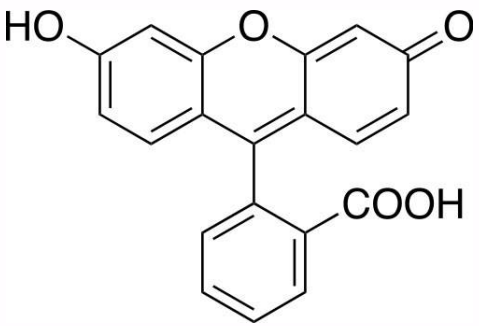
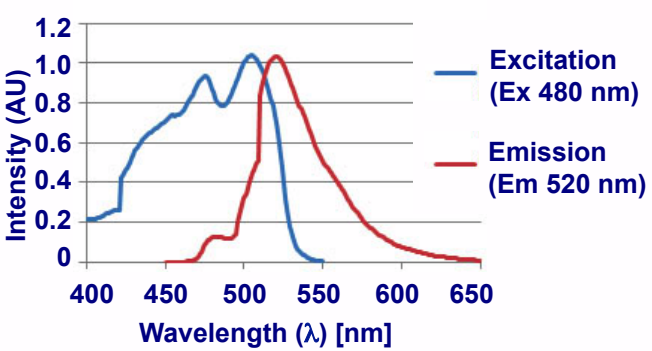

Tracking the inner workings of cells
using fluorescent dyes and proteins

Fluorescein, a green fluorophore

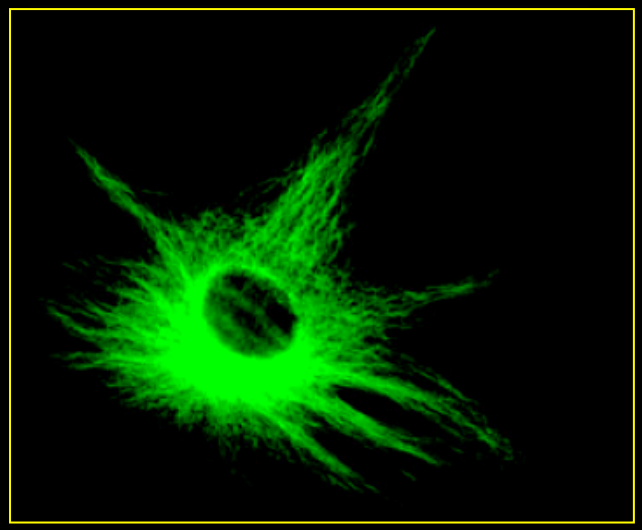


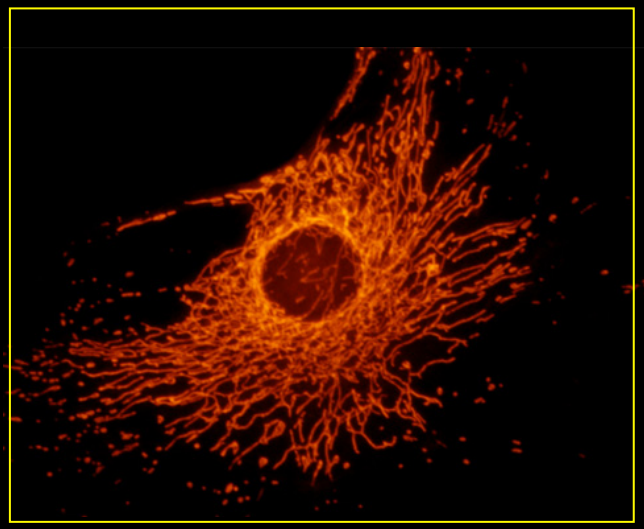
Fluorescein Chemical Structure



Fluorescein is a synthetic organic compound available as a dark orange/red powder soluble in water and alcohol. It is a fluorophore which is widely used as a fluorescent tracer for many applications.

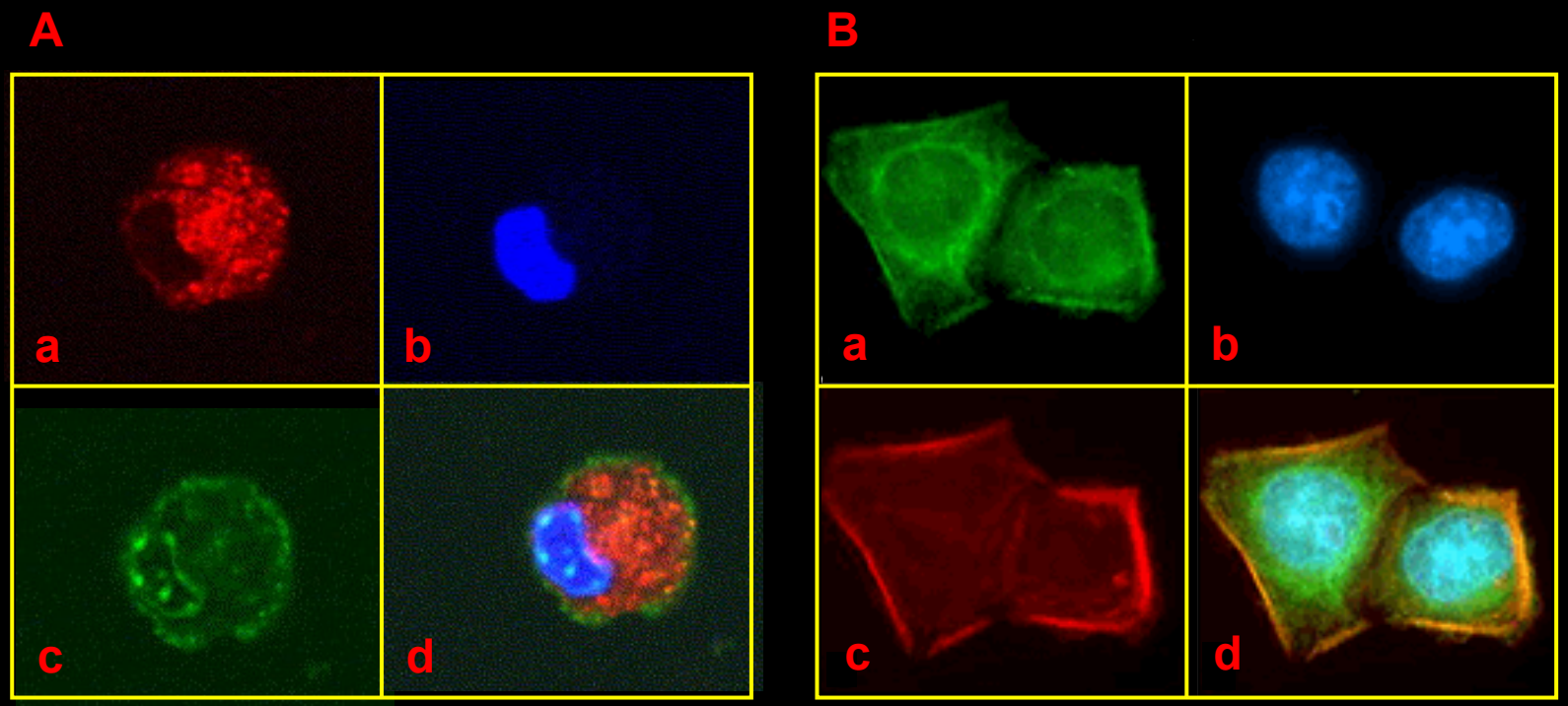
Immunofluorescence





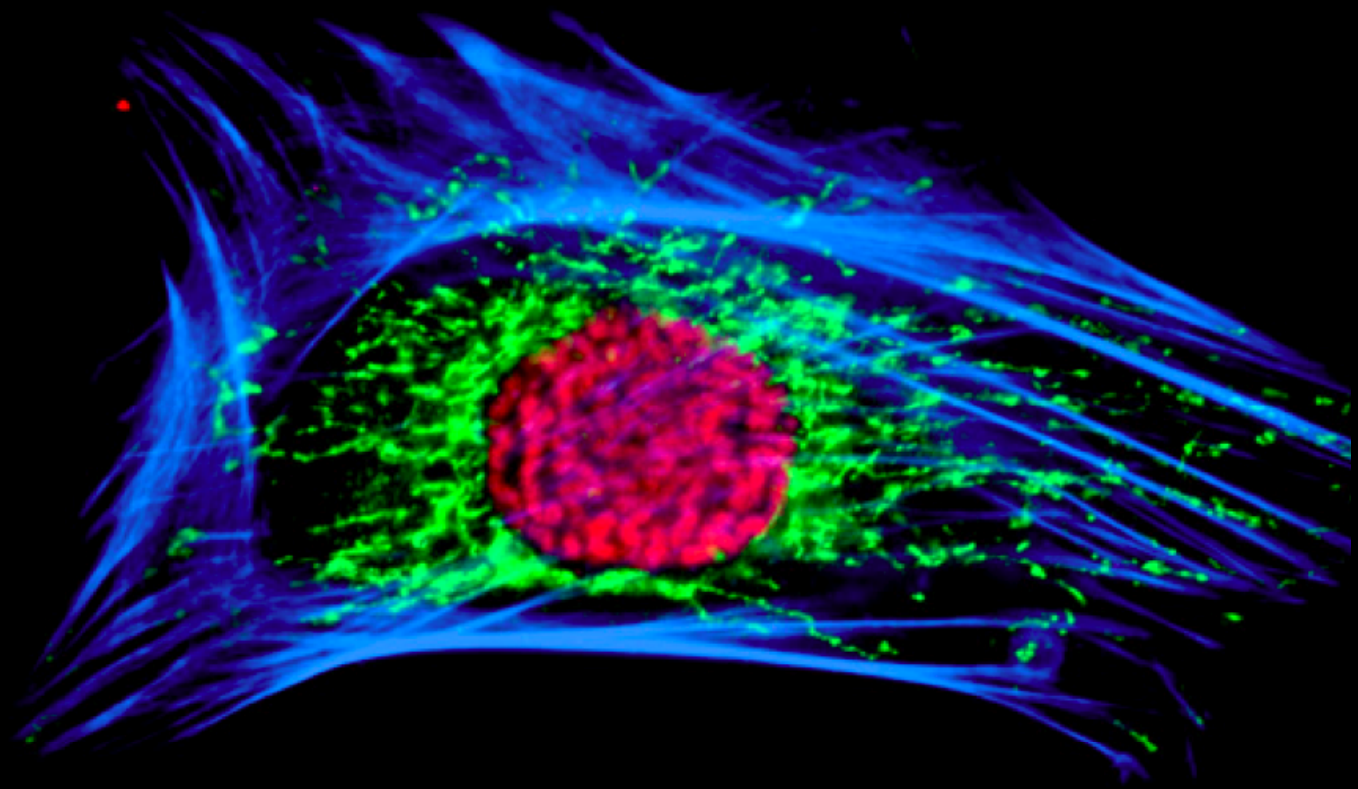
Staining of bovine pulmonary artery endothelial cell microtubules with a mouse anti-α-tubulin monoclonal antibody (mAb) conjugated to different fluorophores.

Immunofluorescence

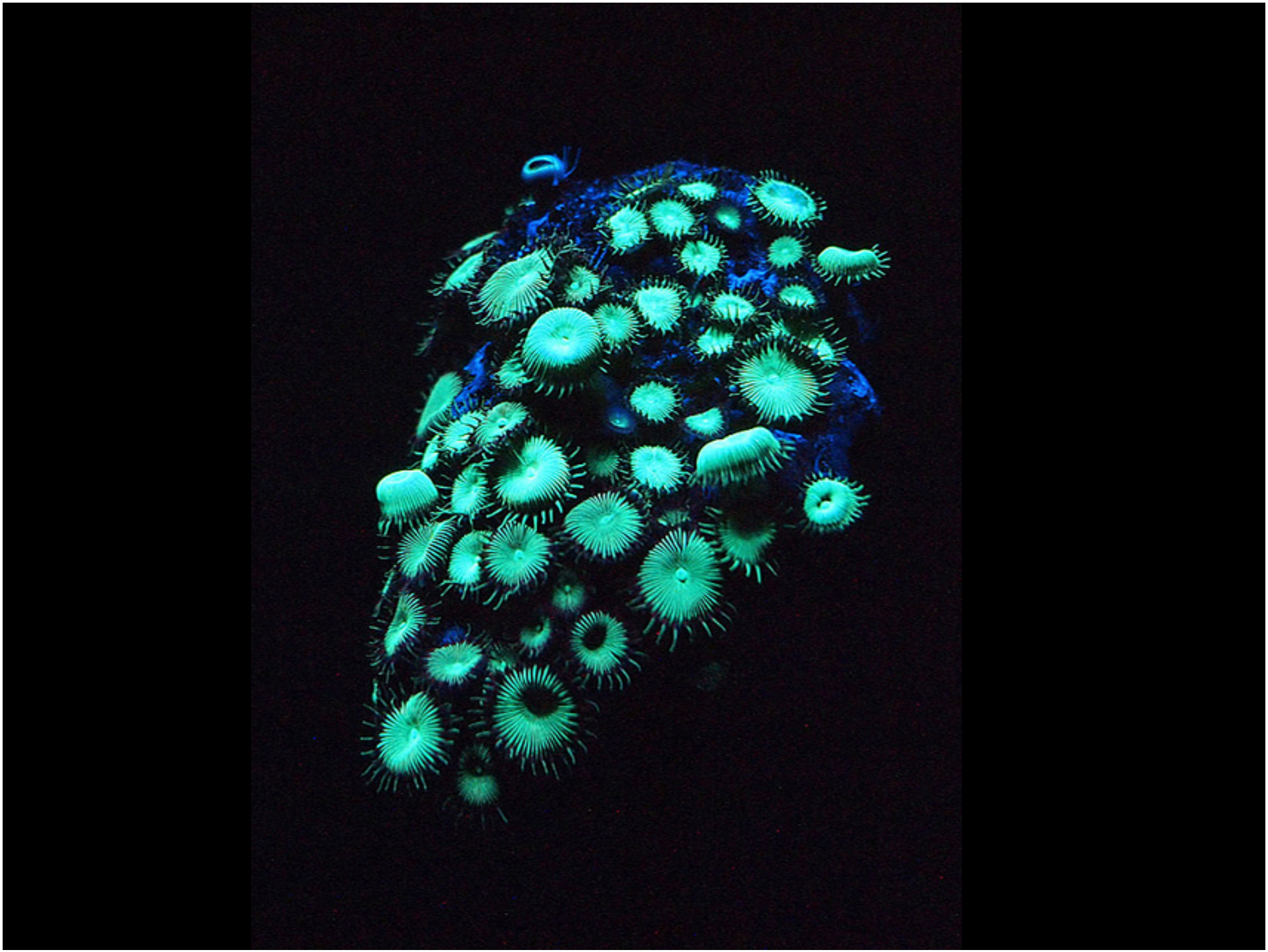


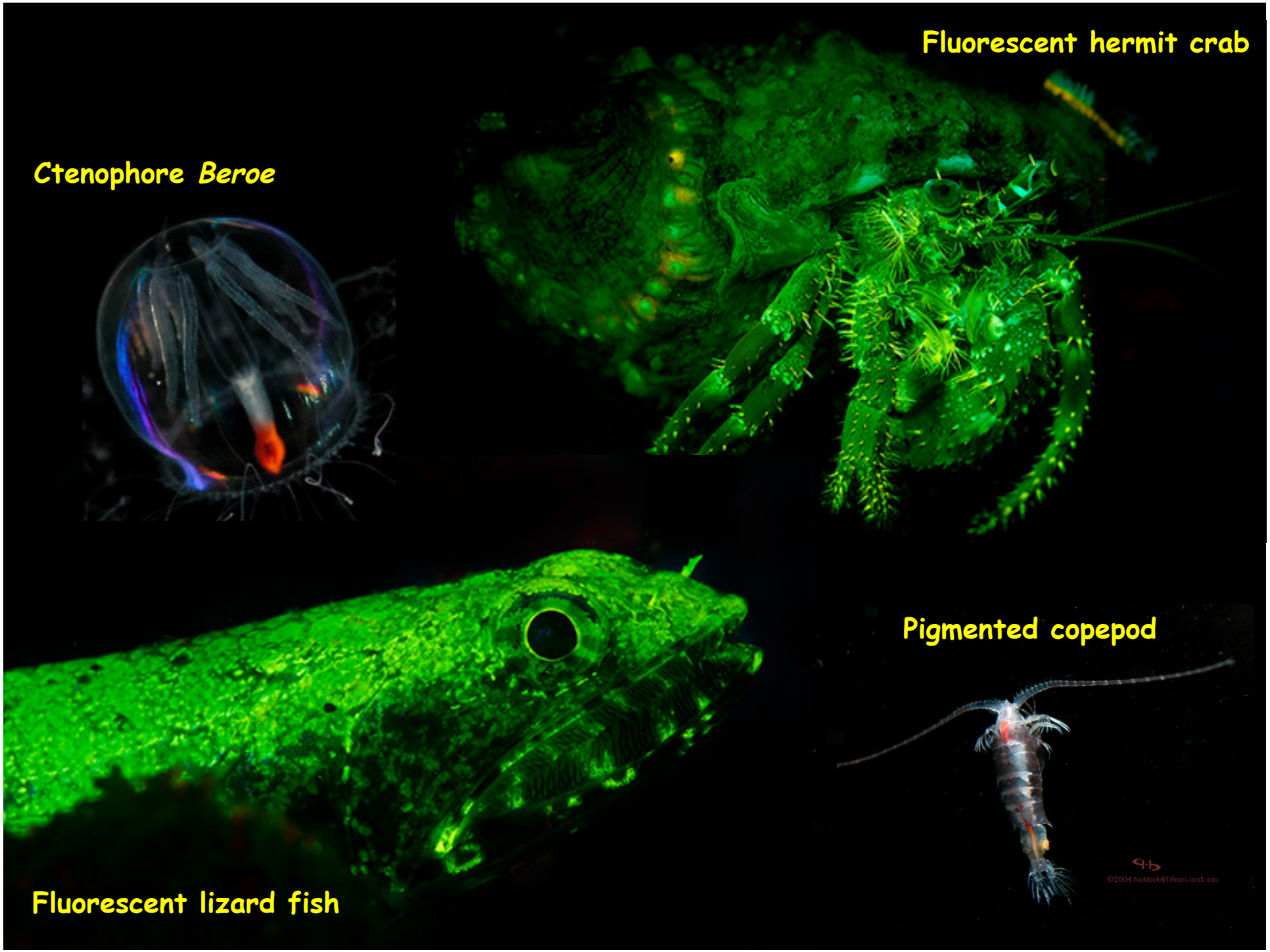
Cell staining of the cytoplasm (a), nucleus (b) and plasma membrane (c). Panel d shows overlay of panels a-c.

Immunofluorescent cell staining



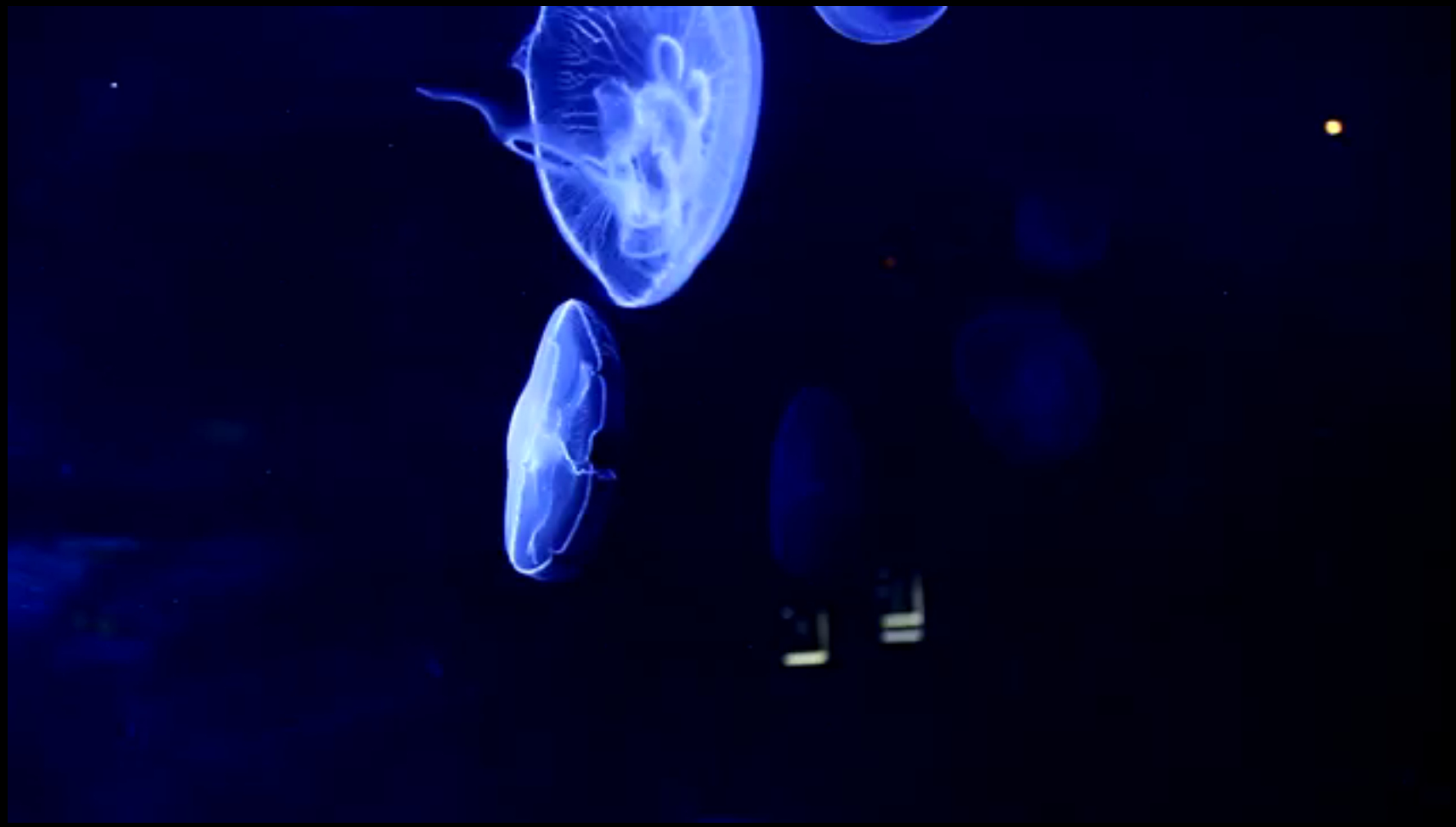
Fixed and permeabilized fibroblast stained with Alexa Fluor 350-phalloidin (stains F-actin (cytoskeleton) in blue), Alexa Fluor 488-conjugated anti-OxPhos Complex V inhibitor protein Ab (stains mitochondria in green), and Alexa Fluor 568-conjugated anti-cdc6 Ab (stains nuclear regions in red).




Fluorescent hermit crab
Ctenophore *Beroe*
Pigmented copepod
Fluorescent lizard fish
©2004 haddock@lifesci.ucsb.edu

A Nudibranch creature stands out against the dark background in Tarragona, Spain

Study of a jellyfish that revolutionized biosciences and lead to a Nobel Prize in Chemistry



A bioluminescent jellyfish Aequorea victoria

The Nobel Prize in Chemistry 2008

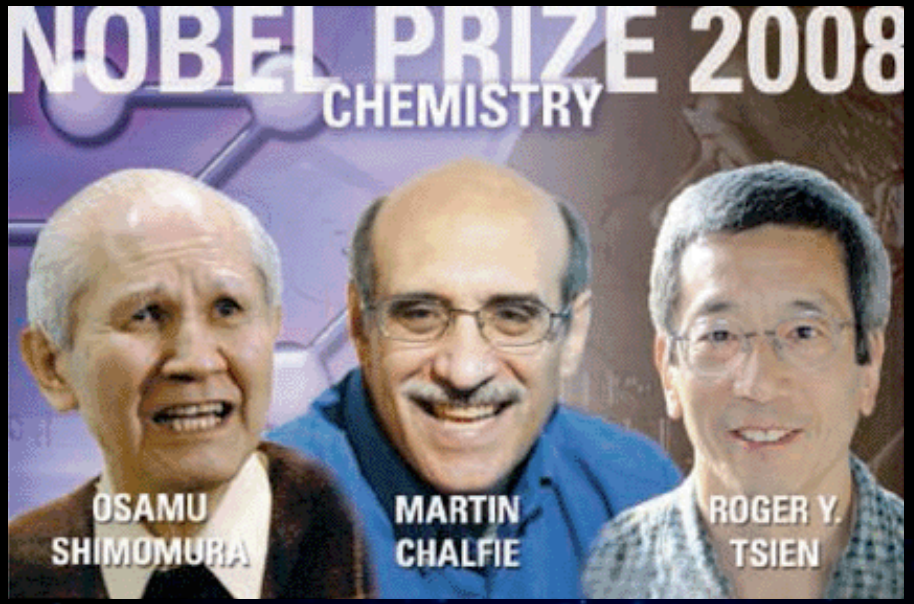
In the 1960s, when the Japanese scientist Osamu Shimomura began to study the bioluminescent jellyfish Aequorea victoria, he had no idea what a scientific revolution it would lead to.

Thirty years later, Martin Chalfie used the jellyfish's green fluorescent protein to help him study life's smallest building block, the cell.

Today, scientists are able to study biological processes that were previously invisible with the aid of Roger Y. Tsien's proteins, which glow in all colors of the rainbow.

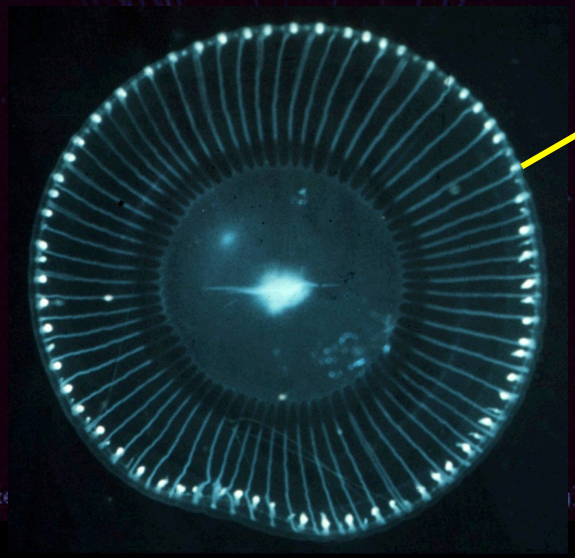
Douglas Prasher, who was the first to clone and use the GFP gene as a means to trace proteins in vivo, did not get the Nobel, which can be shared by up to 3 scientists.

The Nobel Prize in Chemistry 2008

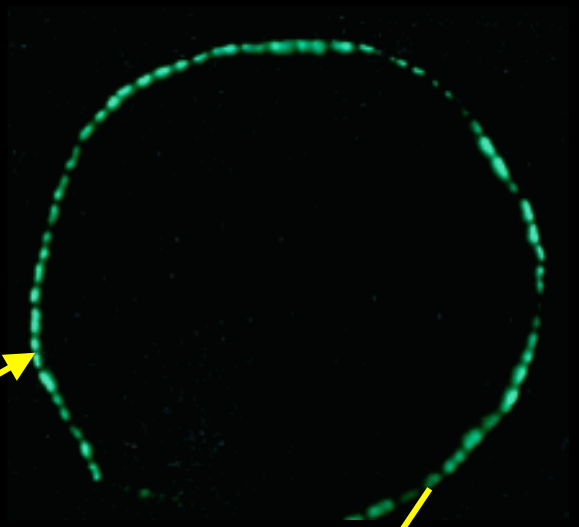


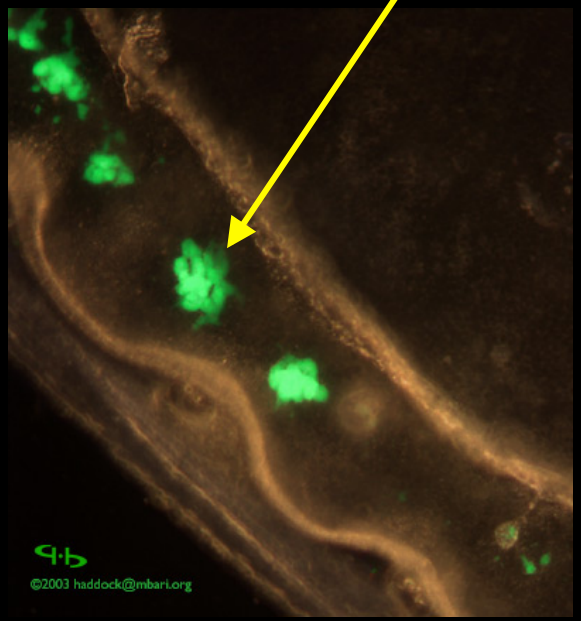
Aequorea Victoria

Side view



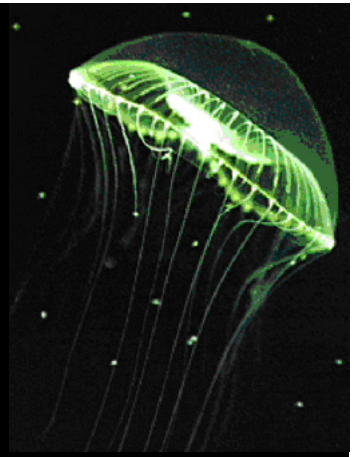
Bottom view





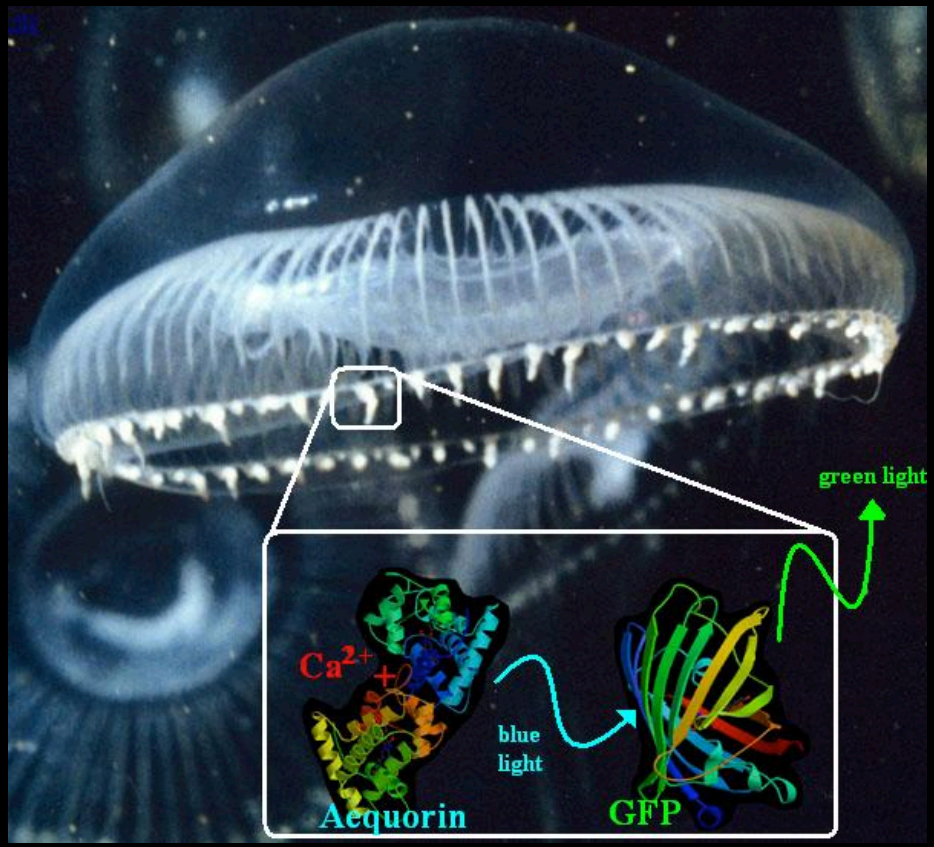
Ca^{2+} > aequorin > blue fluorescence > GFP > green fluorescence

Green fluorescence protein (GFP)



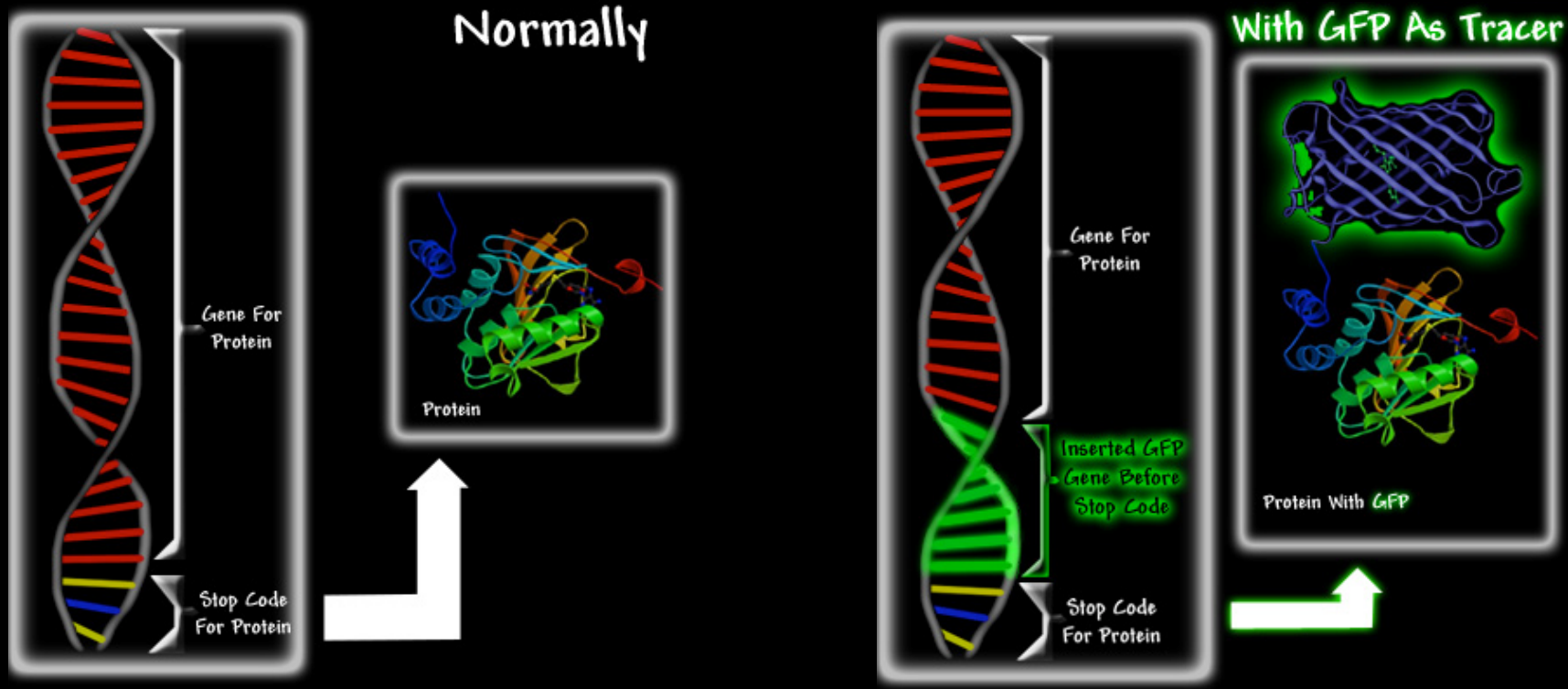
Osamu Shimomura studied the bioluminescence of the crystal jellyfish, *Aequorea victoria*, and was the first to isolate GFP and to find out which part of GFP was responsible for its fluorescence.

The *Aequorea victoria* jellyfish produces green bioluminescence from small photoorgans located on its umbrella. Osamu Shimomura found that in order to bioluminesce Aequorea releases calcium ions. These bind to a protein that he called aequorin, which releases blue light upon calcium binding. The blue light is absorbed by the green fluorescent protein, which in turn emits the green light.

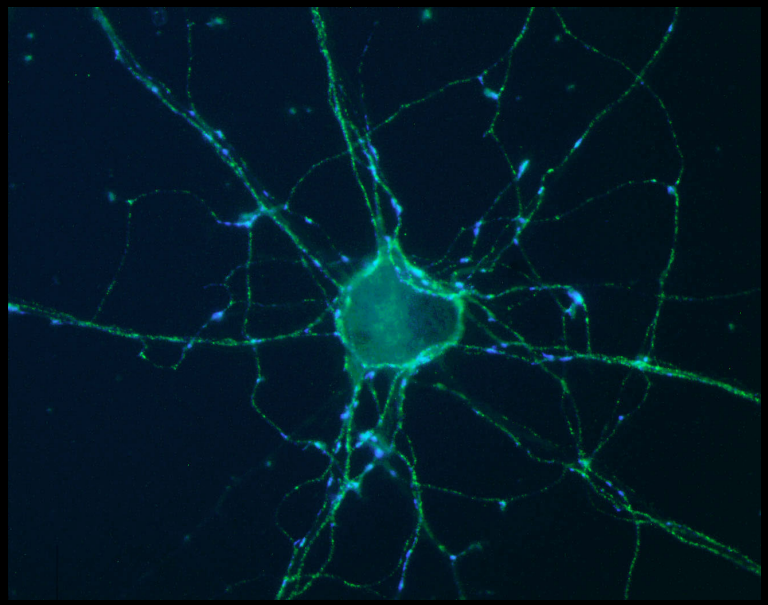


Douglas Prasher was the first to realize the potential of GFP as a tracer molecule.

He was the first to use biomolecular techniques to insert the GFP gene at the end of the hemoglobin gene, right before the stop codon. The cell uses the information encoded in the gene to make hemoglobin, but instead of stopping when the hemoglobin is made, the cell carries on making GFP until it reaches the stop codon at the end of the GFP gene. The result is a chimeric protein that includes hemoglobin with an attached GFP.



GFP in use



www.physiology.wisc.edu/ chapman.html

GFP can be used to tag proteins within a cell or visualize specific cell types in an organism.

Fluorescence lasts ~10 mins when illuminated by 450-490nm.

Tobacco

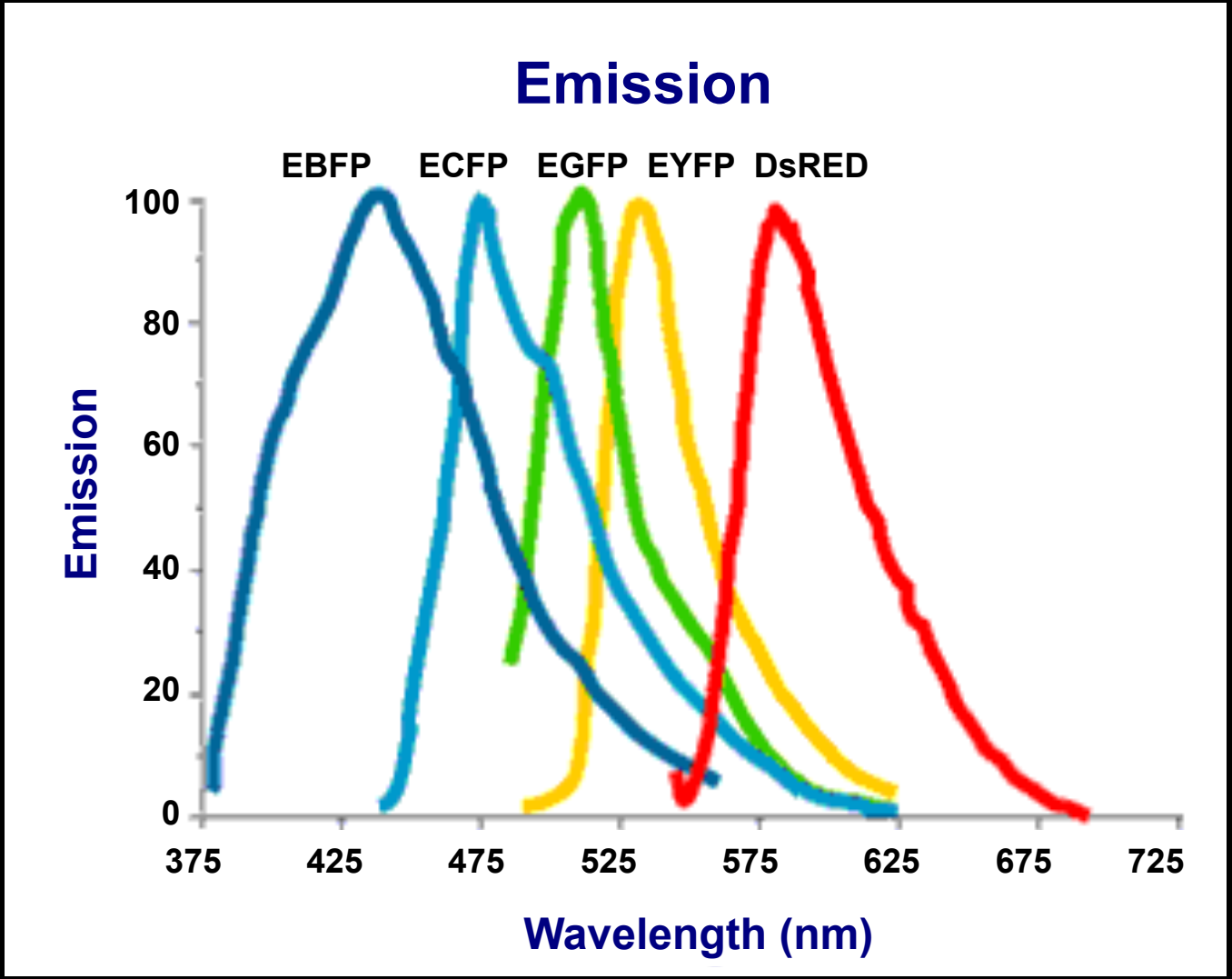
How does it glow?
Firefly luciferase gene, introduced via a virus into tobacco DNA (1986)

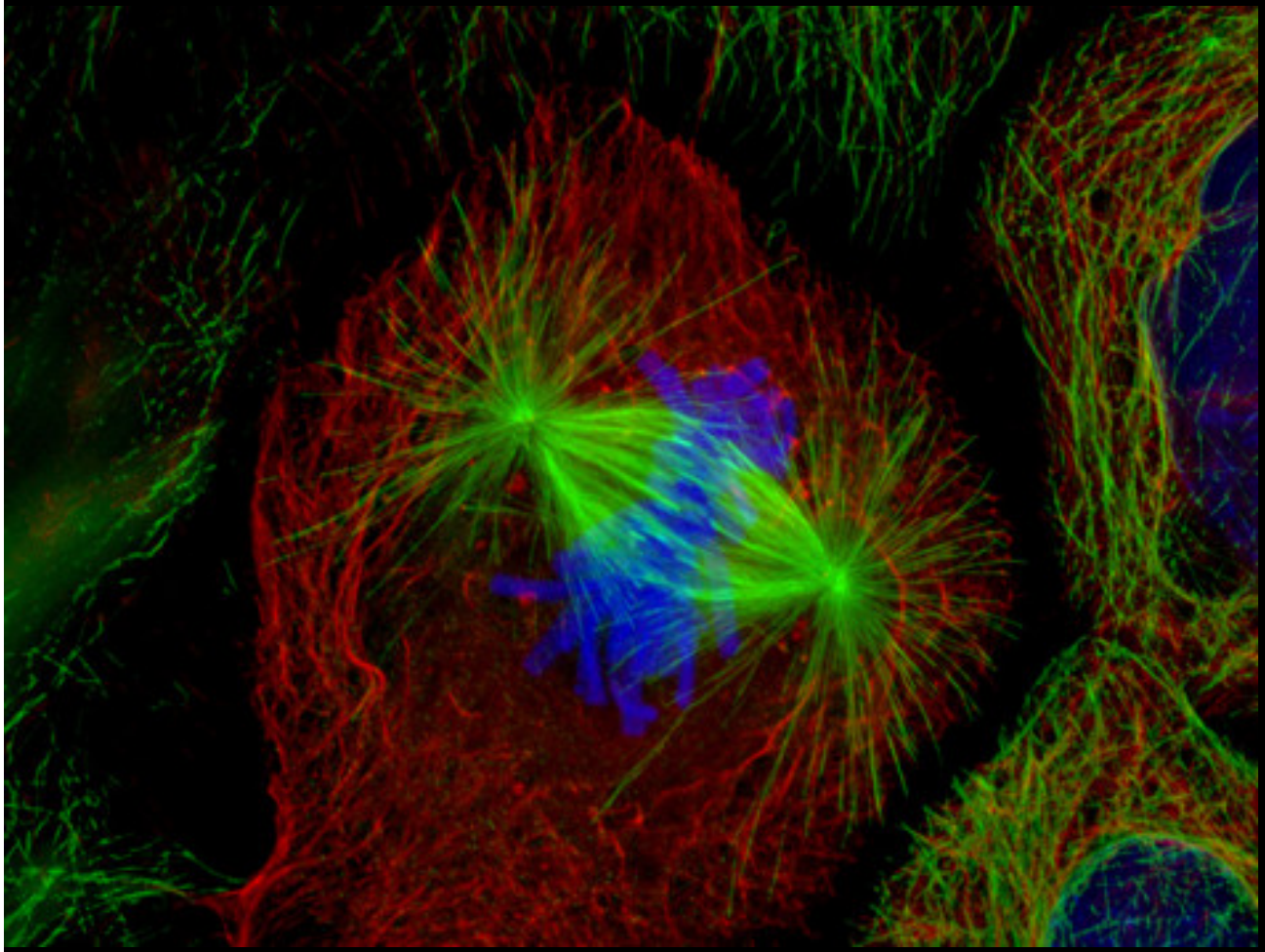
What can we learn?

Iowa State University scientists inserted a genetic structure from fireflies into a tobacco plant, causing it to glow.

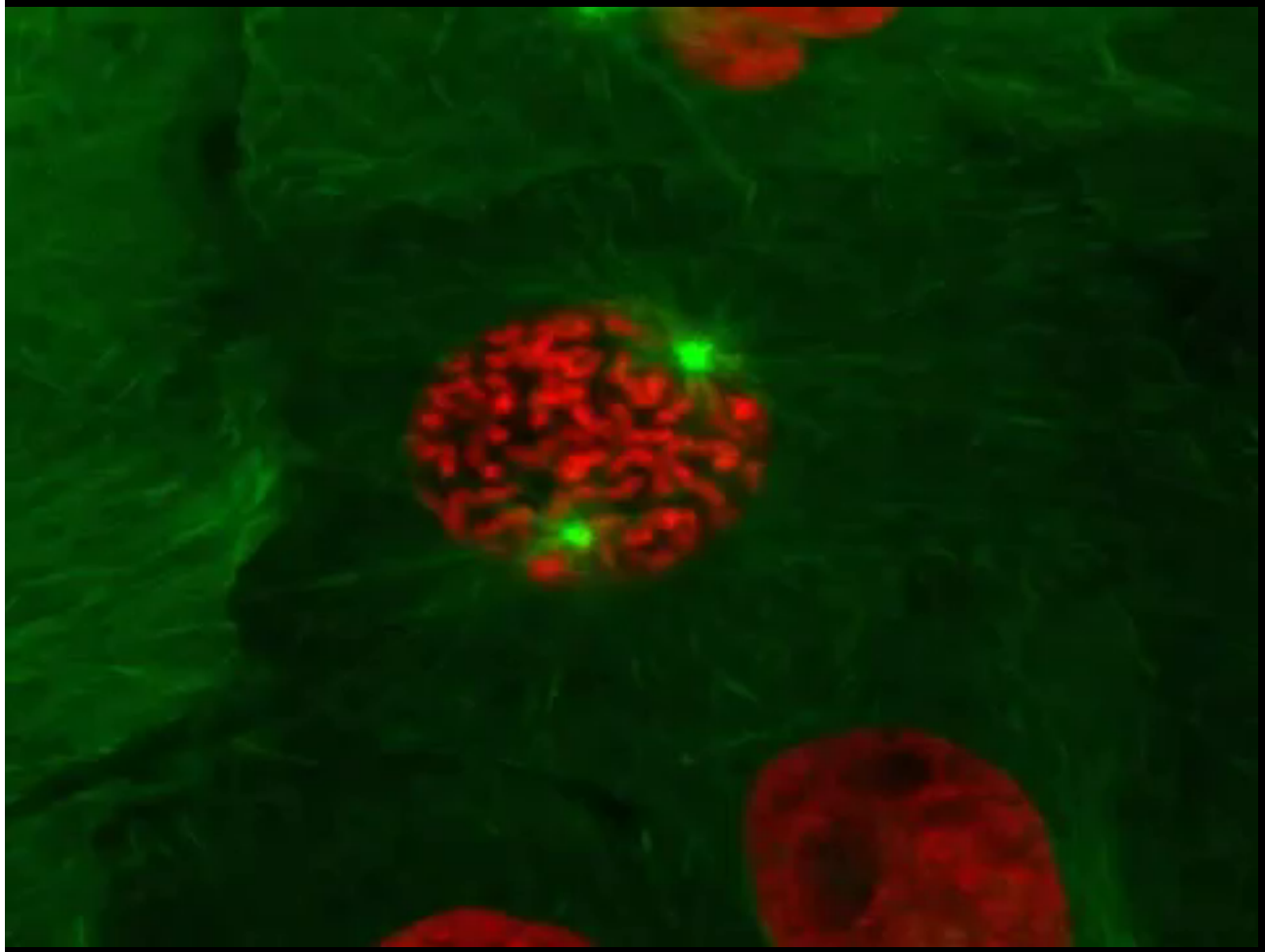
Unlike the gleam spurred by green fluorescent protein, the firefly-derived glow, caused by the pigment luciferin and the enzyme luciferase, does not require ultraviolet light to fluoresce. The firefly light requires oxygen and, under some conditions, ATP, a molecule involved in energy storage inside cells.

A Rainbow of Fluorescent Proteins



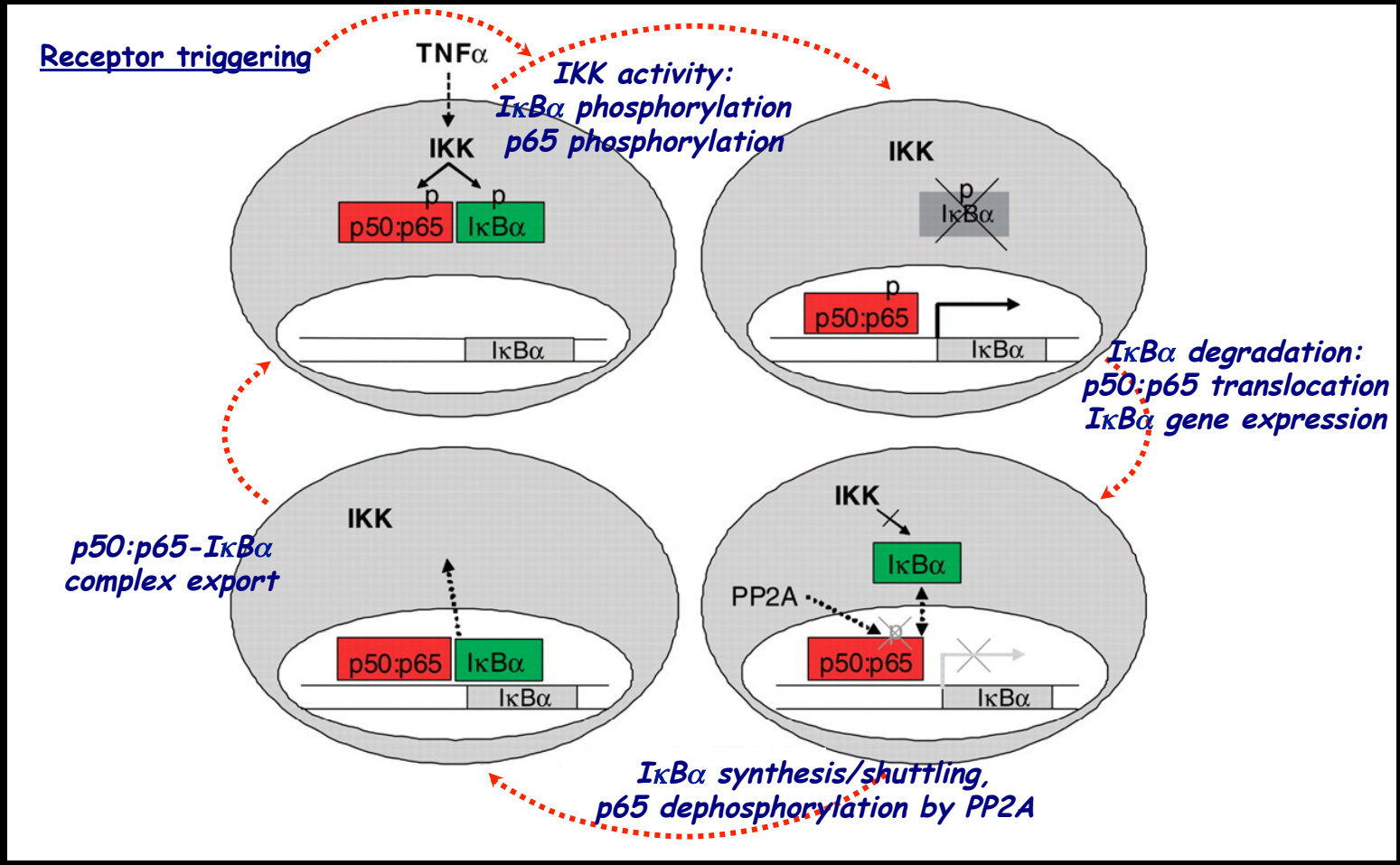


Microtubules and chromosomes can be labelled with different fluorescent colors and their rearrangements during the process of cell division can be studied using time-lapse images of the living cells.



Microtubules and chromosomes can be labelled with different fluorescent colors and their rearrangements during the process of cell division can be studied using time-lapse images of the living cells.

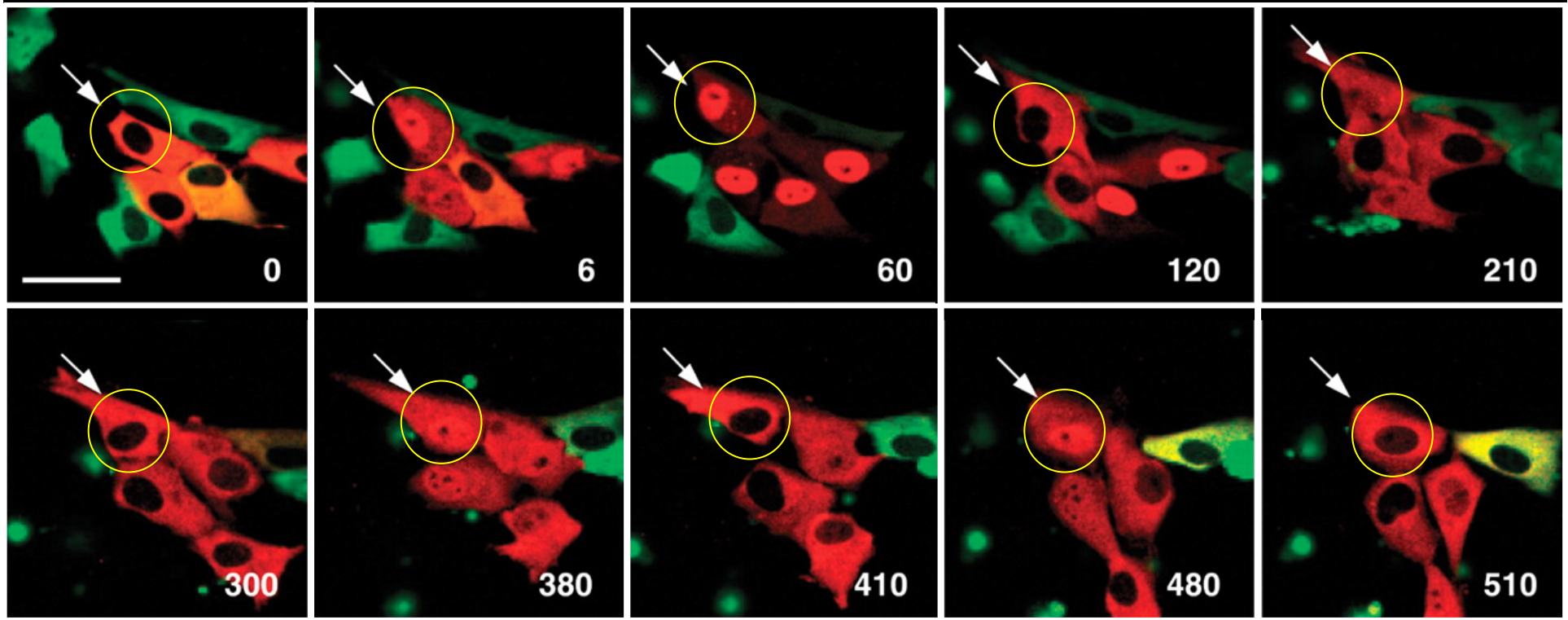
Oscillations in NF-κB localization



Schematic diagram illustrating the potential mechanism for repeated oscillations in NF-κB (p65/RelA) N-C localization.

Nelson, D. E., et al., 2004. Oscillations in NF-kB signaling control the dynamics of gene expression. Science, 306:704-708.

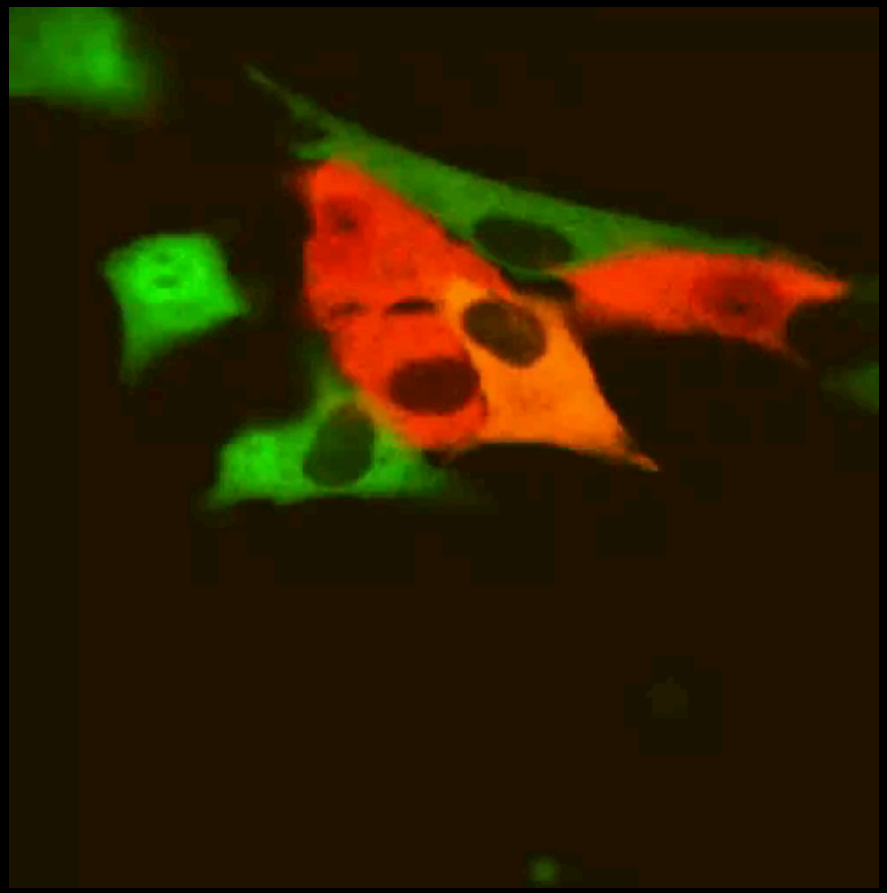
Oscillations in NF-κB localization



Time-lapse confocal images of cells expressing RelA-DsRed (red) and IκBα-EGFP (green) showing single-cell asynchronous N:C oscillations in RelA-DsRed localization after stimulation with 10 ng/ml TNFα. The arrow and circle mark one oscillating cell. Times, min; scale bar, 50 μm.

Nelson, D. E., et al., 2004. Oscillations in NF-kB signaling control the dynamics of gene expression. Science, 306:704-708.

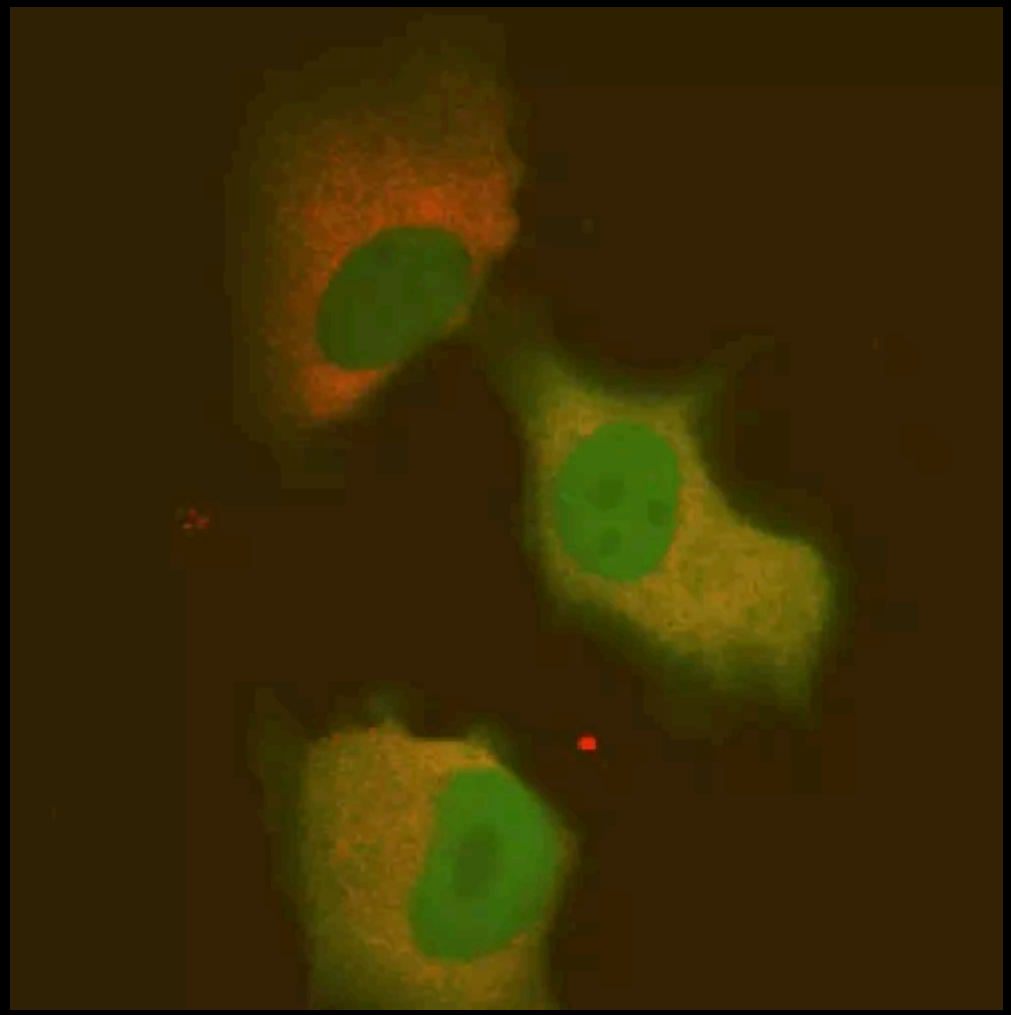
Oscillations in NF-κB localization



Time-lapse confocal images of cells expressing RelA-DsRed (red) and hCMV- IkBα-EGFP (green) over a period of 10 h following continual stimulation with 10 ng/ml TNFα.
Images were taken at 3 min intervals.

Nelson, D. E., et al., 2004. Oscillations in NF-kB signaling control the dynamics of gene expression. Science, 306:704-708.

Oscillations in NF-κB localization

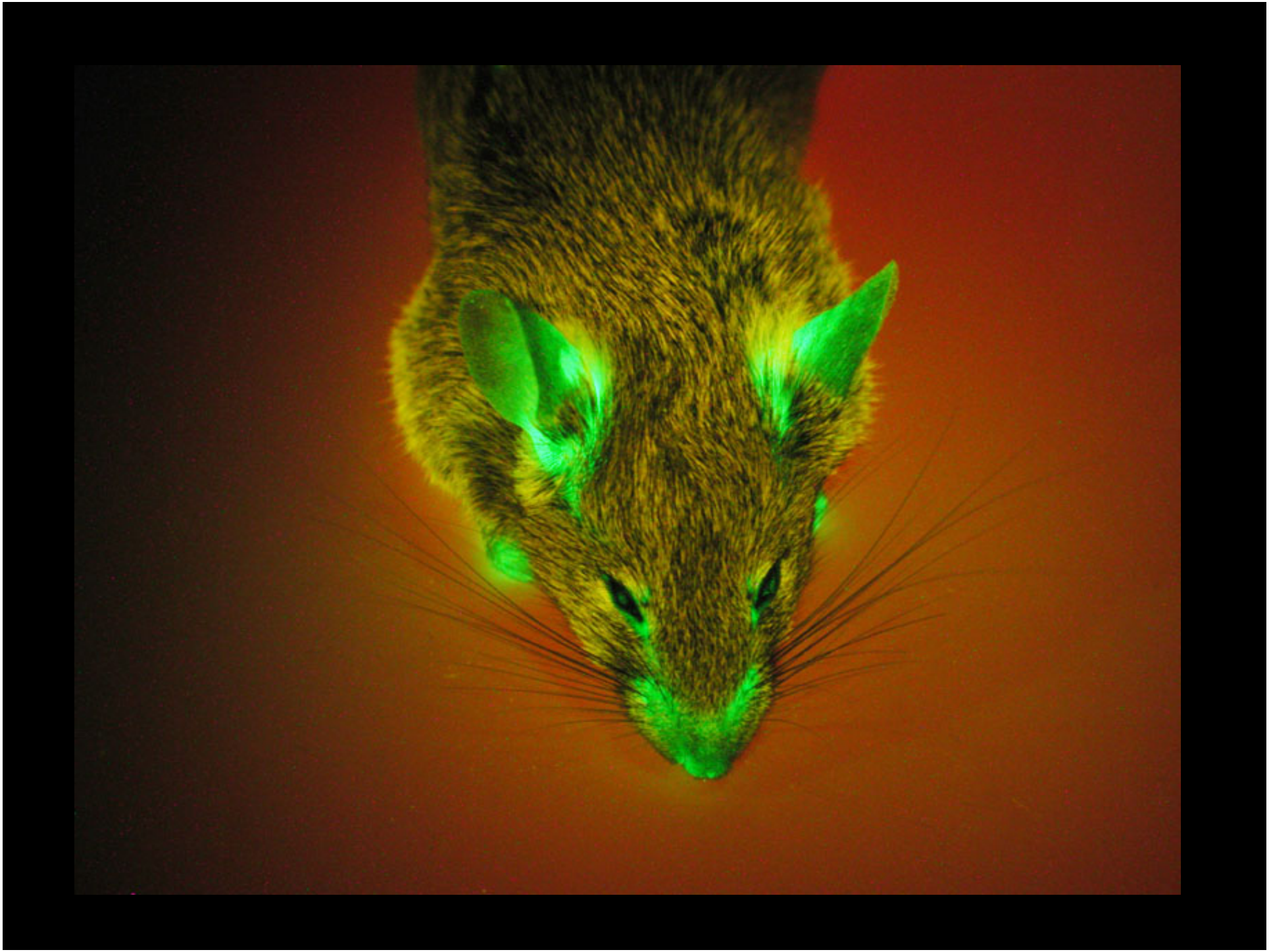


Time-lapse confocal images of cells expressing RelA-DsRed (red) and EGFP (green) over a period of 10 h following continual stimulation with 10 ng/ml TNFα.
Images were taken at 3 min intervals.

Oscillations in NF-kB Signaling Control the Dynamics of Gene Expression.
D. E. Nelson et al., Science, 306:704-708, 2004.

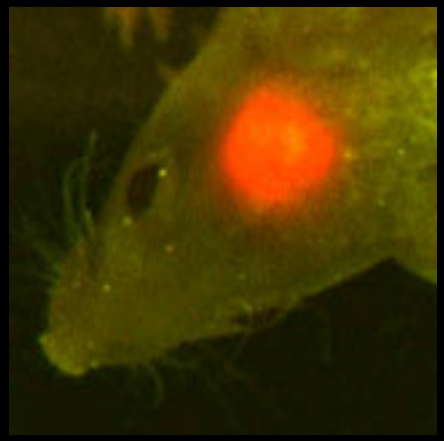
Drosophila

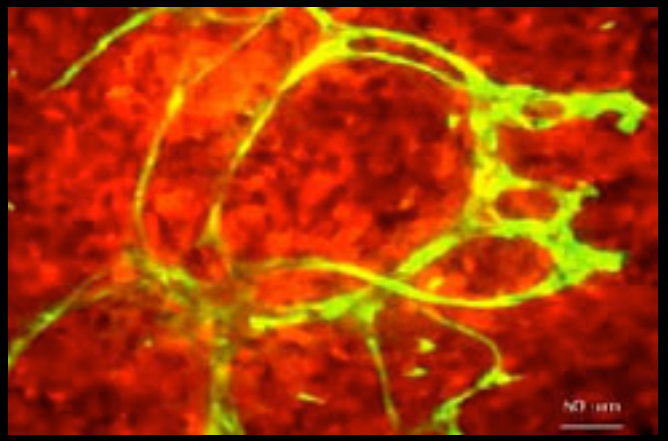
Zebra fish



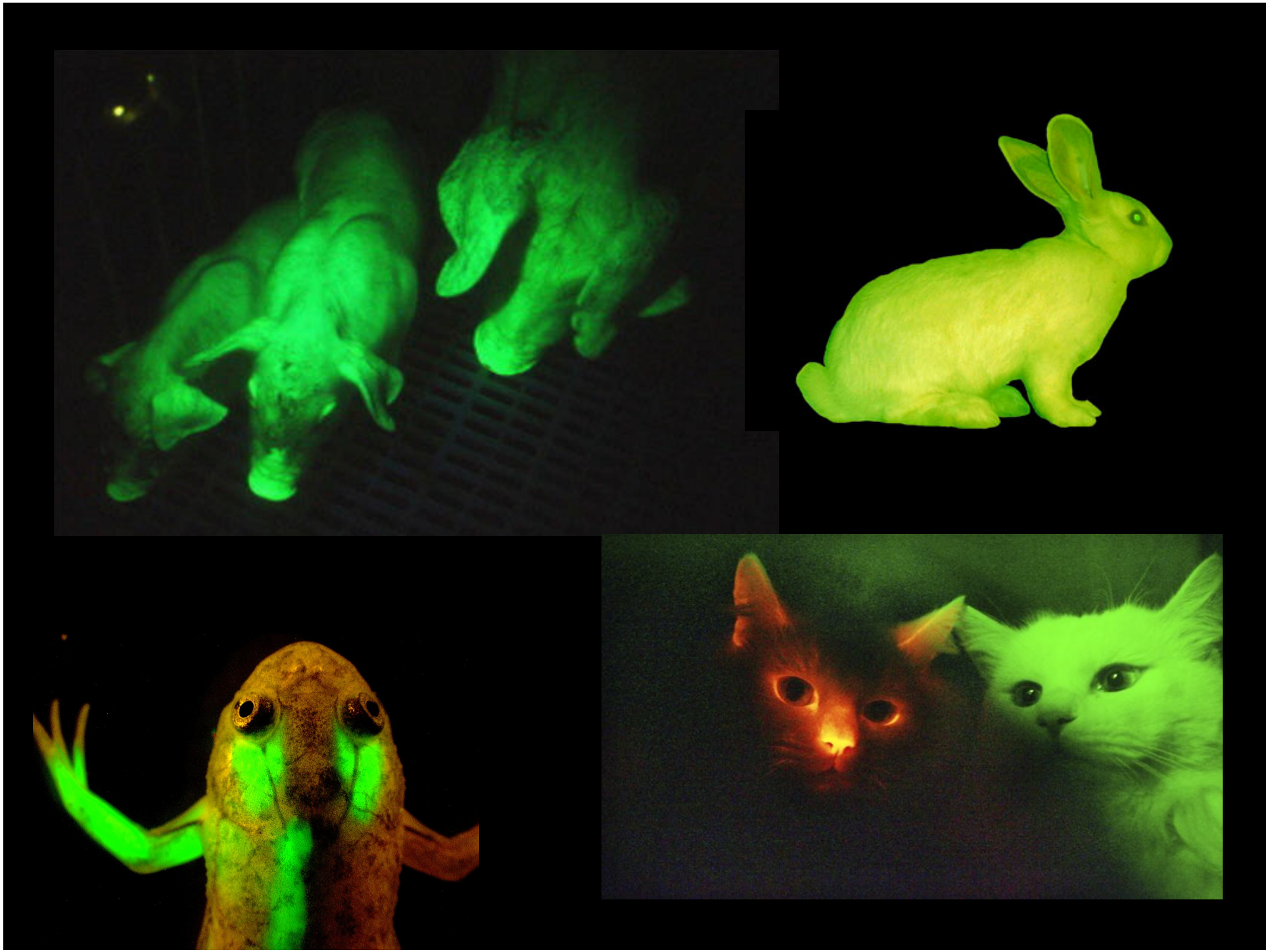

New born mice carrying a GFP gene

Mouse with brain tumor (red-DsRed) and its inner blood vessels (green-GFP).





Human cancer cells into which DsRed has been introduced can be implanted into a nude mouse. The fluorescent cancer cells will be easily monitored in a live mouse, allowing researchers to observe metastasis and angiogenesis.



Malaria

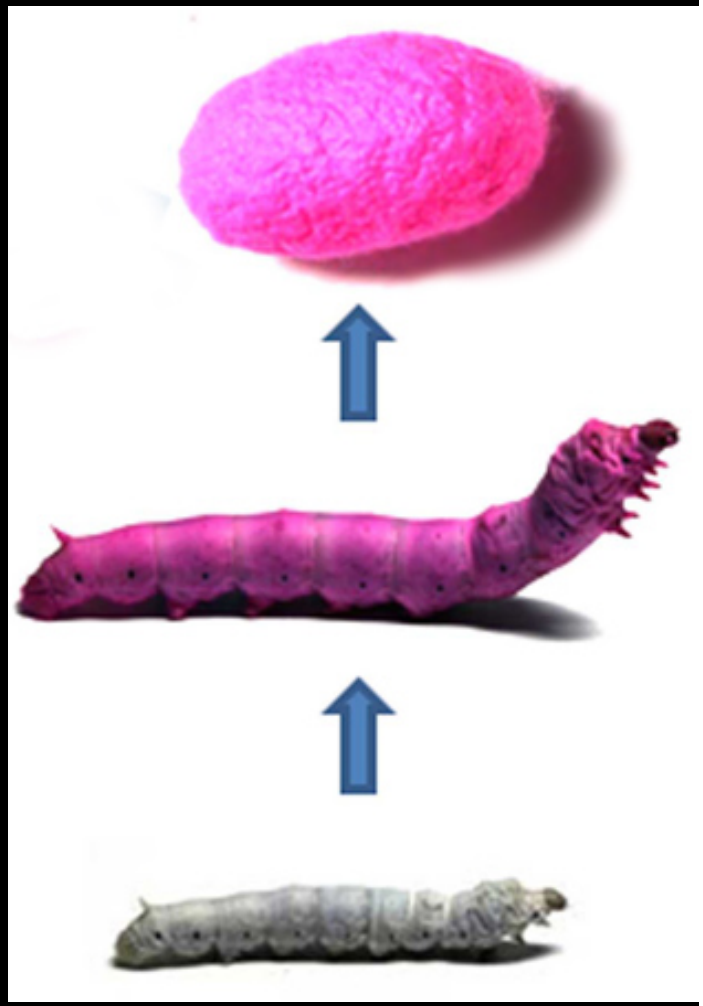
Mosquitoes and mosquito larvae with fluorescent testicles can be used to eradicate malaria

Creation of mosquitoes with green fluorescent testicles enables laser sorting of a large number of male mosquito larvae.

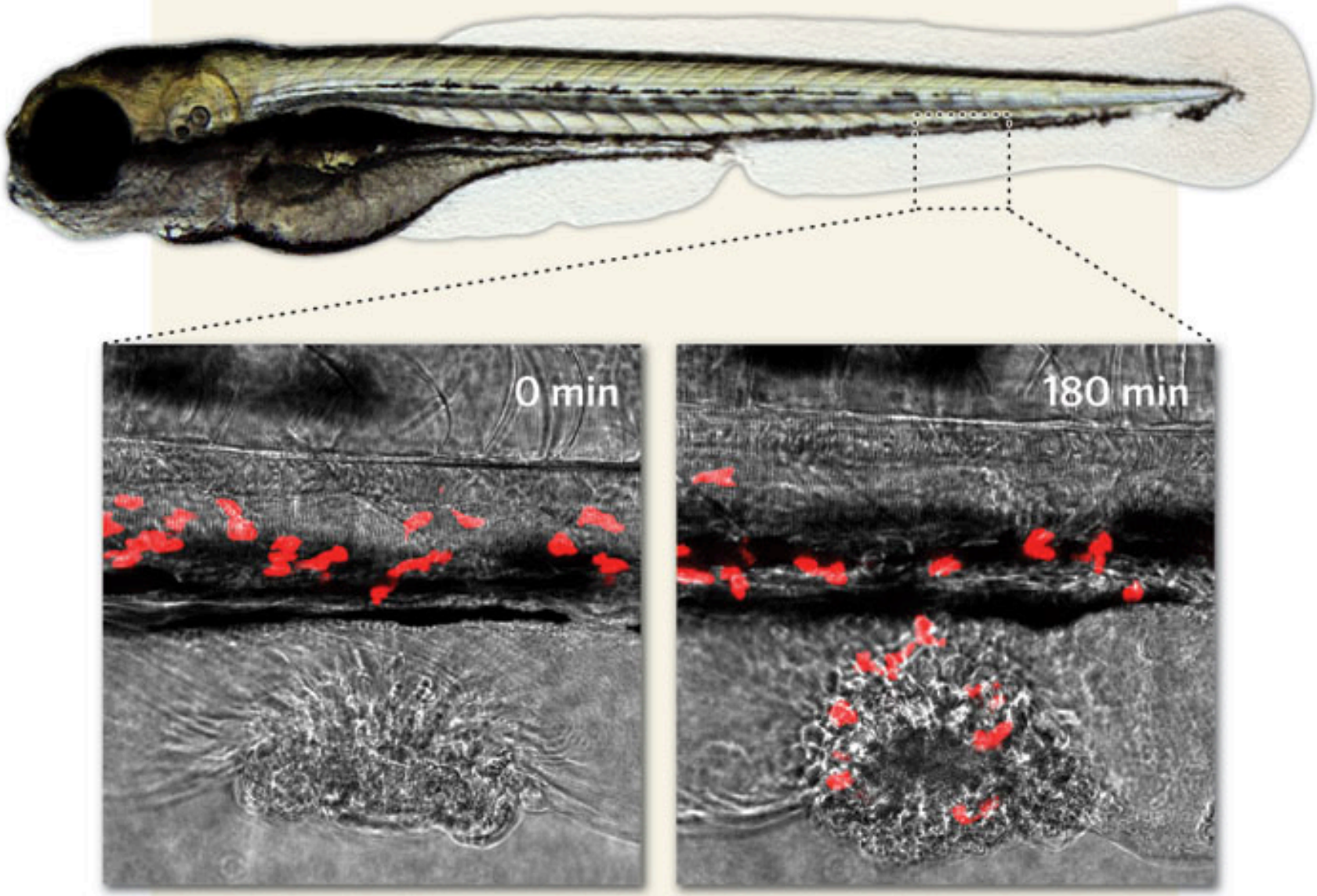
Once separated from the females, the males are sterilized and released into the environment to mate with wild females. These sterile males compete with the wild-type males. Female mosquitoes mate once in their two-week cycle. If a large enough population of sterilized males is released into the wild, the disease can be eradicated in a fairly short time.

Economical and industrial benefit:

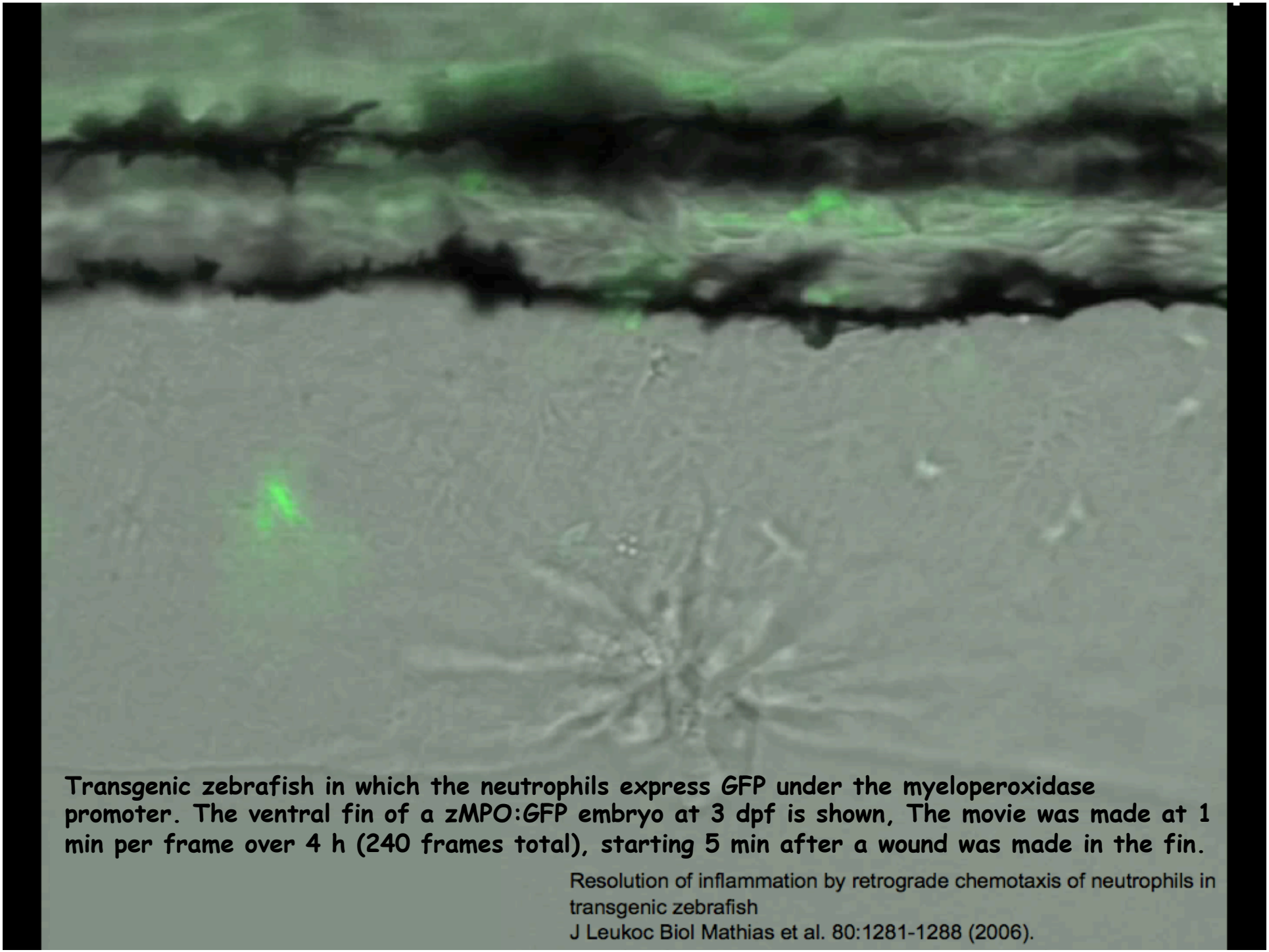
Addition of fluorescent dyes into the silkworm diet during the last four days of the larva stage results in colored cocoons that can then be harvested and processed giving rise to colored silk.



http://www.nature.com/nature/journal/v459/n7249/fig_tab/459921a_F1.html



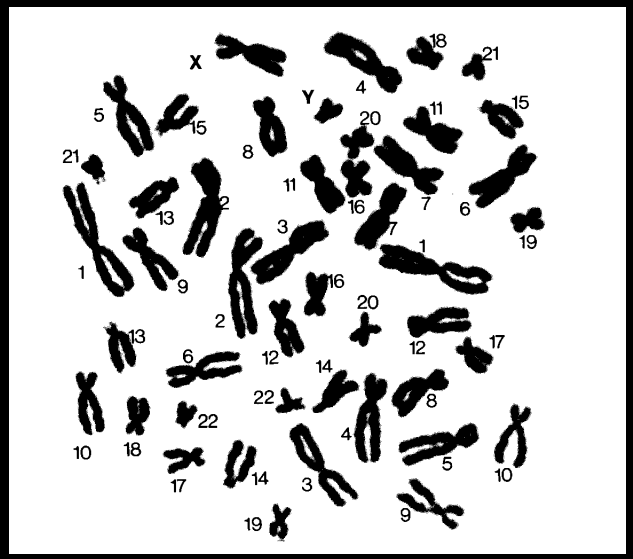
A small wound made in the ventral tail fin of a 4-day-old zebrafish larva results in recruitment of fluorescently tagged neutrophils (red) to the wound site within minutes, where they persist for several hours, providing a miniature model of the human wound inflammatory response.

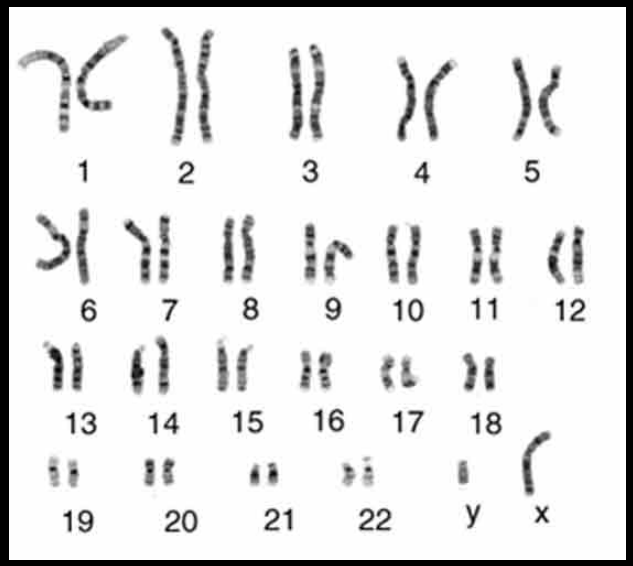


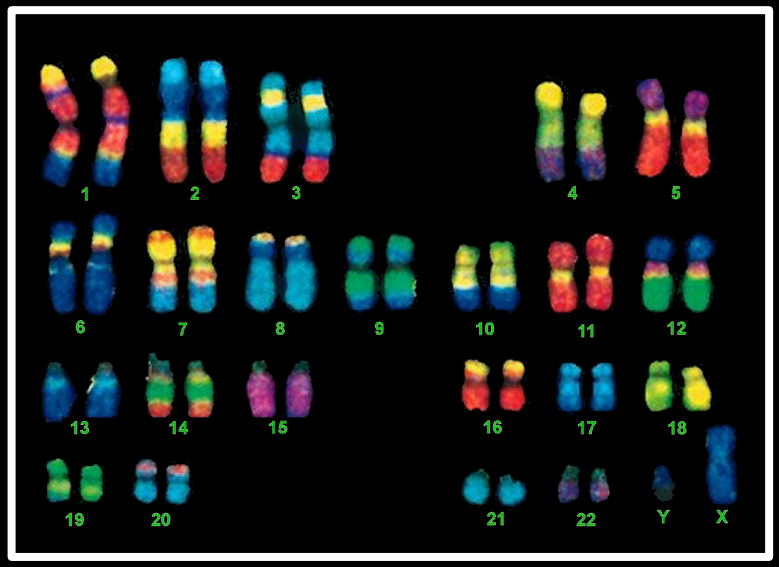
Transgenic zebrafish in which the neutrophils express GFP under the myeloperoxidase promoter. The ventral fin of a zMPO:GFP embryo at 3 dpf is shown, The movie was made at 1 min per frame over 4 h (240 frames total), starting 5 min after a wound was made in the fin.

Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish
J Leukoc Biol Mathias et al. 80:1281-1288 (2006).

A human karyotype of somatic cells includes (2n) 46 chromosomes



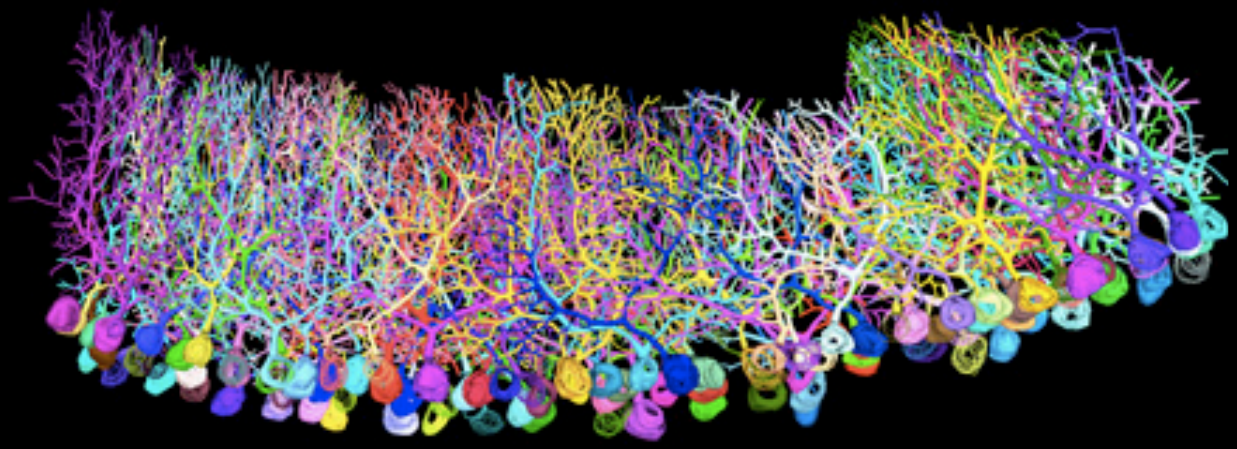




Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system

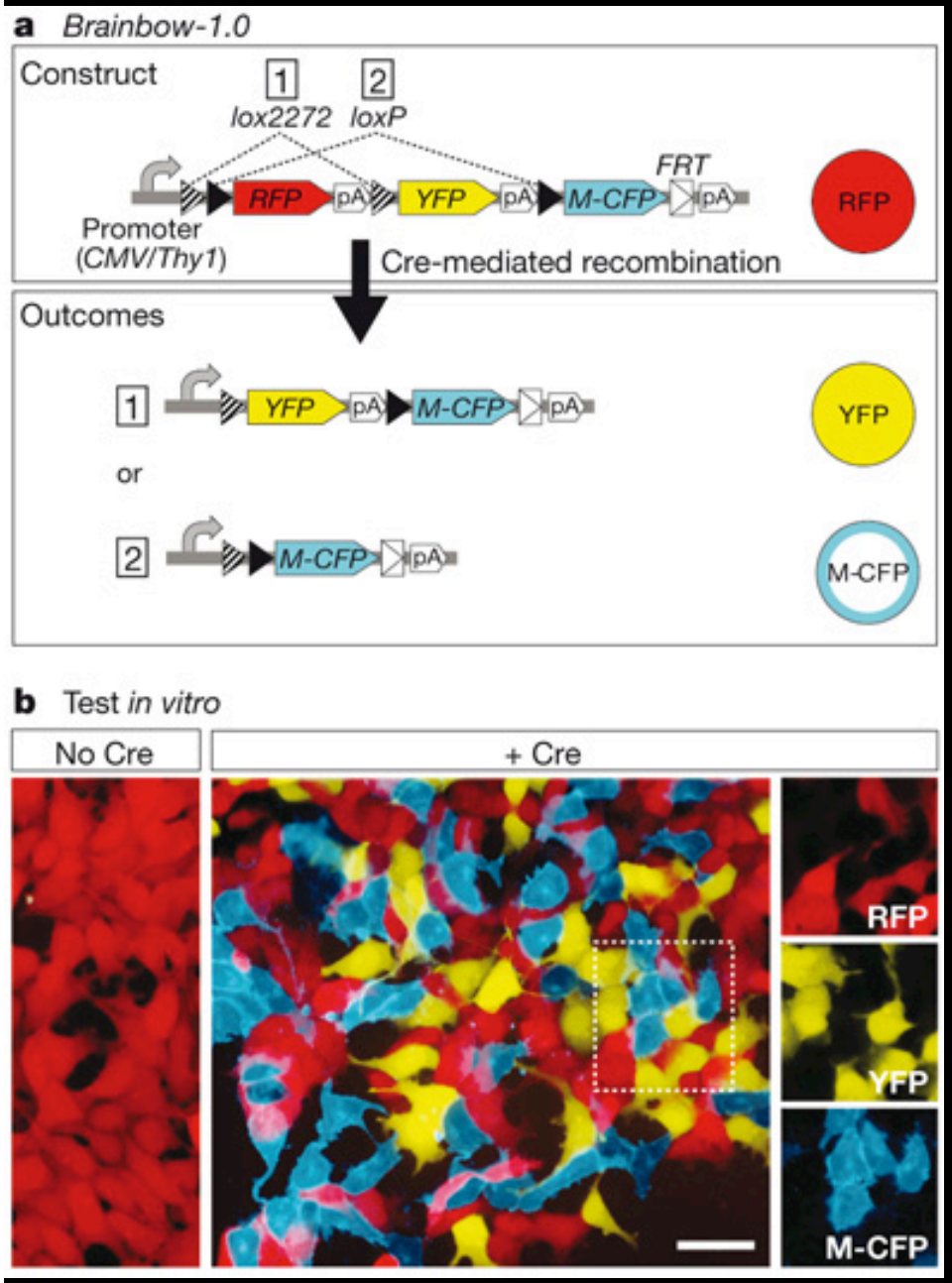
Jean Livet, Tamily A. Weissman, Hyuno Kang, Ryan W. Draft, Ju Lu, Robyn A. Bennis, Joshua R. Sanes & Jeff W. Lichtman

Nature 450, 56-62, 2007



The Brainbow Mouse

Visual analysis of neuronal circuits and network architecture by genetically labelling of neurons with multiple distinct colors



Brainbow-1: stochastic recombination using incompatible lox variants.

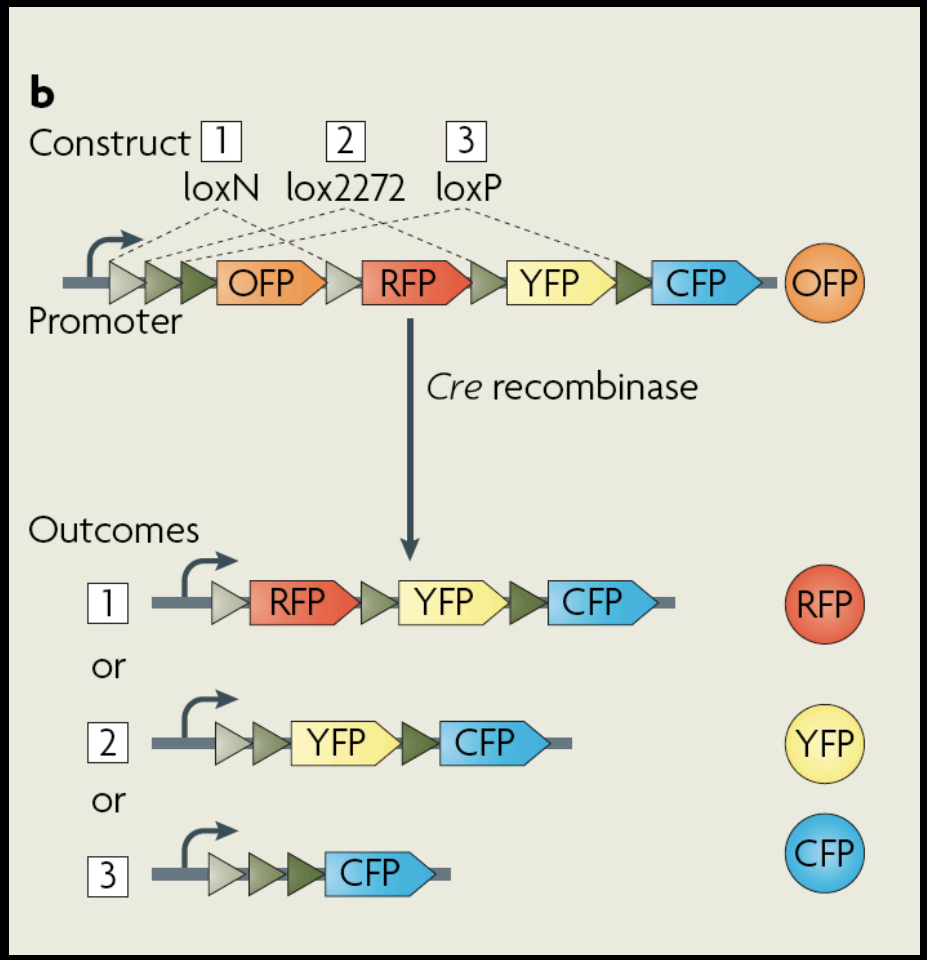
a) In Brainbow-1, incompatible sets of lox sites alternate: Cre chooses between excision events 1 or 2. Before Cre action, only the gene following the promoter is expressed (RFP). Recombination switches expression to either YFP (1) or M-CFP (2).

b) HEK cells stably transfected with CMV-Brainbow-1 express RFP. On transient transfection with Cre, these cells randomly switch to YFP or M-CFP expression.

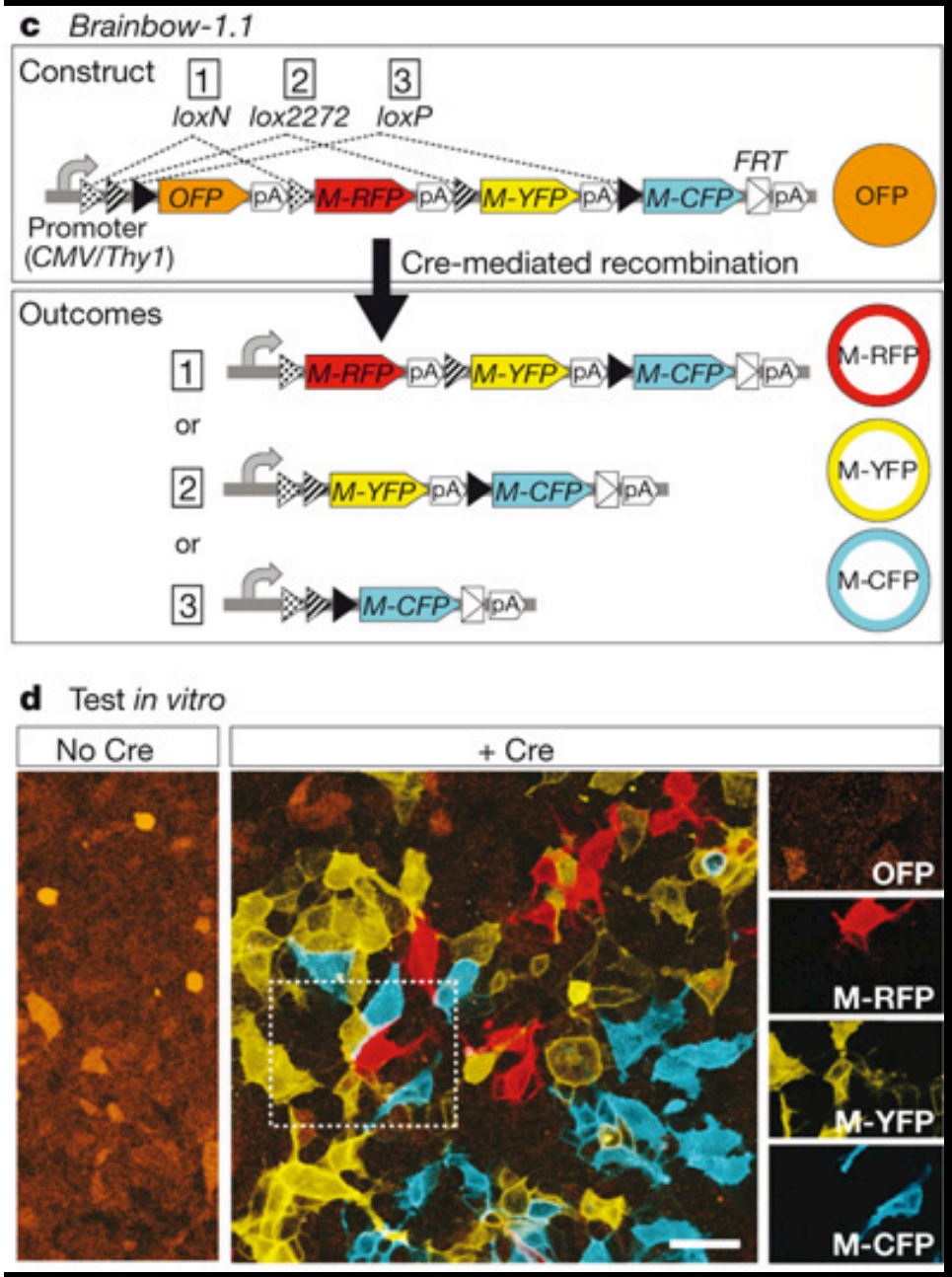
RFP - Red Fluorescence protein
YFP - Yellow Fluorescence protein
CFP - Cyan (Blue) Fluorescence protein

Alternating canonical (wild-type) loxP sites with variant sites creates mutually exclusive excision possibilities.

Implementation of this strategy with three lox variants creates four possible outcomes following excision by Cre recombinase: three different products of recombination plus the unrecombined initial state. In all cases, only the XFP directly following the promoter is expressed.



OFP - Orange Fluorescence protein
RFP - Red Fluorescence protein
YFP - Yellow Fluorescence protein
CFP - Cyan (Blue) Fluorescence protein



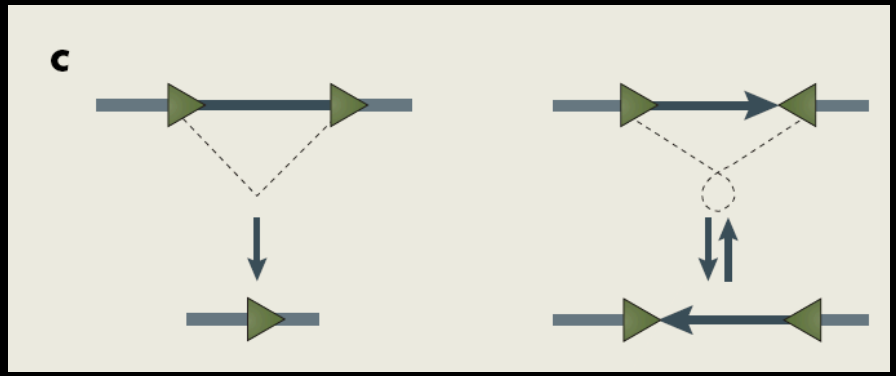
Brainbow-1: stochastic recombination using incompatible lox variants.

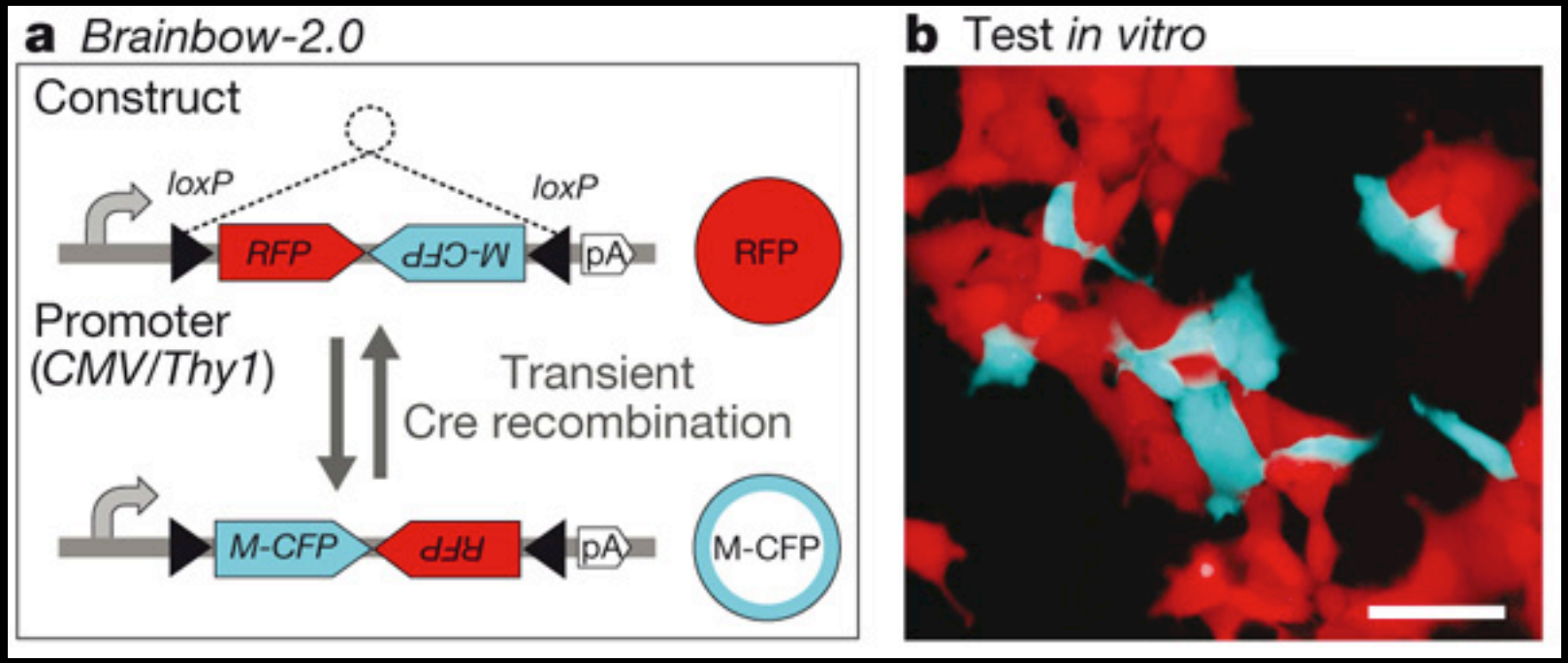
c) In Brainbow-1.1, a third set of incompatible lox sites (loxN) is added, creating three recombination possibilities (1, 2 or 3), switching OFP expression to RFP, YFP or CFP expression.

d) Cells stably transfected with Brainbow-1.1 express OFP. Cre recombination leads to expression of M-RFP, M-YFP or M-CFP. pA, polyadenylation signal; M-XFP, membrane-tethered XFP. FRT site allows reduction of transgene arrays. Scale bar, 50 μm.

OFP - Orange Fluorescence protein
RFP - Red Fluorescence protein
YFP - Yellow Fluorescence protein
CFP - Cyan (Blue) Fluorescence protein

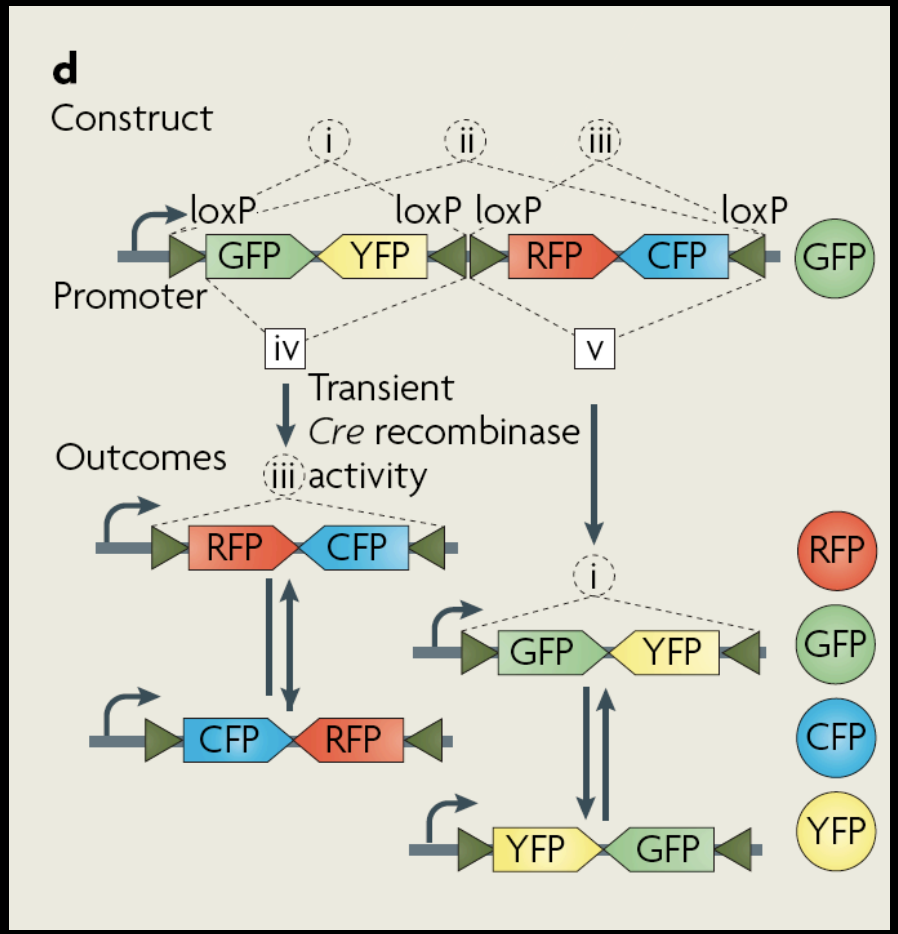
A second strategy, Brainbow-2, uses Cre-recombinase-mediated DNA inversion. The DNA segment can invert repeatedly while Cre recombinase is present but will stabilize in a random orientation when Cre recombinase stops acting, offering two possibilities of expression.



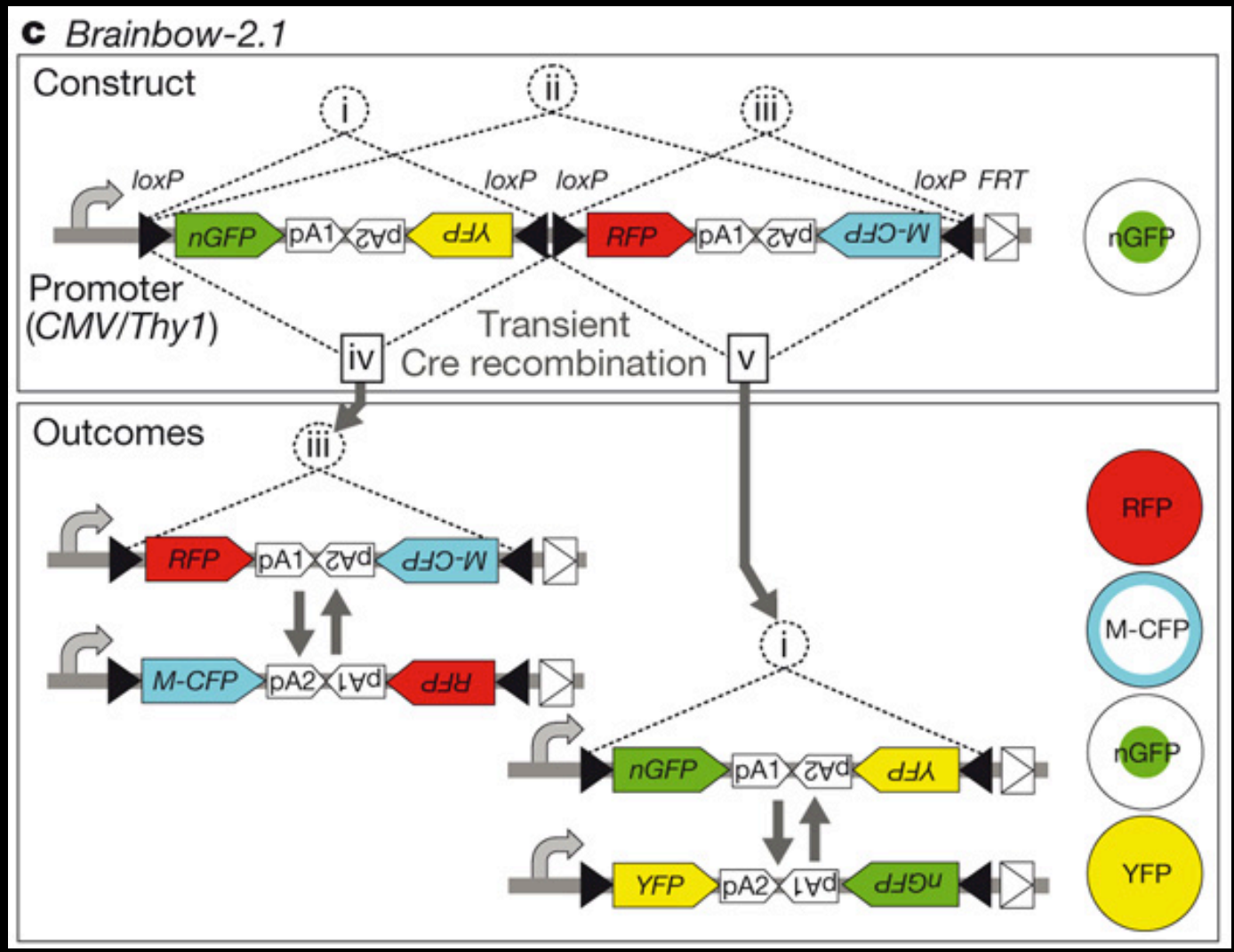


Brainbow-2: Stochastic recombination using Cre-mediated inversion.
a) In Brainbow-2.0, Cre triggers inversion of a DNA segment flanked by loxP sites in opposite orientation. In 50% of cells, inversion should end in an antisense orientation and switch gene expression. B) HEK cells stably expressing CMV-Brainbow-2.0 produce RFP, and stochastically switch to CFP expression when transfected with Cre.

When two invertible segments are arranged in tandem, the number of possibilities fir recombination increases: additional inversions and excisions create a total of four different expression possibilities.

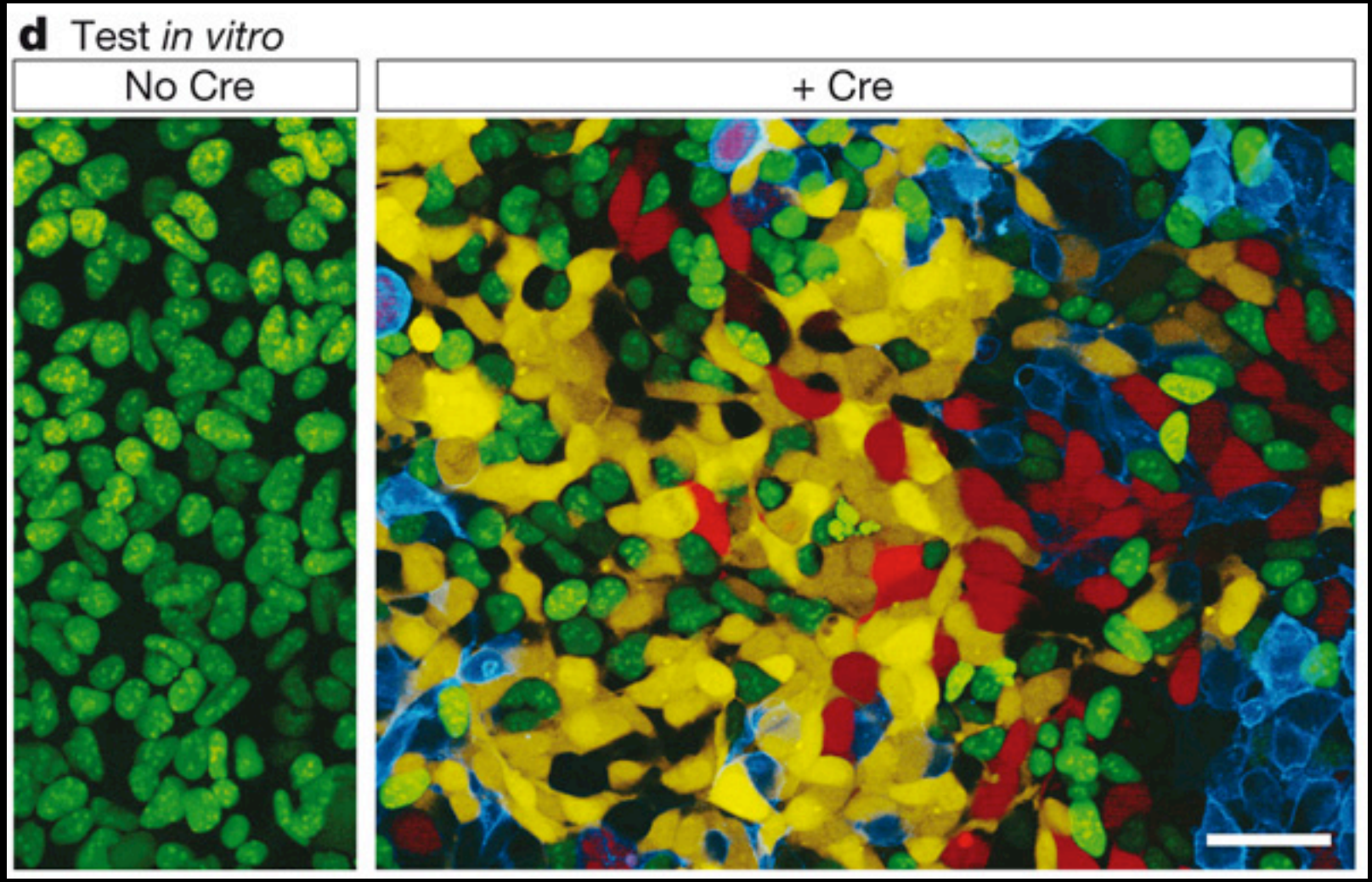


OFP - Orange Fluorescence protein
RFP - Red Fluorescence protein
YFP - Yellow Fluorescence protein
CFP - Cyan (Blue) Fluorescence protein



Brainbow-2: stochastic recombination using Cre-mediated inversion.

c) The Brainbow-2.1 construct contains two tandem invertible DNA segments. Inversion (i–iii) and excision (iv, v) recombination events create four expression possibilities.



Brainbow-2: Stochastic recombination using Cre-mediated inversion.

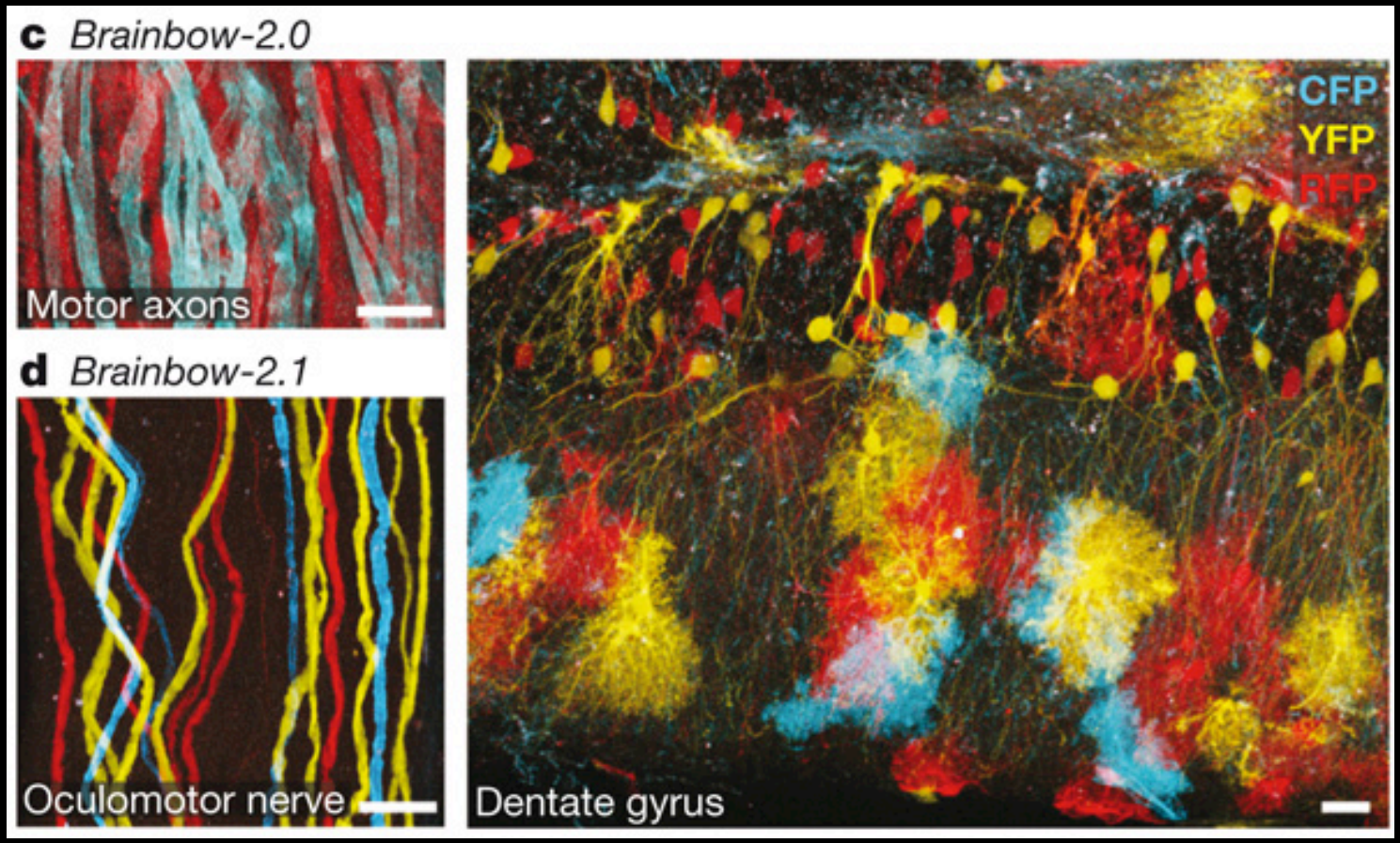
d) Stable CMV-Brainbow-2.1 transfectants express nuclear GFP (nGFP). Cre recombination triggers expression of YFP, RFP or M-CFP. pA1 and pA2, SV40 and bGH polyadenylation signals. Scale bars, 50 μm.

In Brainbow-2, presence of Cre will result in repeated gene conversions, as long as the Cre enzyme exist. This will result in constitutive changes in the type of fluorescence protein expressed within the cell, and a change in its color.

To prevent this problem scientists have used a mouse model in which the Cre was expressed in a temporary fashion.

Thus, to induce recombination in the Thy1-Brainbow mouse lines, the mice were crossed with *CAGGS-CreERT2* mice, in which *CAGGS* drives broad expression of an estrogen receptor–Cre fusion specifically activated by injection of the ligand tamoxifen.

Newborn mice were injected with a single dose of tamoxifen at postnatal day 0–3 (P0–P3) to activate recombination in cells co-expressing Brainbow and CreER. This results in temporary expression of Cre, and recombination of the XFP in majority of cells in all mice tested.



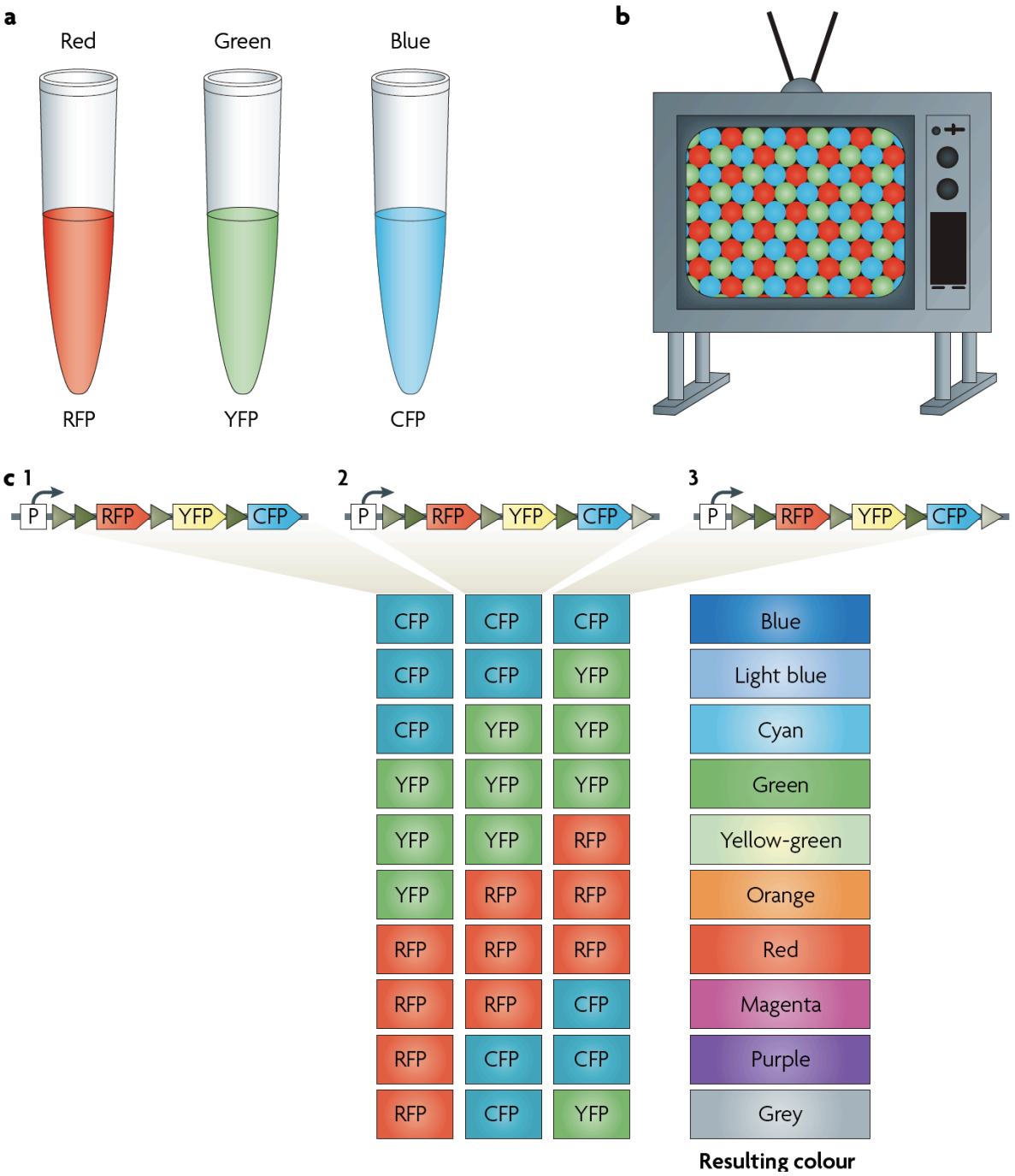
XFP expression in Brainbow transgenic mice.

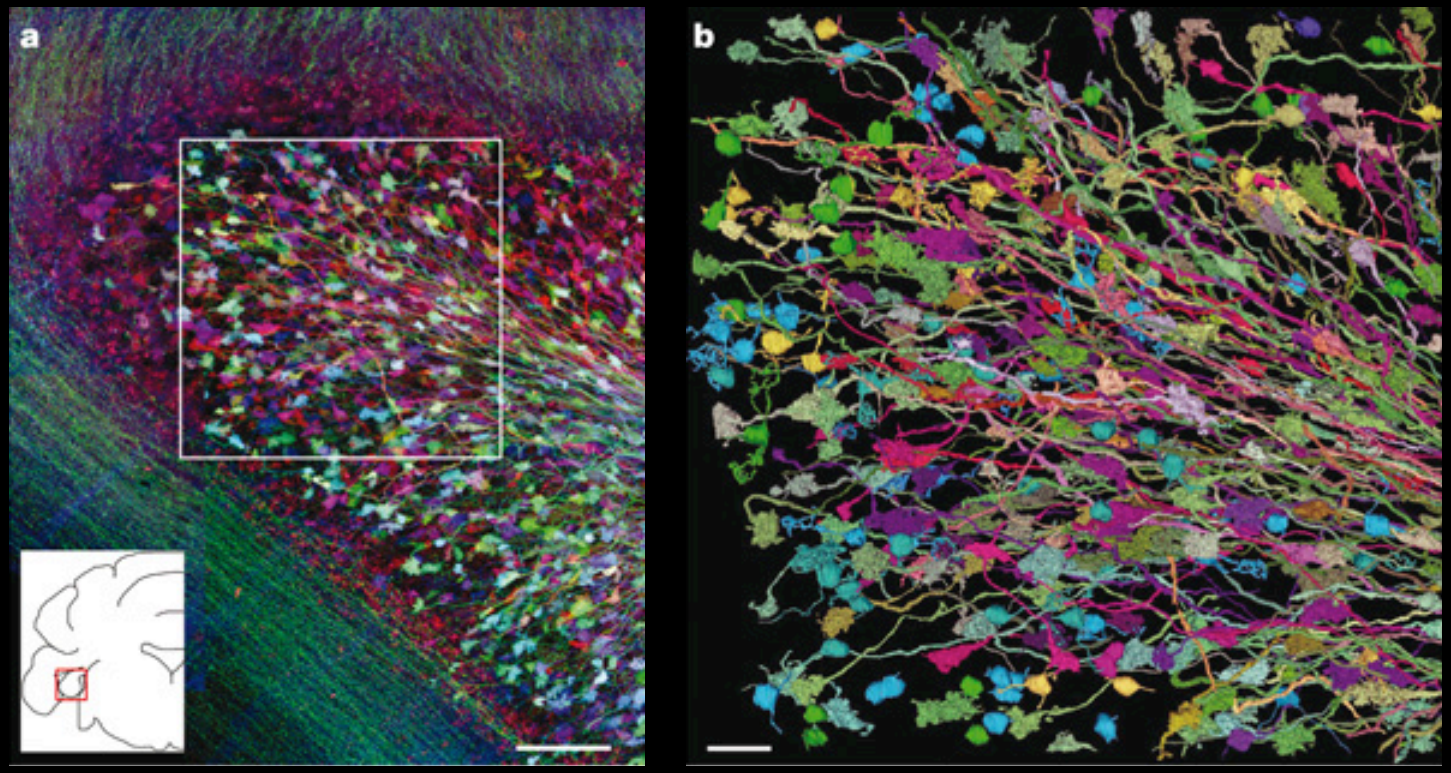
c) In Thy1-Brainbow-2.0 mice, transient recombination with the CreERT2/tamoxifen system triggers expression of M-CFP (peripheral motor axons, line N). d) In Thy1-Brainbow-2.1 mice, CreERT2-mediated recombination leads to expression of multiple XFPs. Left: oculomotor nerve. Right: dentate gyrus (neurons and astrocytes are labeled).

Combinatorial expression of three distinct fluorescent proteins can generate a large spectrum of colours.

a) Several spectrally distinct fluorescent proteins (XFPs) are now available, including ones that emit in red (RFP), green (YFP) and blue (CFP) frequencies. b) The combinatorial expression of red, green and blue XFPs at various levels is sufficient to encode a colour space analogous to the one that is generated by an RGB video monitor. c) An example showing how ten distinct colours can be generated by expressing a trimeric combination of three different XFPs. In Brainbow mice, this outcome would result if three copies of a trichromatic transgene (illustrated at the top of the panel) each recombined independently.

Triangles represent lox sites. CFP, cyan fluorescent protein; P, promoter; RFP, red fluorescent protein; YFP, yellow fluorescent protein.





Cerebellar circuit tracing and colour analysis.
a) Cerebellar flocculus. Inset shows coronal location. b) Three-dimensional digital reconstruction of region boxed in a (341 axons and 93 granule cells).

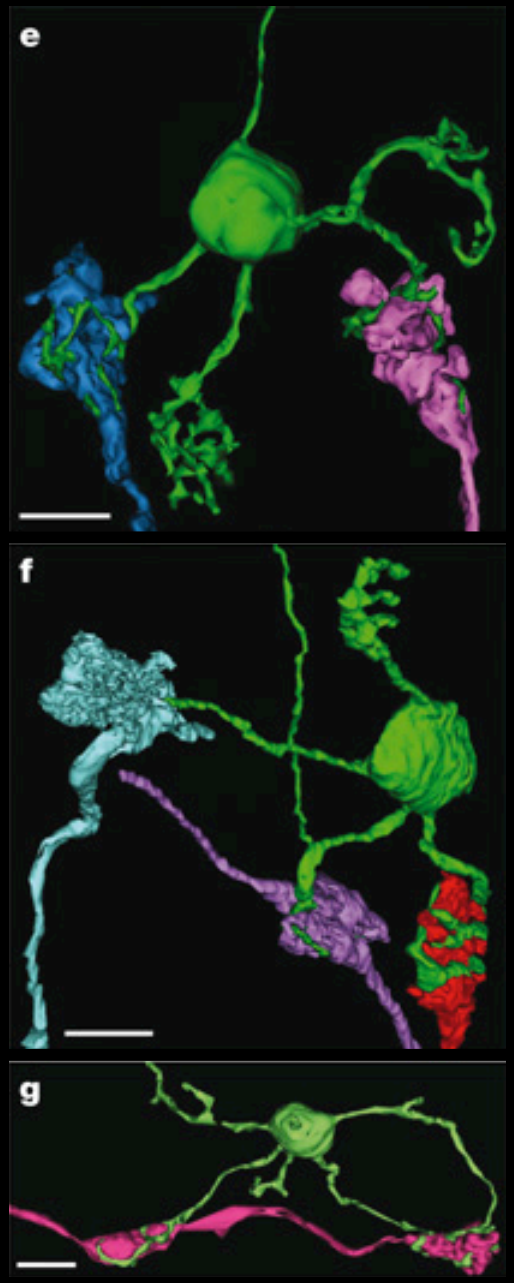
Cerebellar circuit tracing and colour analysis.

e) Reconstructed granule cell receives input from greater than or equal to 3 different mossy fibres (blue, pink and at least 1 unlabeled). The granule cell axon projects upwards.

f) Each granule cell dendrite is innervated by a different presynaptic neuron (three labeled, one unlabeled).

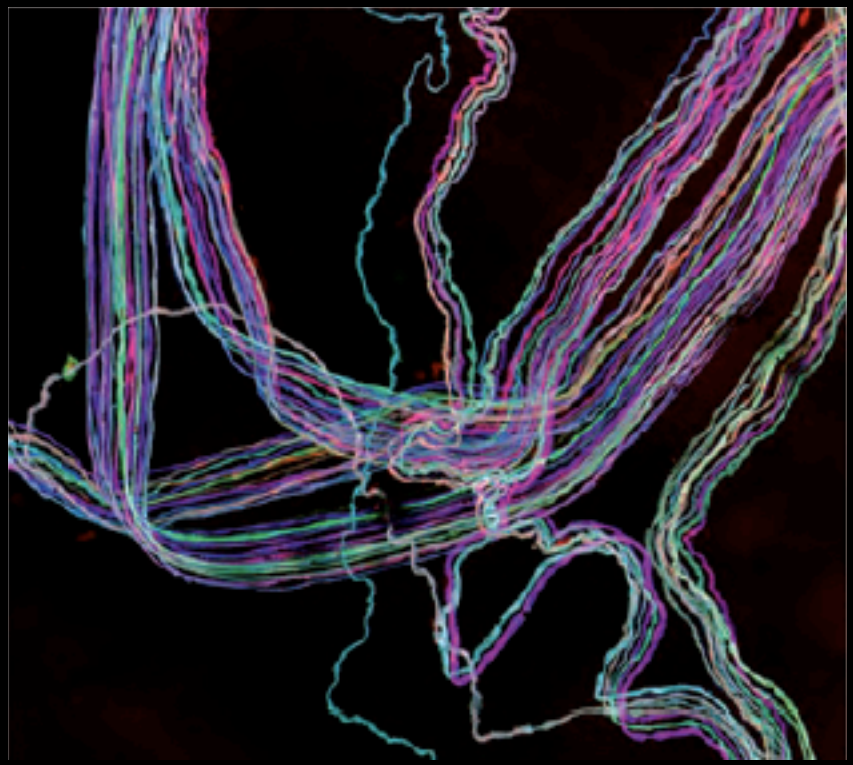
g) Two granule cell dendrites are innervated by the same presynaptic mossy fiber.

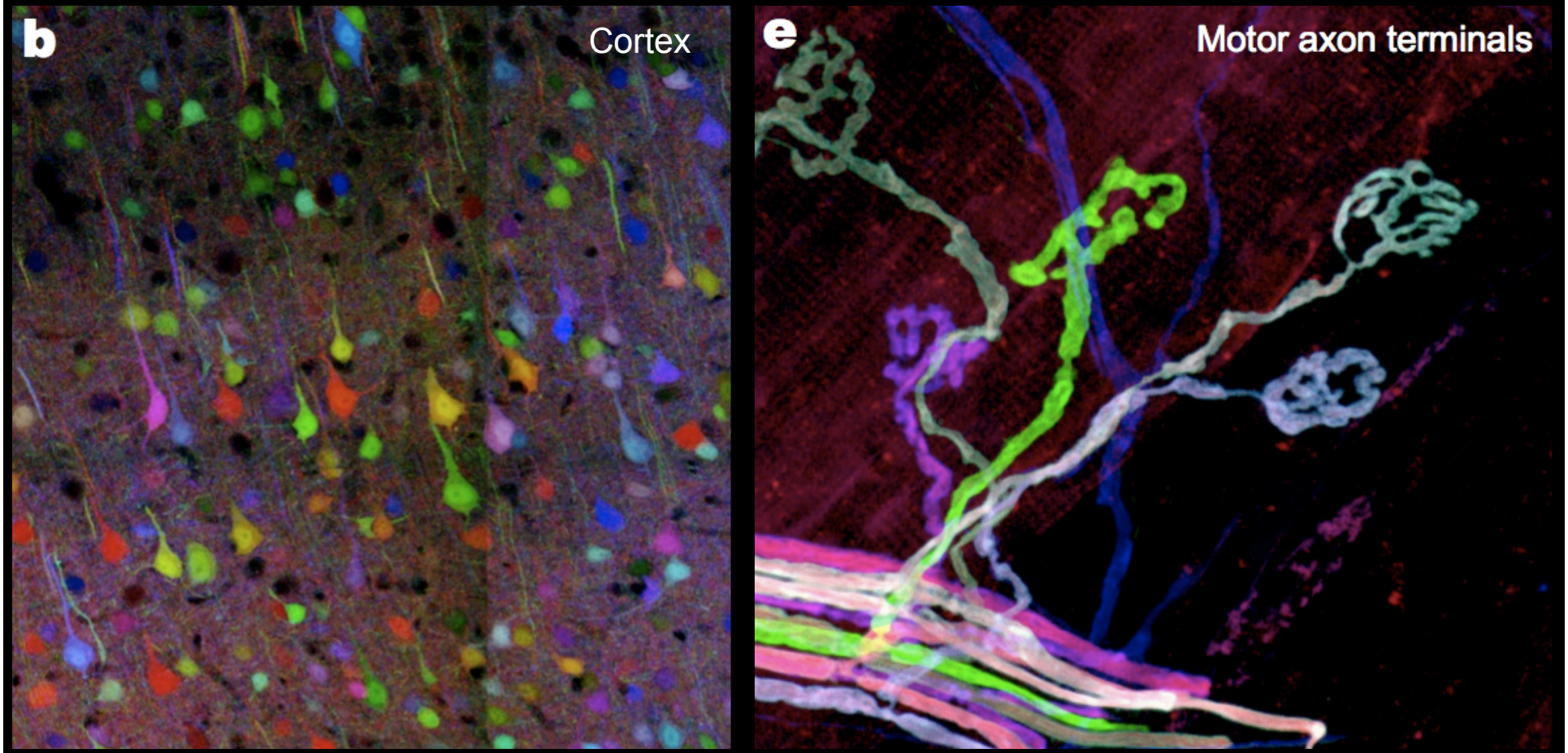
Scale bars: a, c, 50 μm; b, 15 μm; e–g, 5 μm.



Multicolor neuronal labeling in Brainbow transgenic mice

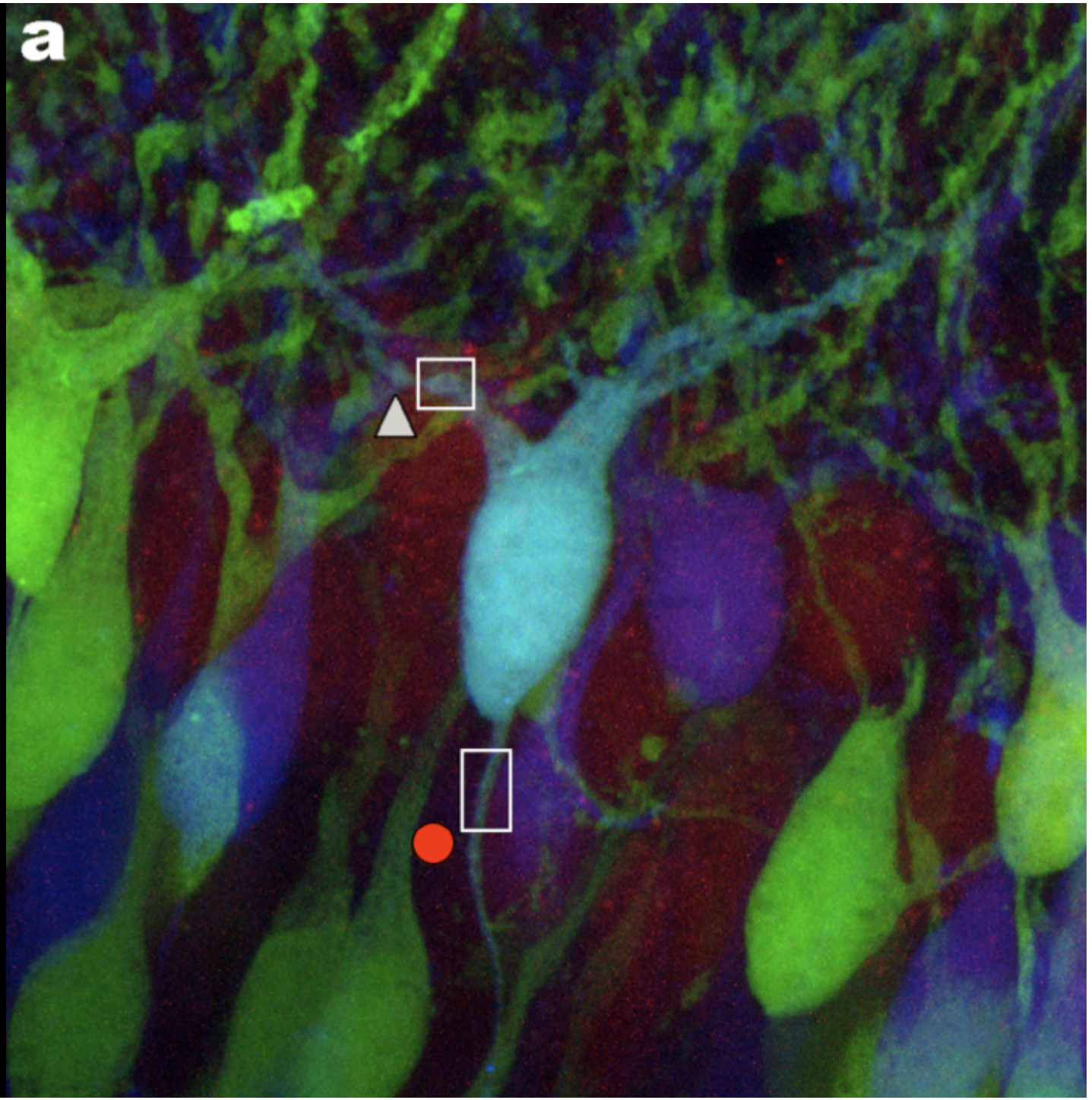
A motor nerve innervating ear muscle



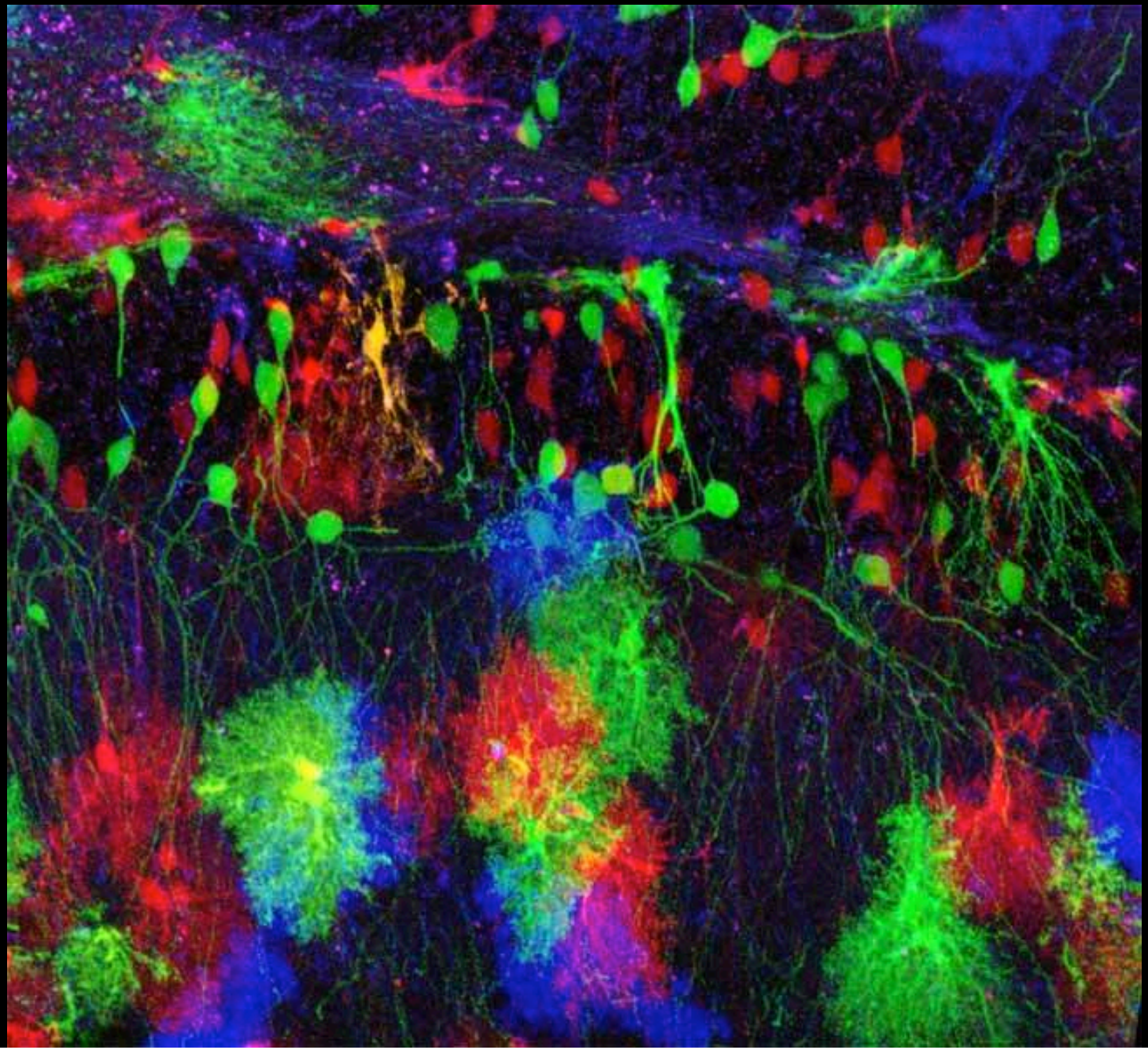


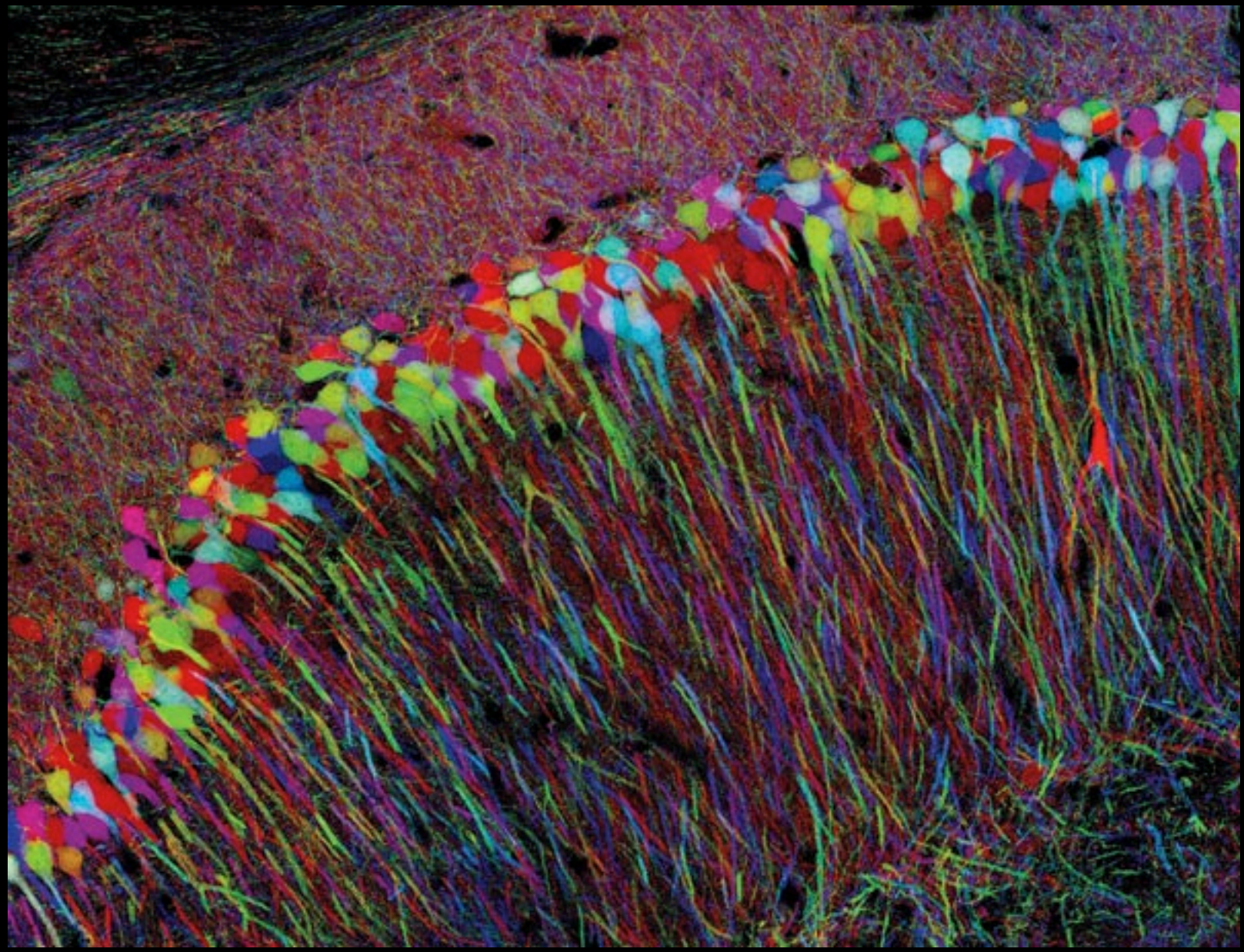
Combinatorial XFP expression in Brainbow mice. Additional examples of combinatorial expression in *Thy1-Brainbow* animals. Recombination was induced with CreERT2 and perinatal Tamoxifen injection. **a-d**, Cortex and hippocampus of *Thy1-Brainbow-1.0*. **b** shows higher magnification of the cortex. **e**, Motor axon terminals in a skeletal muscle of *Thy1-Brainbow-1.0*.

Color consistency between axons and dendrites of a given neuron.
Hippocampal granule neuron in dentate gyrus from Brainbow-1.0.
Sampled regions from dendrite (triangle) and axon (circle) are indicated.

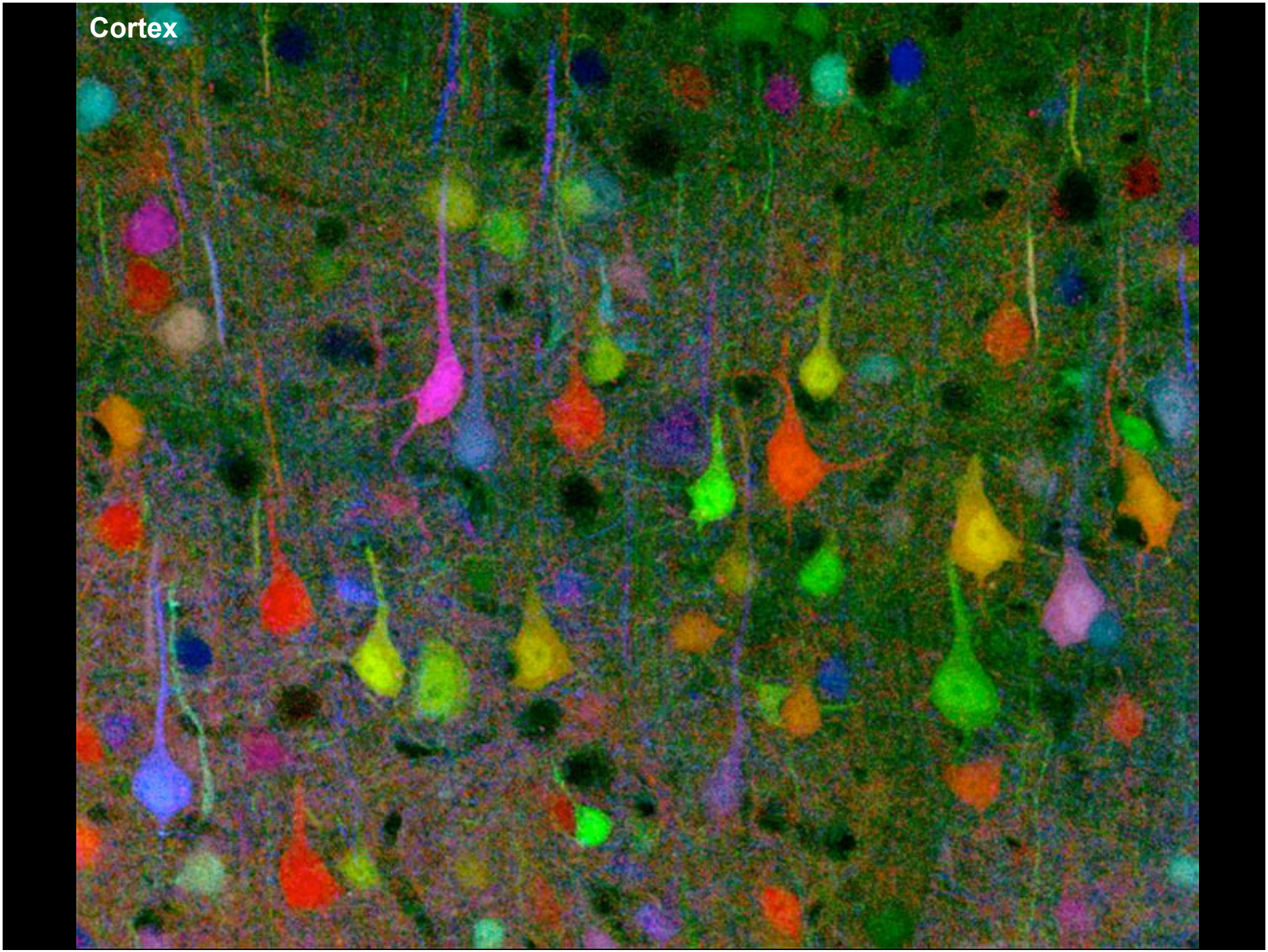


Portion of a mouse cerebellum. The multicolor labeling reveals the intricate meshwork created by ~mossy fiber" axons forming synapses in the area





Neurons of the dentate gyrus


Cortex

Painting of the ‘gray matter’
at the cerebral cortex