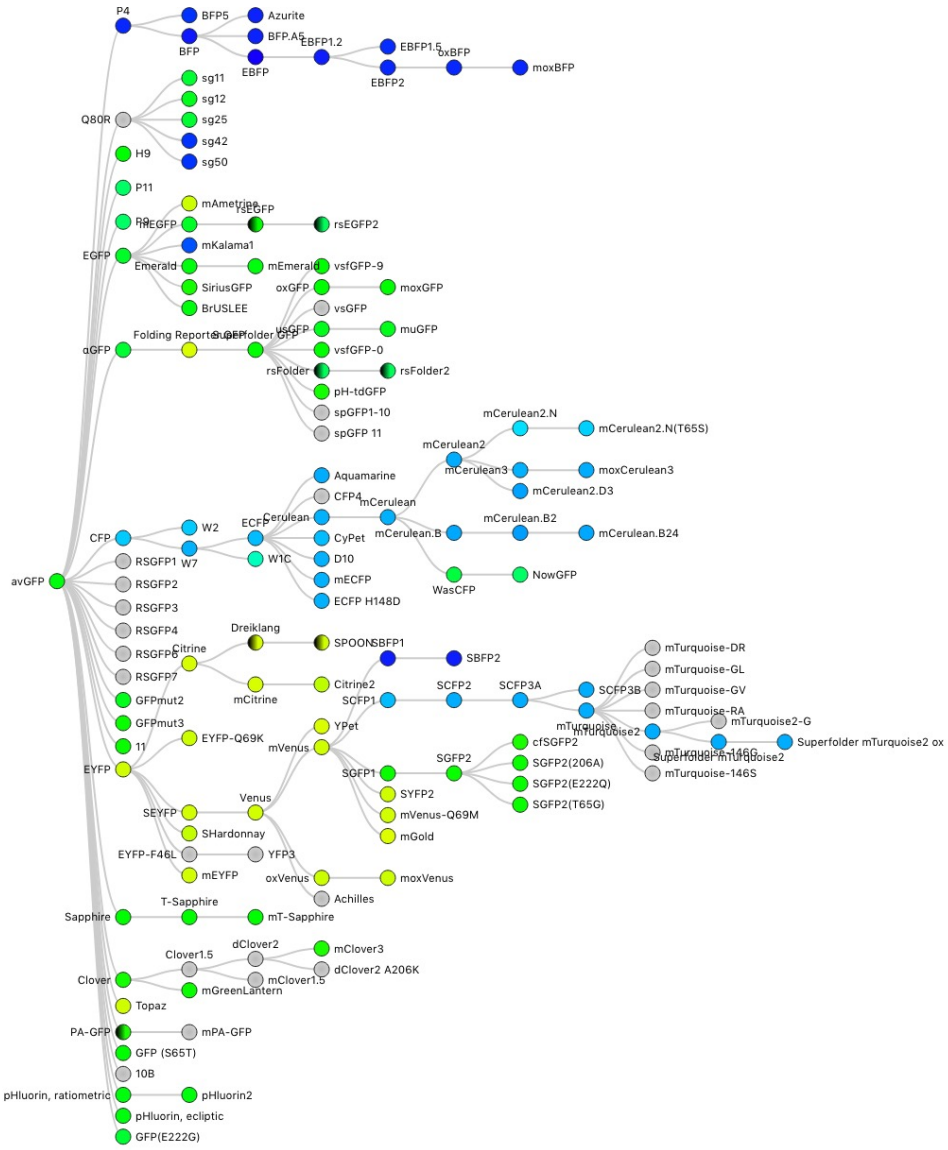
- Different fluorescent proteins have different excitation and emission spectra





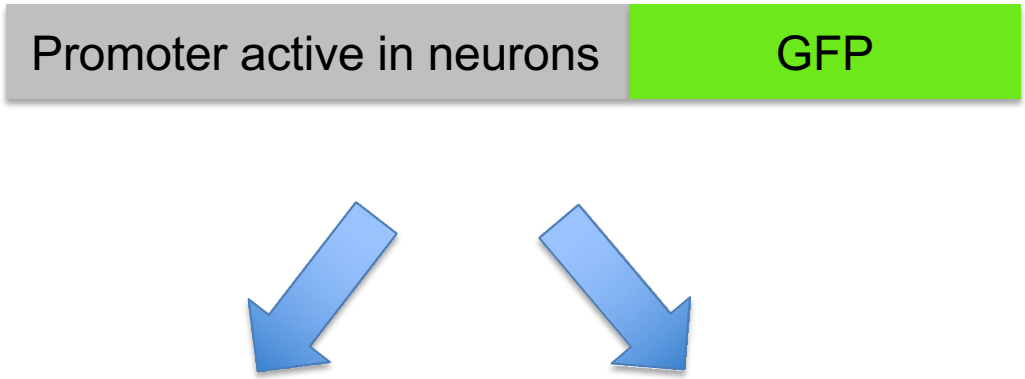
The “evolution tree” of GFP



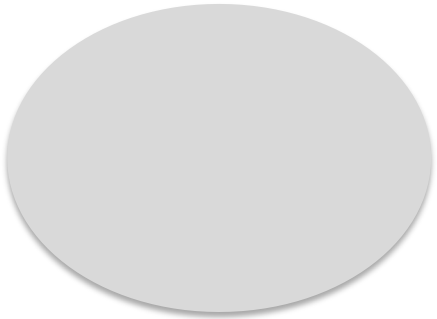


→ The fluorescent protein database (https://www.fpbase.org/): for info about spectra, properties, microscope set-ups…

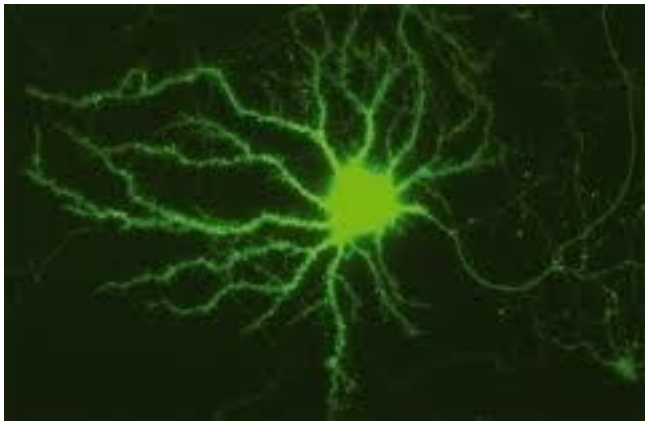
Fluorescent proteins can be used as cell-specific reporters



Non-neuronal cell: no GFP expressed

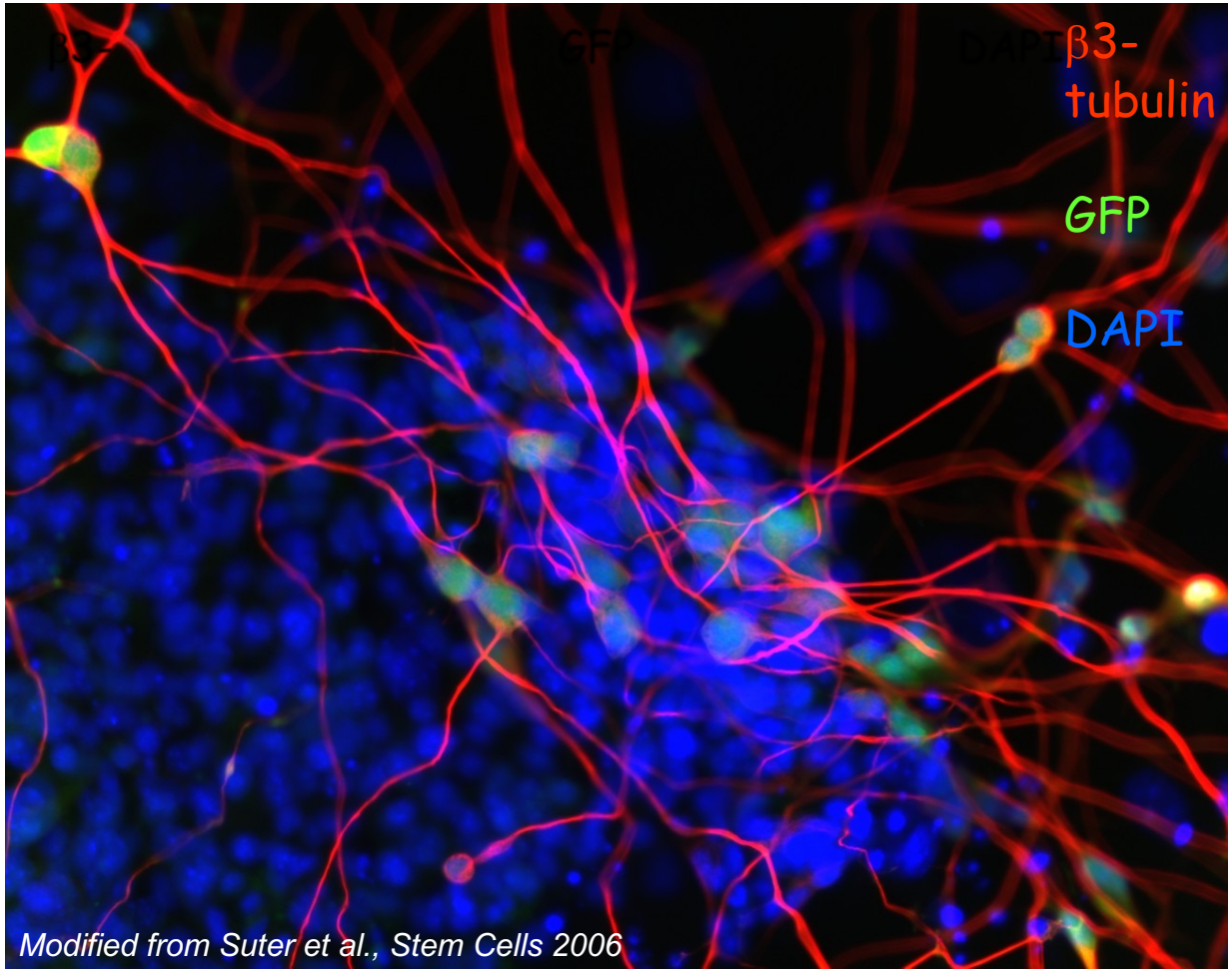


Neuron: GFP expressed



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 β3-tubulin (expressed in neurons) and DAPI staining

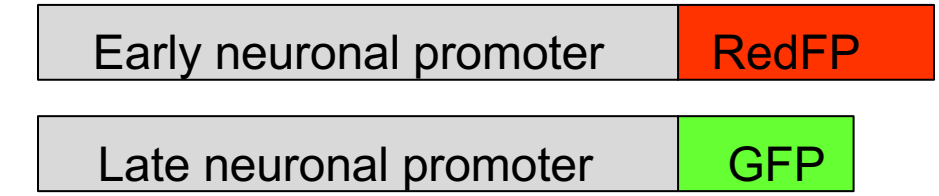


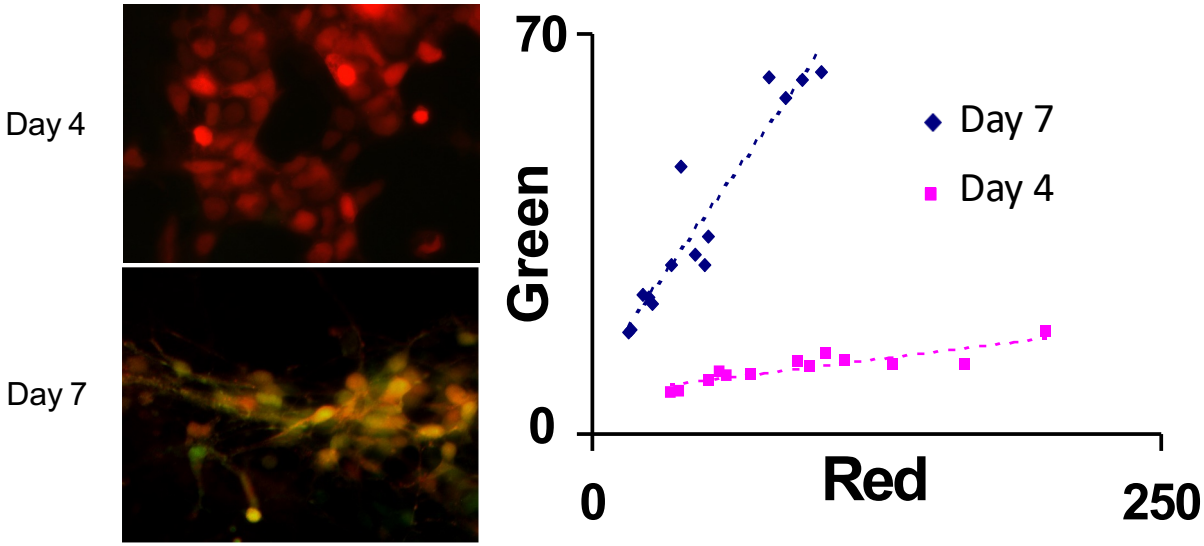
Modified from Suter et al., Stem Cells 2006

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

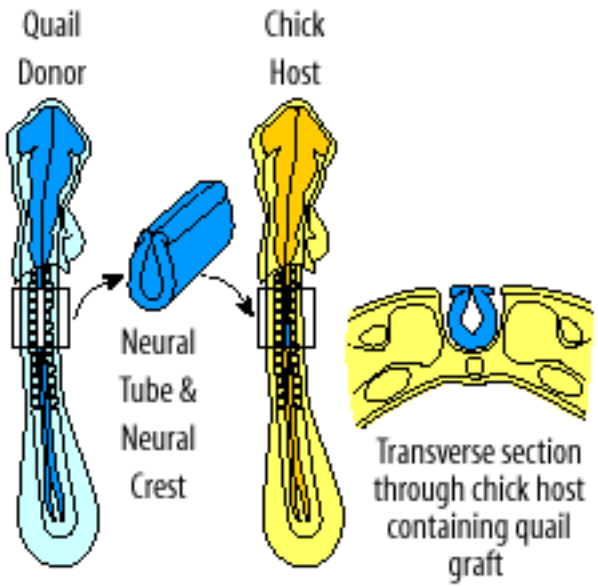


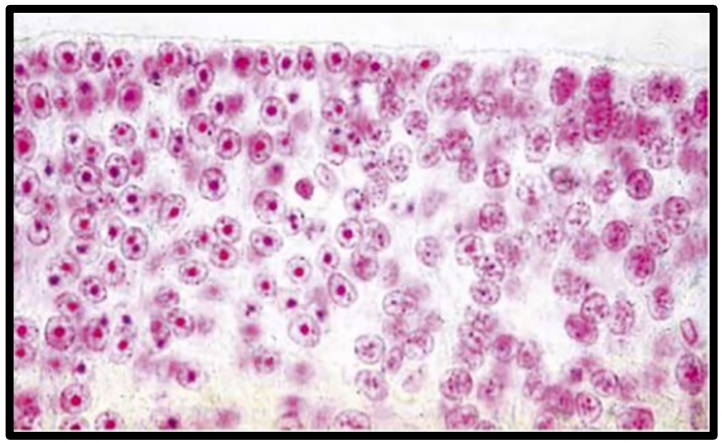


Modified from Suter et al., Stem Cells 2006

Application: tracking cell fate after transplantation

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





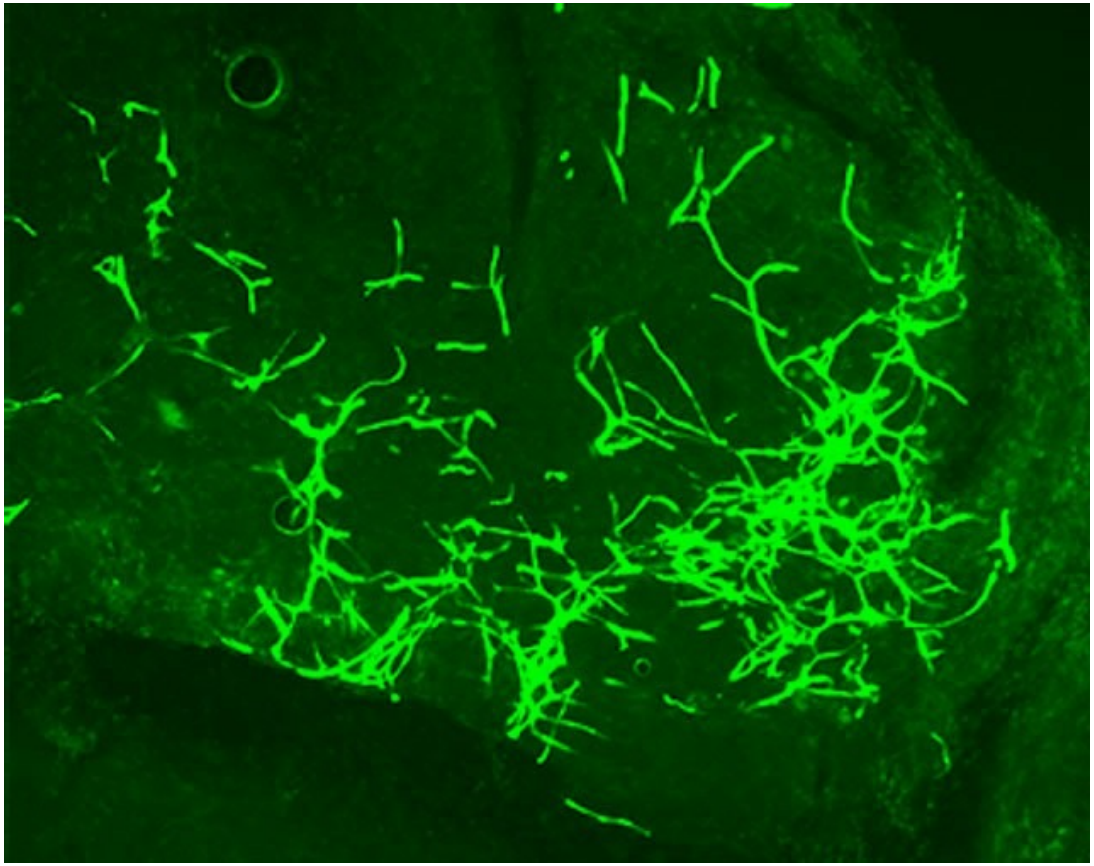
Key concept: quail cells have a prominent nucleolus that serves as a permanent marker

→ **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

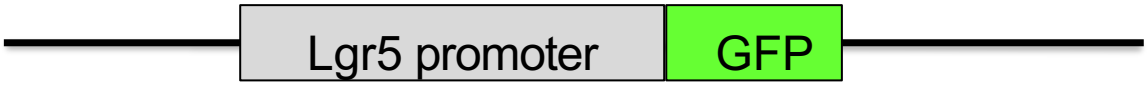
→ **This allows to track the fate of the transplanted cells**

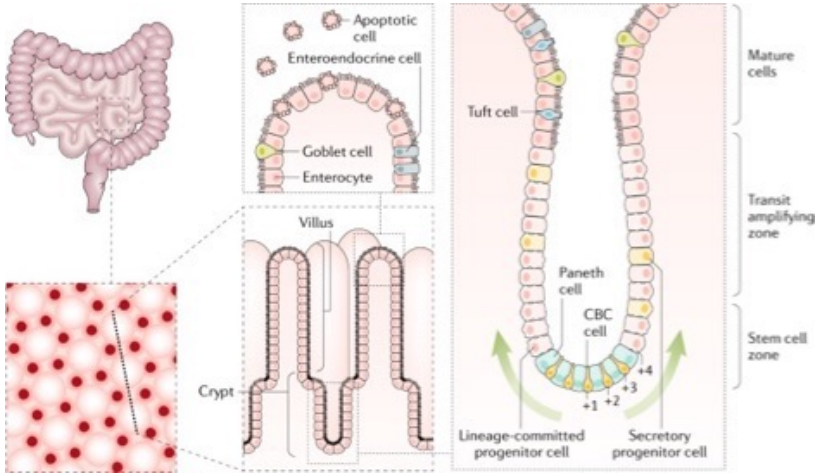


Example: transplantation of vascular endothelial stem cells
→ The resulting vessels can be directly seen by GFP fluorescence in the recipient mouse

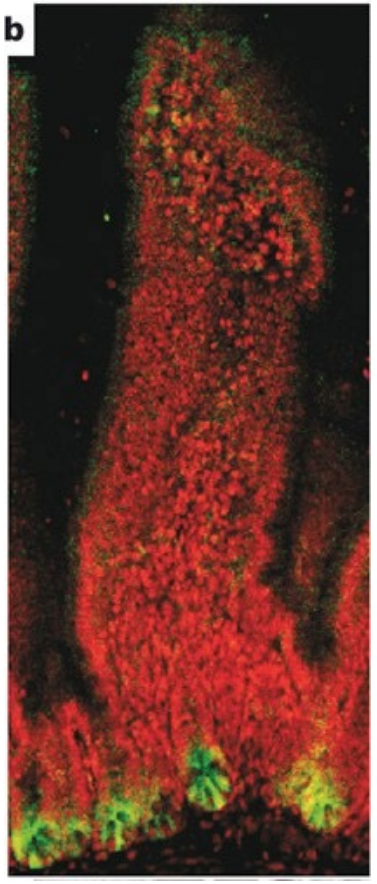
Application: In vivo labeling of stem cells

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

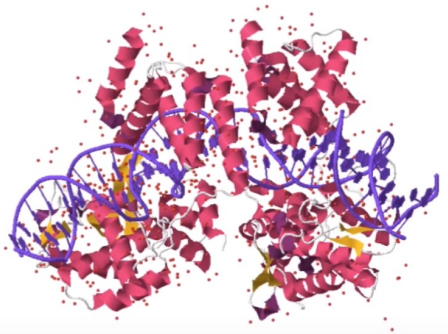


Barker et al., Nature 2007

4. Reporter-based lineage tracing

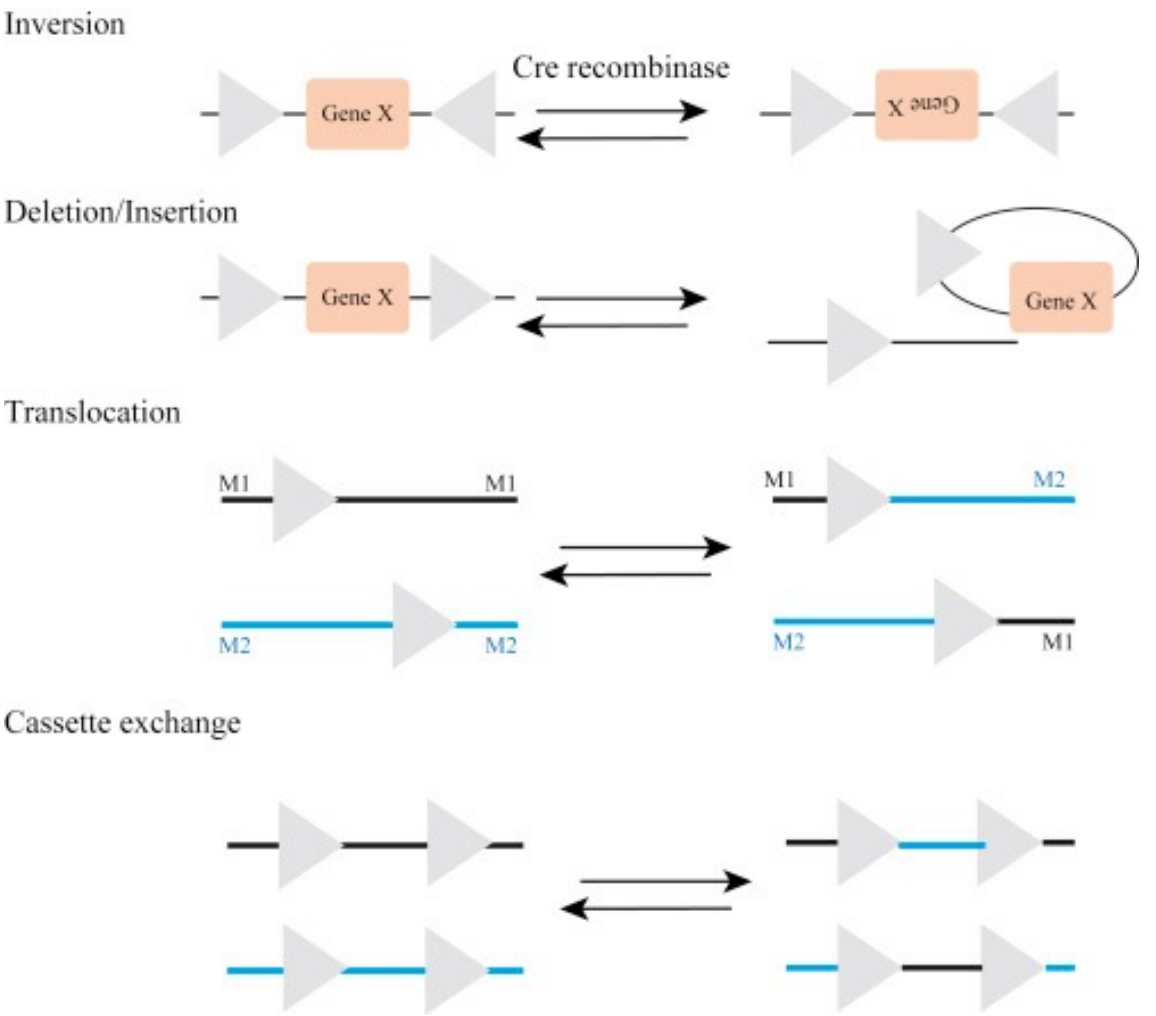
The Cre-Lox recombination system

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



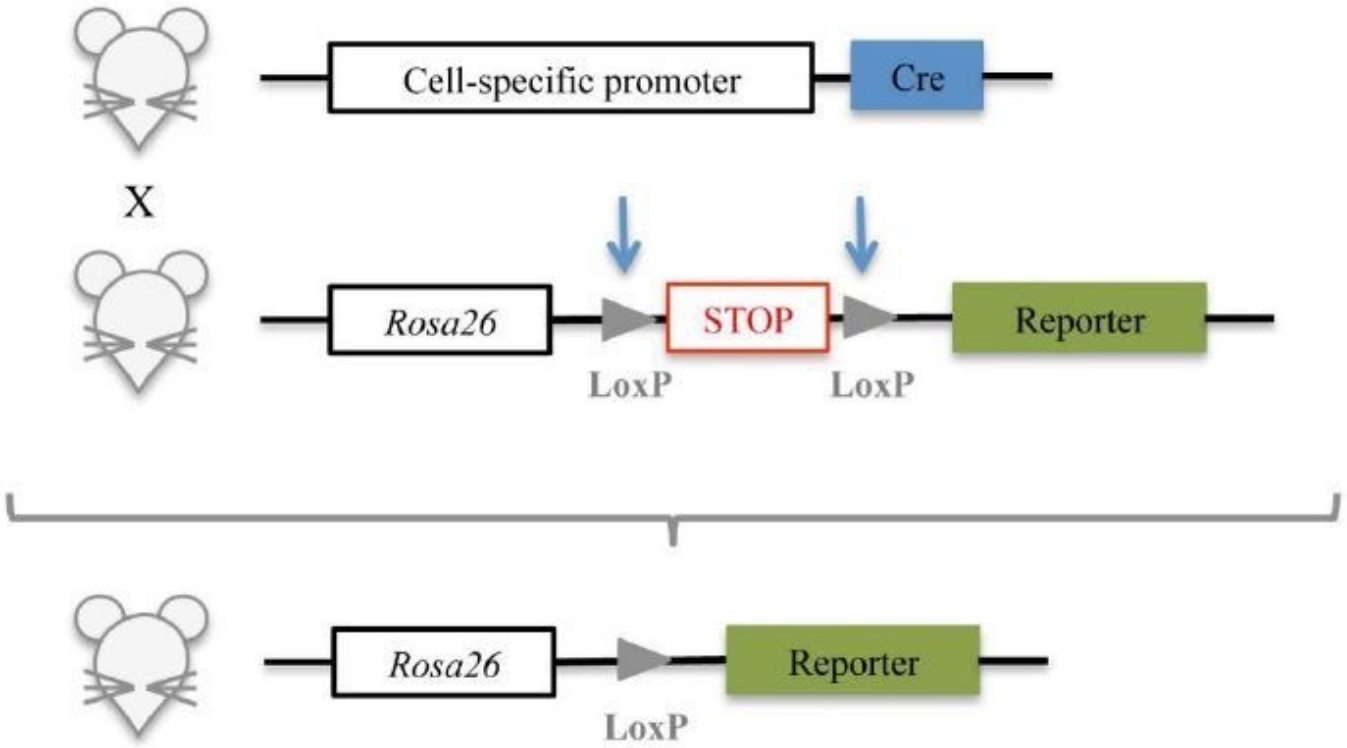
LoxP

5′-ATAACTTCGTATA-GCATACAT-TATACGAAGTTAT-3′
3′-TATTGAAGCATAT-CGTATGTA-ATATGCTTCAATA-5′



Marintcheva 2018

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



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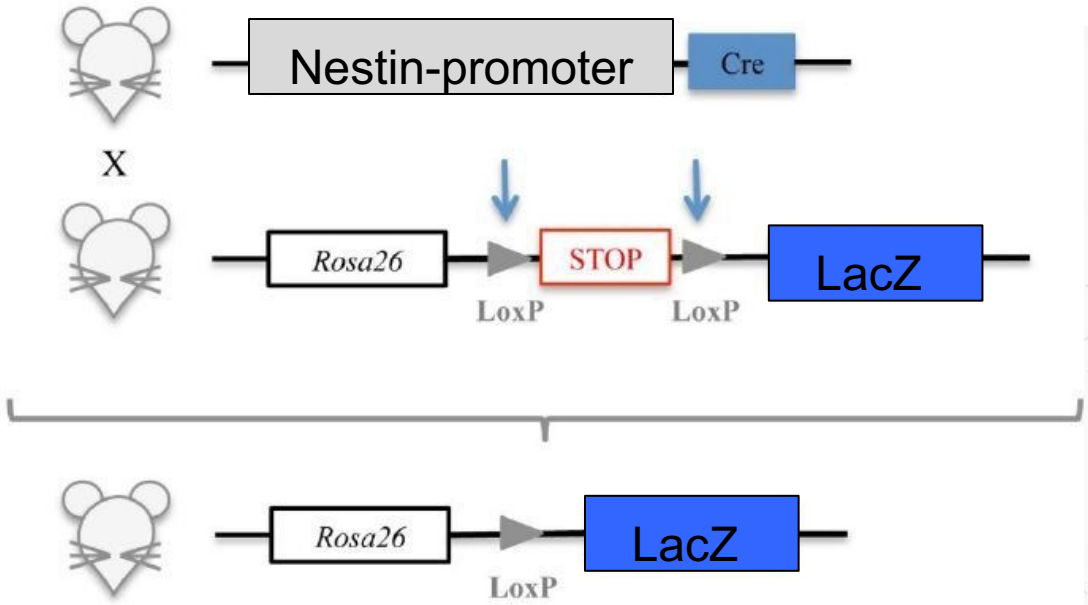
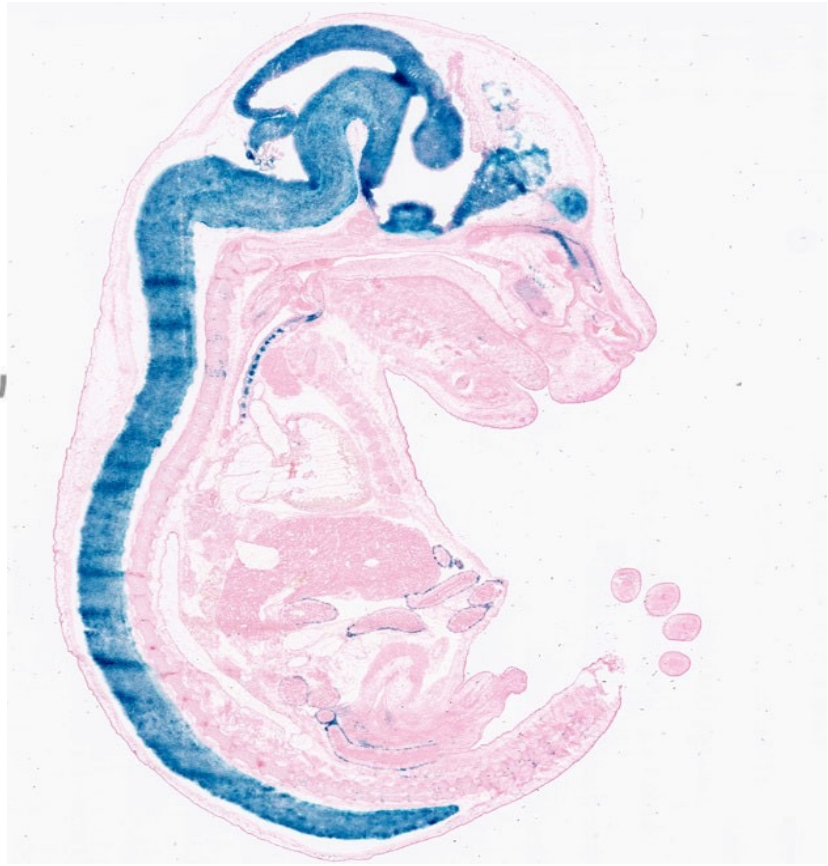
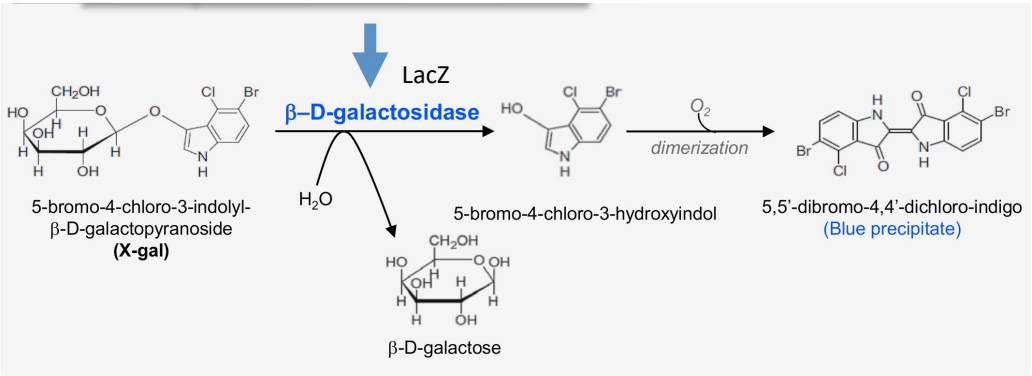






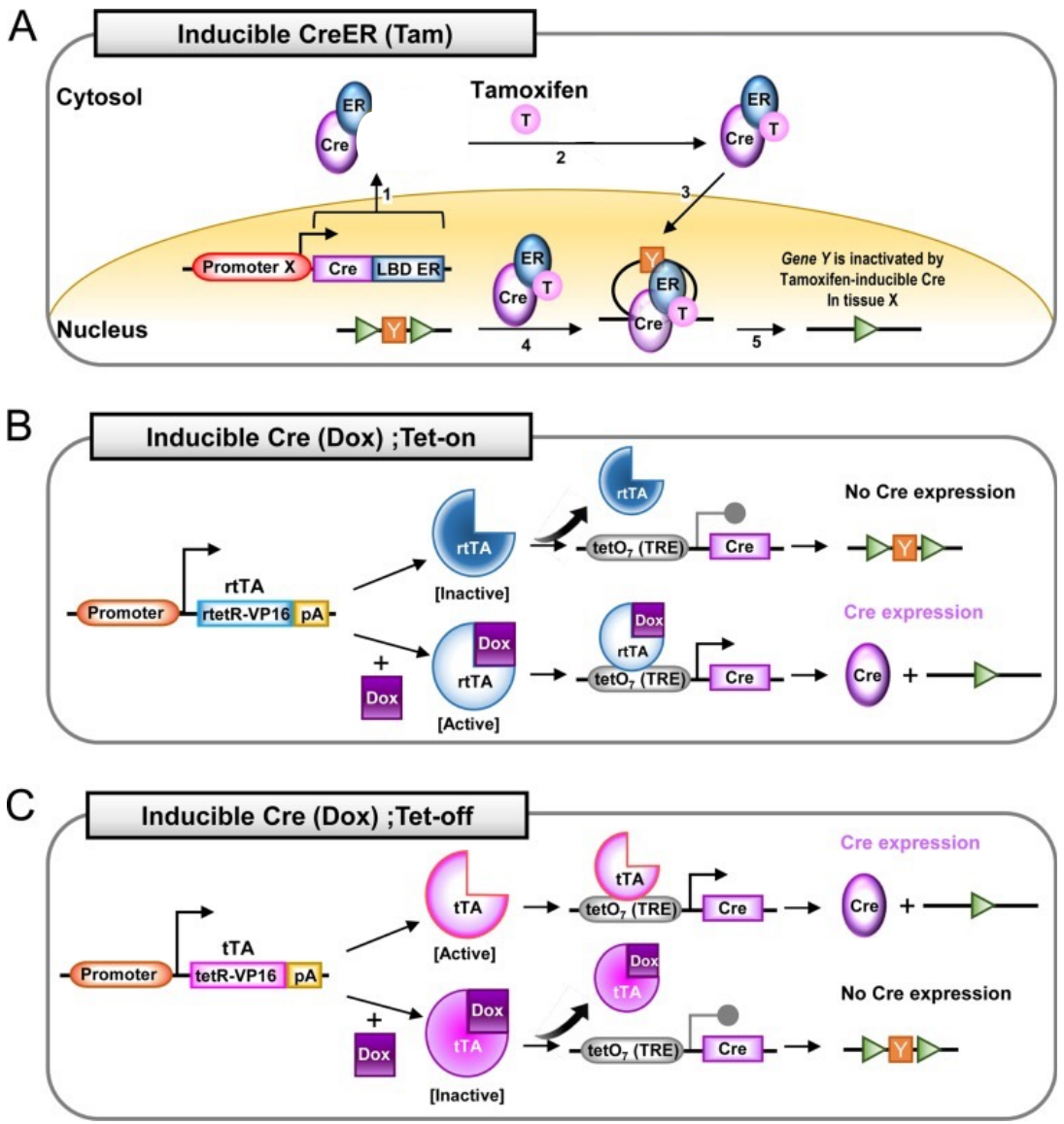
Inducible Cre-Lox recombination

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:

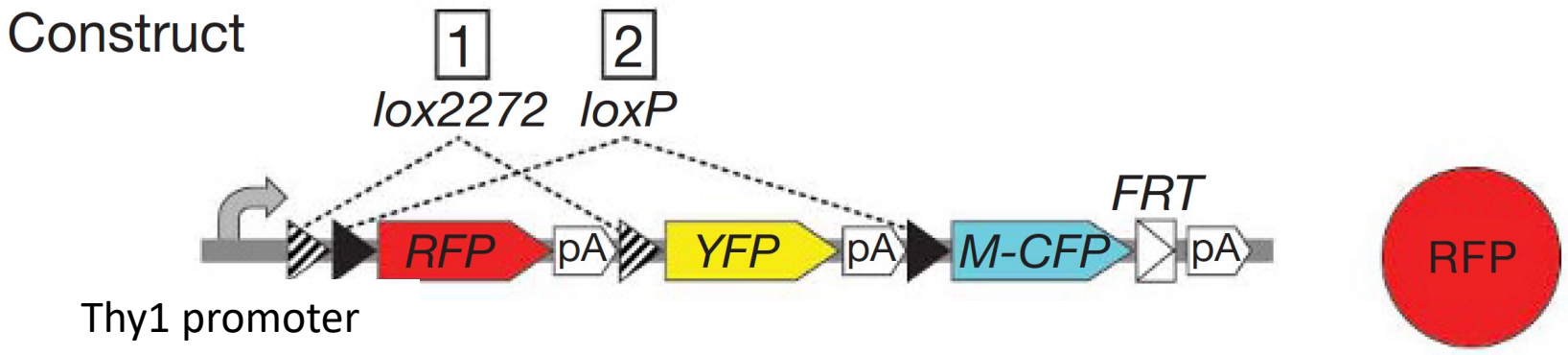
- Cre expression is driven by a tetO promoter
- (B) In presence of Dox, rtTA induces Cre expression
- (C) In absence of Dox, tTA induces Cre expression
- (C) In presence of Dox, Cre expression is inhibited



Kim et al., 2018

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

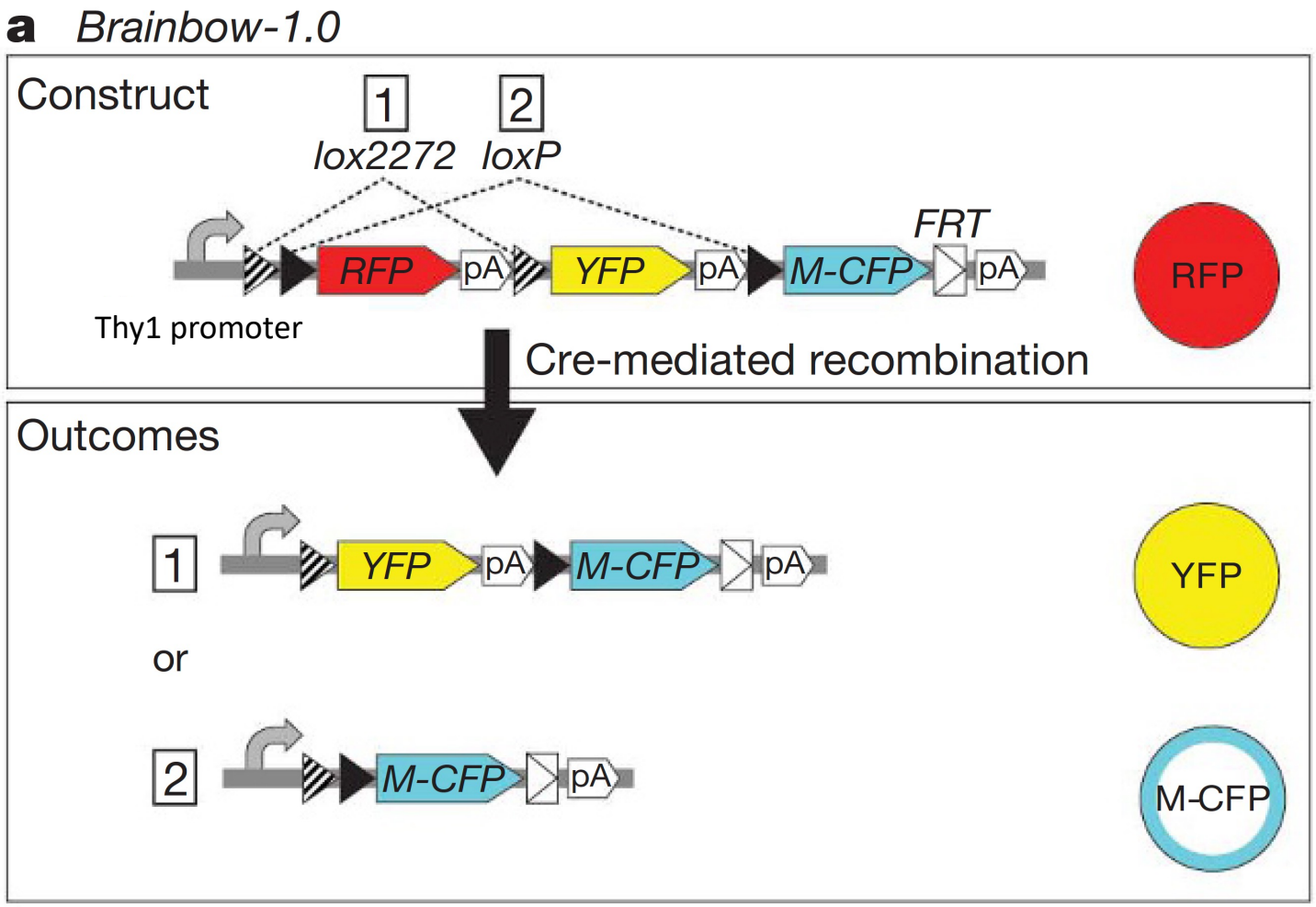


Modified from Livet et al., Nature 2007

https://www.nature.com/articles/nature06293

Multicolor lineage tracing: the brainbow mouse

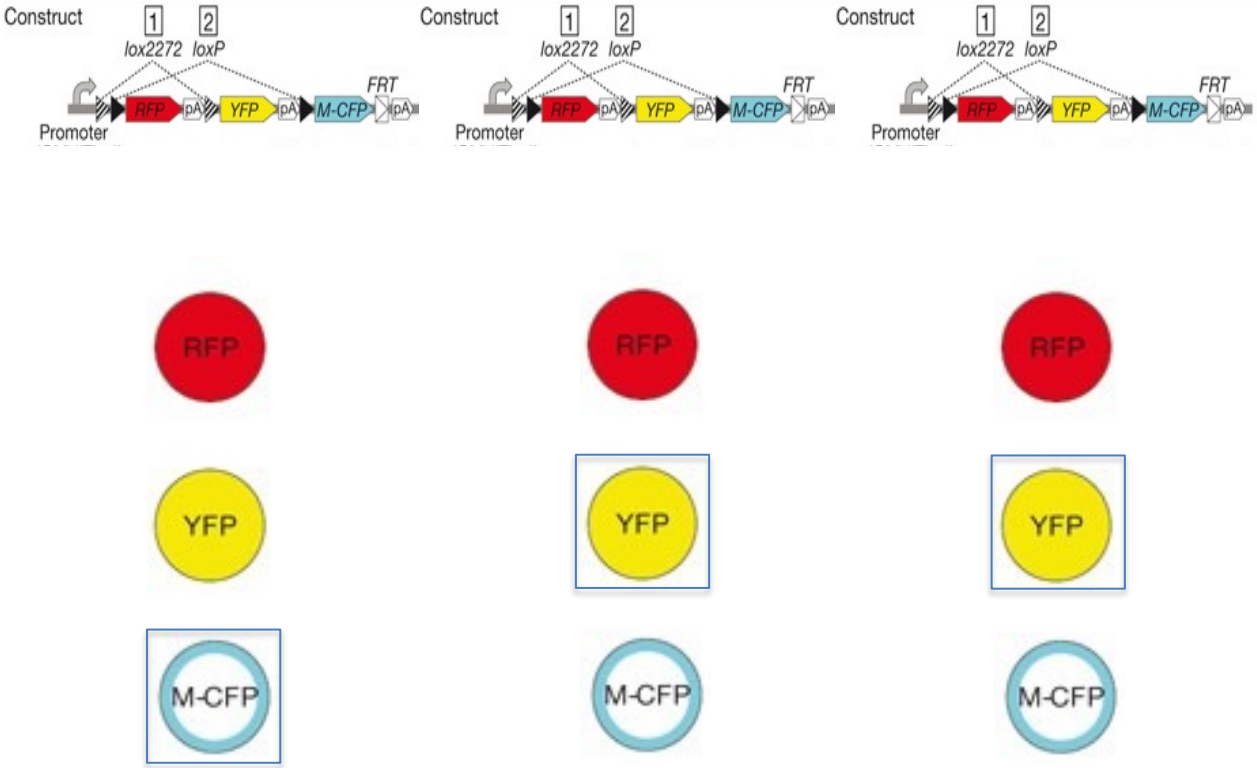
Each permutation results in expression of a single fluorescent protein



Modified from Livet et al., Nature 2007

Multicolor lineage tracing: the brainbow mouse

If the DNA sequence is present in 3 copies:
→ 3 different fluorescent proteins are expressed in each cell!

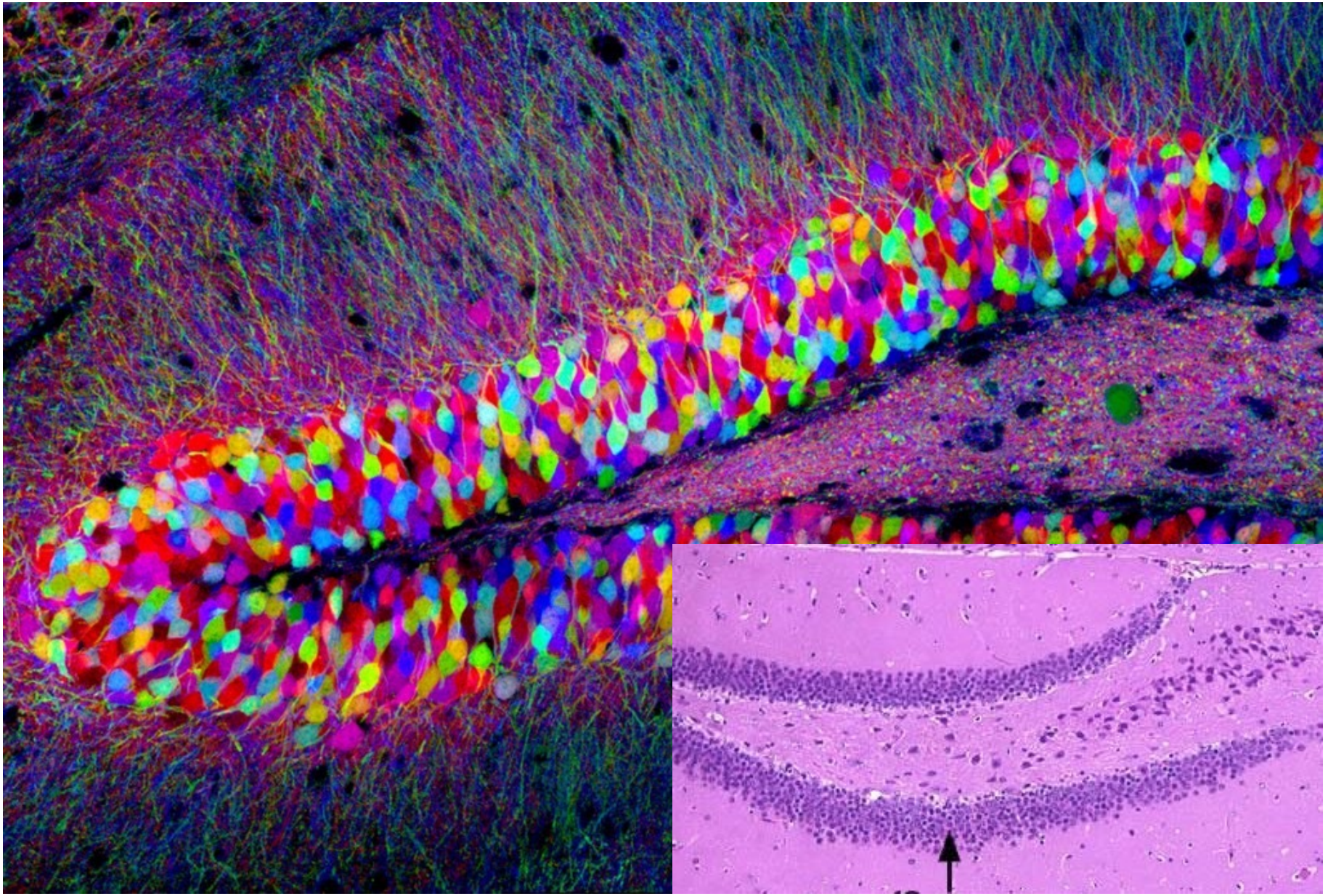


a XFP combinations

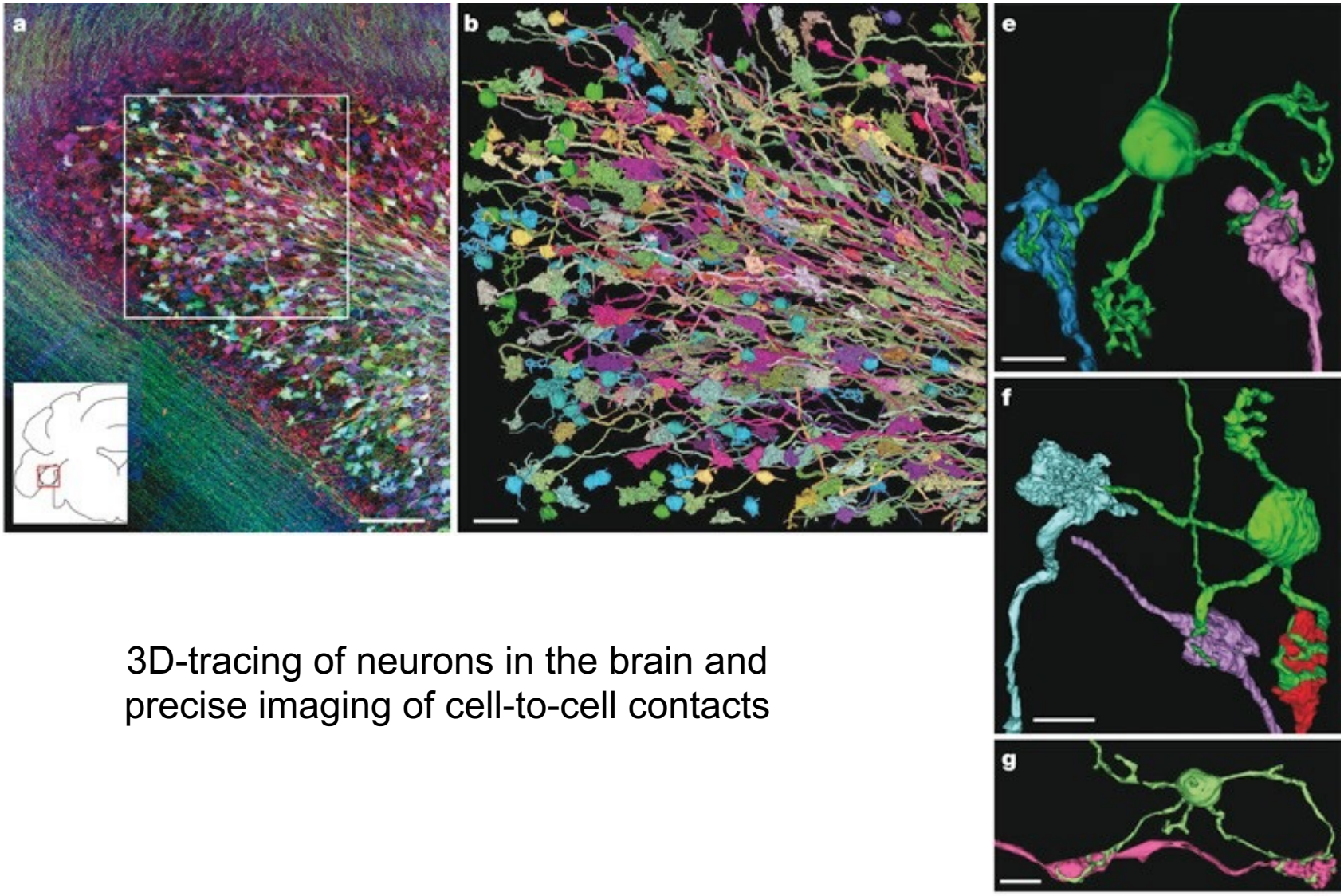
Outcome for each copy 1	2	3	Resulting colour
C	C	C	Blue
C	C	Y	Light blue
C	Y	Y	Blue-green
Y	Y	Y	Green
Y	Y	R	Light green
Y	R	R	Orange
R	R	R	Red
R	R	C	Magenta
R	C	C	Purple
R	C	Y	Grey

Modified from Livet et al., Nature 2007

Neurons of the brainbow mouse



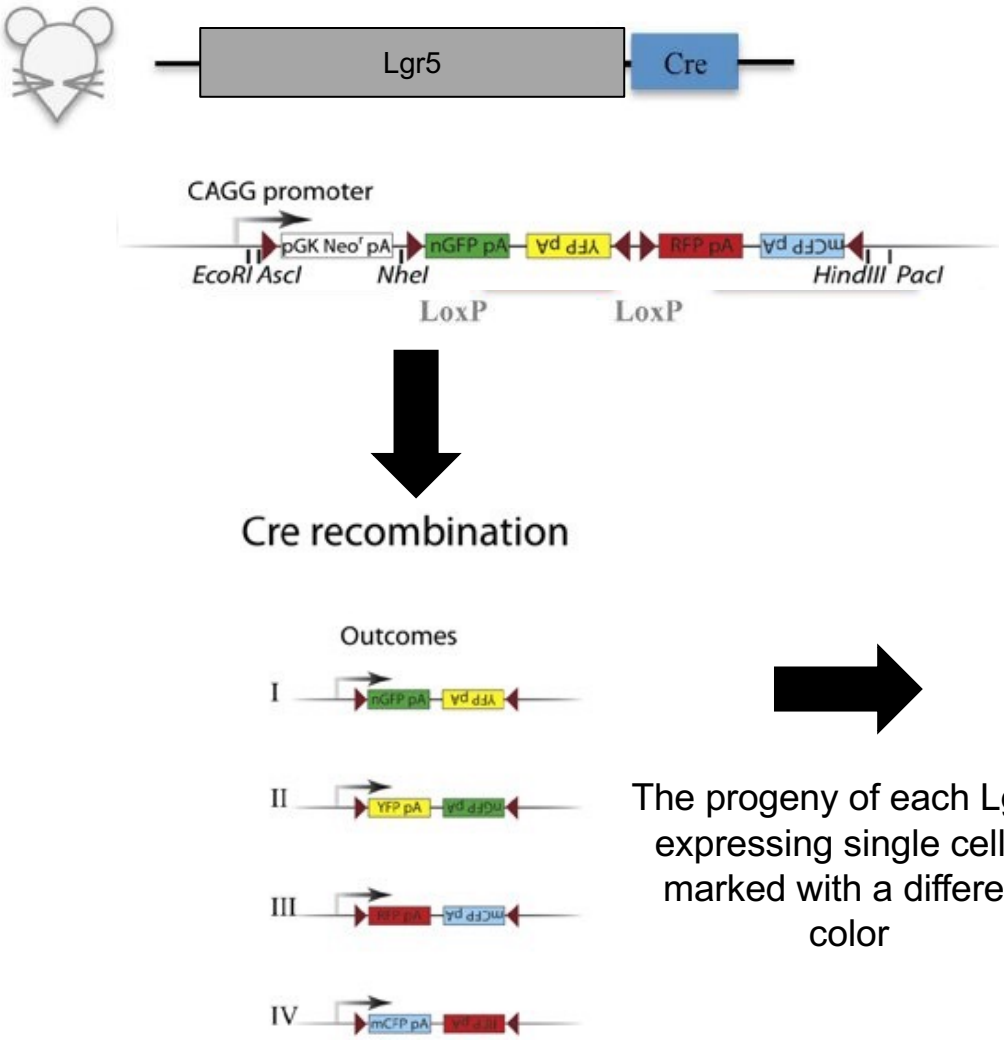
Neurons of the brainbow mouse



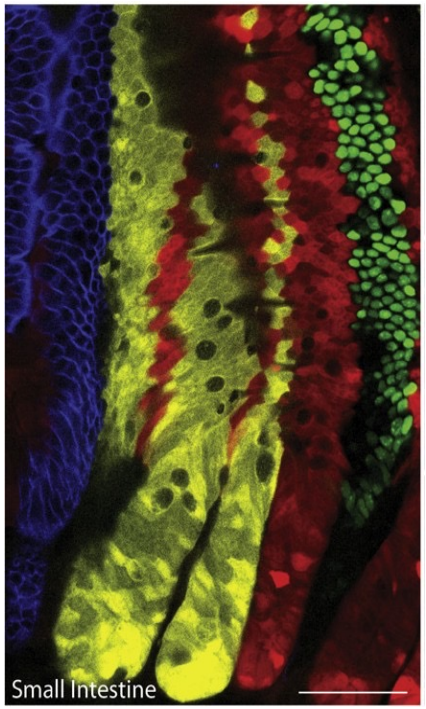
3D-tracing of neurons in the brain and precise imaging of cell-to-cell contacts

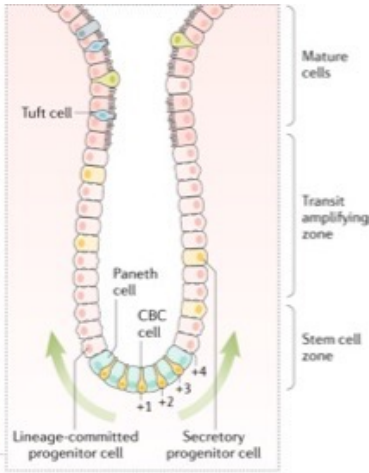
Modified from Livet et al., Nature 2007

Multicolor lineage tracing: the confetti mouse



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



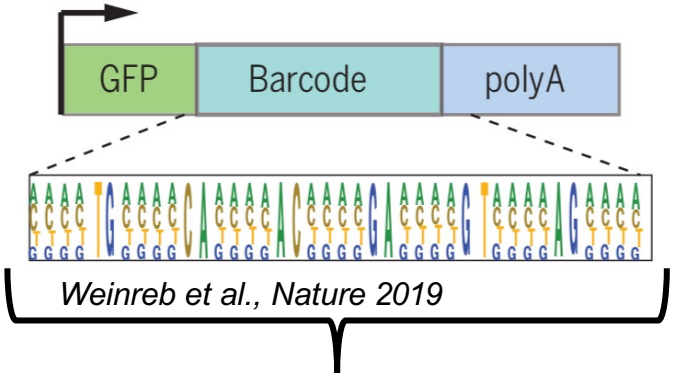


Modified from Snippert et al., Cell 2010 https://www.cell.com/fulltext/S0092-8674(10)01064-0

5. Sequencing-based lineage tracing

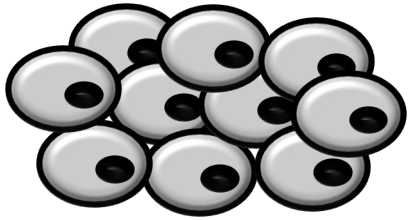
The principle of cellular barcoding

Labelling cells with nucleic acid sequences

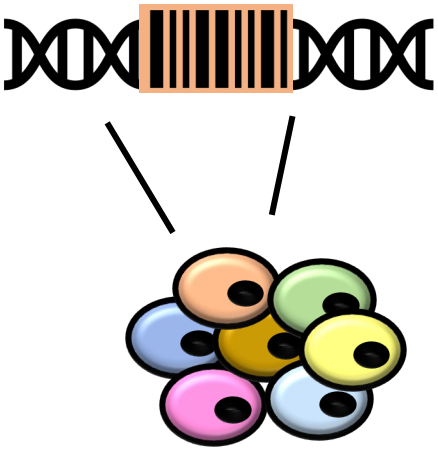


Lentiviral library

Cell population of interest

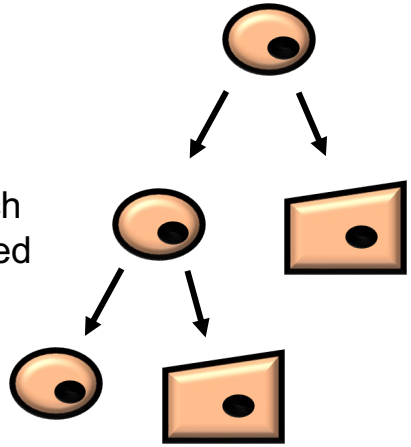


Selection of barcoded cells (FACS/antibiotics)

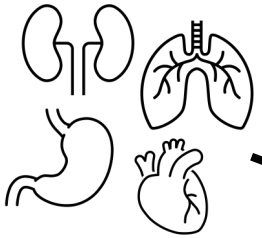


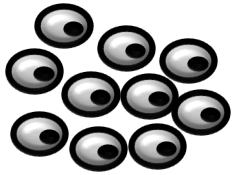
Differentiation

The unique barcode of each cell is transmitted to its progeny

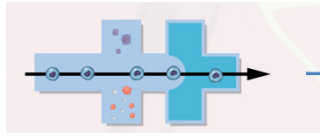


Single cell analysis (scRNA-Seq)

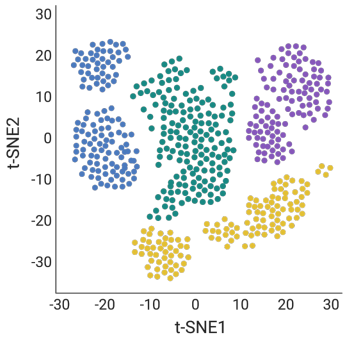




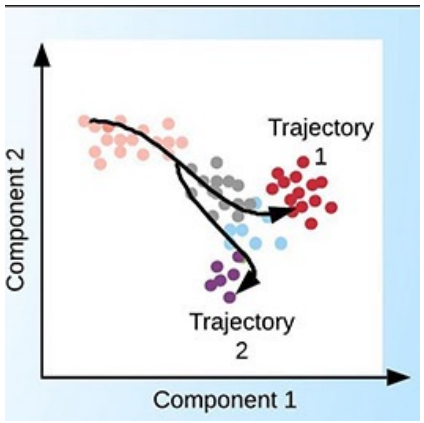
Single cell suspension



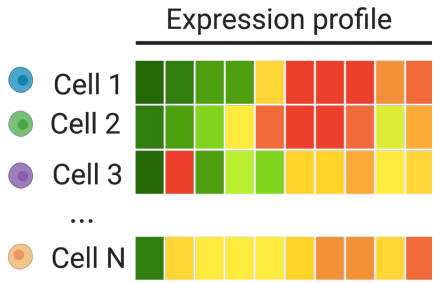
High-throughput sequencing



Characterizing complex cell populations

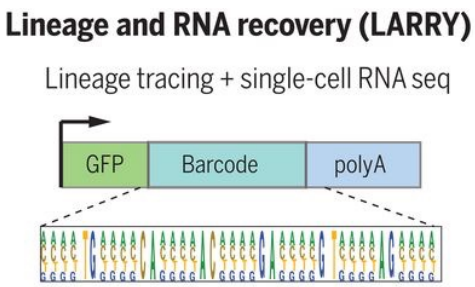


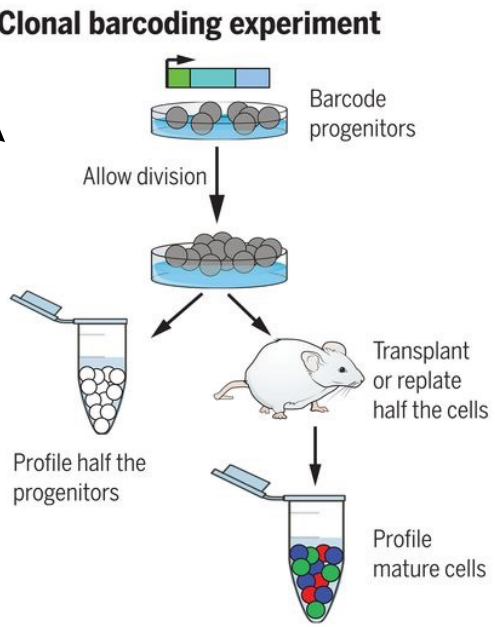
Inferring cell lineages and differentiation trajectories

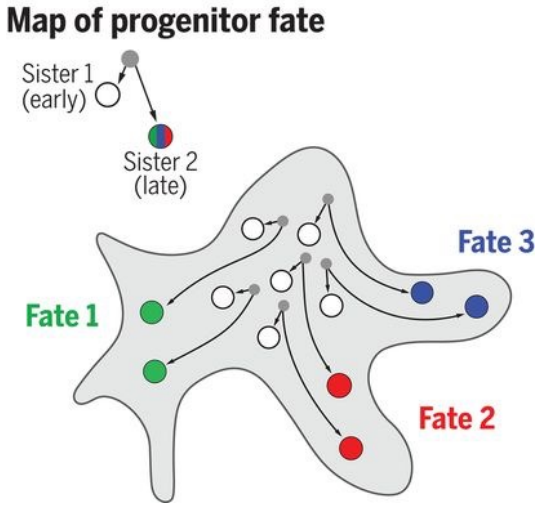


Transcriptome of each cell

Application: Cellular barcoding to trace HSC differentiation



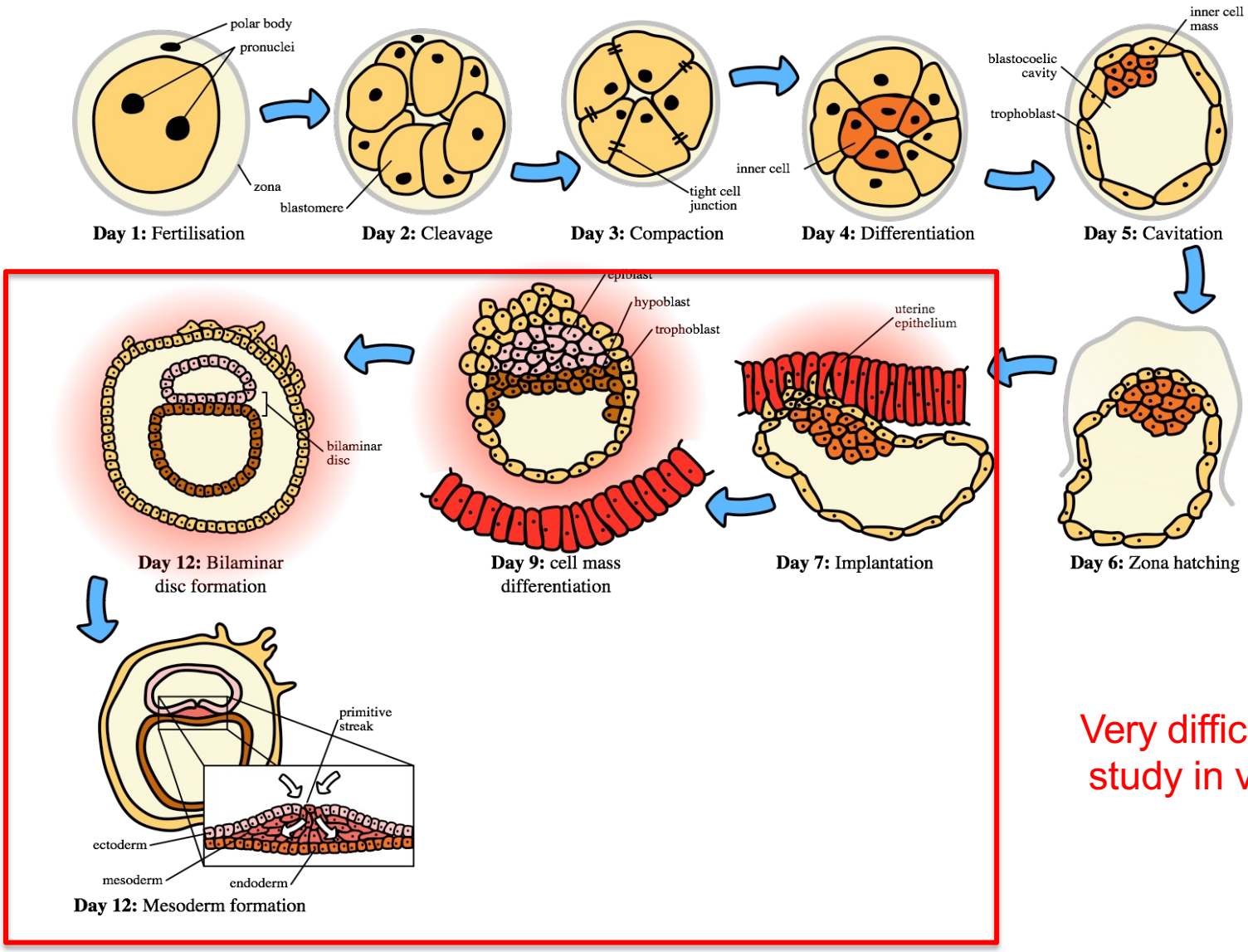




Weinreb et al., Nature 2019

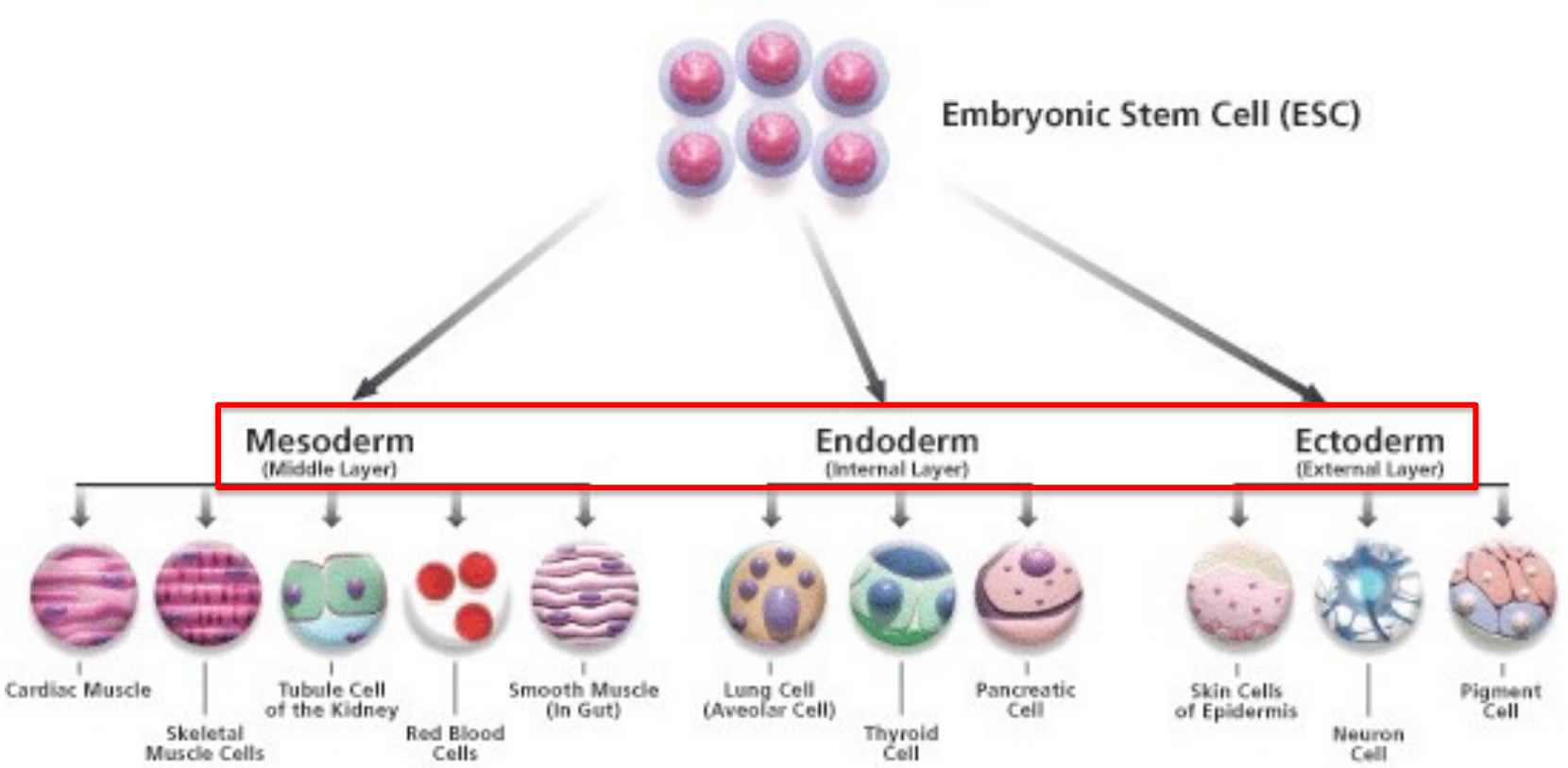
II. Using stem cells to model early embryogenesis

How can we study early embryogenesis?

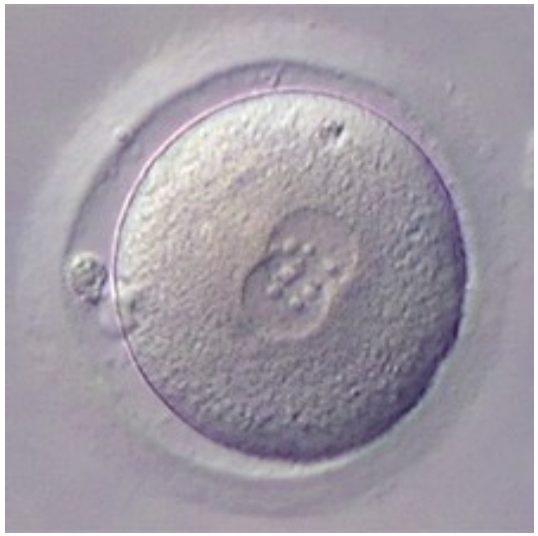


Very difficult to study in vivo !

How can we study early embryogenesis?



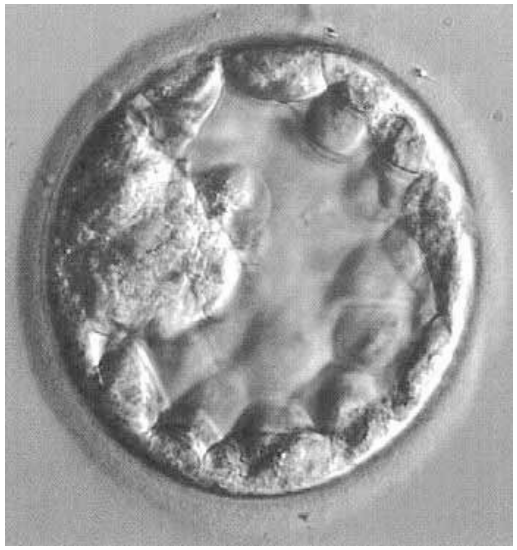
Timing of embryogenesis..



3 weeks

40 weeks

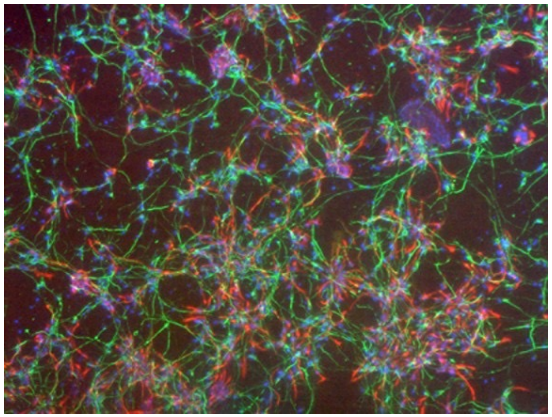
..parallels ES differentiation timing



5 days

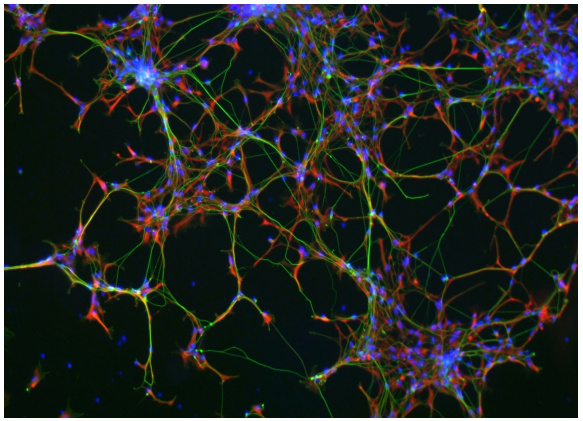
3 weeks

Mouse ES cell-derived neurons



www.csc.mrc.ac.uk

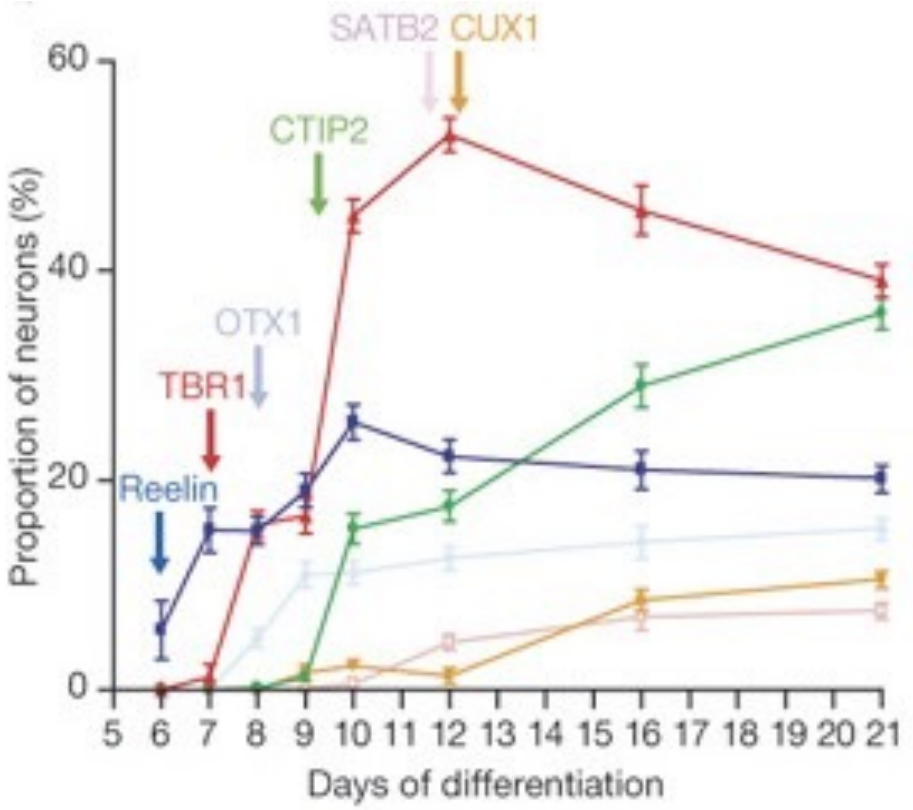
Human ES cell-derived neurons



Suter et al., unpublished

How can we study early embryogenesis?

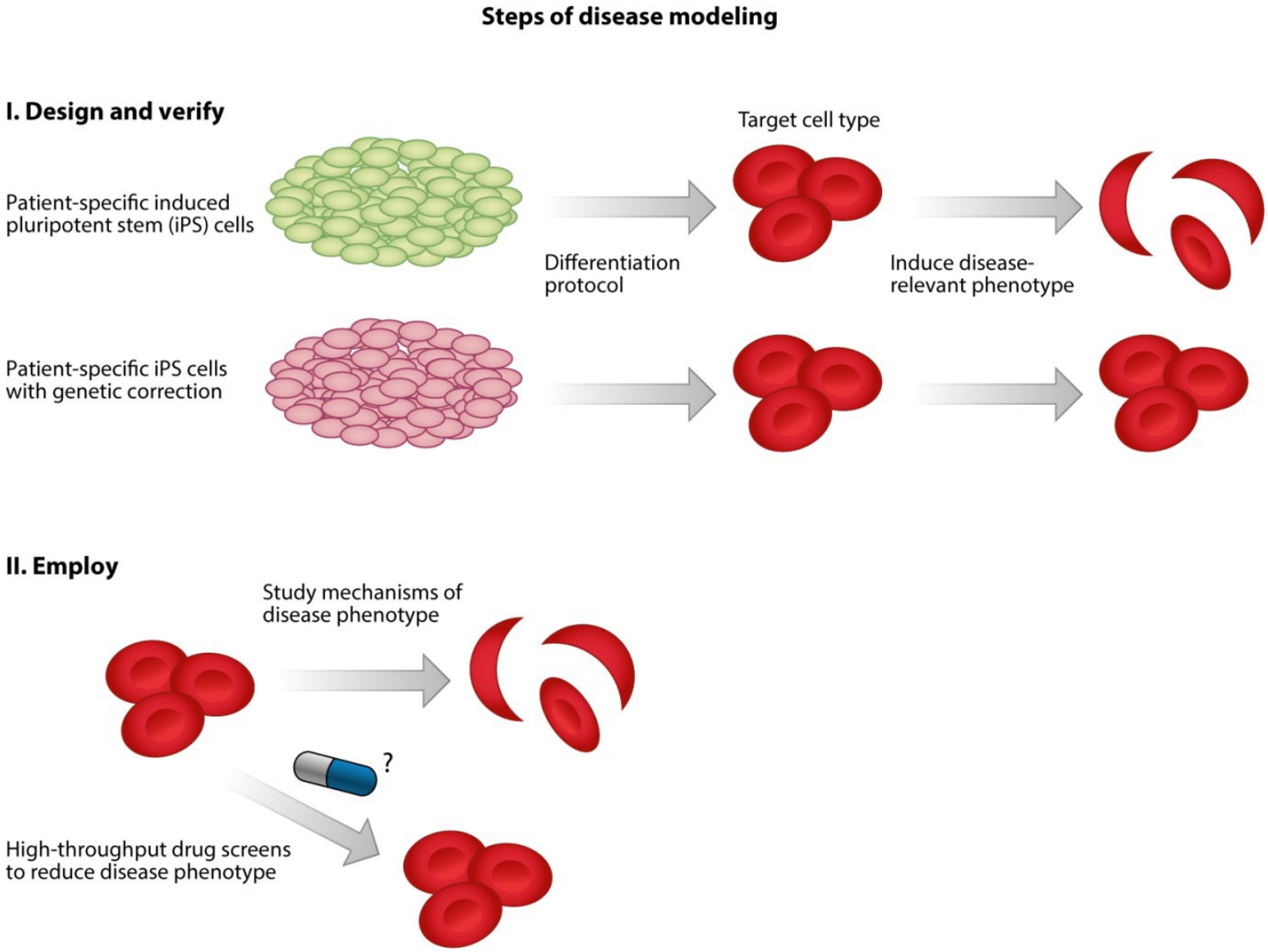
Layers		Markers	Birthdate
Cajal–Retzius neurons		Reelin, calretinin, p73, TBR1	E10.5–11.5
Upper layers		SATB2, CUX1	E13.5–16.5
Deep layers		CTIP2, SOX5, OTX1, ER81, TBR1, TLE4, FOXP2	E11.5–14.5
Subplate		TBR1, calretinin, reelin	E10.5–13.5



Gaspard et al., Nature 2008

III. Using stem cells to model disease

Using pluripotent stem cells to model diseases



Cherry & Daley, Annual Reviews in Medicine 2013

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

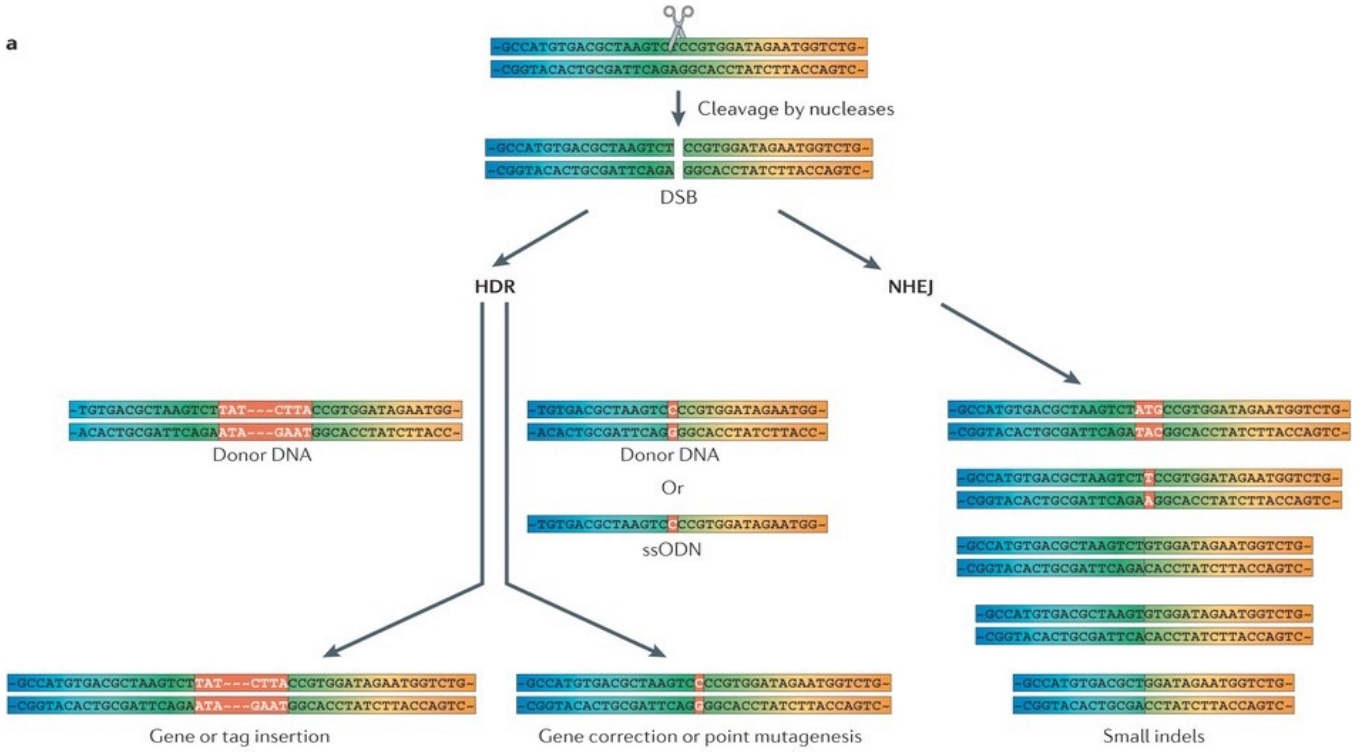
→ Starting from a given pluripotent cell line that is modified **specifically** at the disease locus allows a much cleaner model

→ How to introduce specific mutations in the genome of pluripotent cells ?

Current genome editing technologies

Principle: cleavage of a specific genomic sequence by nucleases

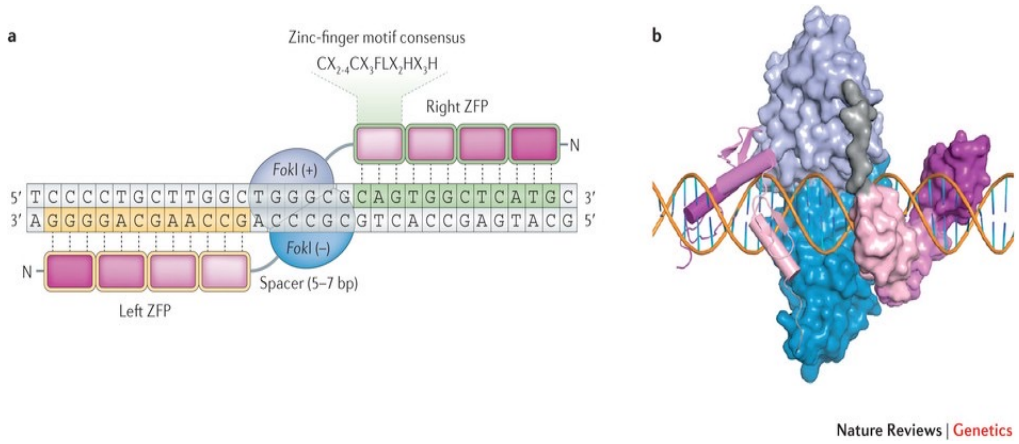
→ The break will be repaired either by non-homologous end-joining (NHEJ) or by homology-directed recombination (HDR)



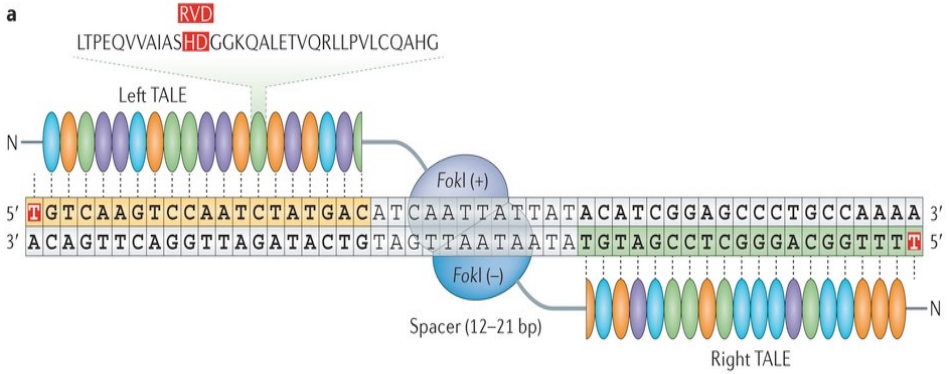
Kim et al., Nat Rev Gen. 2014

Three different generations of nucleases

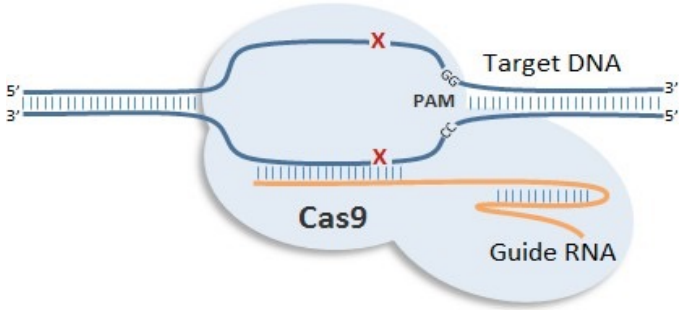
1. Zinc-finger nucleases: specific aa motifs recognize specific triplets of base pairs



2. Transcription activator-like effector nucleases (TALEN)

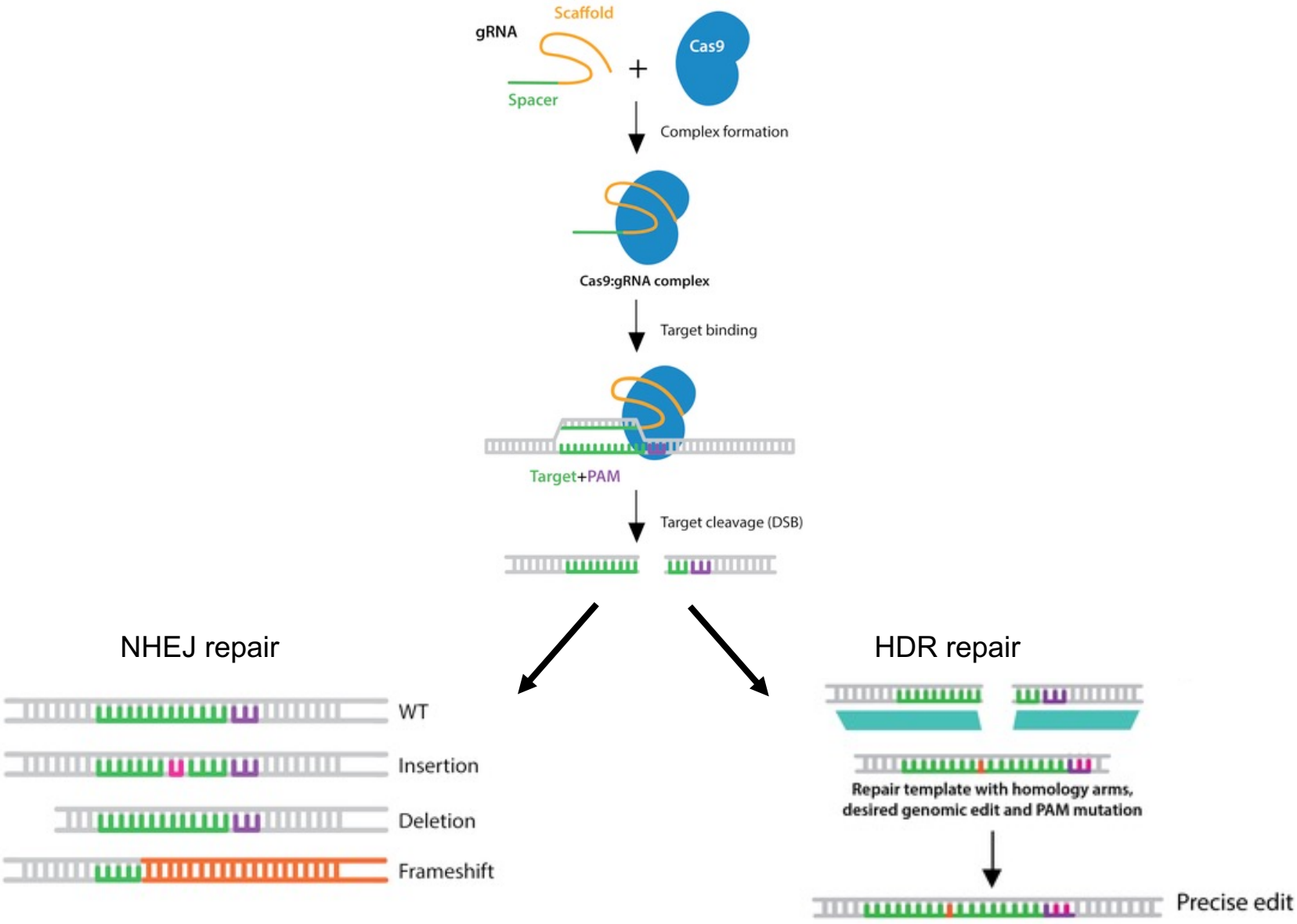


3. CRISPR-Cas9 nucleases



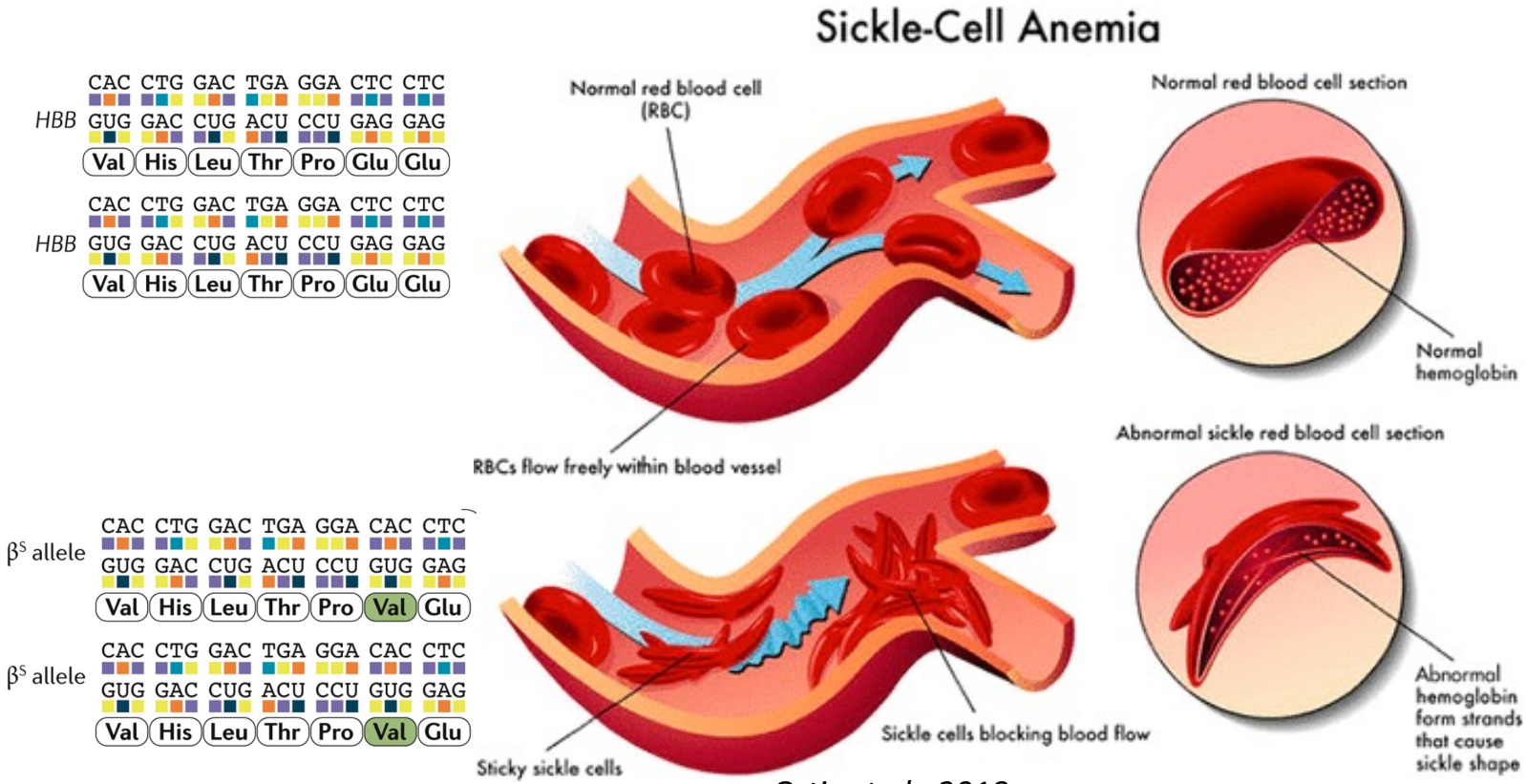
Kim et al., Nat Rev in Gen. 2014

CRISPR-Cas9 nuclease system



https://www.addgene.org/guides/crispr/

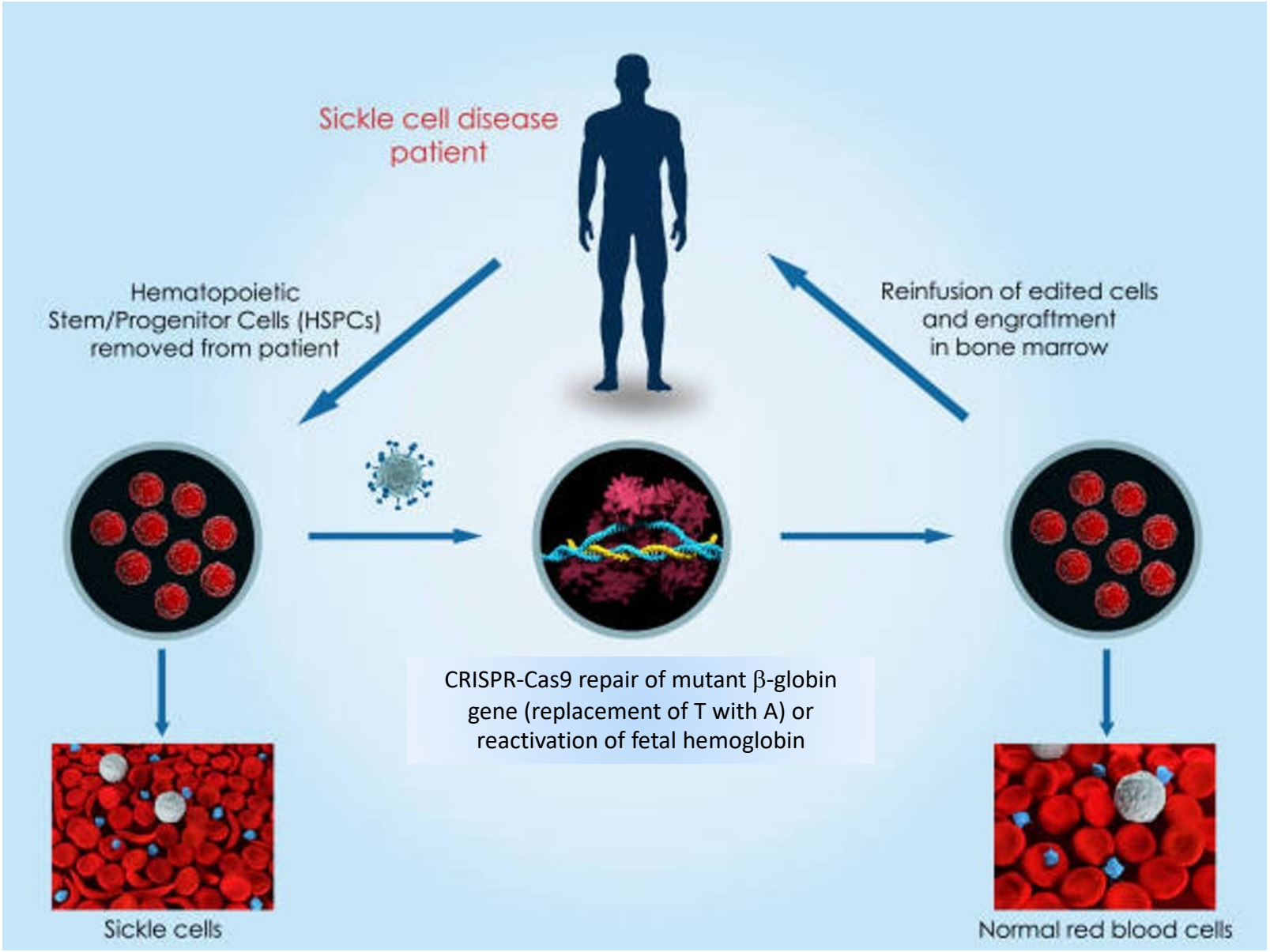
CRISPR-Cas9 based gene therapy for sickle cell anemia



Ortiz et al., 2018

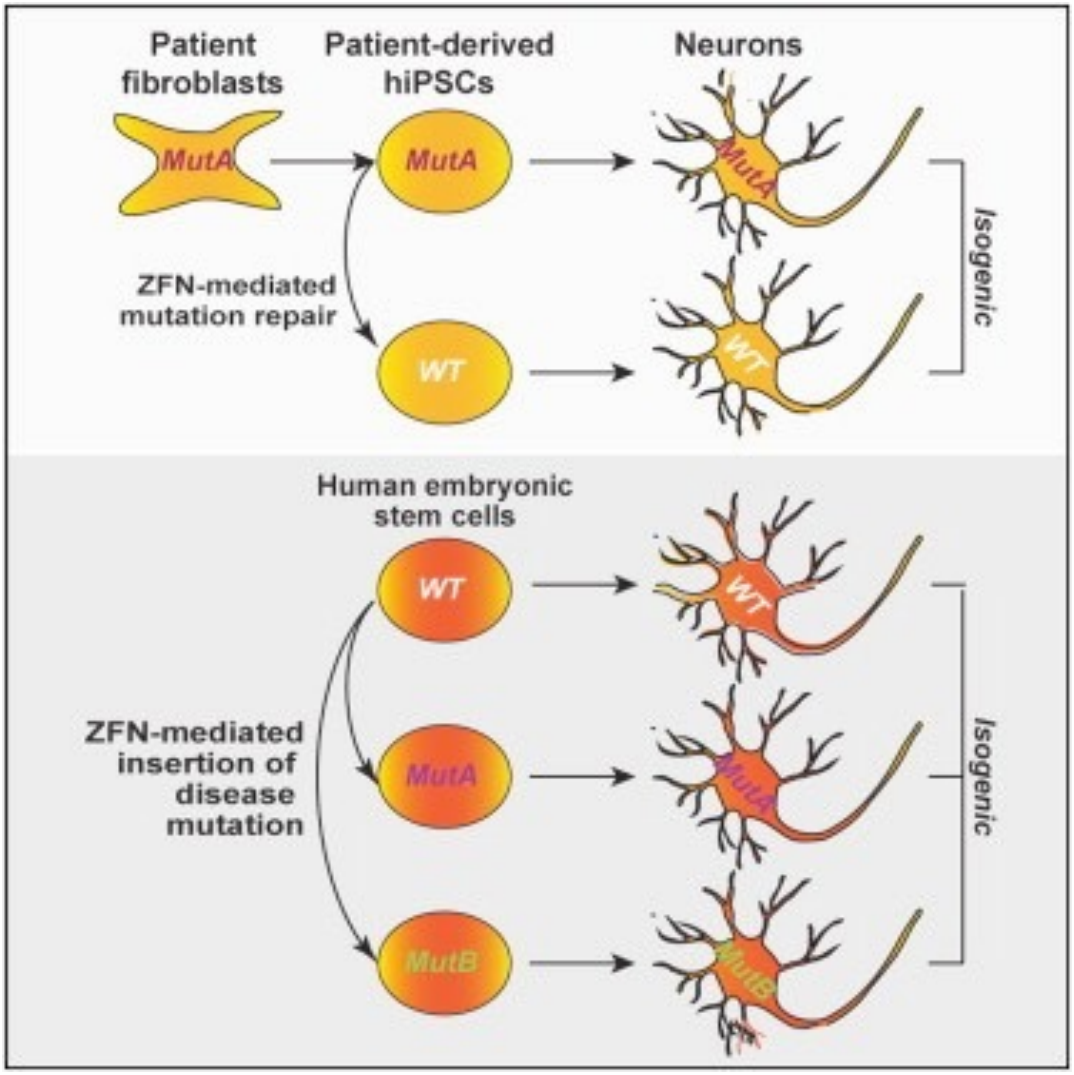
Kato et al., Nat. Rev. Dis. Primers 2018

CRISPR-Cas9 based gene therapy for sickle cell anemia



https://www.synthego.com/

Genome editing model of Parkinson's disease

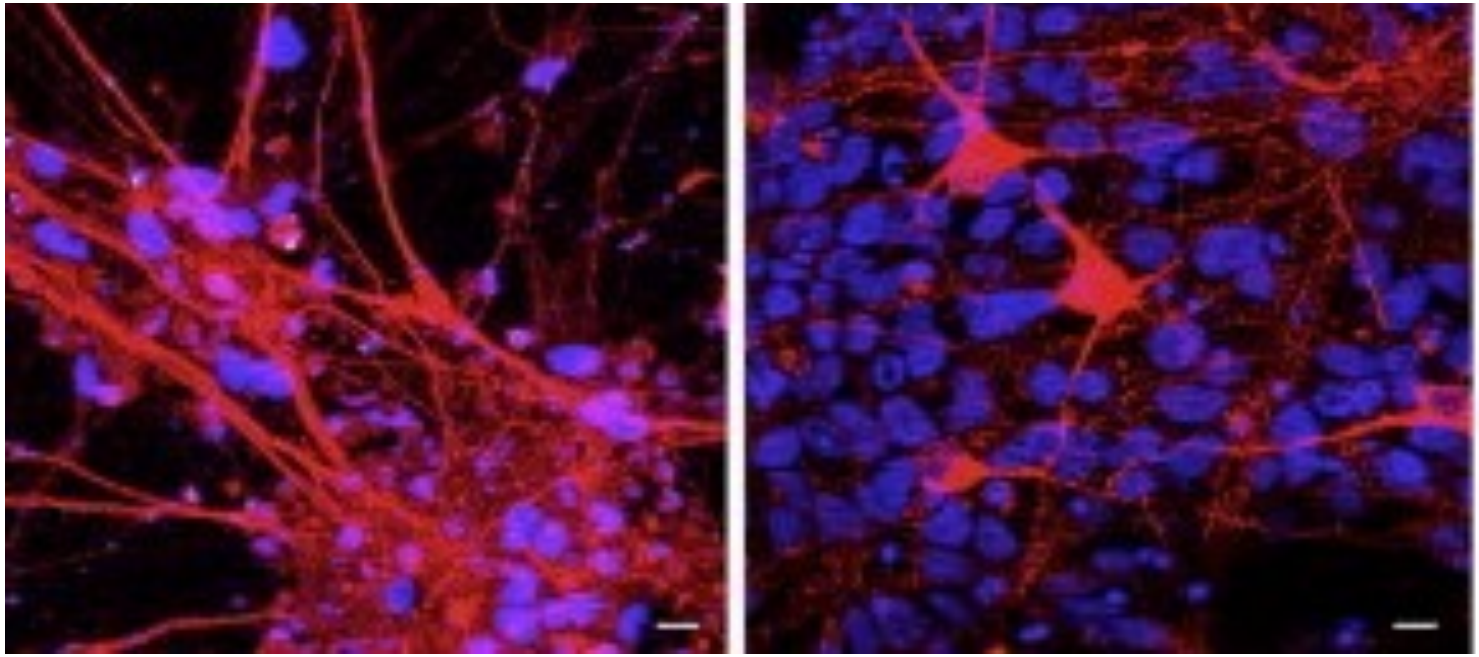


Soldner et al., Cell 2011

Genome editing model of Parkinson's disease

Corrected point mutation

Point mutation causing Parkinson's disease



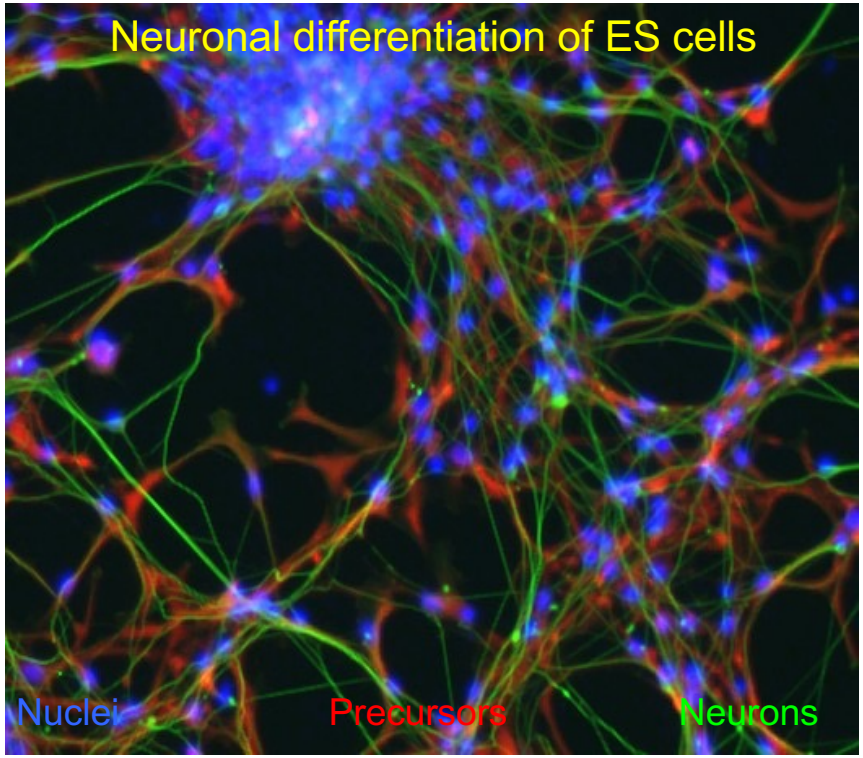
TH+ neurons

DAPI

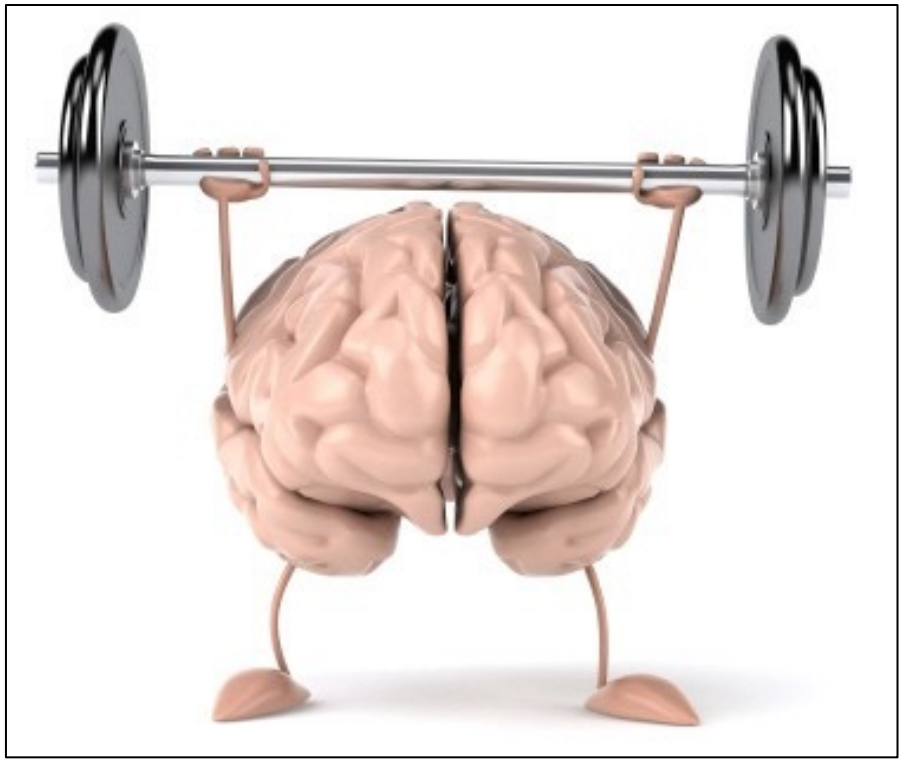
Ryan et al., Cell 2013

Limitations of in vitro differentiation of ES cells to model diseases

ES cell differentiation in a dish is “messy”

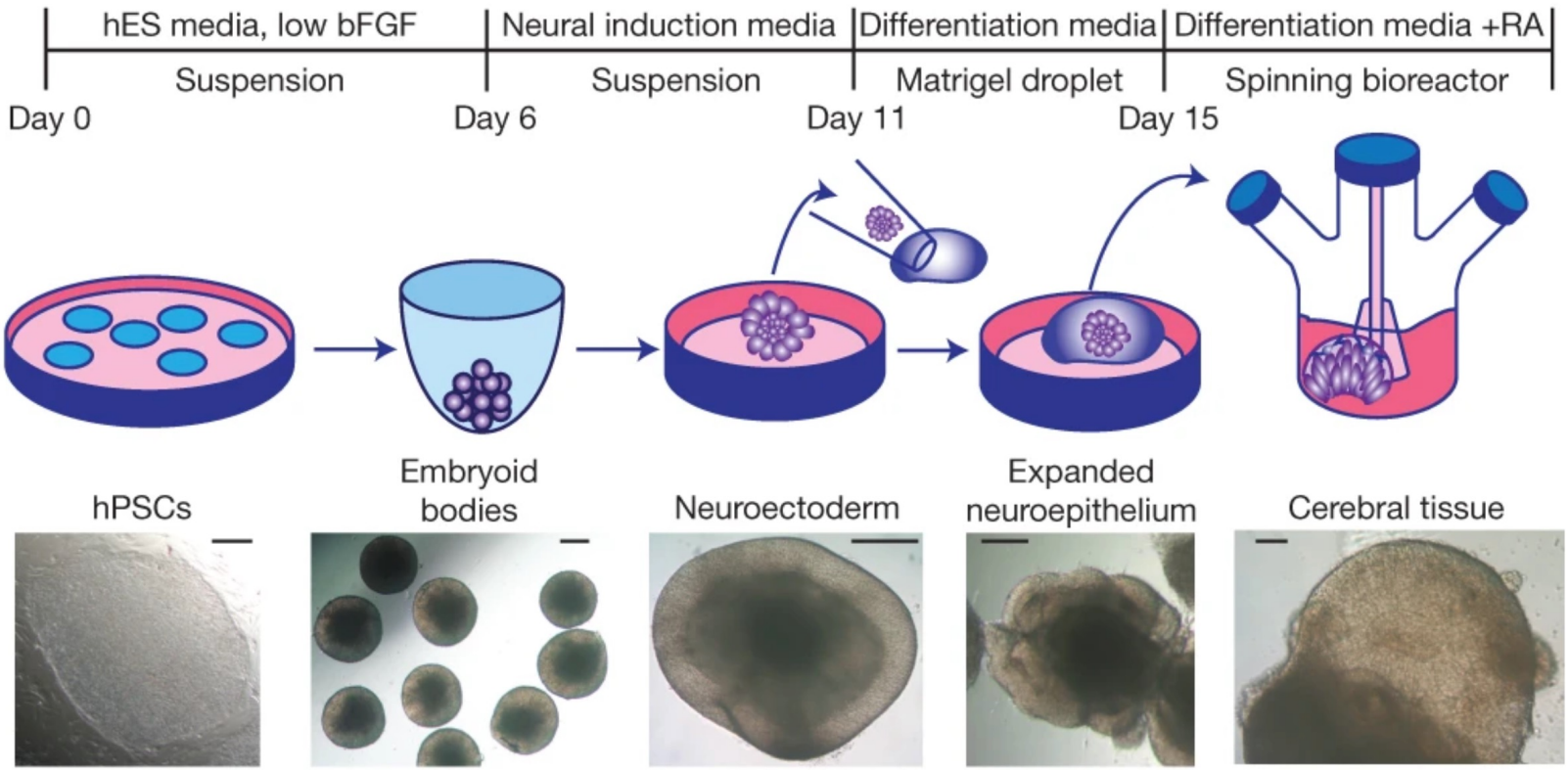


David Suter, unpublished



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

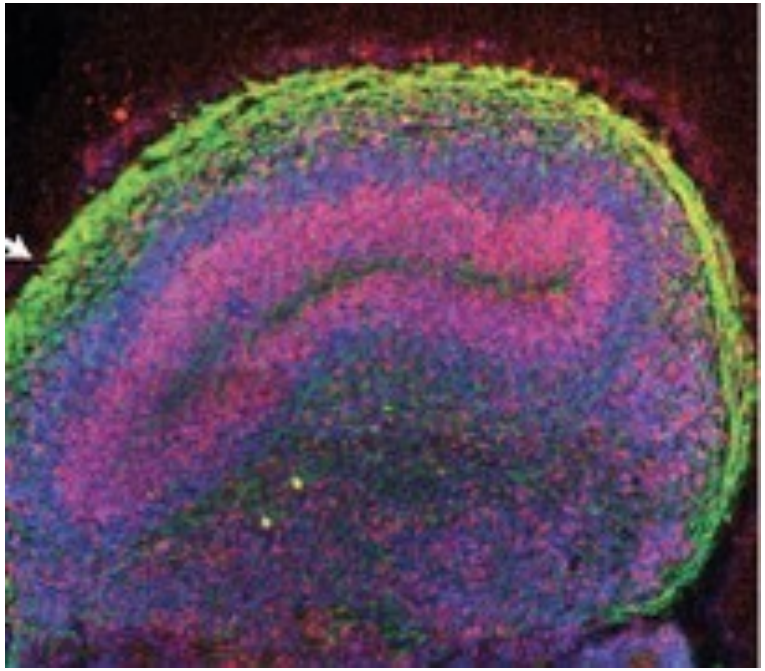
“Minibrains” generated from human ES cells



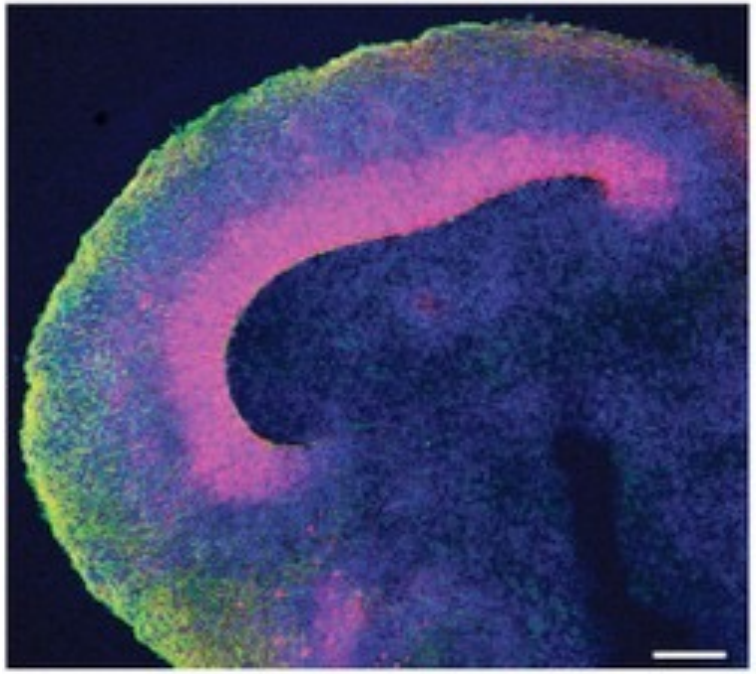
Lancaster et al., Nature 2013

“Minibrains” generated from human ES cells

Human ES cell-derived minibrain



Human fetal brain



Red: neuronal progenitors

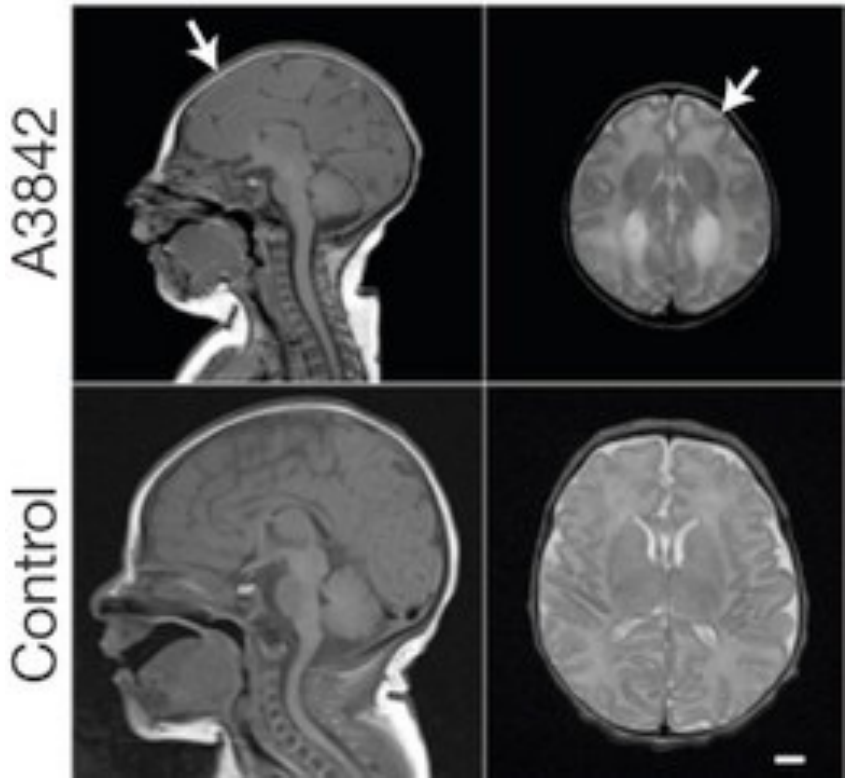
Green: mature neurons

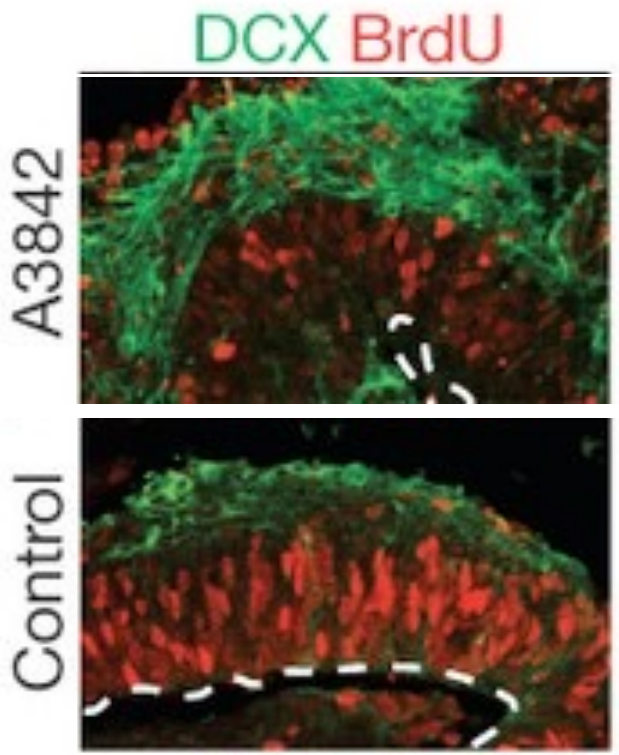
Blue: cell nuclei

Lancaster et al., Nature 2013

“Minibrains” to model human disease

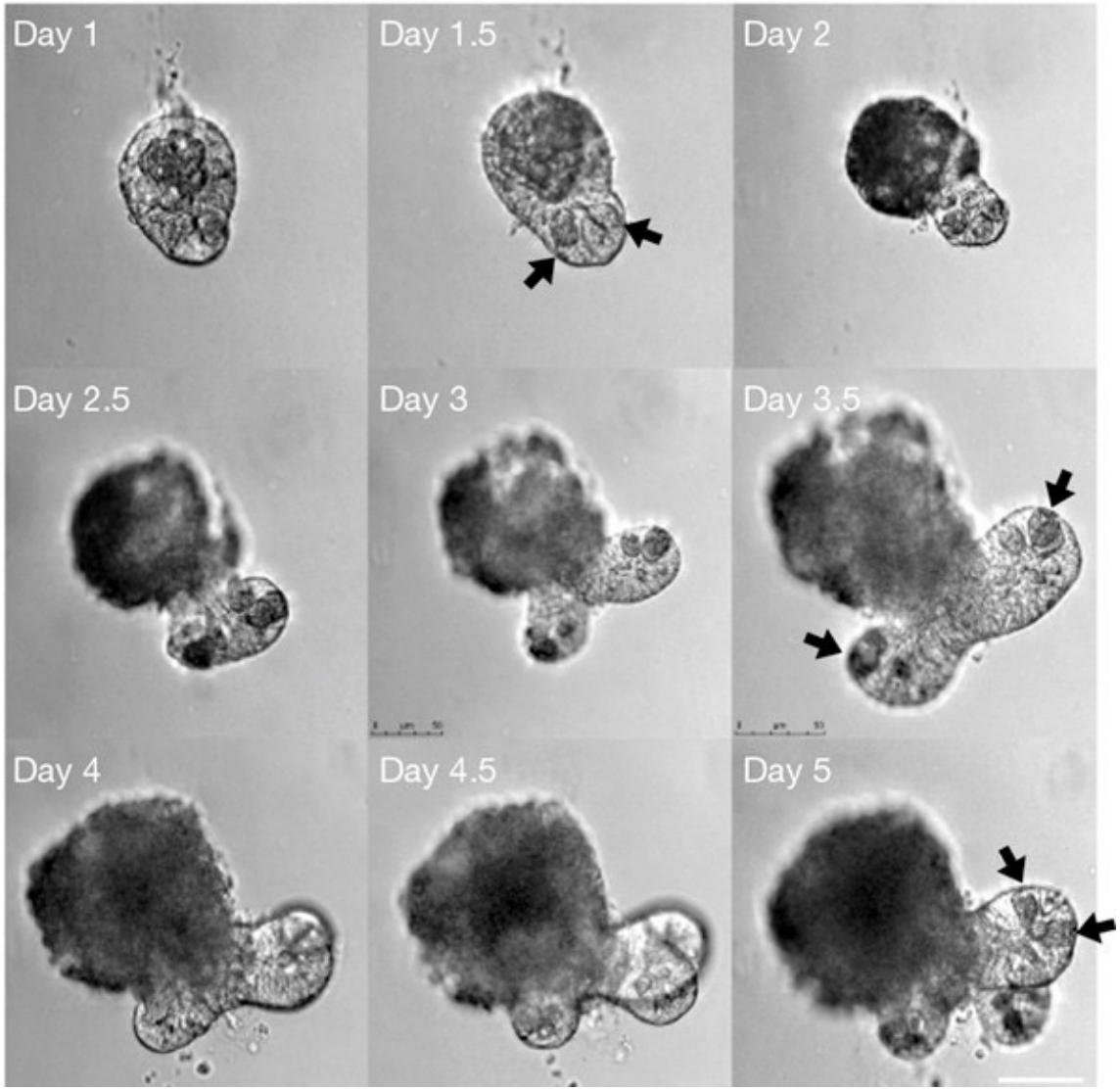
Human iPS cells generated from a patient with a mutation in the CD5RAP2 protein

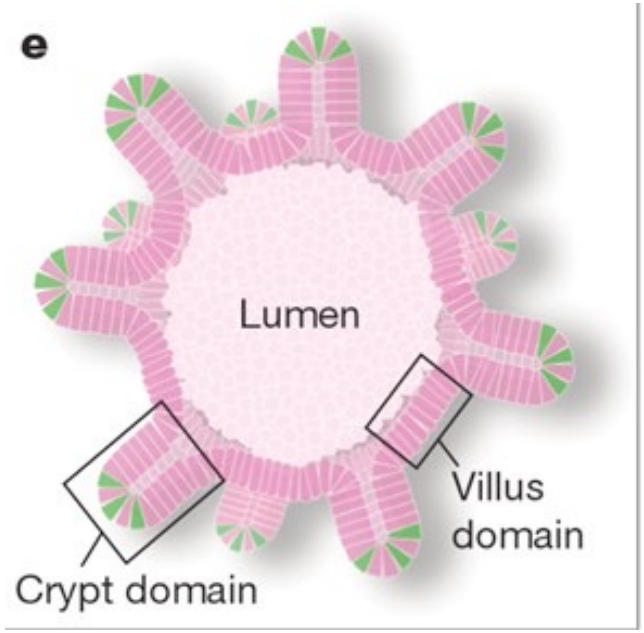




Lancaster et al., Nature 2013

In vitro intestine from intestinal stem cells





Sato et al., Nature 2009

Next lecture 11.10
Wouter Karthaus: Adult stem cells