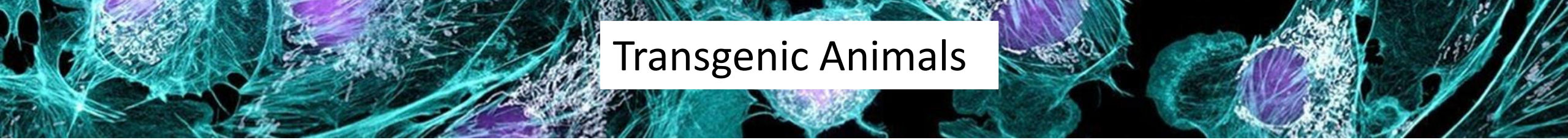
Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of November 10th is about to begin…



Transgenic Animals

1- DNA microinjection
- random insertion

2- embryonic stem cell-mediated gene transfer
- random insertion
- homologous recombination (double selection)
- Rosa26 locus
- genomic analysis to identify genetically modified animals

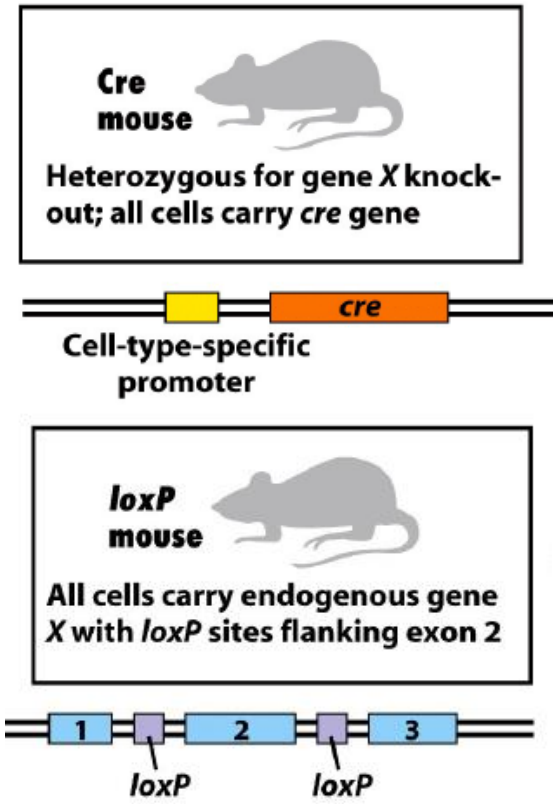
- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)
- siRNA
- CRISPR-CAS9

STEPS TO OBTAIN A CRE/LOX MOUSE

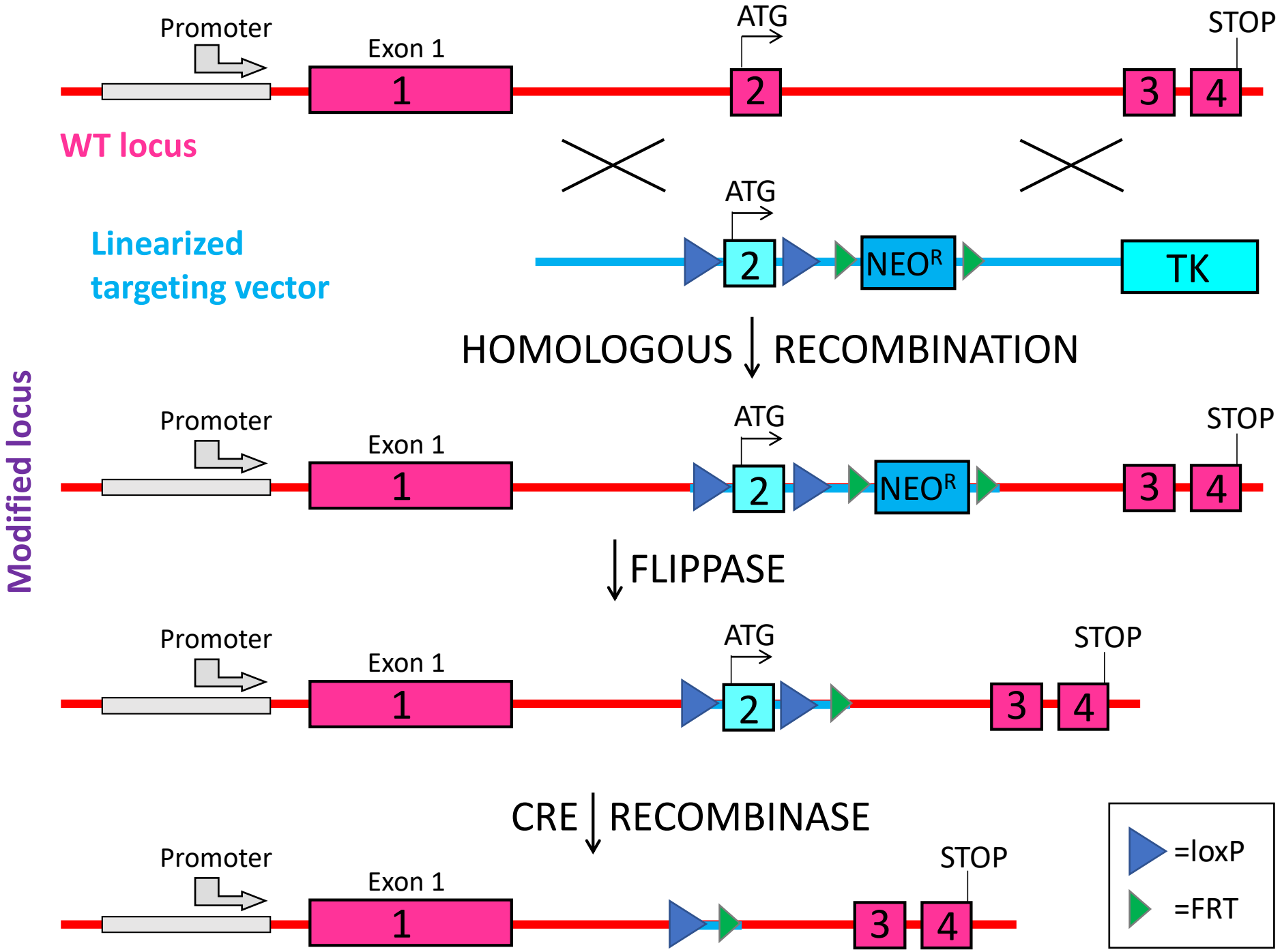
- Preparation of the construct containing **specific promoter + CRE**
- injection in the pronucleus of the zygote
- screen mice to identify CRE-transgenic mice
- Verify CRE expression

- Preparation of the construct containing **loxP-exon X-neo-TK**
- Transfection in ES cells for homologous recombination
- Selection and screening of the clones
- Injection in blastocysts
- Chimeric mice -> +/- floxed mice: only one allele is floxed
- Screen mice

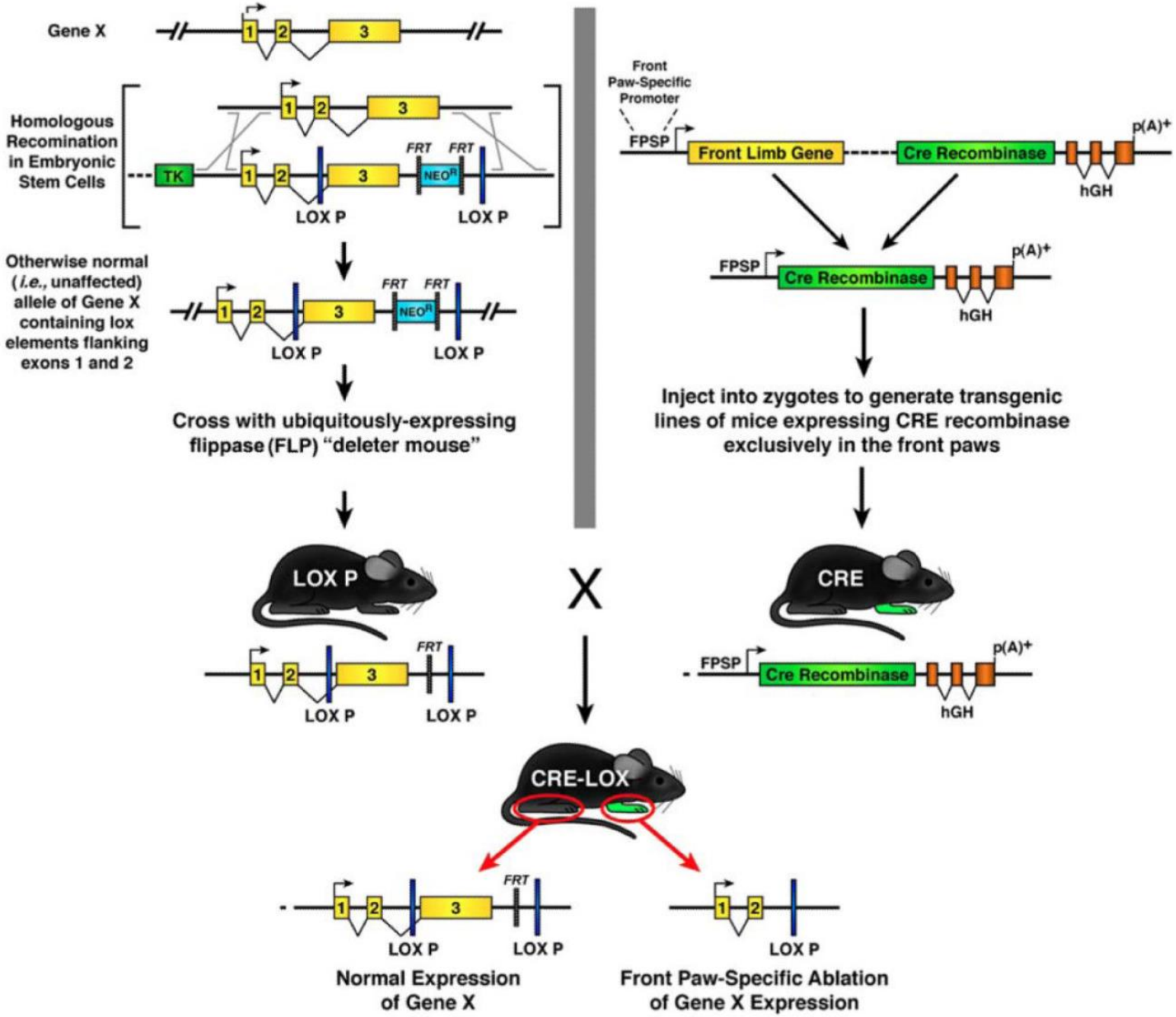
- Cross mice to obtain -> -/- floxed mice
- Screen mice
- Cross -/- floxed mice with CRE-transgenic mouse
- verify KO in the specific cells



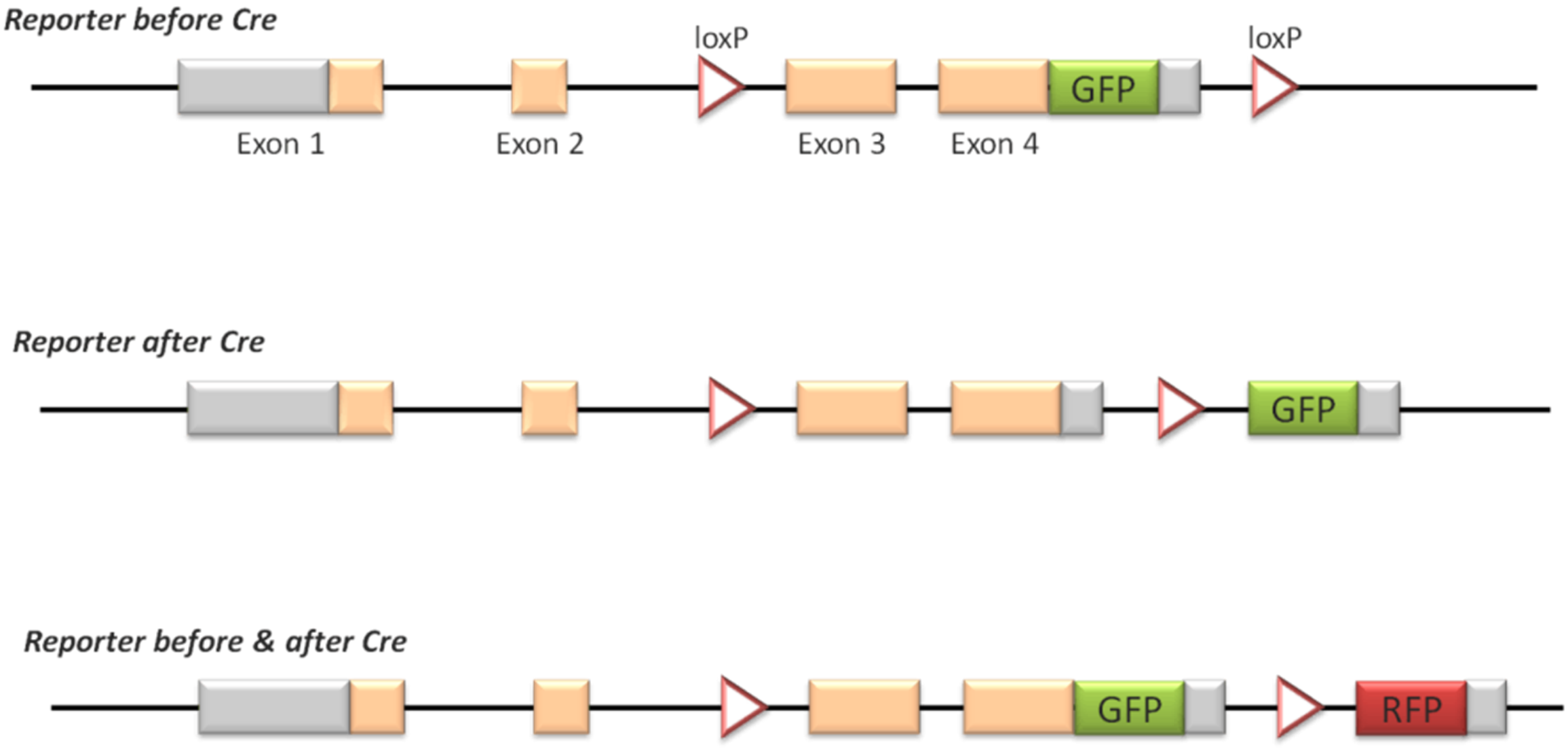
- CRE animals can be crossed with different floxed animals!
- Floxed animals can be crossed with different CRE animals!
- CRE expression can be obtained also by virus infection


Promoter
Exon 1
ATG
STOP
1
2
3
4
WT locus
Linearized
targeting vector
NEOR
TK
HOMOLOGOUS RECOMBINATION
Modified locus
FLIPPASE
CRE RECOMBINASE
=loxP
=FRT

Conditional knock out mice with tissue specific ablation of gene expression



Conditional knock-out with reporter, driven by native promoter



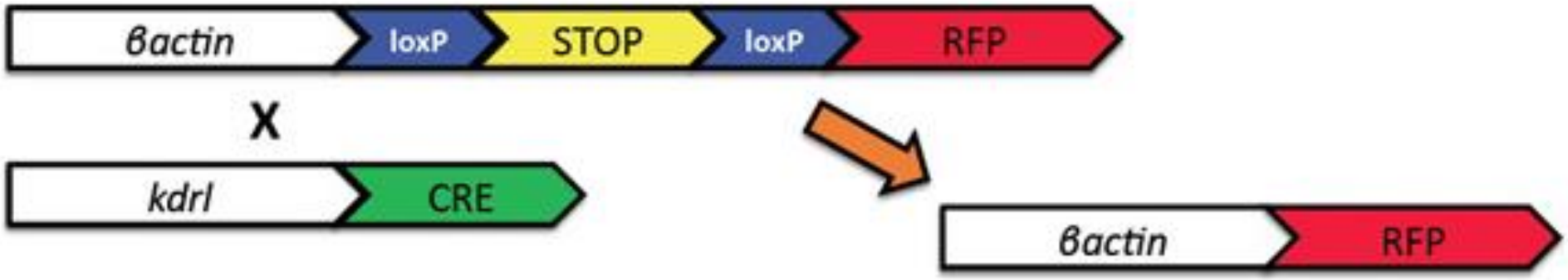
Fate mapping & cell lineage tracing

TSP = tissue specific or inducible promoter

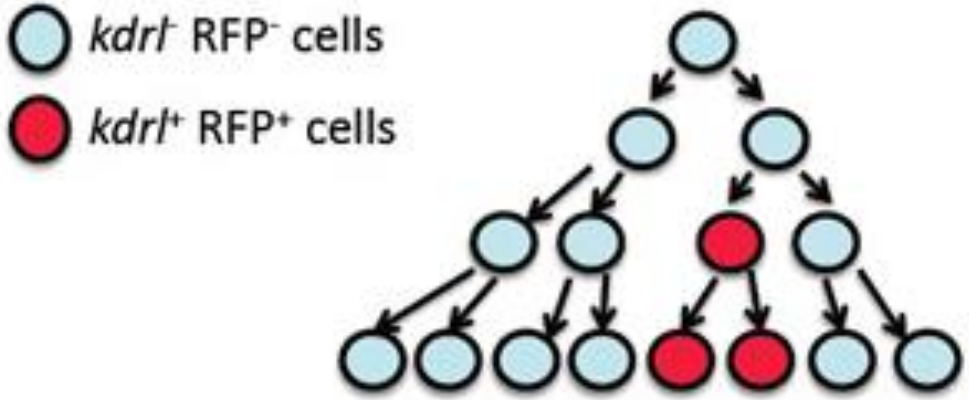
- Which is the colour of the transfected cells when TSP is OFF?
- Which is the colour of the transfected cells when TSP is ON?

In developmental biology, **fate mapping** is a method to identify the embryonic origin of various tissues in the adult organism by establishing the correspondence between individual cells (or groups of cells) at one stage of development, and their progeny at later stages of development. When carried out at single-cell resolution, this process is termed **cell lineage tracing**.

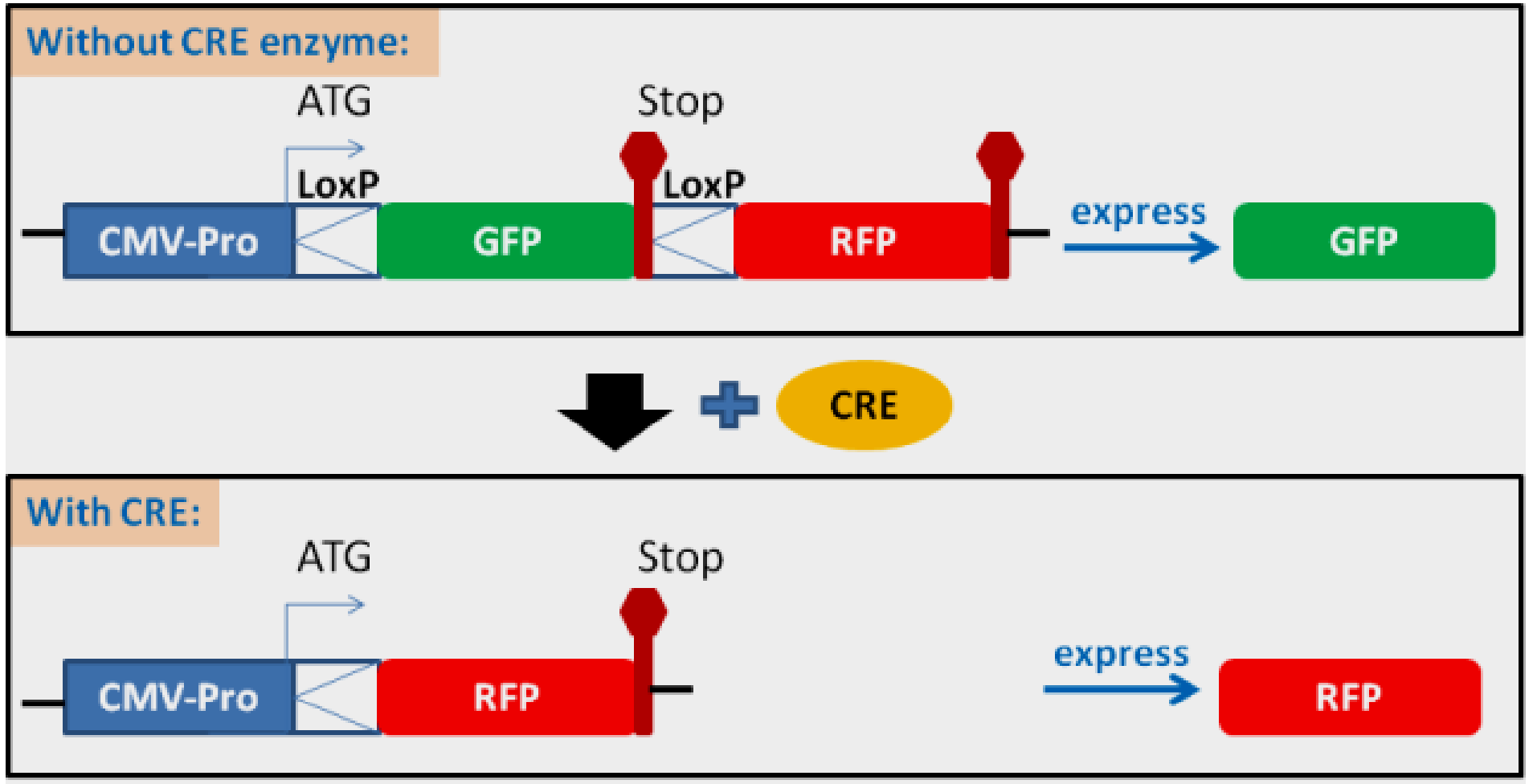
Figure2: Bertrand & Chi use Cre-lox System to find the endothelial origin of hematopoietic stem cells.



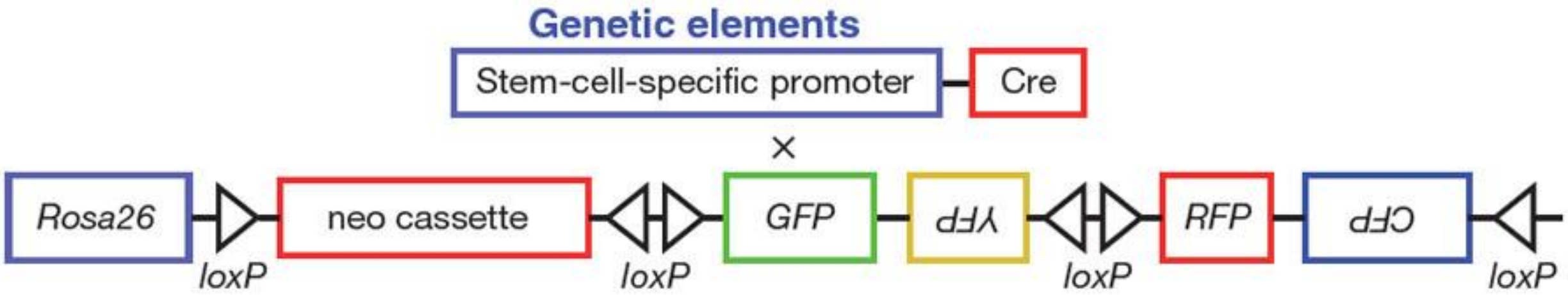
Hierarchical Cell Fate Map, all progeny of a *kdrl*$^{+}$ cell will be permanently RFP^{+}, even once cells turn off kdrl and turn on hematopoietic stem cell markers such as *cmyb*.



kdrl=vascular endothelial growth factor receptor kdr-like

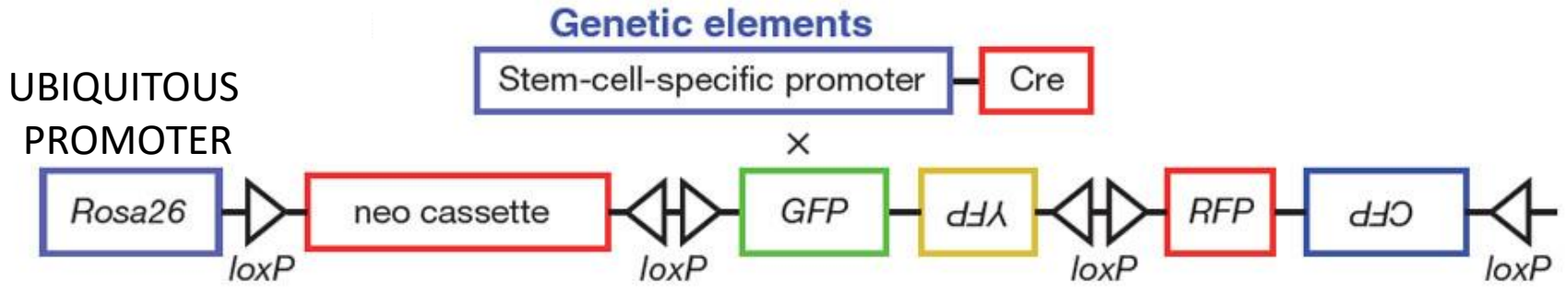

Without CRE enzyme:
ATG
Stop
LoxP
LoxP
CMV-Pro
GFP
RFP
express
GFP
CRE
With CRE:
ATG
Stop
CMV-Pro
RFP
express
RFP

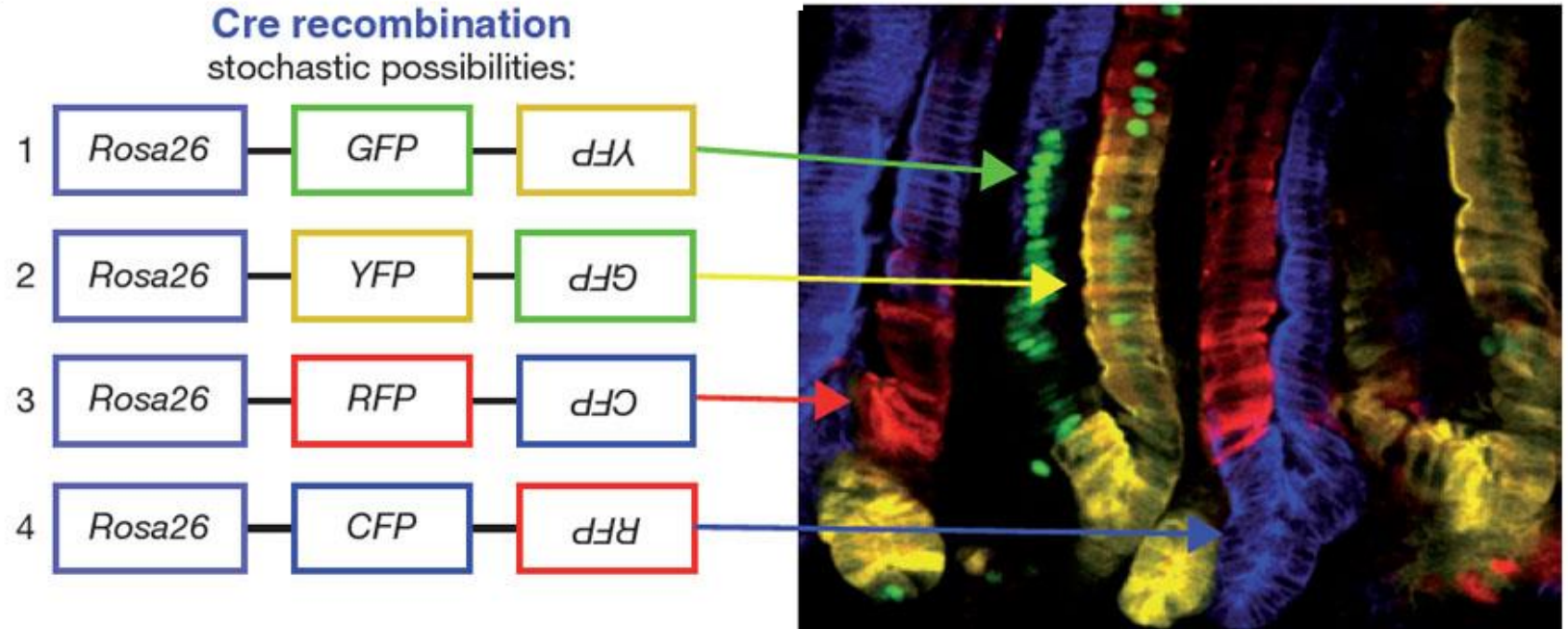
What color will the cells be? **GREEN**, **YELLOW**, **RED** OR **CIANO**?



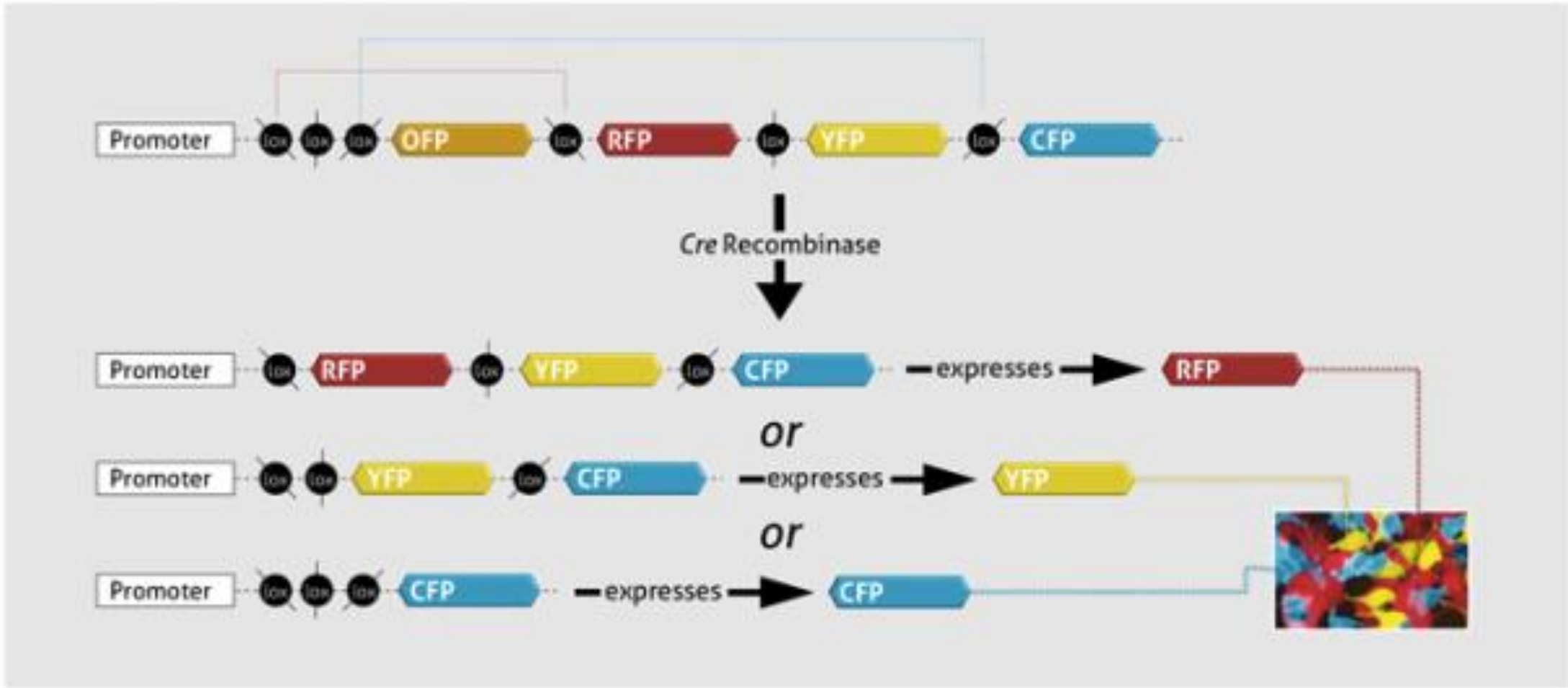
wooclap 1

Cre-recombinase-mediated multicolour reporter transgenic line for lineage tracing and clonal analysis, as a genetic strategy to mark cells with multiple fluorescent proteins.

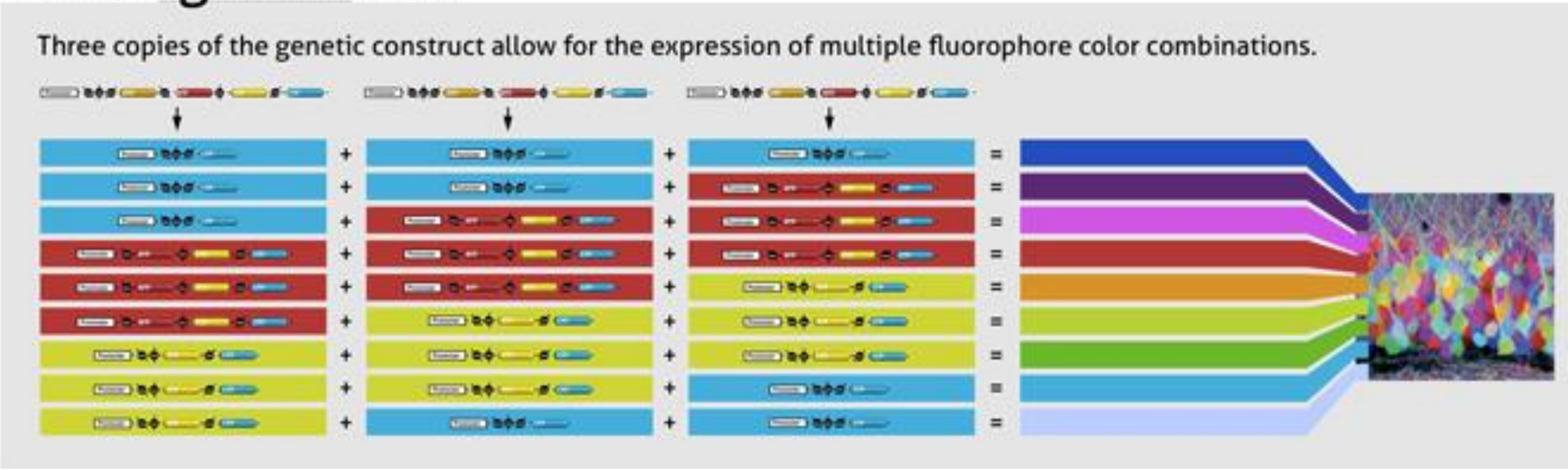




Basic Genetic Construct



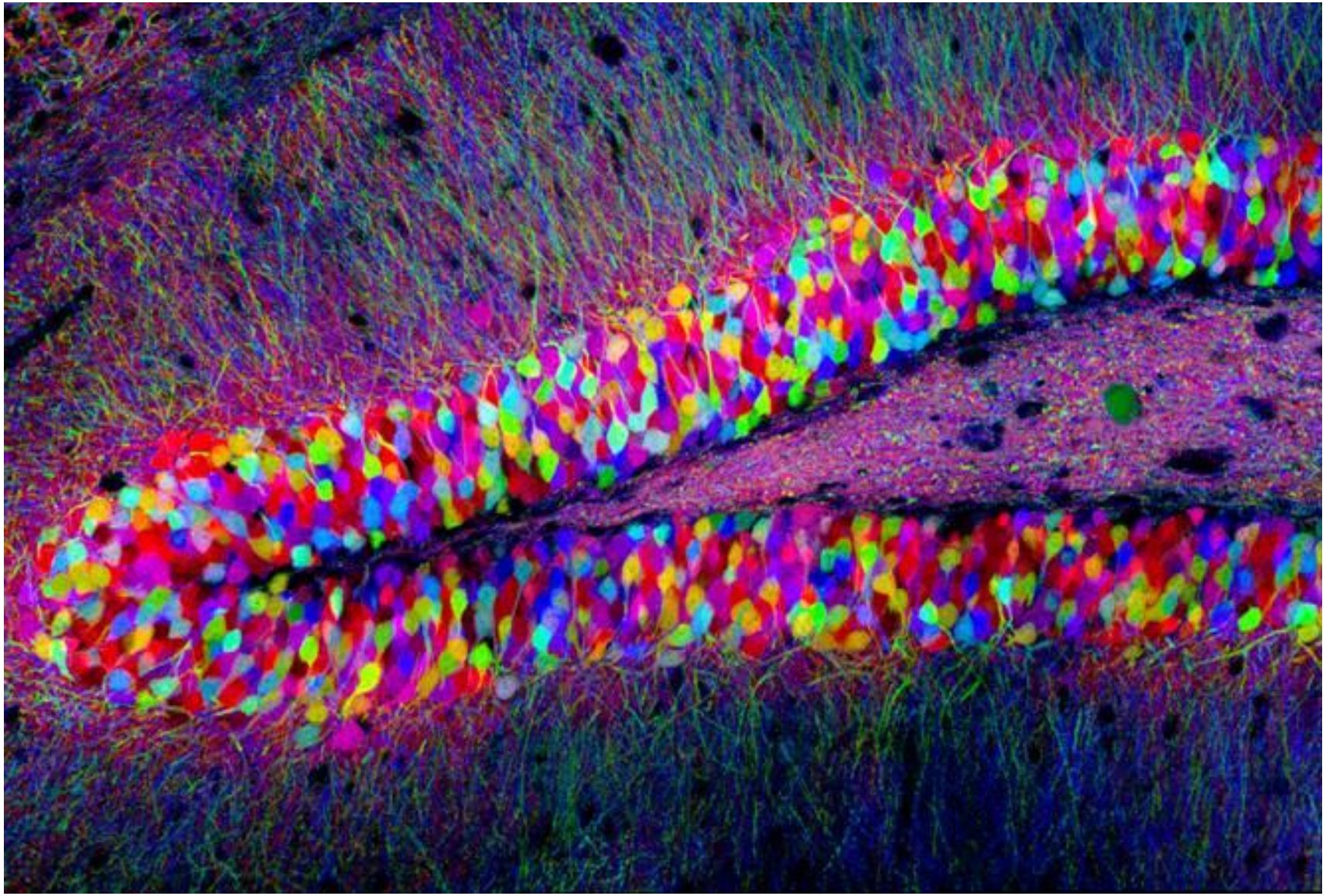
Building Brainbow



"Brainbow" mice are engineered with a gene that includes three different fluorescent proteins, but only one color is actually expressed from each copy of the DNA construct. Pairs of "incompatible lox sites" are nested around different portions of the gene, allowing for recombination to snip out different parts of the gene randomly. Depending on what DNA is excised, a different color results.

https://www.cell.com/pictureshow/brainbow

Hippocampus



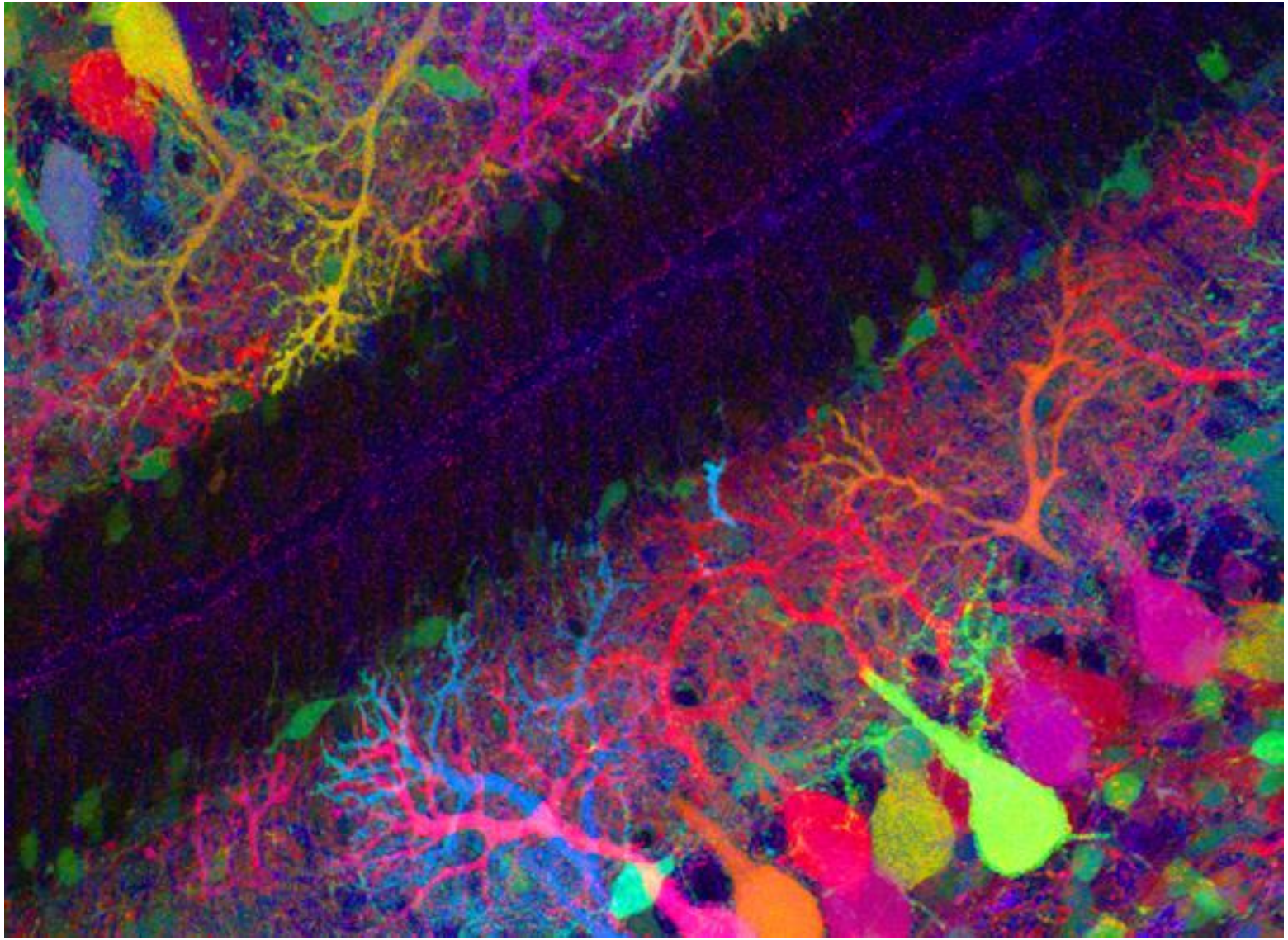
by Tamily Weissman, Harvard University

"Brainbow" mice engineered with a gene that includes three different fluorescent proteins, but only one color is actually expressed from each copy of the DNA construct.

Image: Here individual neurons of the dentate gyrus, a layer of the hippocampus, project their dendrites to the outer layer, where they receive input from the cortex. Neurogenesis occurs inside the "V," where neurons are born and then migrate outward toward the dentate gyrus.

Purkinje Neurons

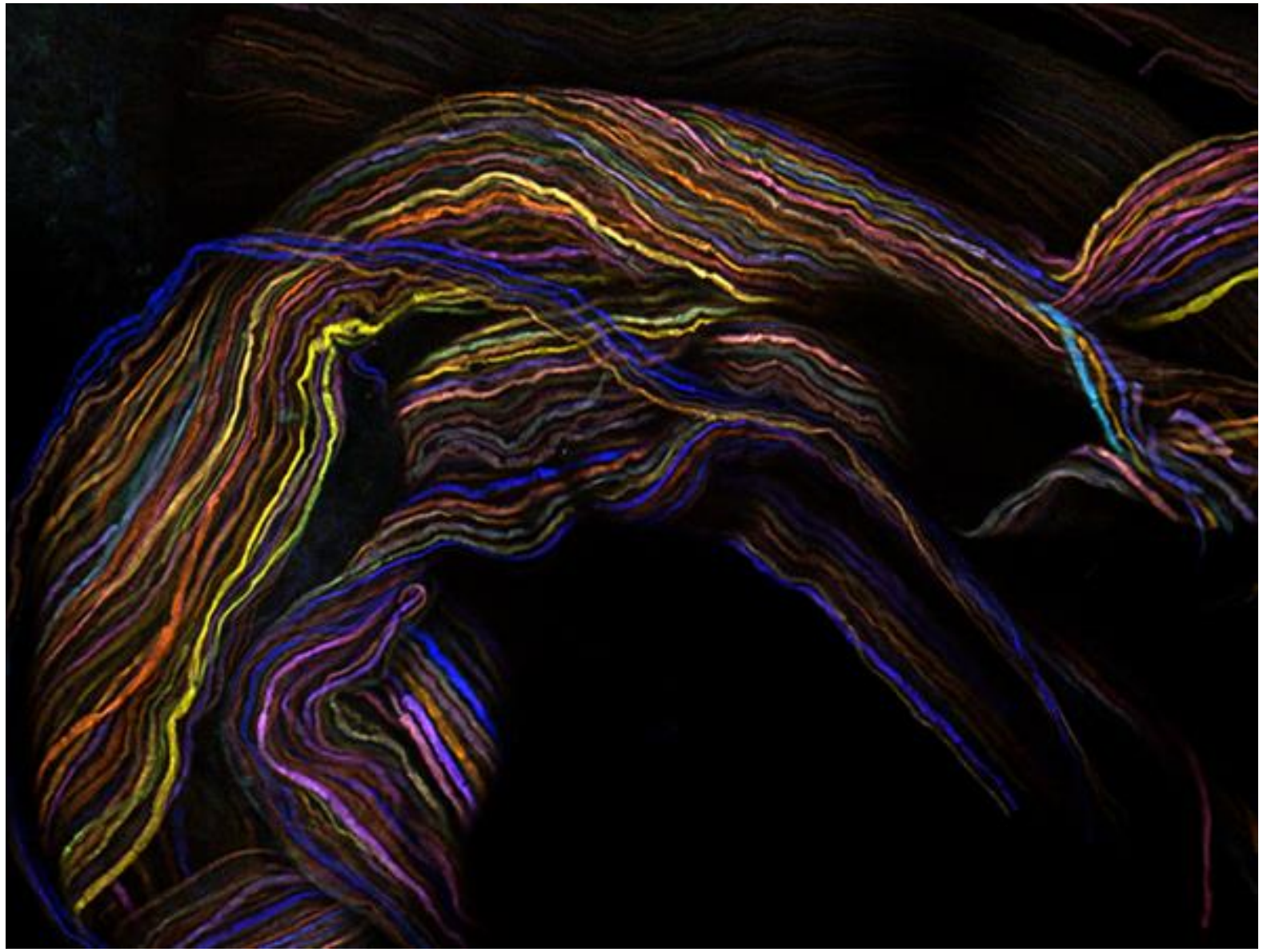
by Tamily Weissman, Harvard University



In some mouse lines, cells express many different copies of the "Brainbow" gene, and each one may be excised differently so that each cell expresses a unique combination of the 3 fluorescent proteins. The 3 primary colors can combine together to produce a huge number of colors, similar to how a color TV works.

Image: Purkinje neurons play an essential role in motor function. Here the Purkinje neurons reach their arbor-like dendrites into the molecular layer of the developing cerebellum of a mouse. The mostly green cells at the bottom left are cerebellar granule cells, which relay information from the nervous system to the Purkinje neurons.

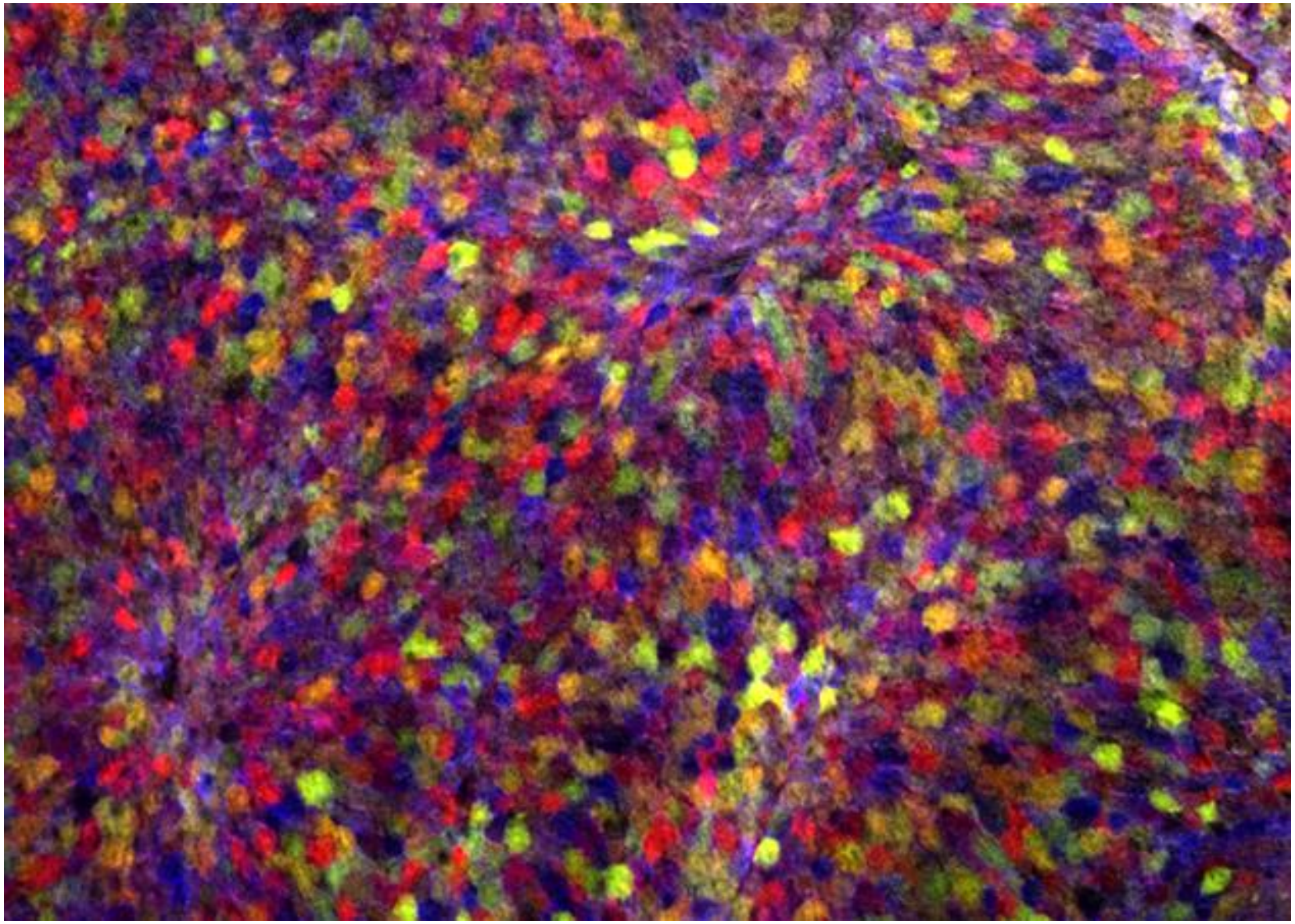
Axonal Waves



by Dawen Cai, Josh R. Sanes, Jeff W. Lichtman, Harvard University

A nerve comprises axons of peripheral sensory neurons bundled together as ribbon. Here the axonal bundles of the dorsal root ganglions are imaged by fluorescence line scanning confocal microscope (Zeiss LSM710) equipped with multiple lasers.

To create this image, a new generation of "Brainbow" mouse was created by crossing the original mouse with one containing the Cre gene under the control of the Islet-1 promoter. Enhanced green fluorescent protein, Kusabira-Orange, and mKate2 fluorescent proteins are digitally colored in blue, green and red, respectively.



Confetti Mice

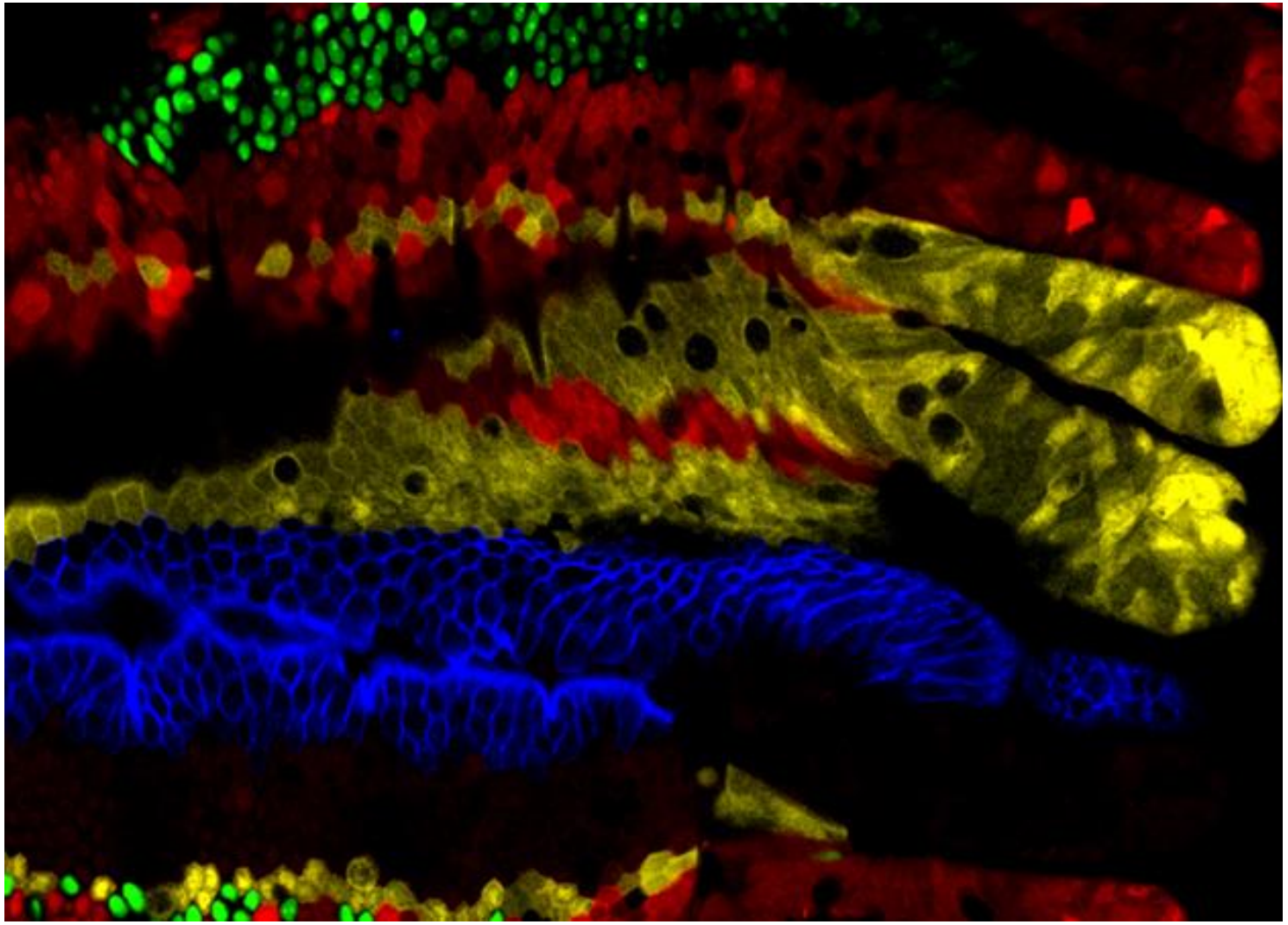
by Hugo Snippert, Hubrecht Institute.

A variation of the "Brainbow" gene adds a transcriptional "roadblock" to inhibit expression of the fluorescent proteins until Cre is activated. By placing Cre under the control of a tissue-specific promoter, the "Brainbow" effect can be activated in one tissue of the mice. This is called the "Confetti" mouse.

Image: Here the "Confetti" gene is transiently activated in all liver cells in a mouse ~4 weeks of age. As a result, each cell independently recombines the "Confetti" gene toward one of four fluorescent proteins. Image was taken after 2 months tracing.

Visualizing the Villi

by Hugo Snippert, Hubrecht Institute

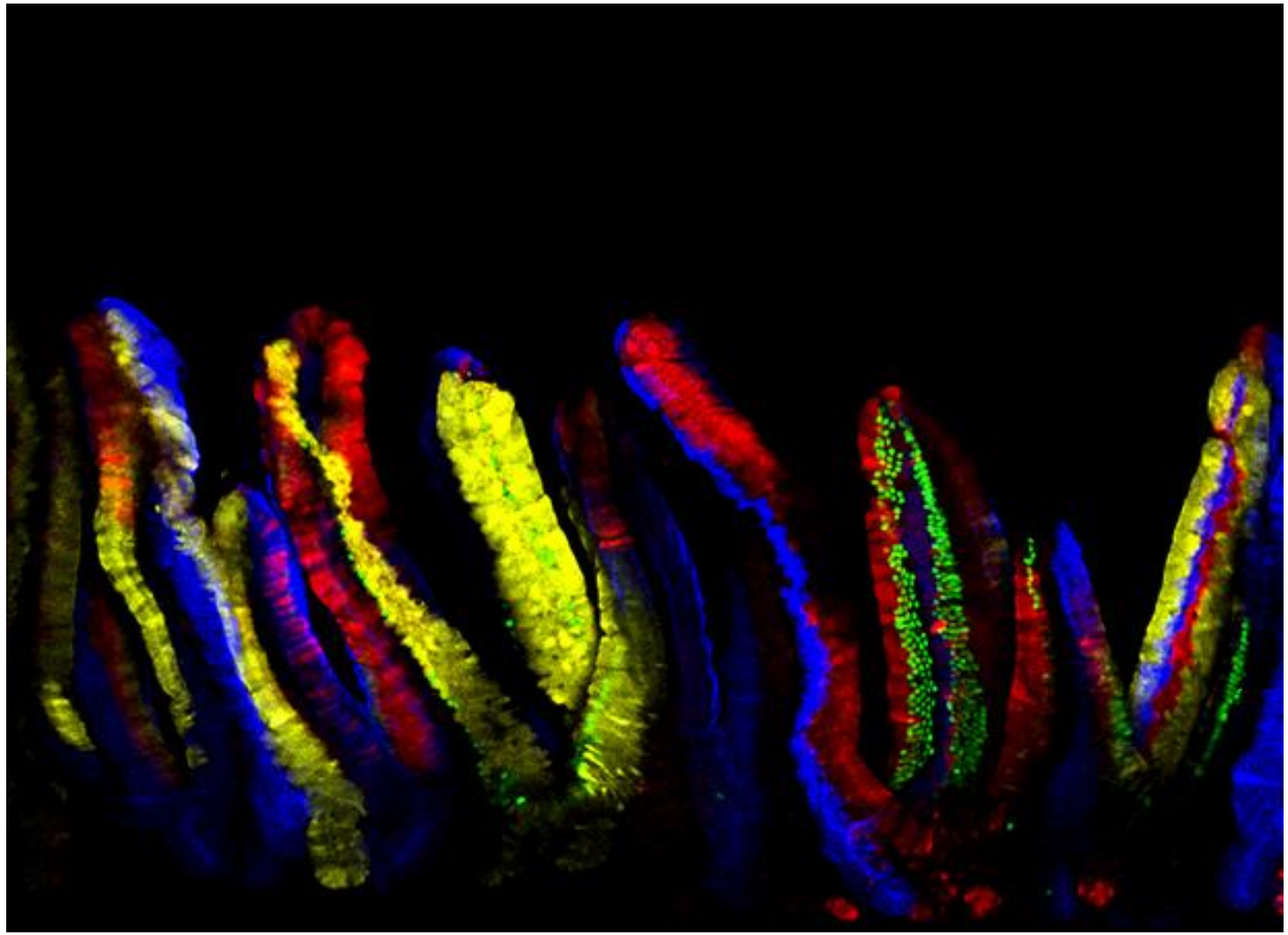


In the "Confetti" mouse, the recombination process is not only independent in each cell but also transient and stochastic. Thus, after recombination each cell continuously produces one fluorescent protein -GFP (green), cytoplasmic YFP (yellow), cytoplasmic RFP (red) or membrane tethered CFP (blue). Eventual daughter cells will produce the same fluorescent protein, creating clonal patches with the same color.

Image: in the small intestine, a layer of epithelial cells covers the finger-like projections of the villi. At the base of each villi, a pocket-like structure called the crypt store intestinal stem cells that replenish lost epithelial cells of the villi. The progeny produced by intestinal stem cells will migrate up to the tip of the villus like clonal conveyor belts.

Competition in the Crypt

by Hugo Snippert, Hubrecht Institute

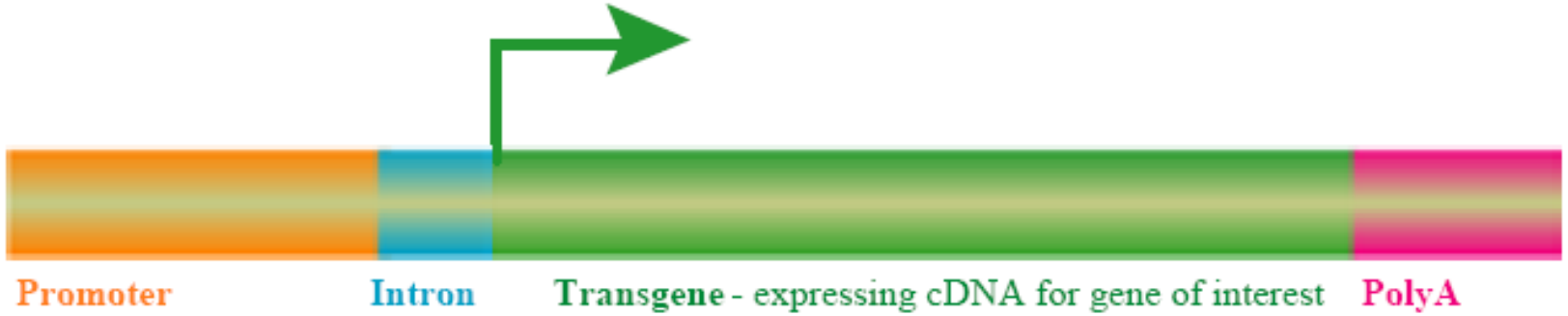


Here is an image of the small intestine, zoomed in on the crypts. The "Confetti" gene was activated in all cells in the intestine, including stem cells. Once the Cre recombination process is over, each stem cell produces progeny that inherit the same fluorescent marking. The number of cells in the crypt is fixed. Thus, over time, stem cells (~14) compete each other out of this niche, and the crypt becomes clonal, visualized here by monochromatic crypts.

Image: This image was taken 4 months after Cre activation using a Leica Sp5 microscope. Clonal conveyor belts from the crypt base to the top of villi are imaged with four different colors at 40x magnification.

CONDITIONAL KNOCK-OUT, CRE-LOX TECHNIQUE, INDUCIBLE SYSTEMS TO STUDY

- **the over-expression of a protein** (ubiquitous / **tissue specific** / **inducible**)
- the regulation of a promoter?
- **the lack of a protein** (ubiquitous / **tissue specific** / **inducible**)
- the localization of a protein?
- ..... ?



- How can you discriminate between endogenous and exogenous proteins?

- How can you study the activity of a mutated exogenous protein without the expression of the wild type endogenous protein?

INDUCIBLE SYSTEMS TO REGULATE CRE RECOMBINASE ACTIVITY

- Tamoxifen

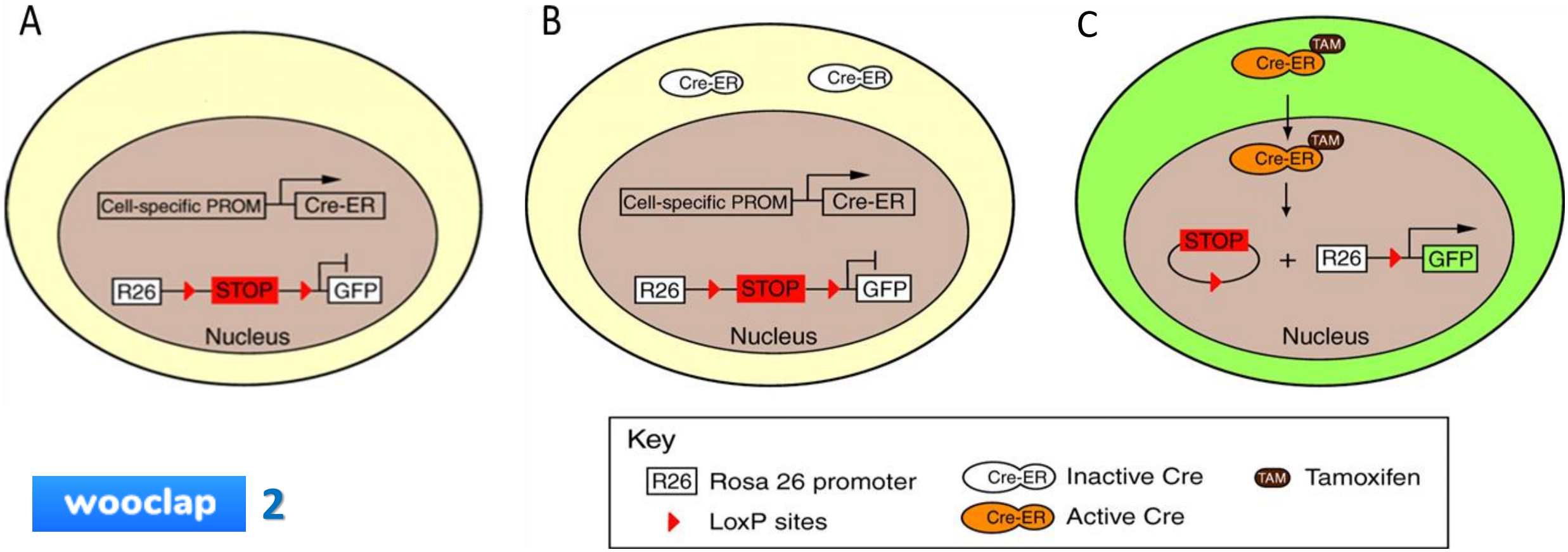
Inducible systems to regulate CRE recombinase expression
or expression of a gene of interest

- Tet-OFF
- Tet-ON

- Virus mediated expression

Inducible Cre activity has been achieved by fusing Cre to a mutant form (**ER™**) of the **estrogen receptor** .

- Cre-ER™ is insensitive to the natural ligand (17β-estradiol), but functions as a specific receptor for the synthetic ligand 4-hydroxy-**tamoxifen**.
- in the absence of tamoxifen, Cre-ER™ is sequestered in the cytoplasm, complexed with hsp90.

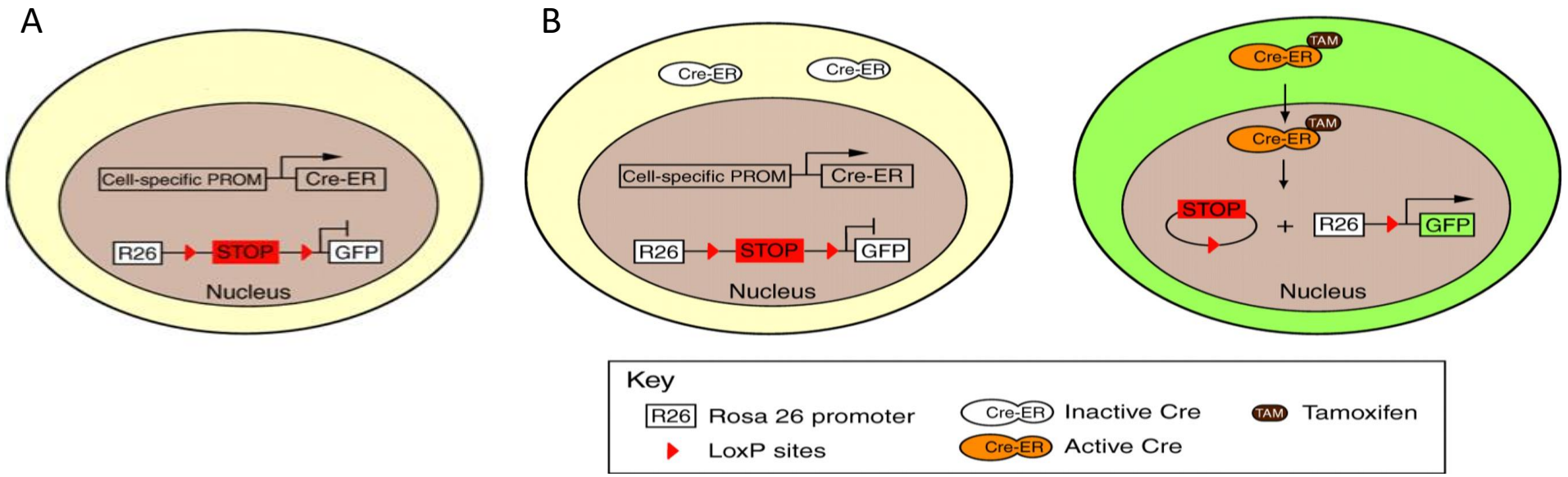


wooclap 2

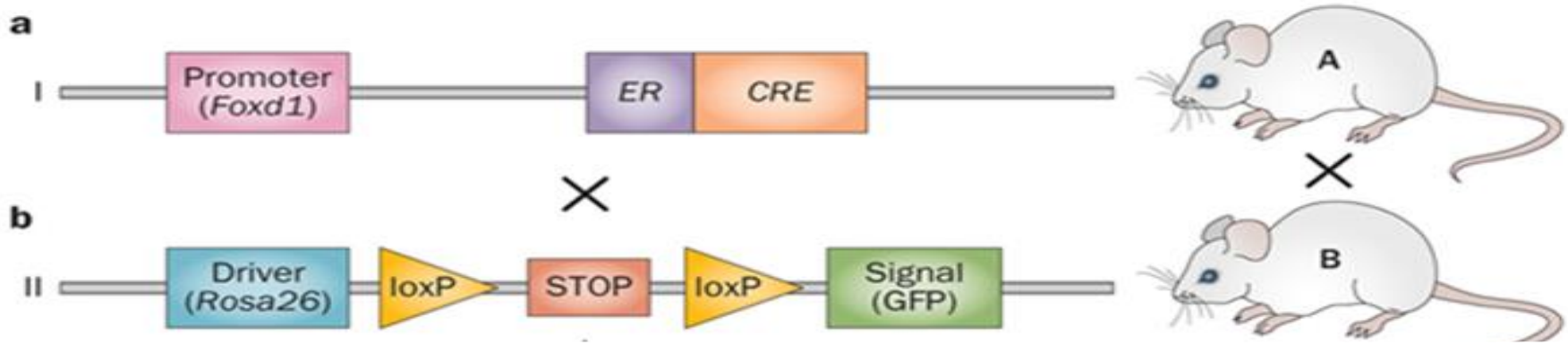
Inducible Cre activity has been achieved by fusing Cre to a mutant form (**ER™**) of the **estrogen receptor** .

Cre-ER™ is insensitive to the natural ligand (17β-estradiol), but functions as a specific receptor for the synthetic ligand 4-hydroxy-**tamoxifen**.

- in the absence of tamoxifen, Cre-ER™ is sequestered in the cytoplasm, complexed with hsp90
- binding to tamoxifen disrupts the interaction with hsp90
- permits the translocation of Cre-ER™ into the nucleus
- where it catalyzes *loxP*-specific recombination events



TISSUE SPECIFIC & INDUCIBLE GENE SWITCHING ON



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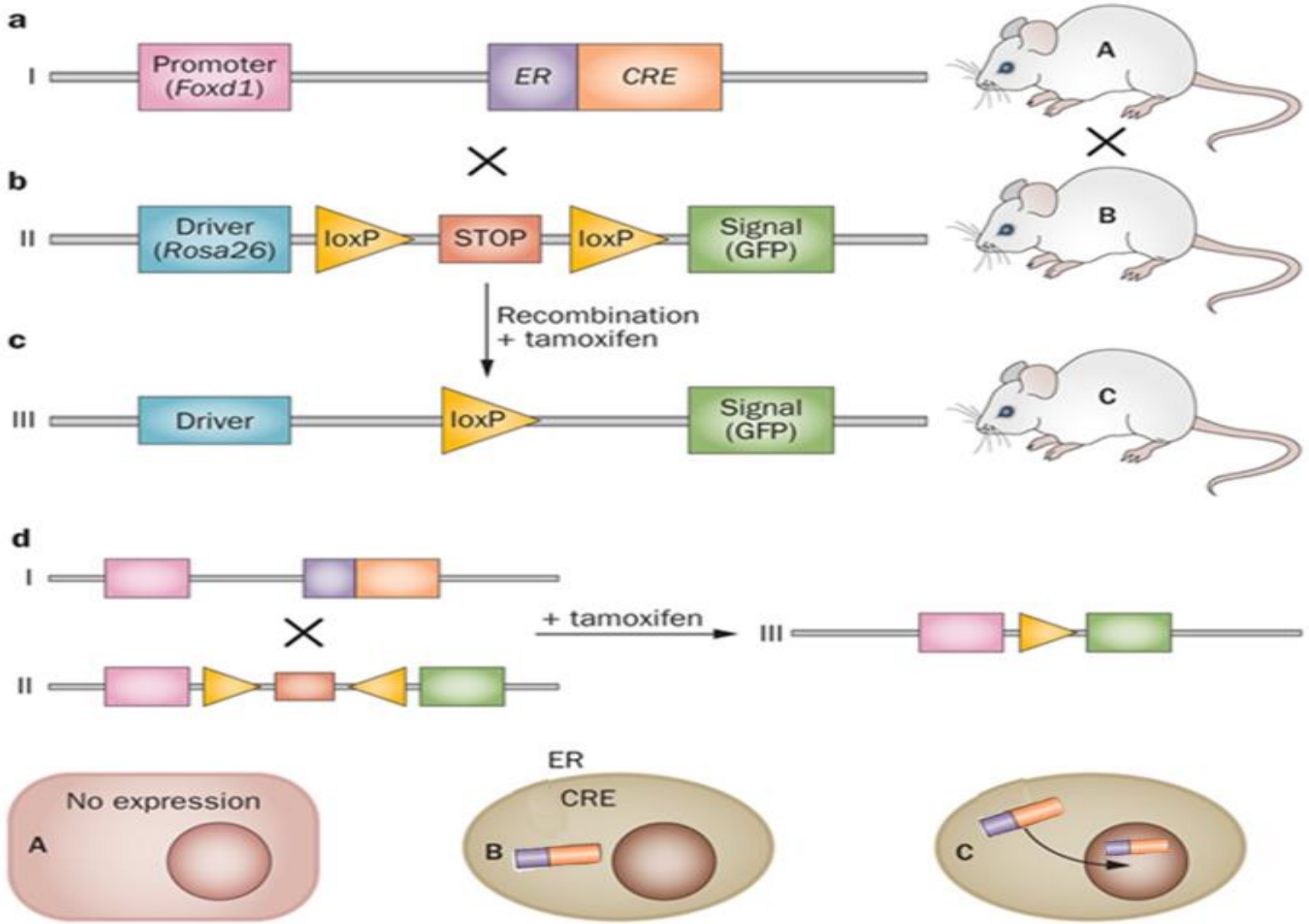

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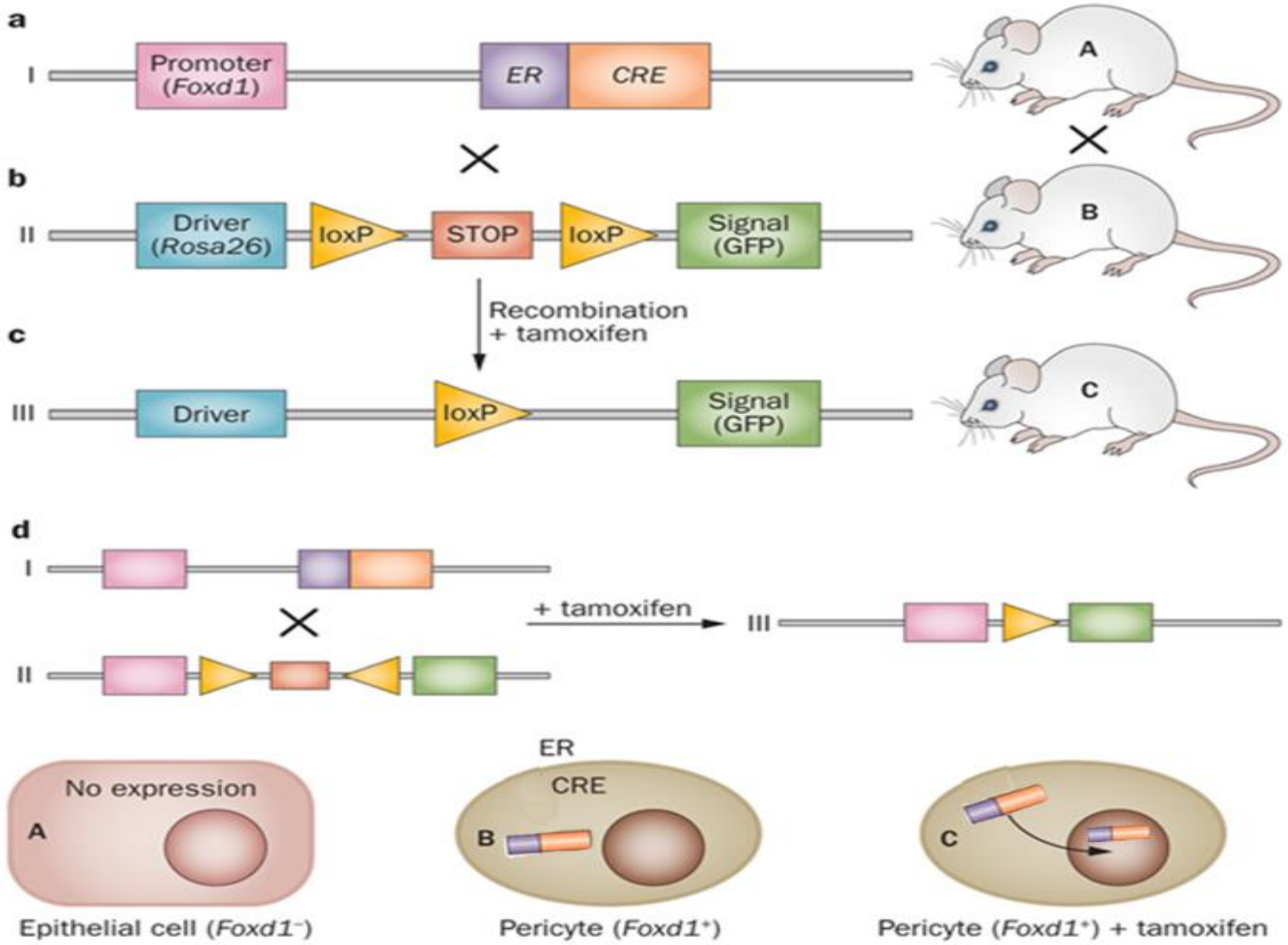

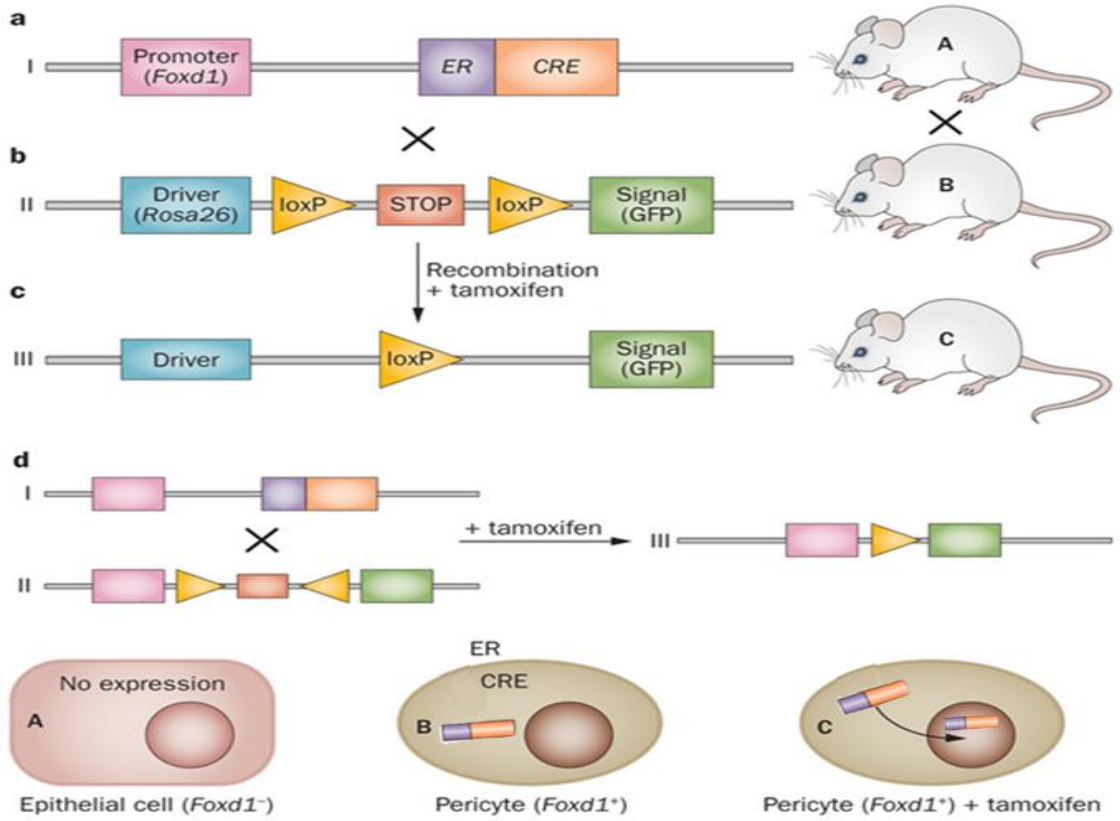

a | Transgenic mouse "A" contains Cre recombinase capable of recognizing *loxP* sites, under the control of a specific tracer–marker promoter (in this case *Foxd1*). The Cre recombinase is only expressed in cells that are positive for the marker.

b | Transgenic mouse "B" contains a signalling sequence (in this case GFP) that is driven by a ubiquitous driver sequence (*Rosa26*) that is normally prematurely terminated by an inserted stop codon. The stop sequence is flanked by *loxP* sites.

c | Crossbreeding mouse "A" and "B" can produce transgenic mouse "C" in which the tracer or marker promoter driven Cre recombinase recognizes the *loxP* sites flanking the stop sequence, upon which the latter is excised. This excision allows the driver sequence to complete transcription and translation of the signalling cassette, thus allowing for specific lineage tracing.

d | An epithelial cell not expressing *FoxD1* does not have a tamoxifen-specific modified oestrogen receptor Cre recombinase (ER/Cre) fusion protein expressed at the cell membrane. Consequently, activation of the ER with tamoxifen does not occur and does not lead to fusion protein translocation to the nucleus and signal protein expression by recombining the stop sequence out of the DNA. In pericytes, however, *FoxD1* driven ER/Cre fusion protein expression does occur. Subsequently, tamoxifen injection ultimately leads to permanent signal protein expression allowing for timed FOXD1 specific tracing of pericytes. Abbreviations: FOXD1, forkhead box D1; GFP, green fluorescent protein.