From Mice to Multiple Levels of Data: How to get the most out of your expensive mouse.

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The problem of cost!

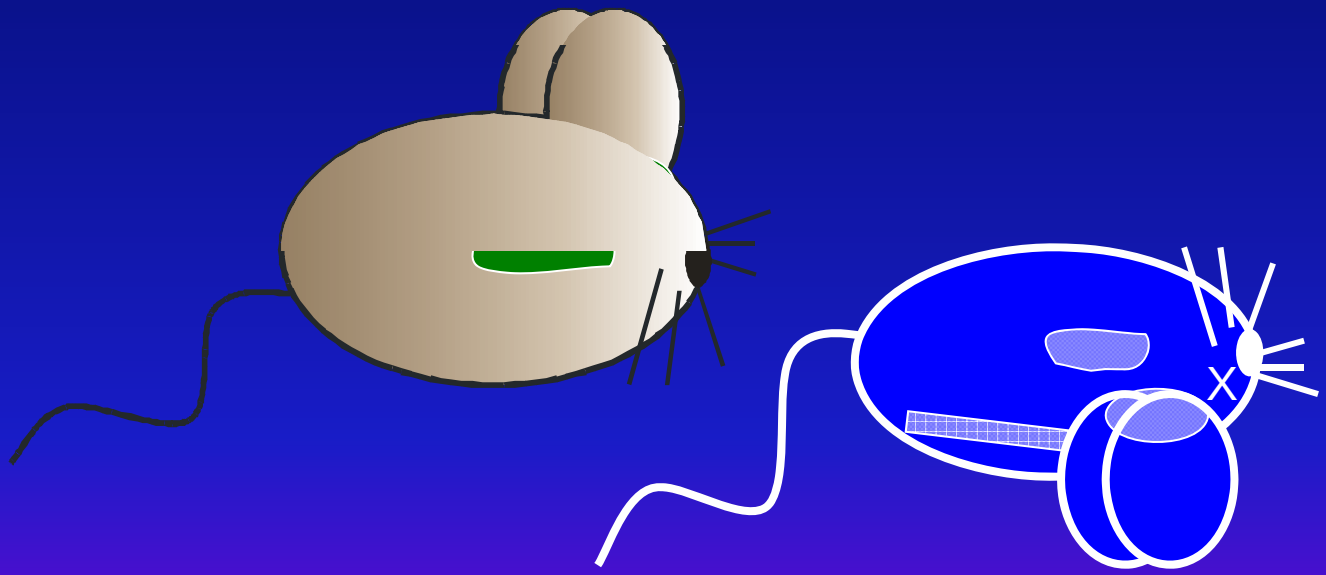
- A mouse line costs around $25,000 or more.
- Simple null (het x het) is 1:4
- Double null (double het x double het) is 1:16.
- Triple null (triple het x triple het) is 1:64
- Costs would be 3 x 25,000 x number of litters to obtain a single mutant

  (~6 litters at 11).
- Solution: use a single mutant multiple times to maximize data collection.

Conditional or tissue-specific knockouts

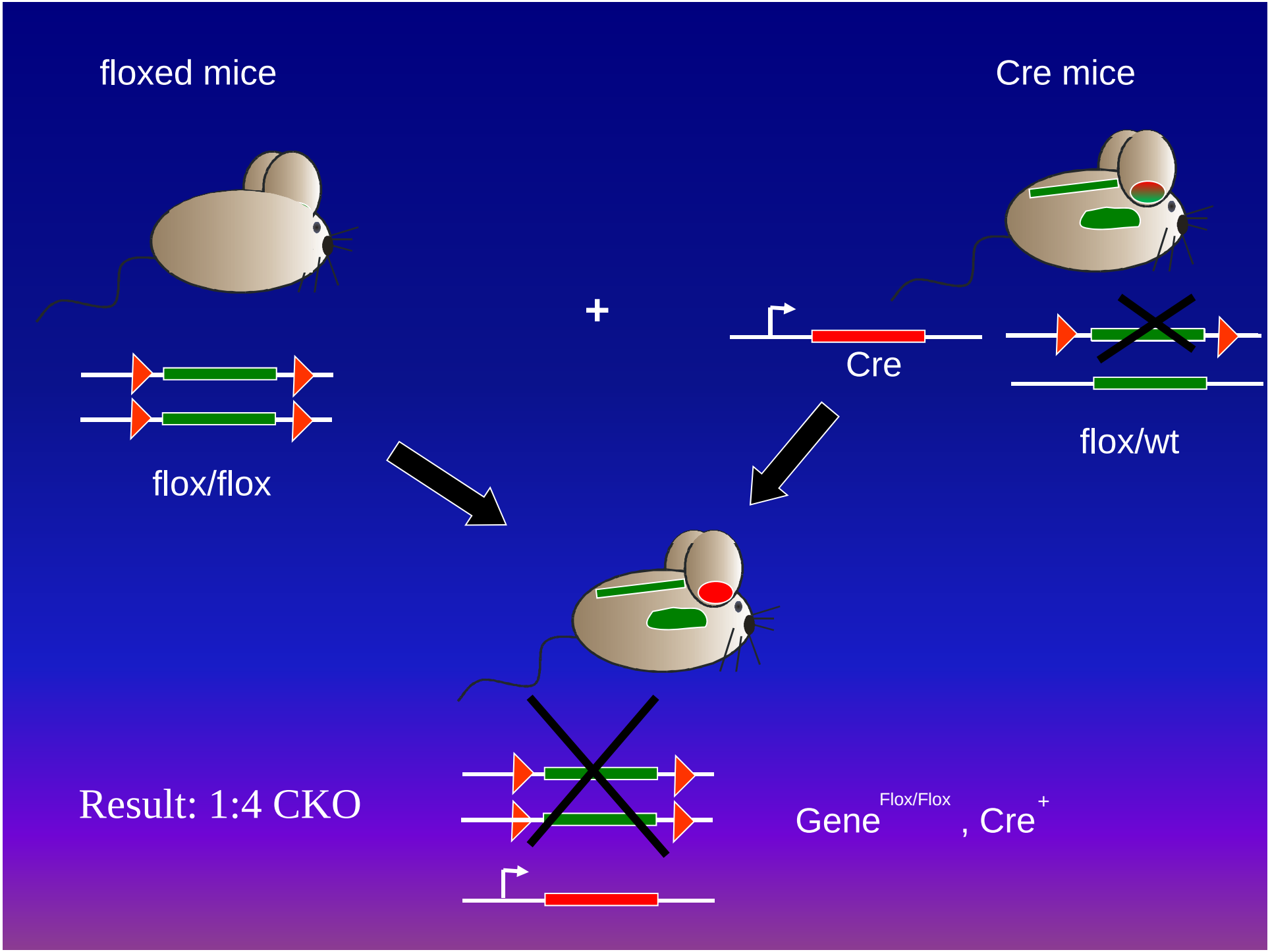
The problem with knockout or mutant mice is that the gene of interest is inactivated in ALL tissues, throughout embryogenesis.

For genes that are expressed in many tissues, especially early in development, the mutant is often early lethal.



The gene of interest is expressed in multiple locations

If we knock out the gene in all tissues, the mouse dies


floxed mice
Cre mice
+
Cre
flox/flox
flox/wt
Result: 1:4 CKO
Gene Flox/Flox , Cre +

Even with floxed genes there is a problem of cost!

- Single CKO: f/f x Cre;f/+ (hom x het x het) is 1:4
- Double CKO (double hom x double het x het) is 1:8.
- Triple CKO (triple hom x triple het x het) is 1:16
- Quadruple CKO (4 hom x 4 het x het) is 1:32
- For quadruple CKO, costs would be 5 x 25,000 x number of litters to obtain a single mutant

  (~3 litters at 11 + breeding to get there).
- Solution: use a single mutant multiple times to maximize data collection.

Outline of presentation

- Lipophilic dyes can be used for tract tracing in fixed tissue.
- Lipophlic dyes can be used in many color combinations, currently up to six.
- Lipophilic dyes can be combined with expression markers such as GFP or LacZ
- LacZ reaction (BCIP) product can be photoactivated using 2 photon excitation.
- Tissue can be processed for in situ hybridization
- Tissue can be used for immunocytochemistry.

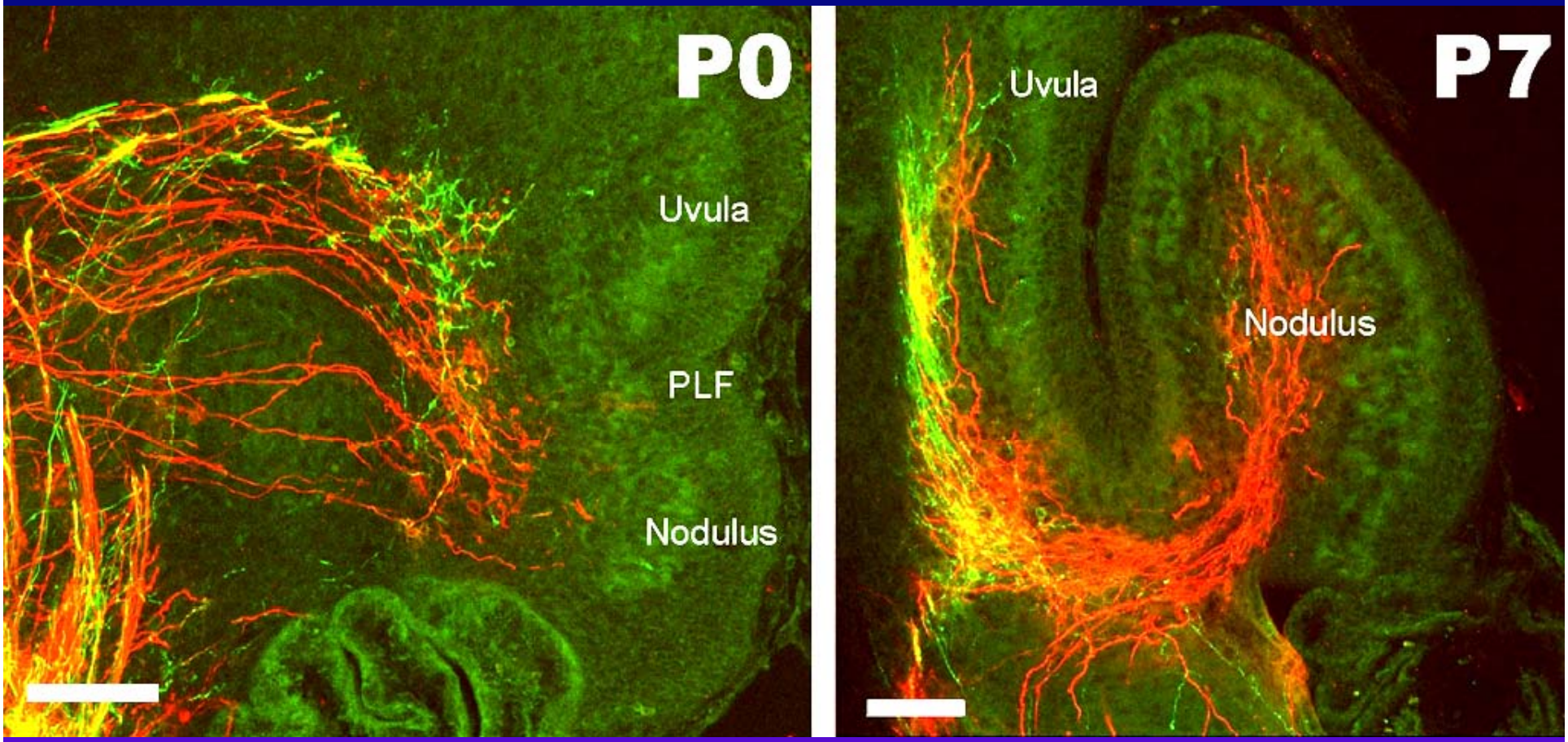
Background and significance of dye tracing

- The lipophilic indocarbocyanine dye DiI has revolutionized fiber tracing in fixed nervous system since its introduction in 1986 with nearly 2000 papers.
- Several lipophilic dyes (www.probes.com; www.mtarget.com/mtti/neurovue.html) exist that allow double labeling under certain circumstances. However, problems such as diffusion time differences, segregation with filters and relative visibility have not been completely overcome by past work (before 2005).
- The almost ubiquitous presence of single photon confocal microscopy has changed epifluorescence imaging as only specific excitation lines exist such as Kr/Ar 488,568, 647 or Red (537) , infrared (635) and Blue (405) diode.

Aims of this part of the presentation

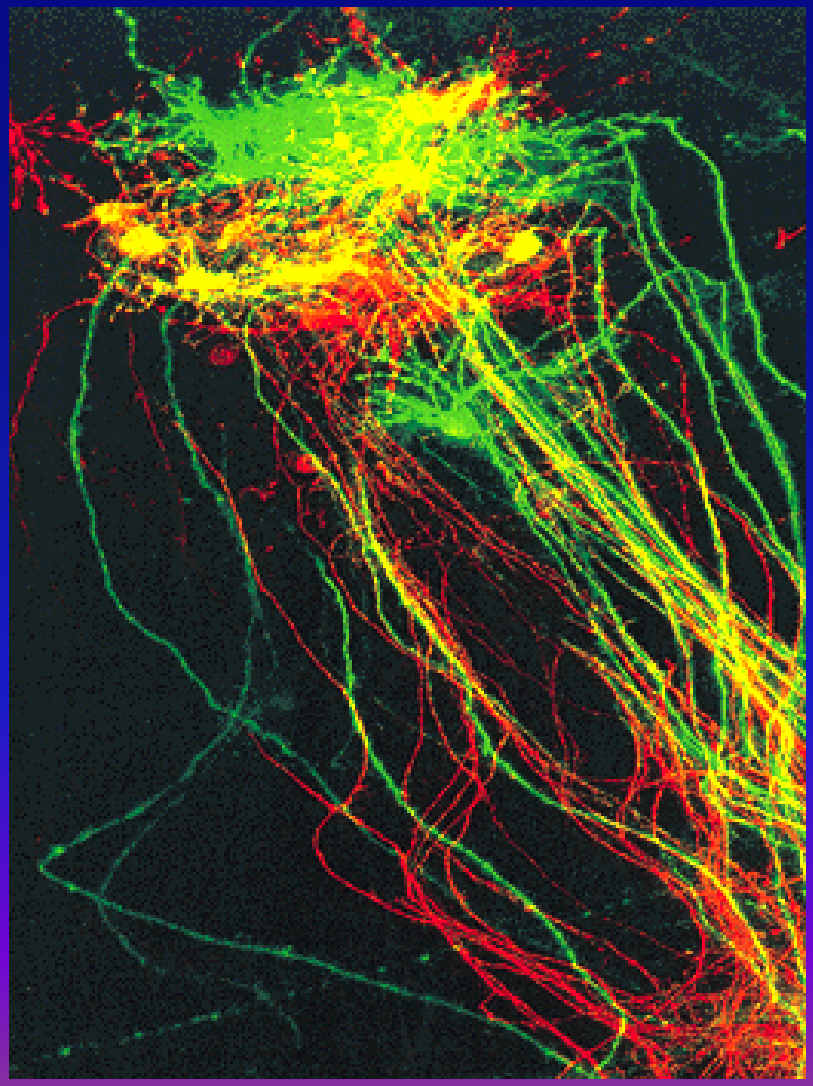
- Provide details of the use of dyes that will allow simple and completely segregated double, triple (up to six colors) labeling using near simultaneous applications in the neurosensory systems of embryos and juveniles to label the over 100 connections made and received by a neuron.
- Find dyes that fit in their excitation maxima closely to the physically specified excitation bands of the most commonly used Kr/Ar laser system and red/IR/blue diodes.

Double labeling can be achieved with existing dyes, but may be cumbersome



Maklad and Fritzsch, Dev. Brain Res 2003
DiI 3 weeks, DiA, 5 weeks 488/568 DM 500; EF515/30; 600/40

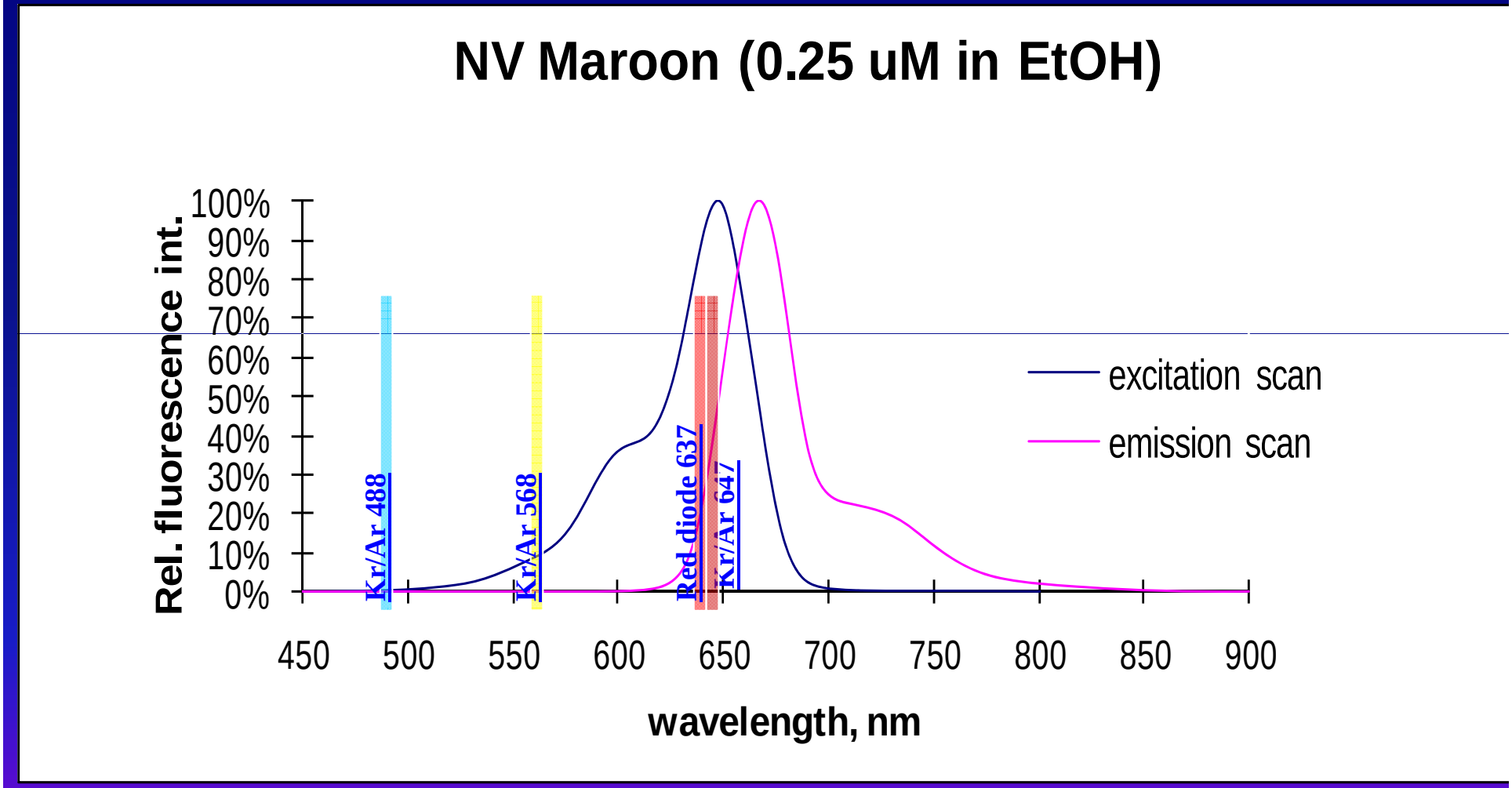
Previous work has identified a near infrared dye (DiD) with properties comparable to those of DiI.



Agmon et al.,
J. Neurosci. (1995)
15: 549-561
Picture provided from
Molecular Probes catalogue

Diffusion appears to be similar
Imaging was with the 647 nm line of a Krypton/Argon laser
Application was to barrel fields

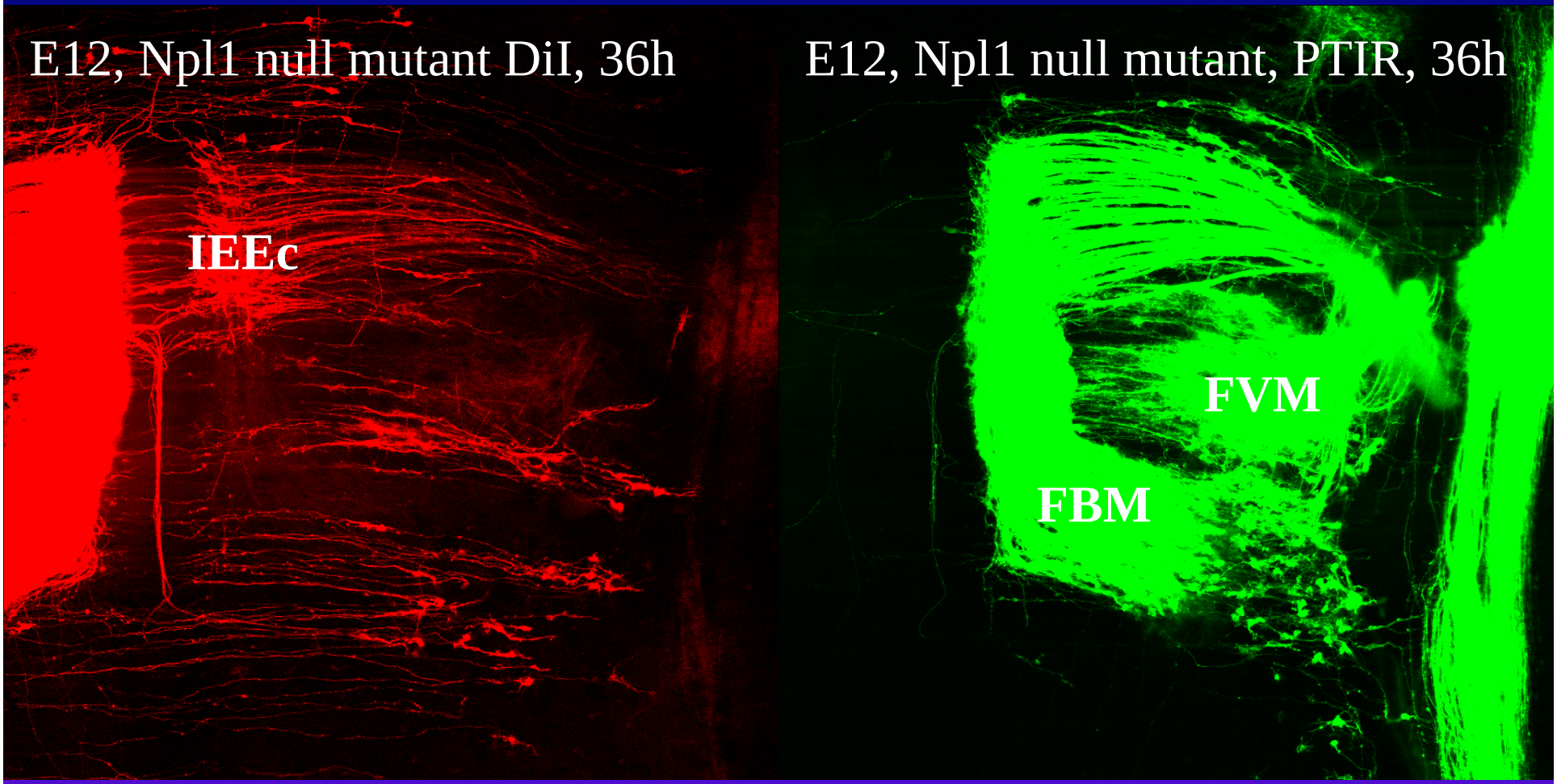
Fluorescence Spectra of NV Maroon



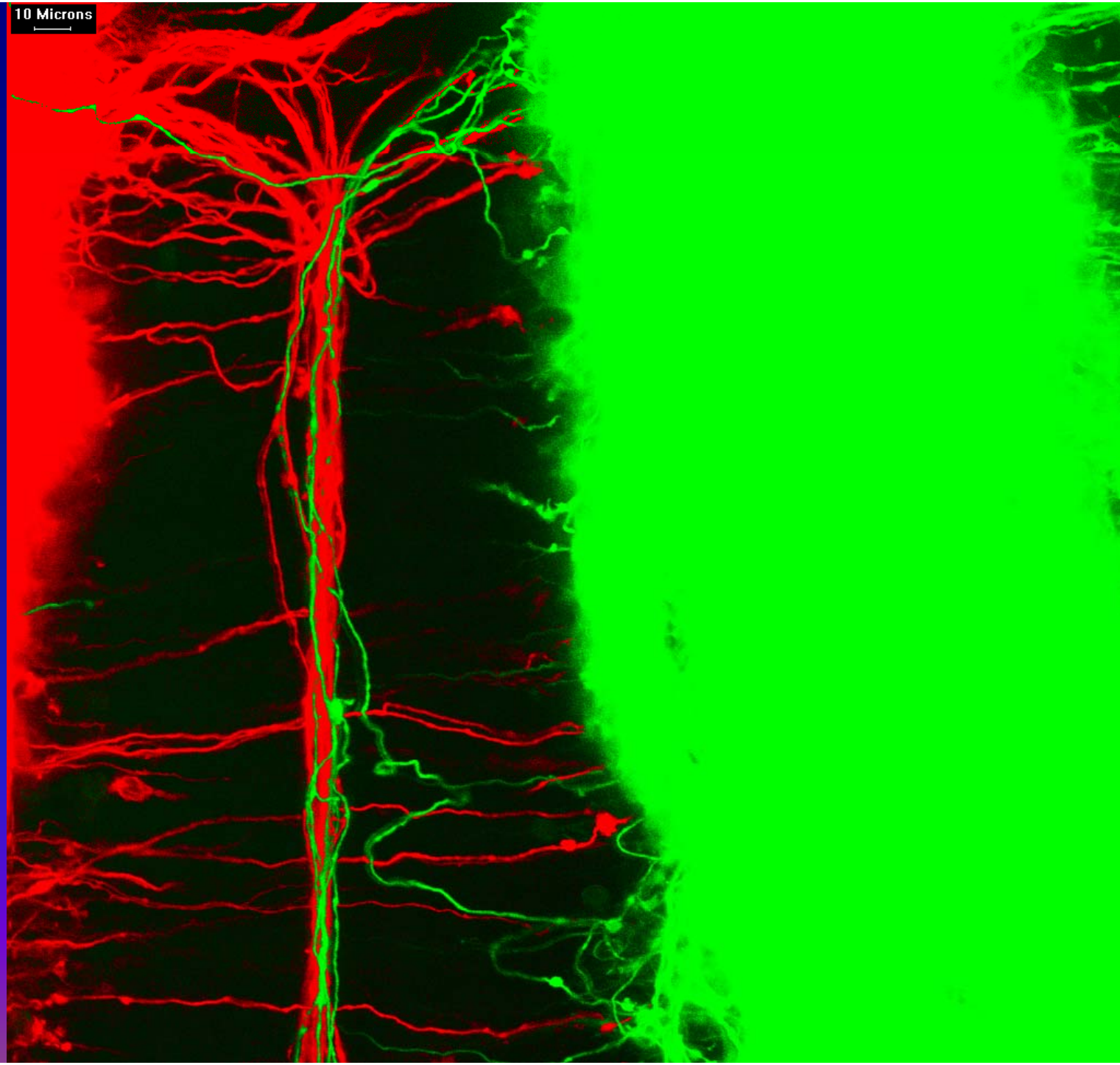
DiD: 644/665

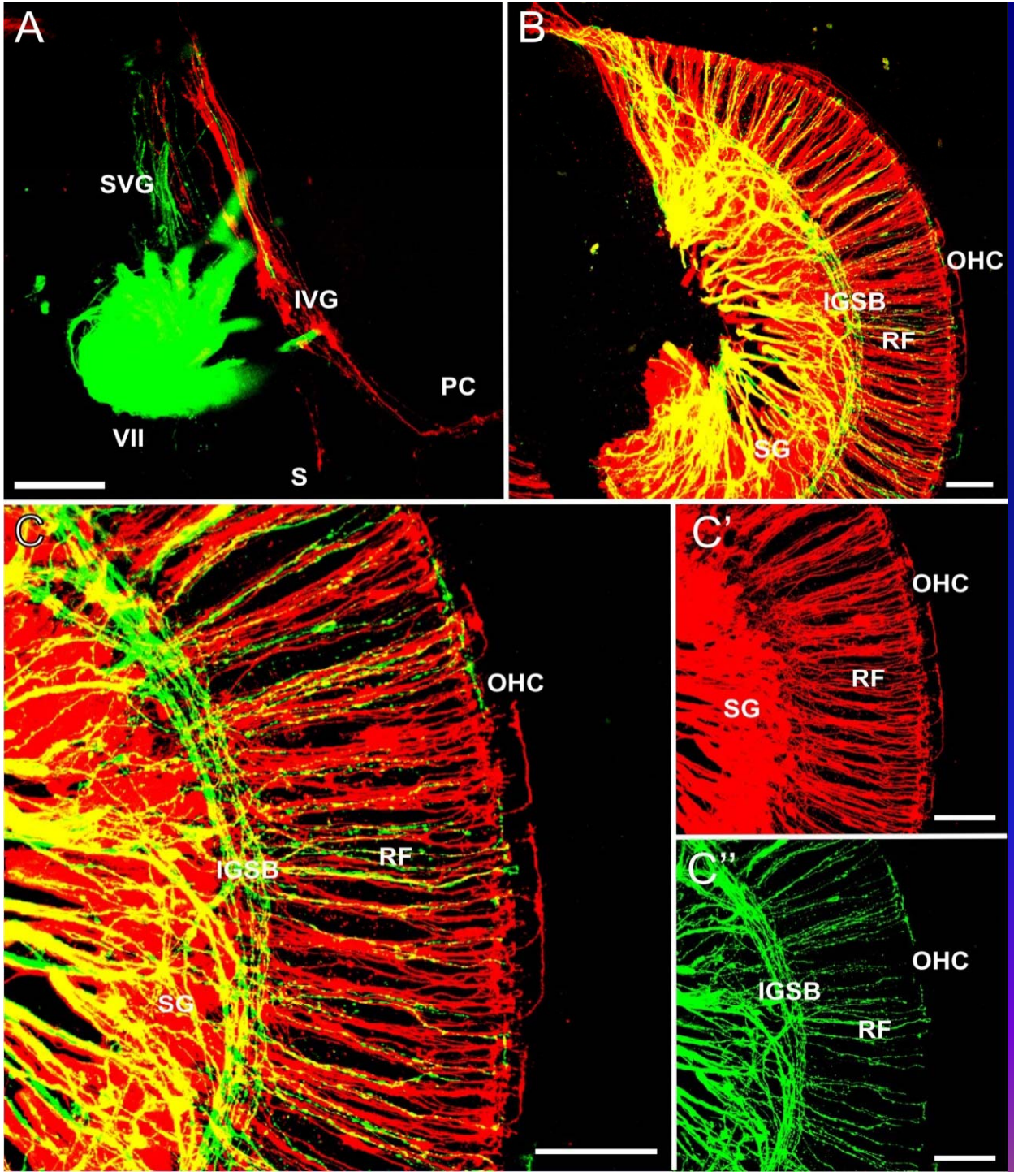
PTIR271: 647/667

PTIR/DiI double labeling: no bleed trough



Diffusion: 36 h at 37 ^{o}C; Imaging: BioRad Radiance 2000;
Excitation: 568/637; Dichroic: 500/650, EF: 600/40; 660LP


10 Microns

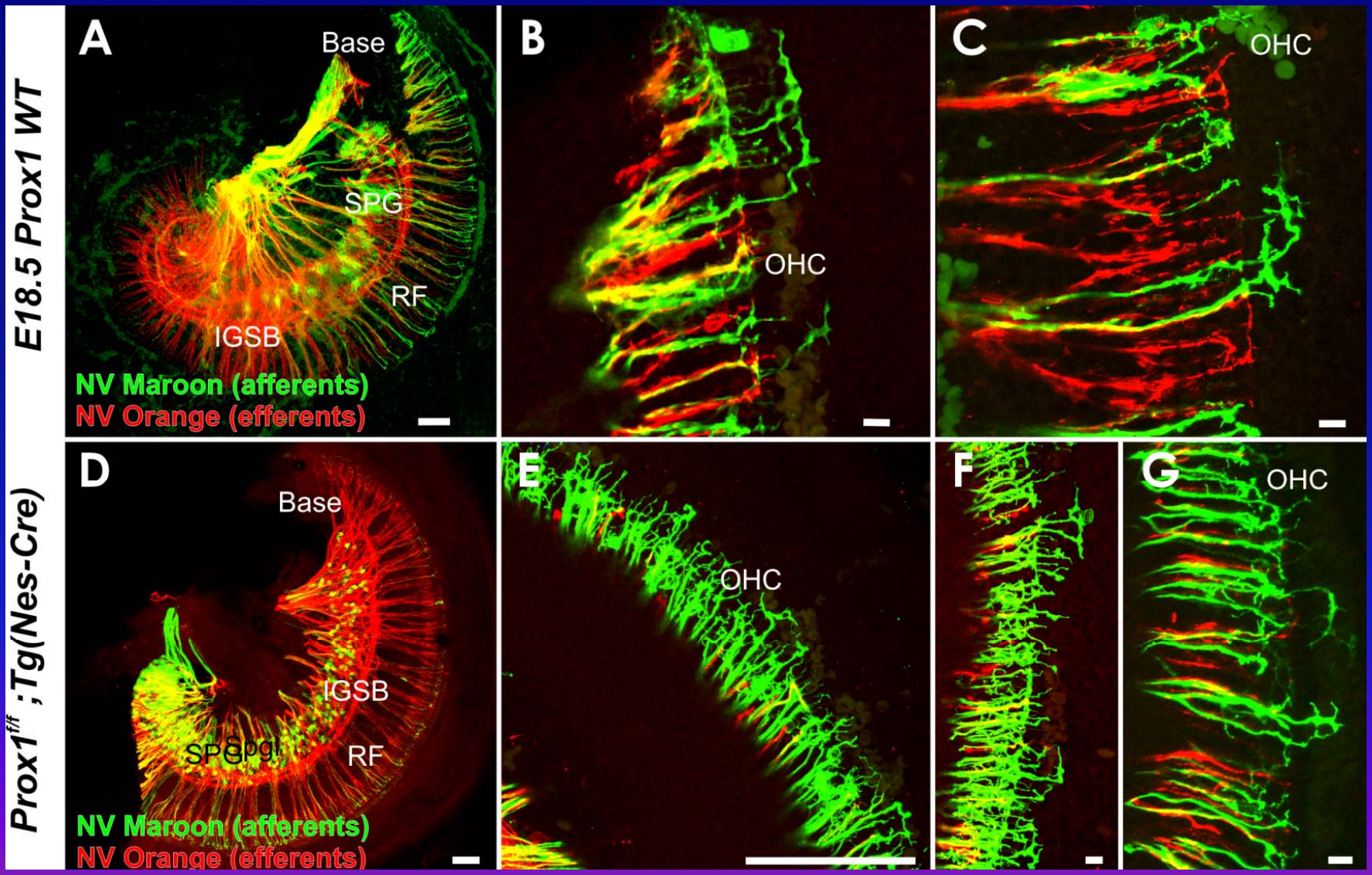


Double labeling reveals details of afferent and efferent projection

By applying NVR and NVM to afferents and efferents, projections to the ear can be studied at the single fiber level to reveal interactions between fibers in normal and mutant mice .

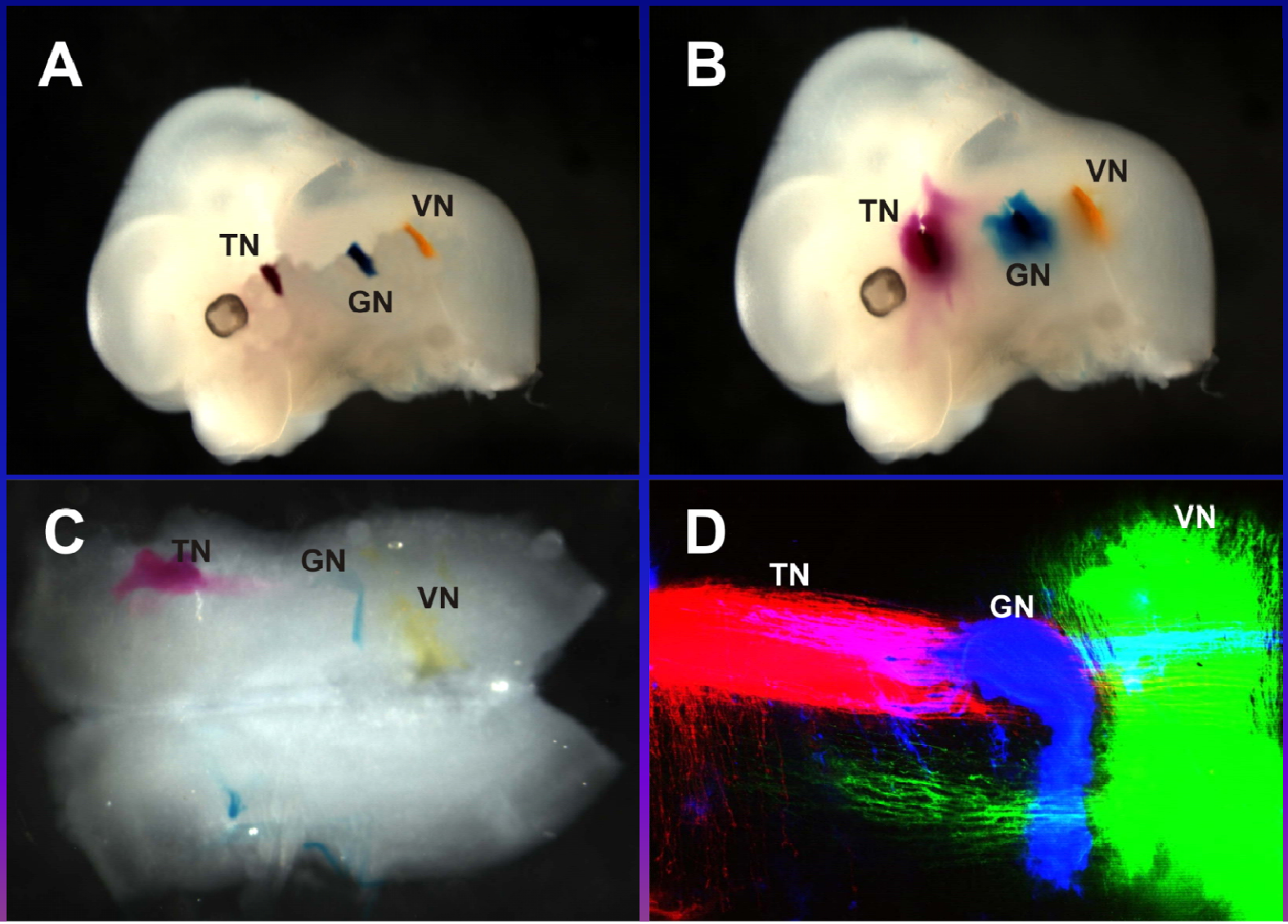
Simmons et al., 2010

Double labeling can help understand mutant phenotypes

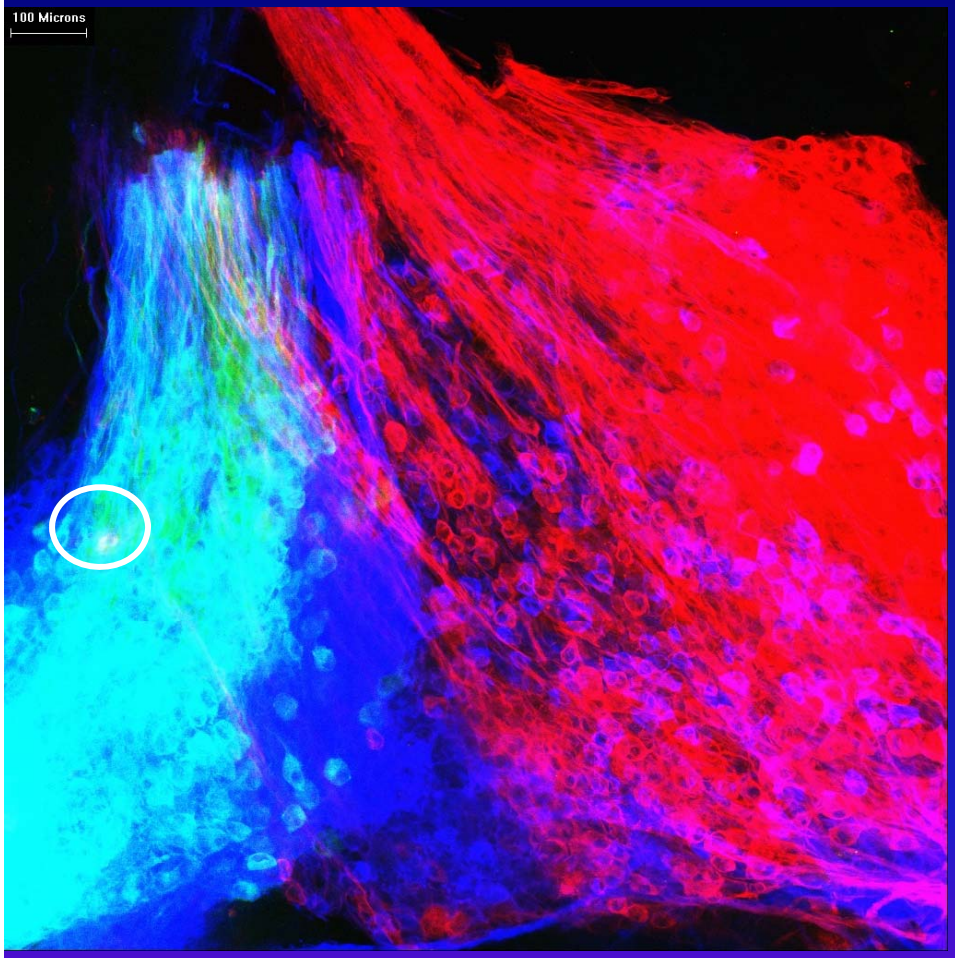


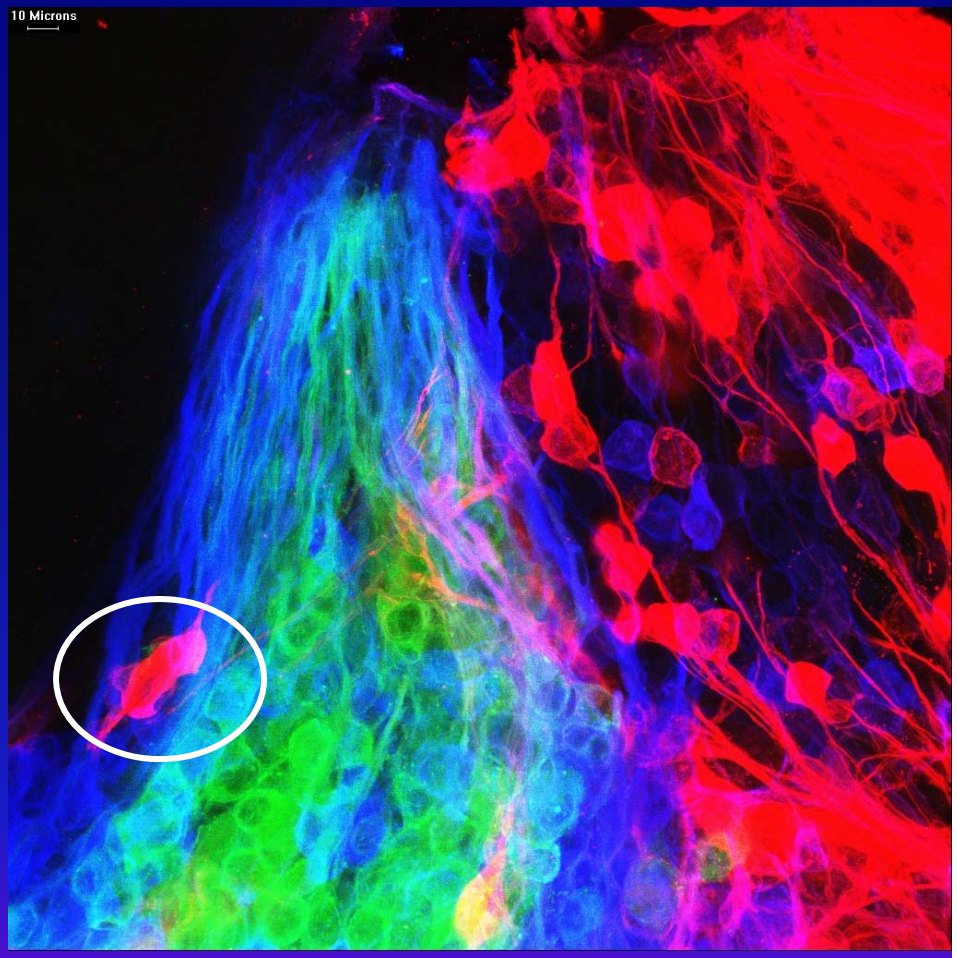
Fritzsch et al., 2010

Lipophilic dye placement and imaging in a mouse embryo using 3 dyes.



Triple labeling of inner ear sensory neurons

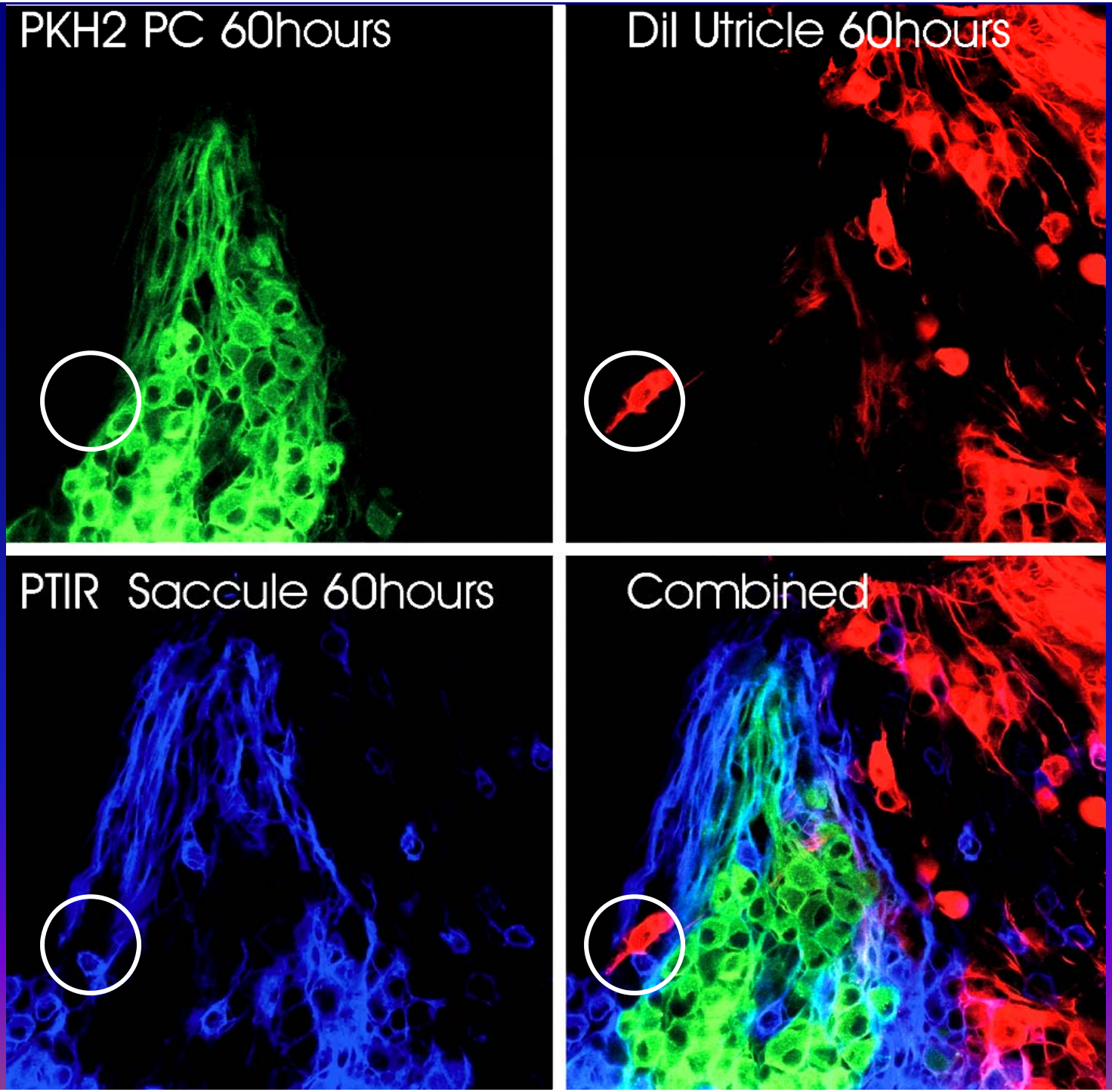


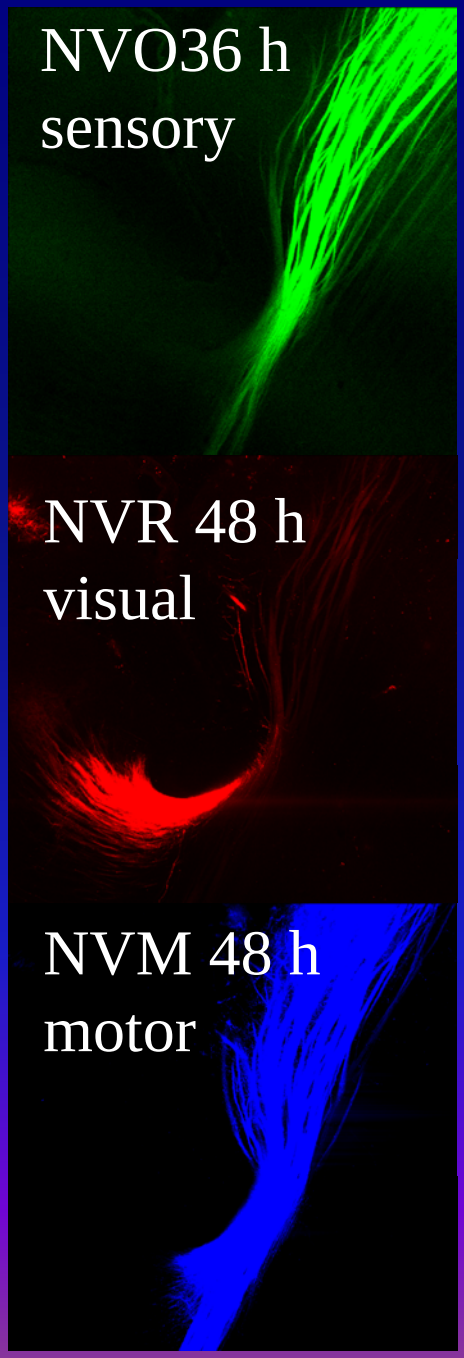


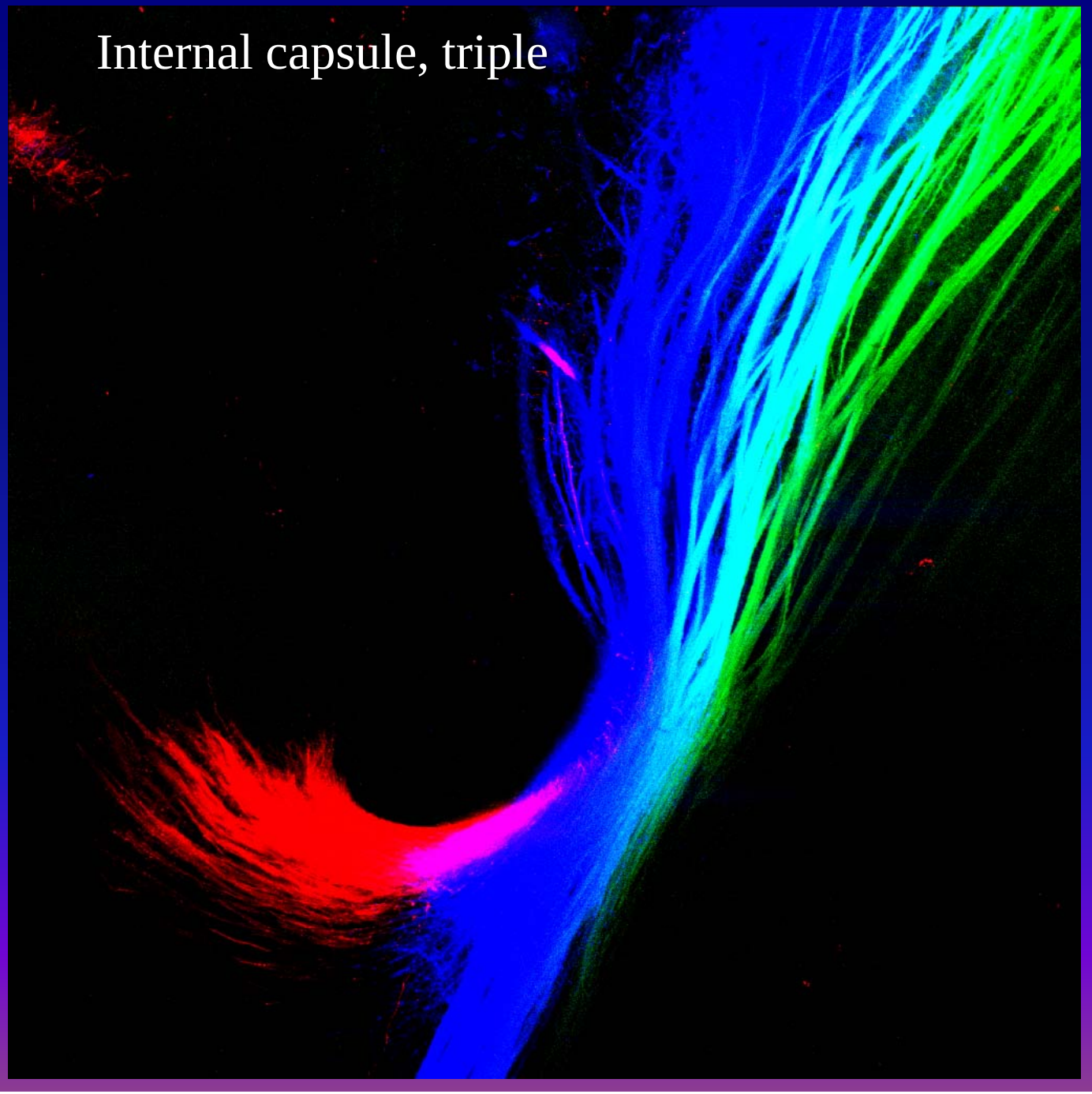
Diffusion: 60 h at 37 ^{o}C

Imaging: BioRad Radiance 2000; Excitation: 488/568/637

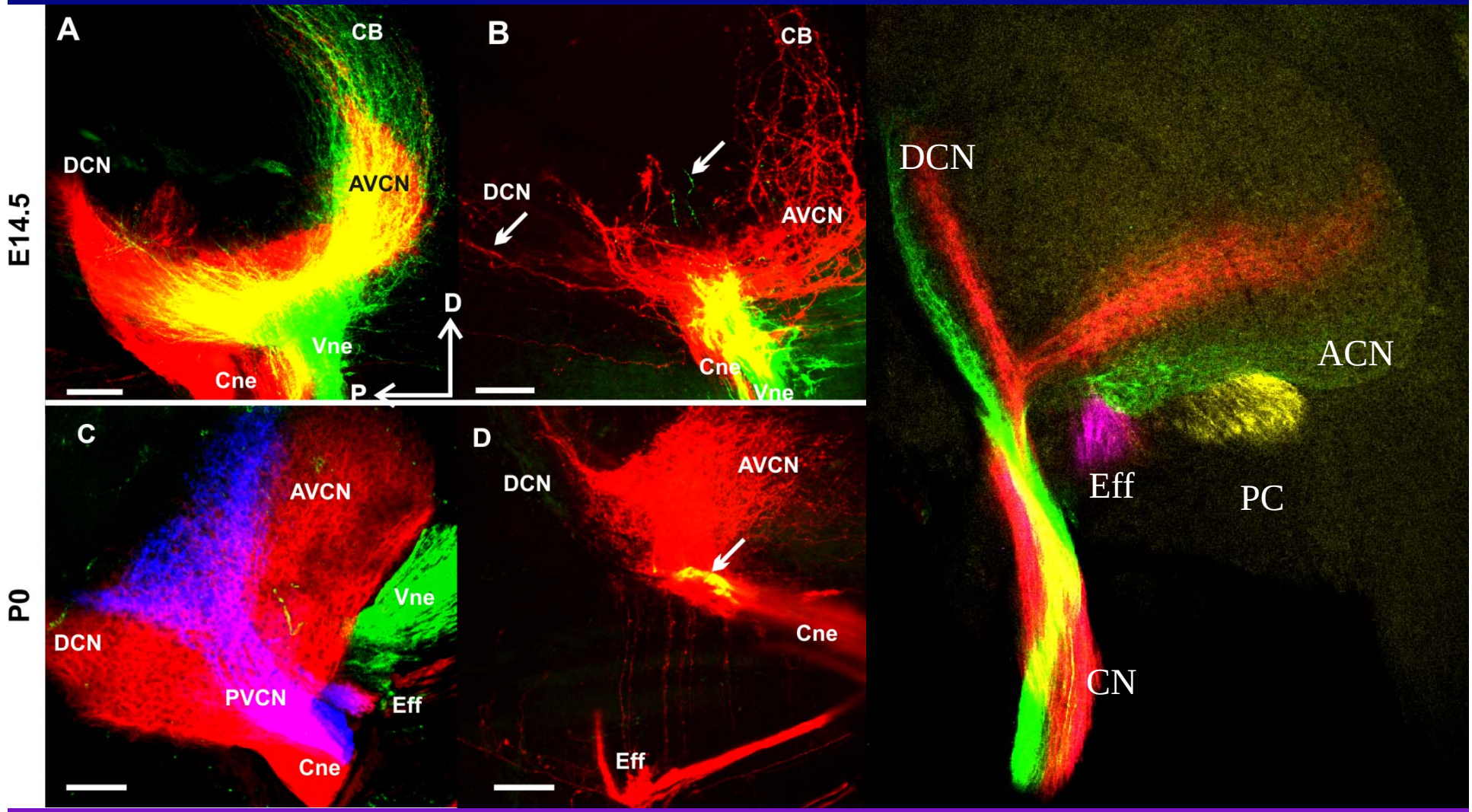
Dichroic: 500/650, Emission Filters: 515/40; 600/40; 660LP


PKH2 PC 60hours
DiI Utricle 60hours
PTIR Saccule 60hours
Combined


NVO36 h
sensory
NVR 48 h
visual
NVM 48 h
motor

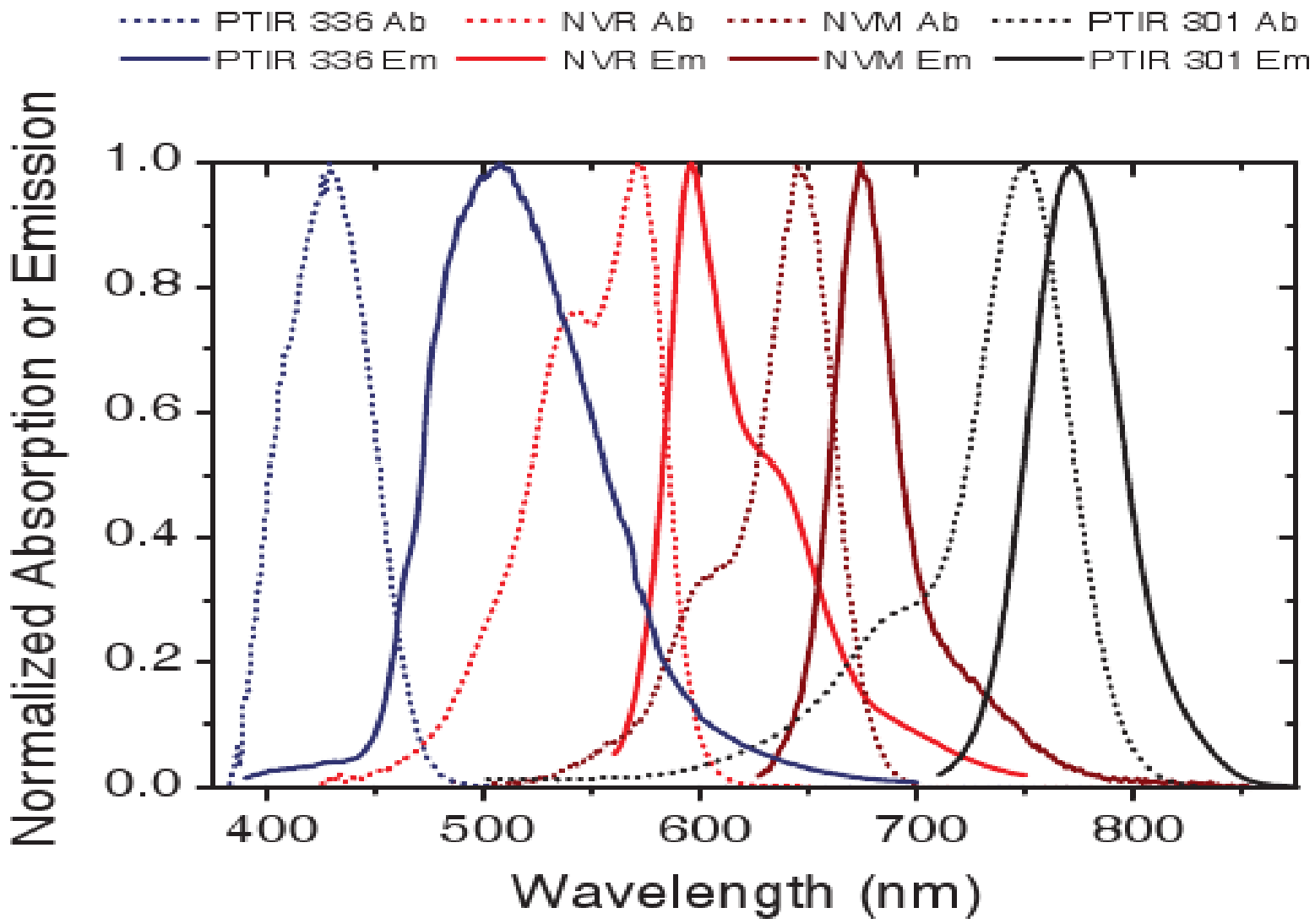

Internal capsule, triple

All ear afferents enter through cochlear nerve, efferents enter through facial nerve in conditional Neurod1 null mice

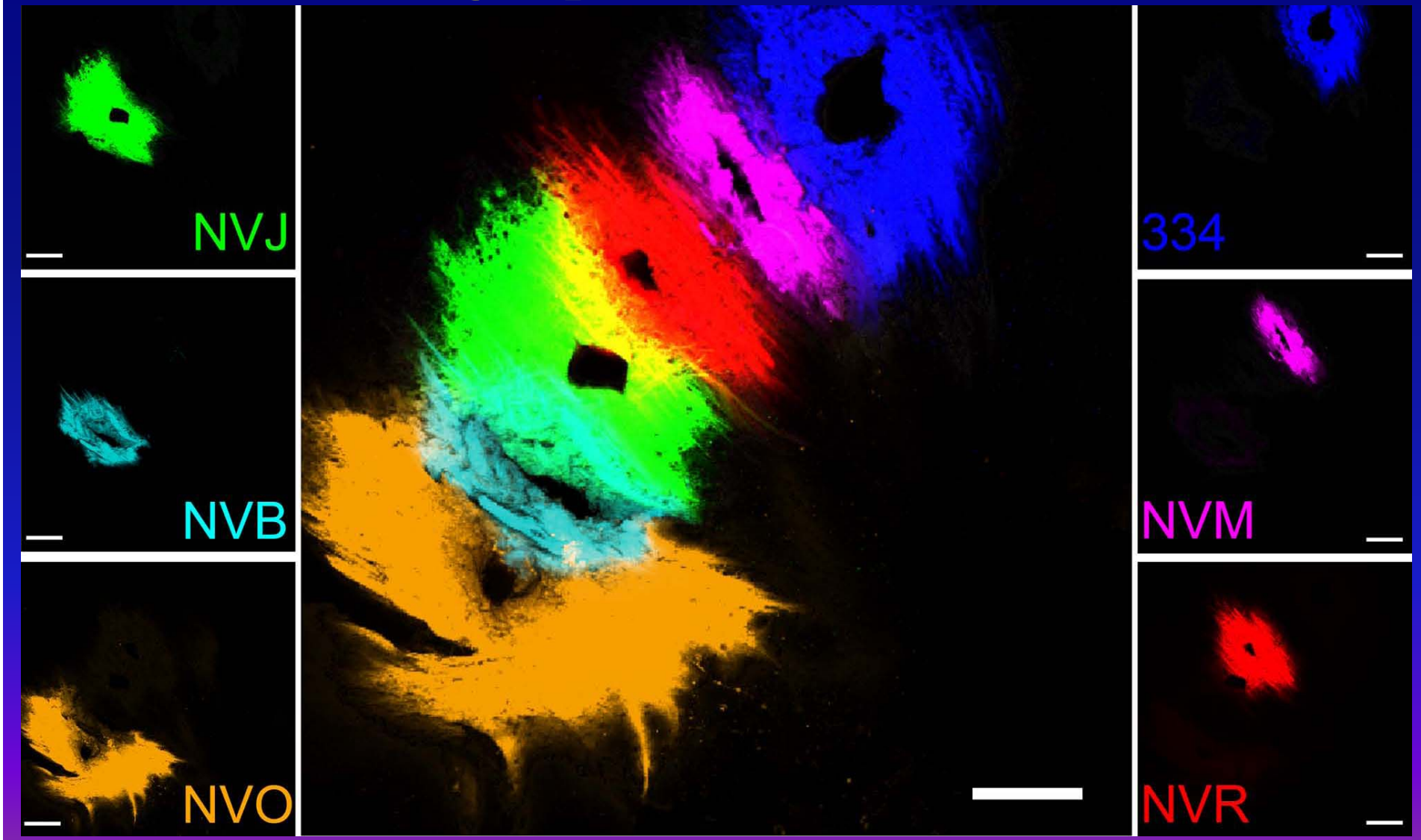


Jahan et al., 2010

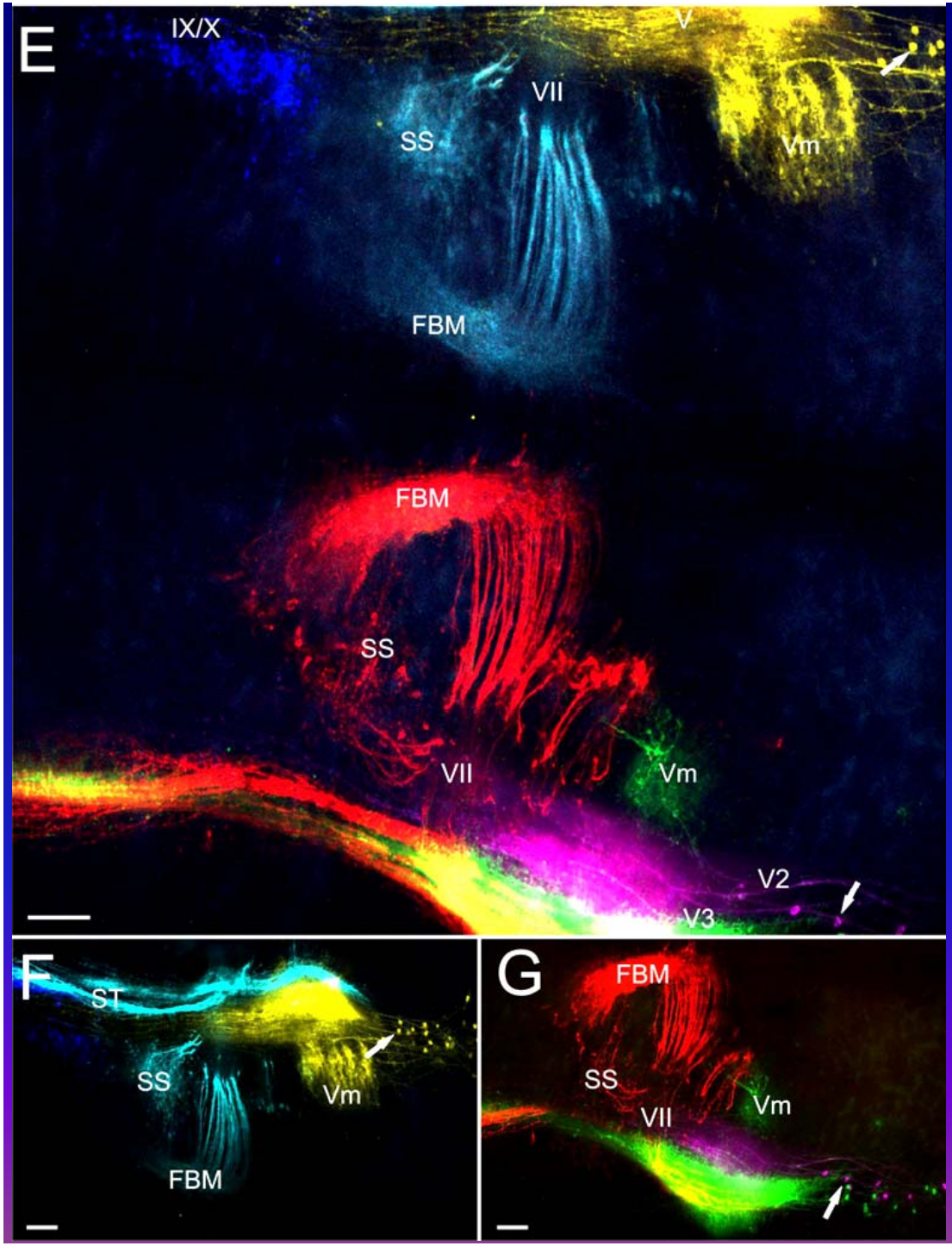
Development of six dyes



Six dyes can be imaged discretely using single photon excitation



Tonniges et al., 2010 J Microsc. 239(2):117-34

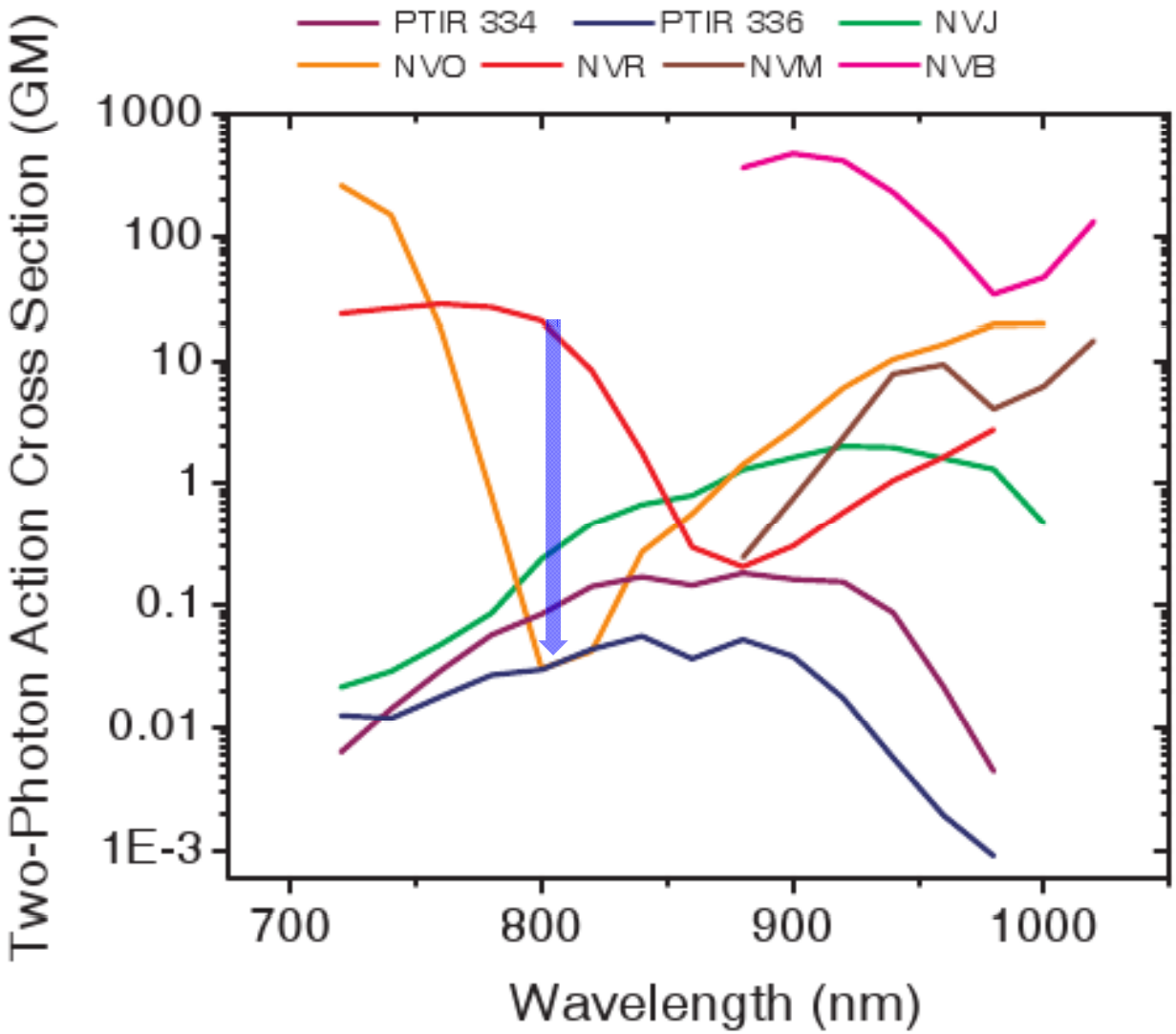


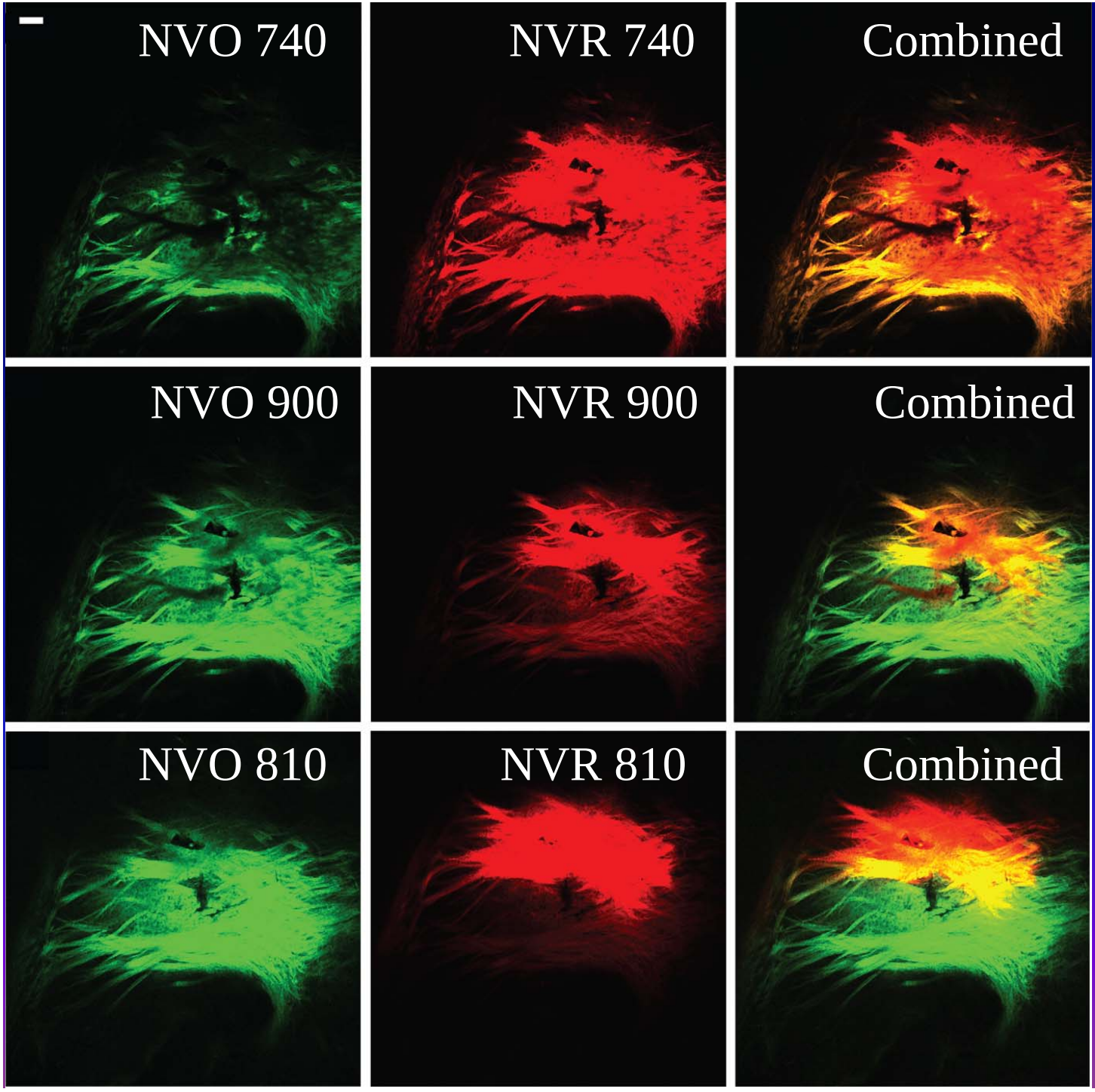
Six color simultaneous imaging allows for better understanding of adjacent and overlapping neuronal populations

Projections can be simultaneously imaged for six cranial nerves at the same or opposing sides of the brain using simultaneous dyes application

Images taken with Leica SP5 confocal microscope.

2P ‘Spectra’

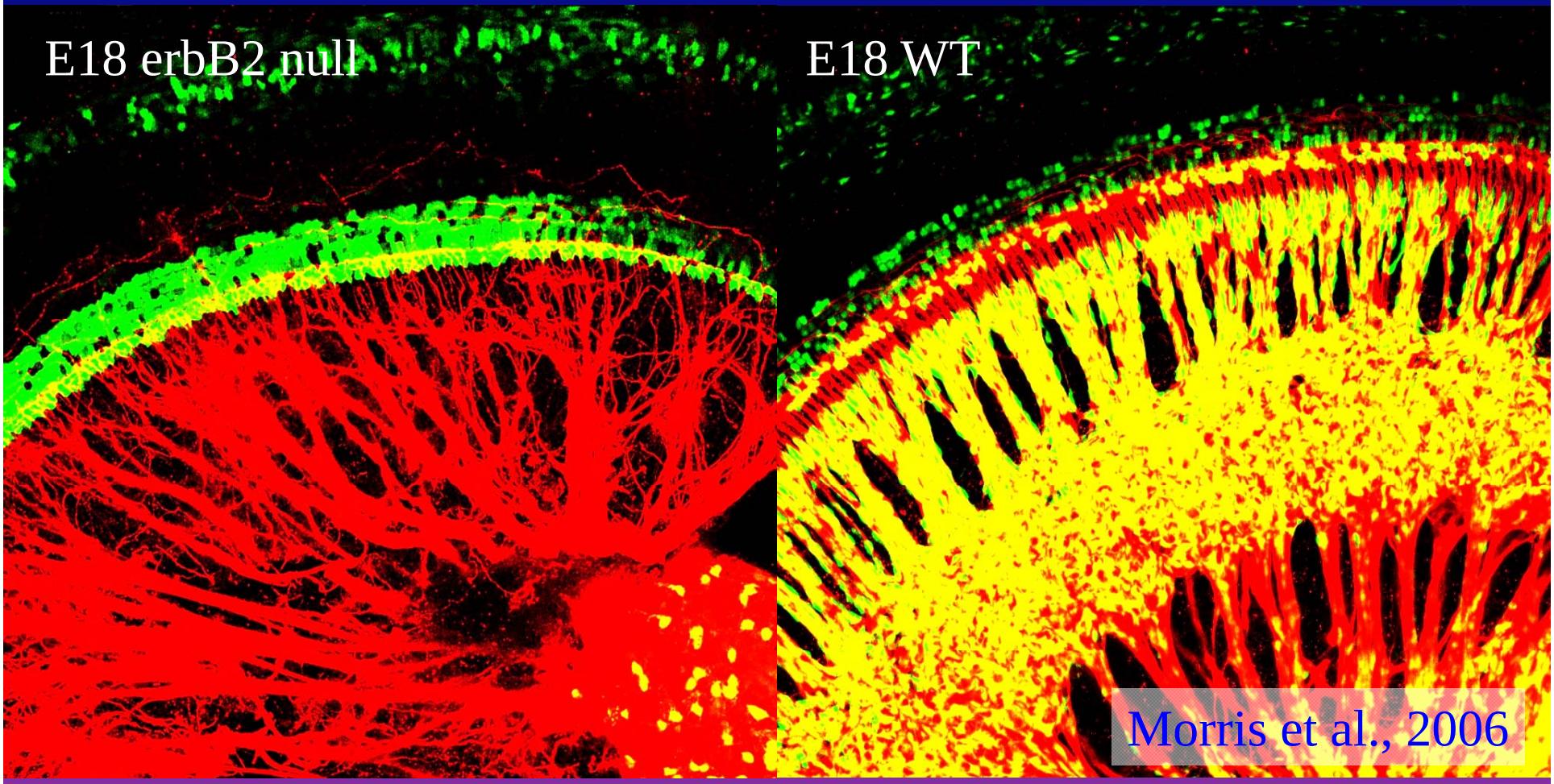

PTIR 334
PTIR 336
NVJ
NVO
NVR
NVM
NVB
Two-Photon Action Cross Section (GM)
1000
100
10
1
0.1
0.01
1E-3
700
800
900
1000
Wavelength (nm)



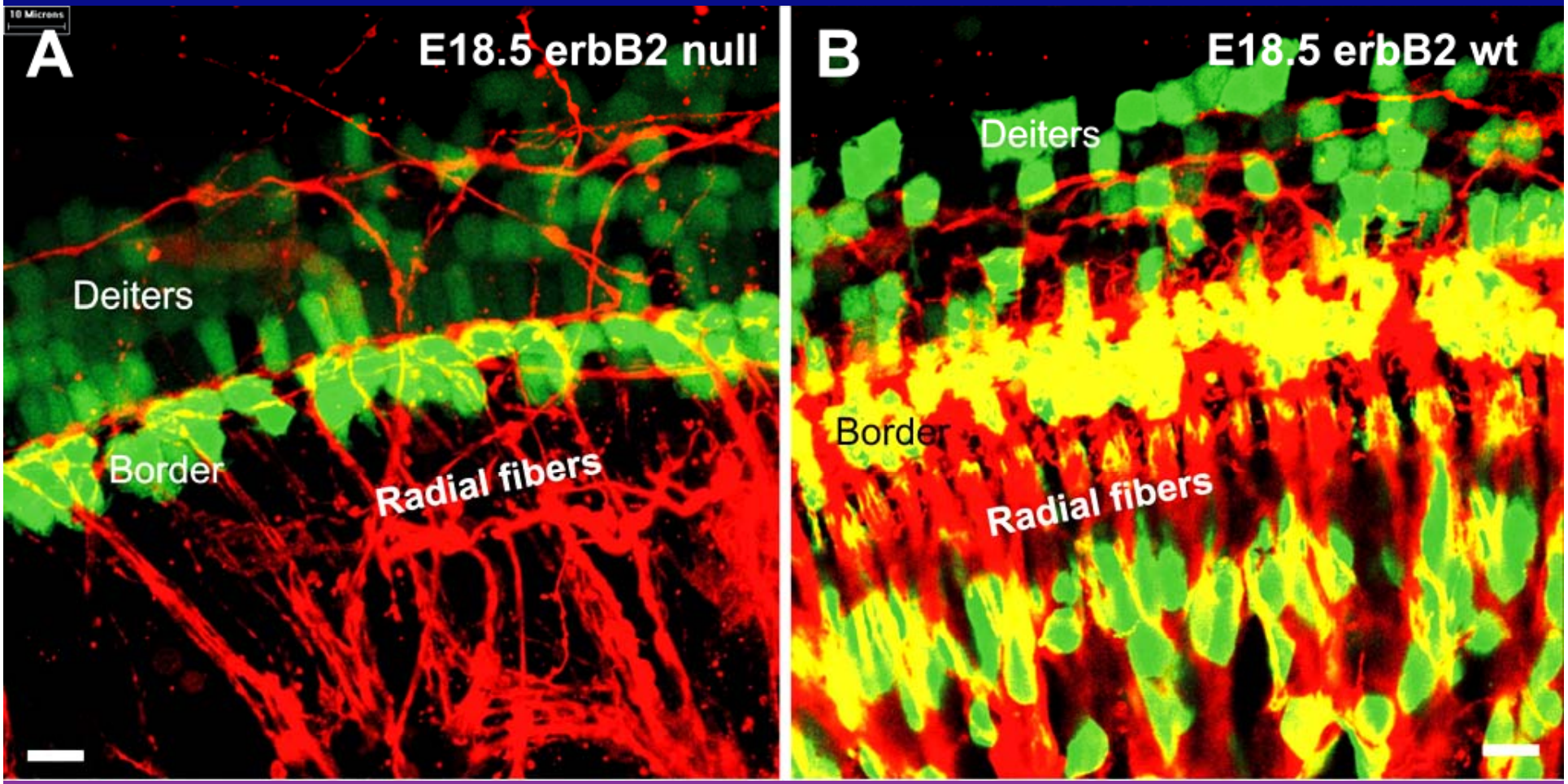
2P excitation can segregate dyes that are difficult for single photon

Overlapping excitation and emission can be segregated using proper 2P excitation and filter settings for complete segregation.

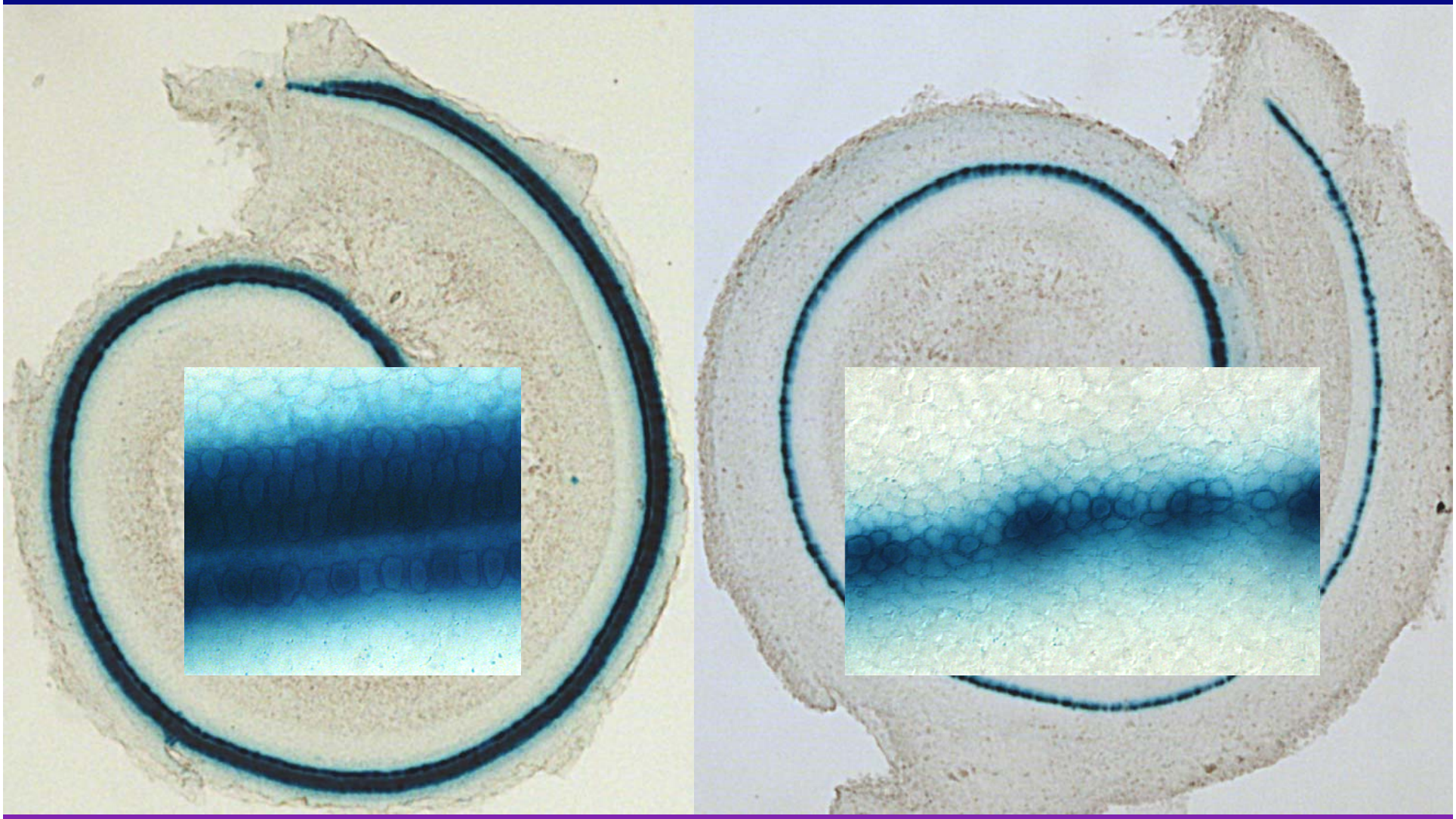
Lipophilic dyes can be combined with GFP to simultaneously image expression of a gene and nerve fibers



PLP-eGFP expression is on in adults and can be used to drive gene expression selectively in supporting cells



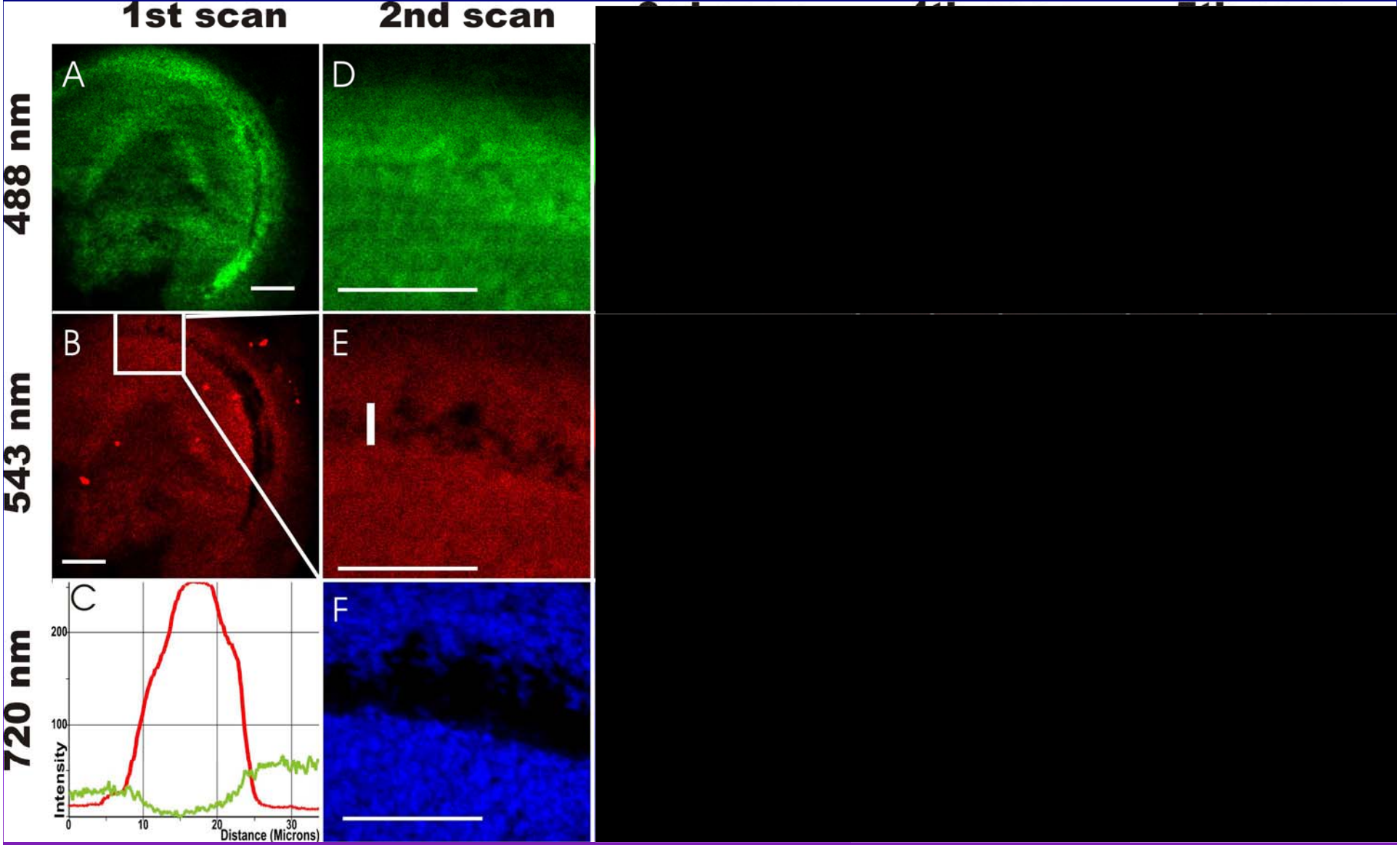
Atoh1 null mice develop almost normal ear morphology and sensory epithelia with LacZ positive cells.



Atoh1-LacZ het

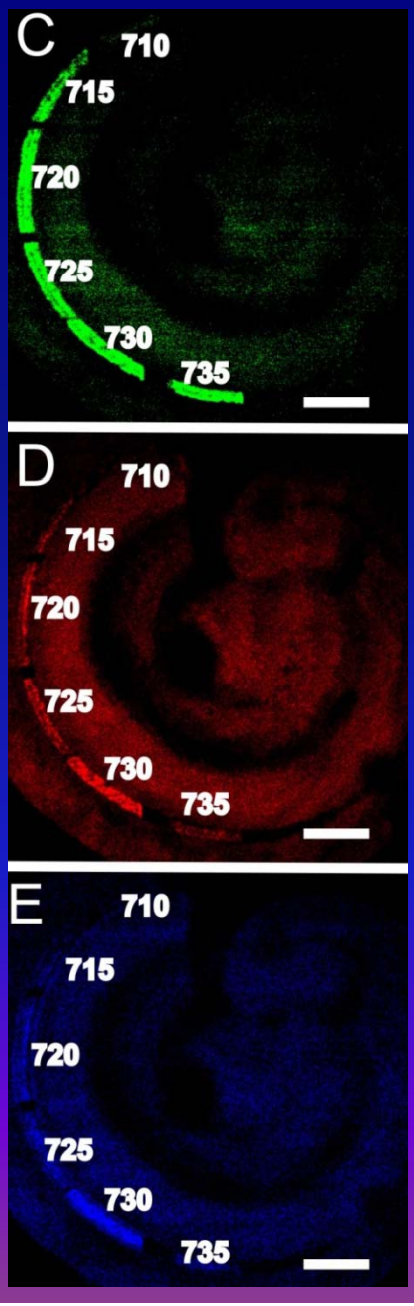
Atoh1-LacZ null

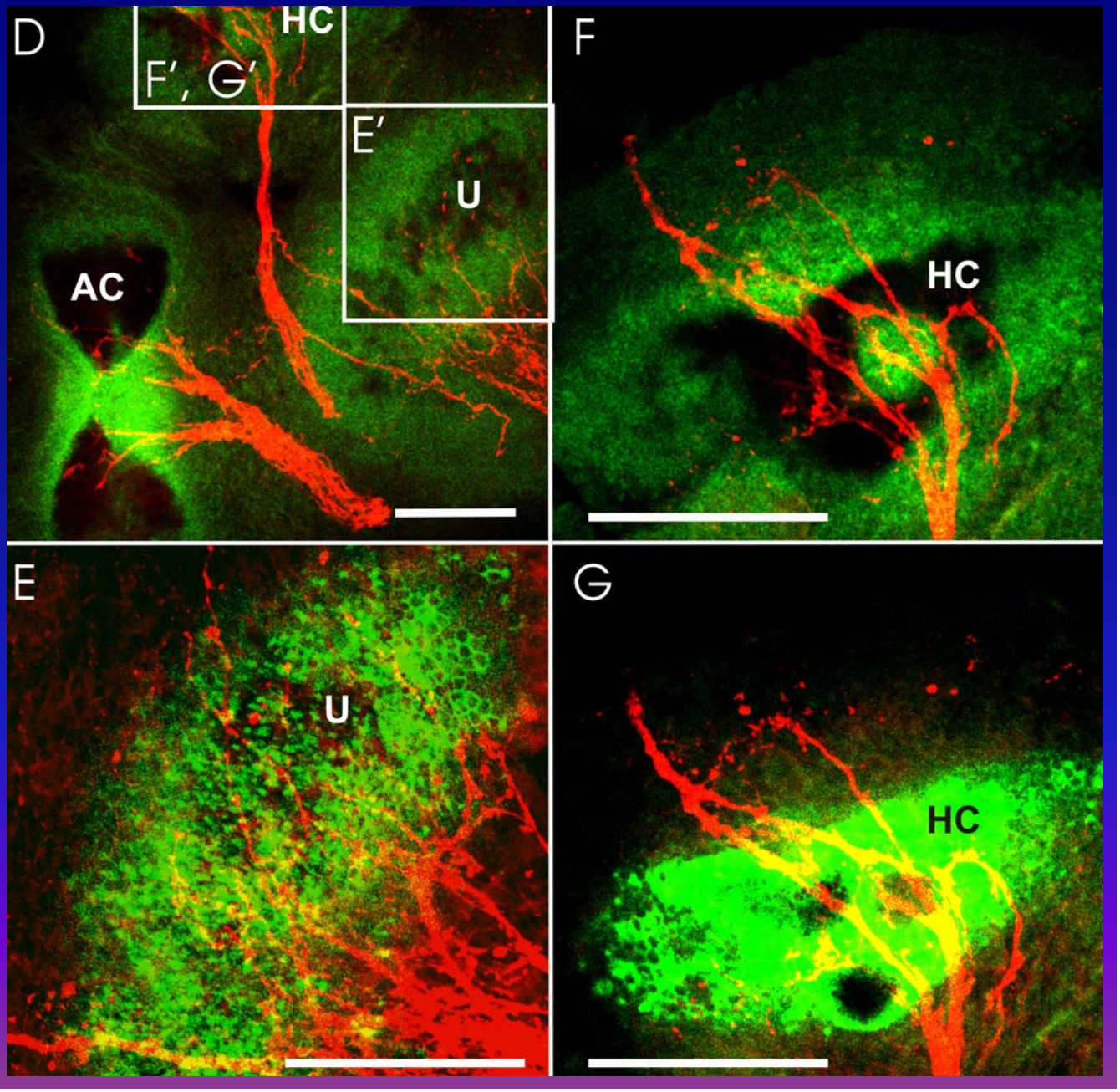
5-bromo-4-chloro-3-indolyl (BCI) responds to 2P with emission



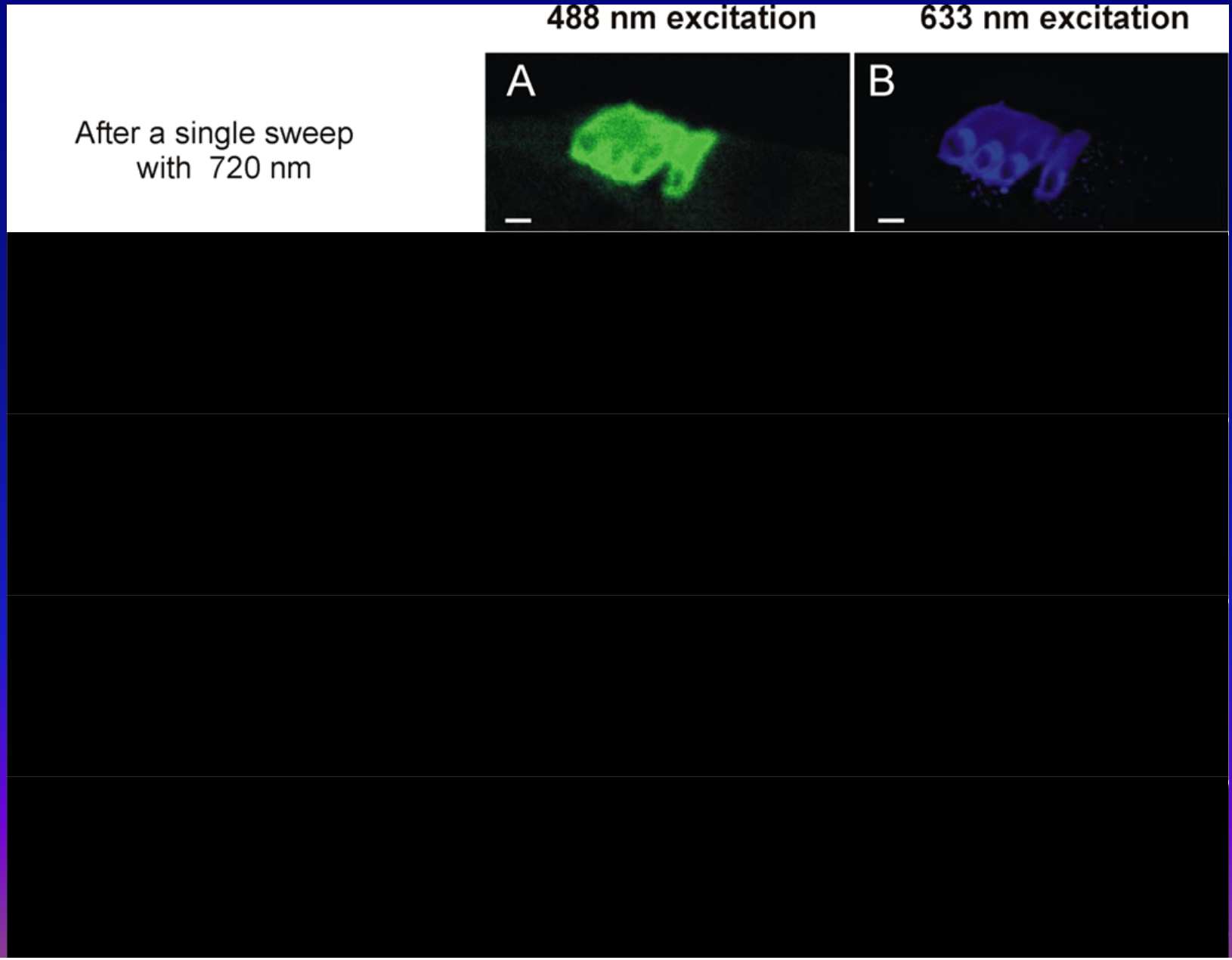
Matei et al., 2006

2P activation works at 730nm and helps image expression





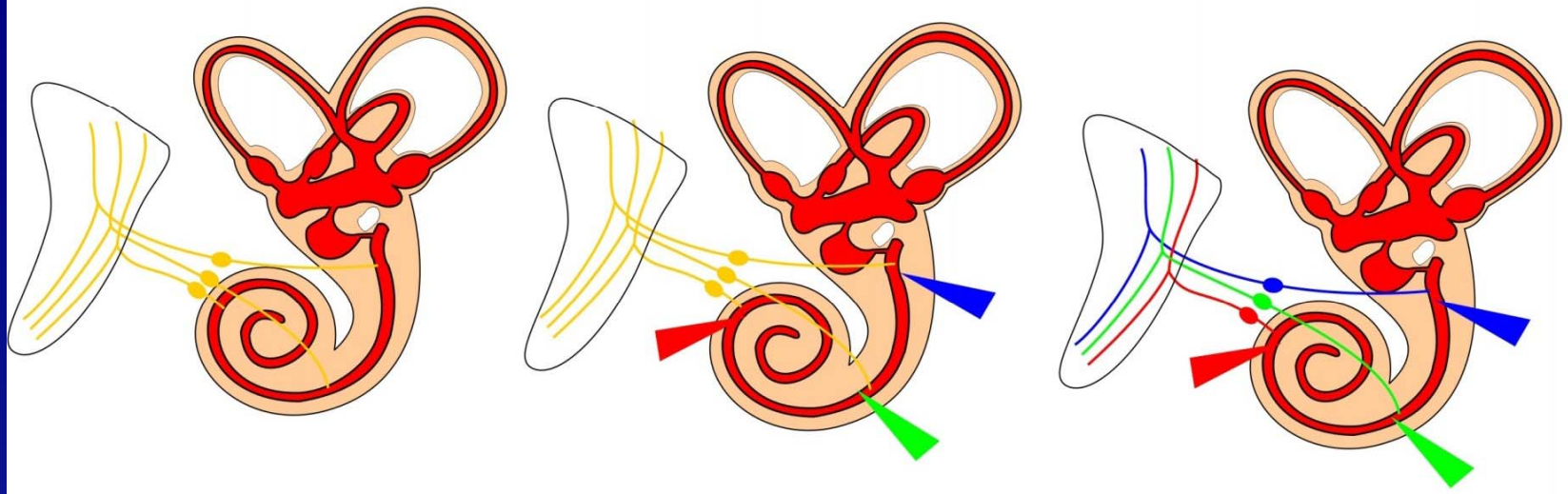
BCI is a 2 bit molecular information store



Organization

Dye application

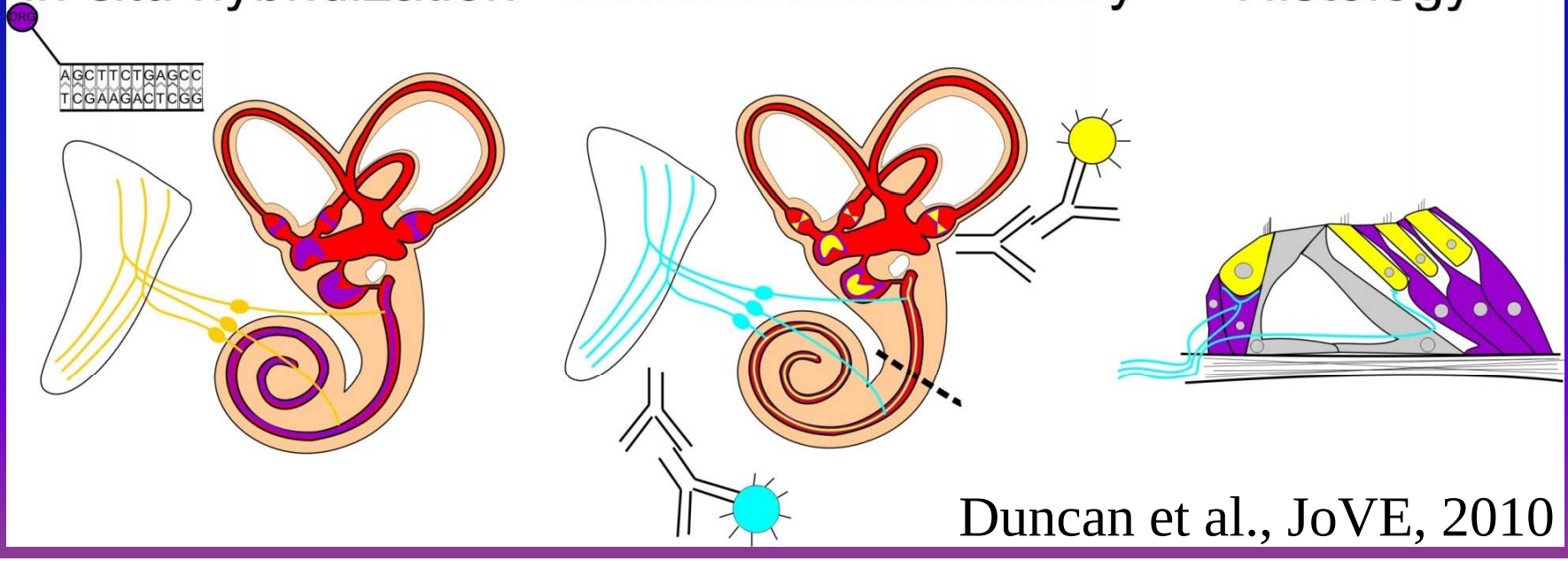
Dye diffusion



in situ hybridization

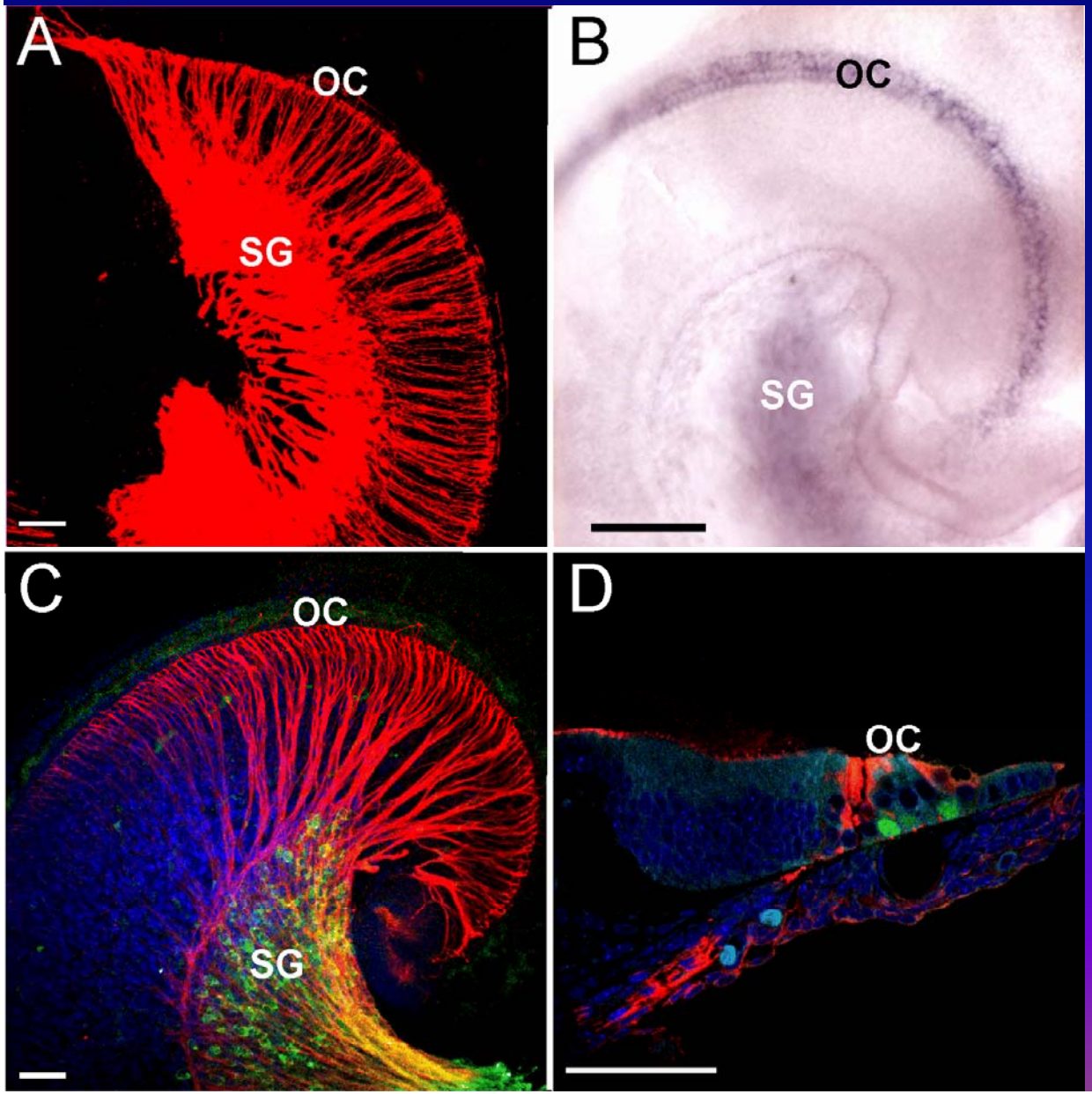
Immunohistochemistry

Histology



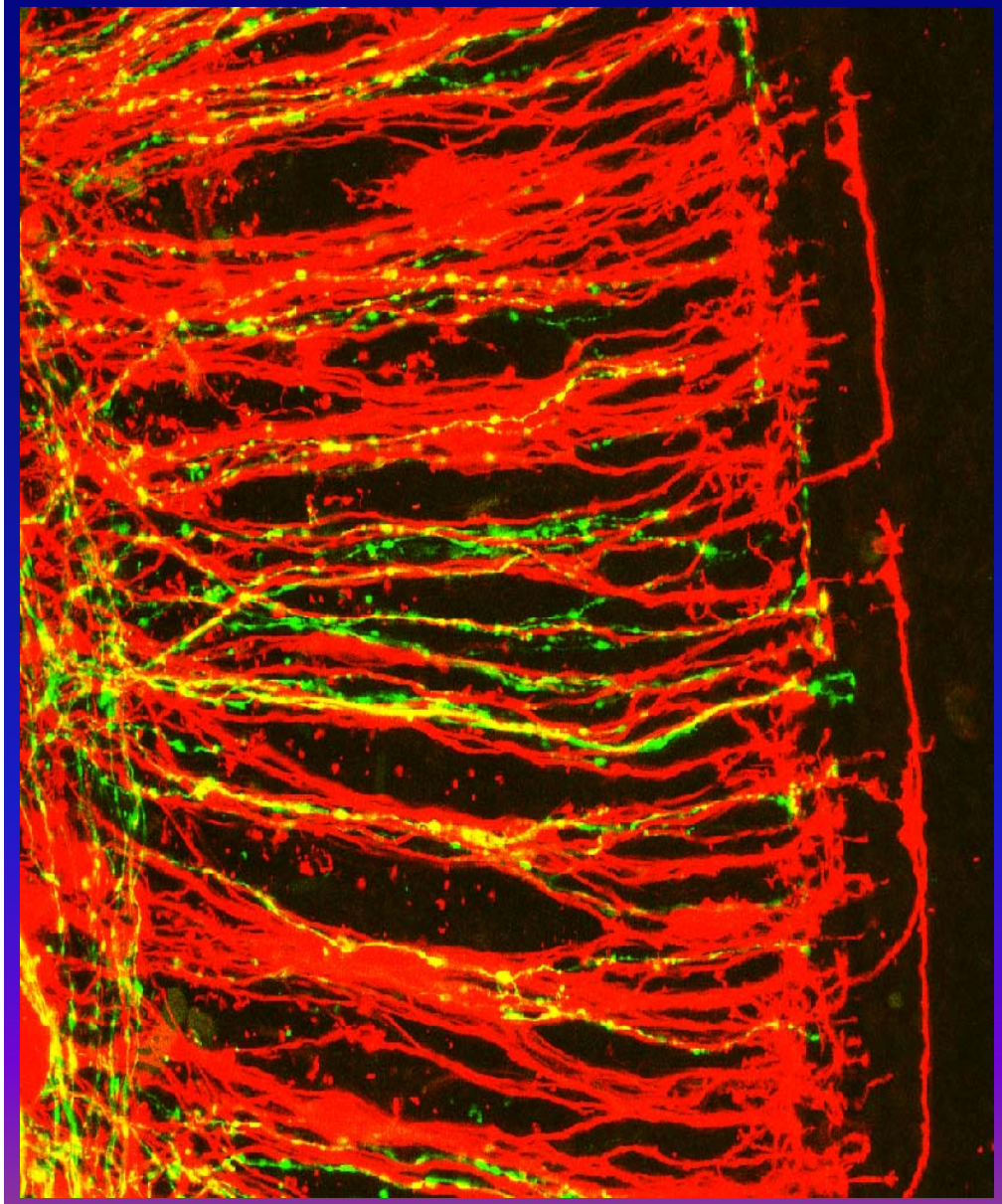
Duncan et al., JoVE, 2010

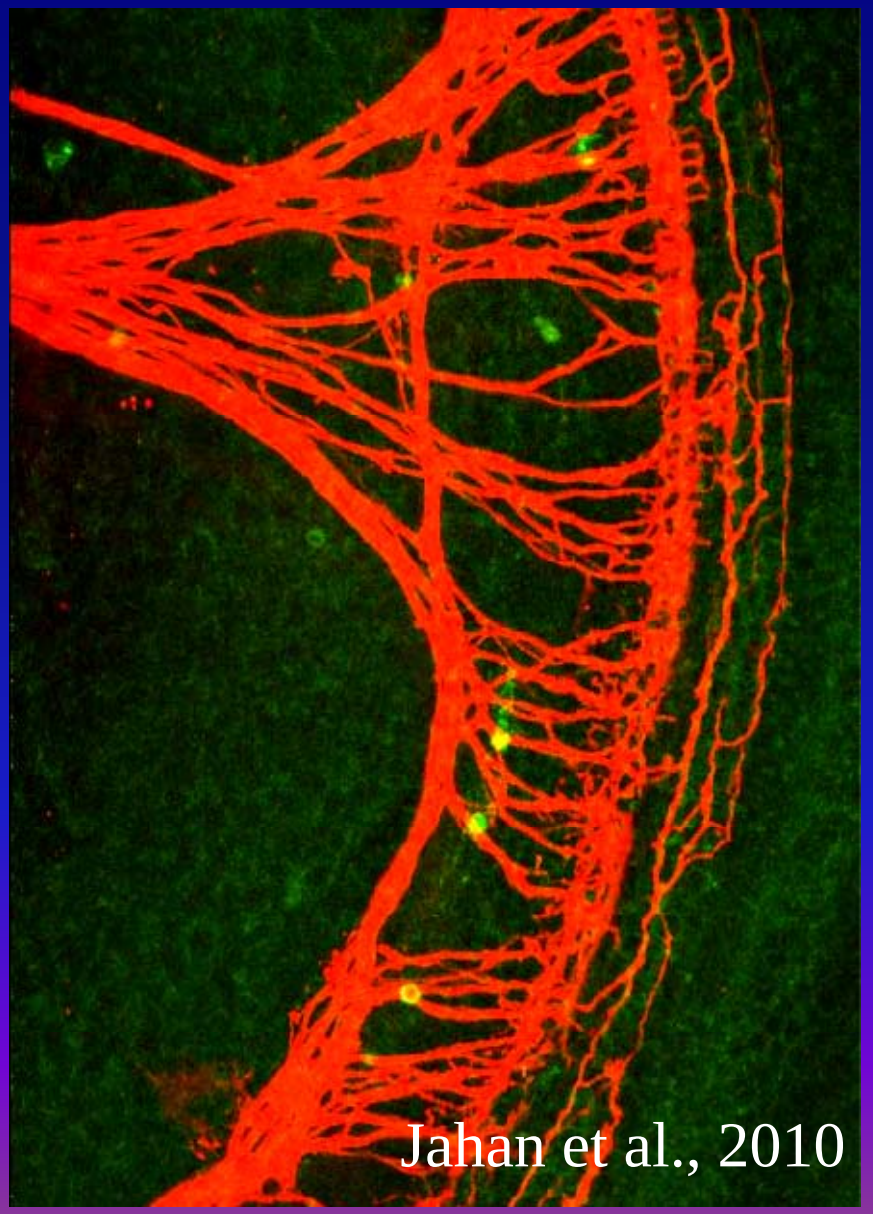
Lipophilic dye tracing, followed by in situ hybridization, than immuno staining and epoxy resin histology.



Sequential use of lipophilic dyes, followed by in situ hybridization, immunocytochemistry and Epoxy resin embedding and thick plastic sections

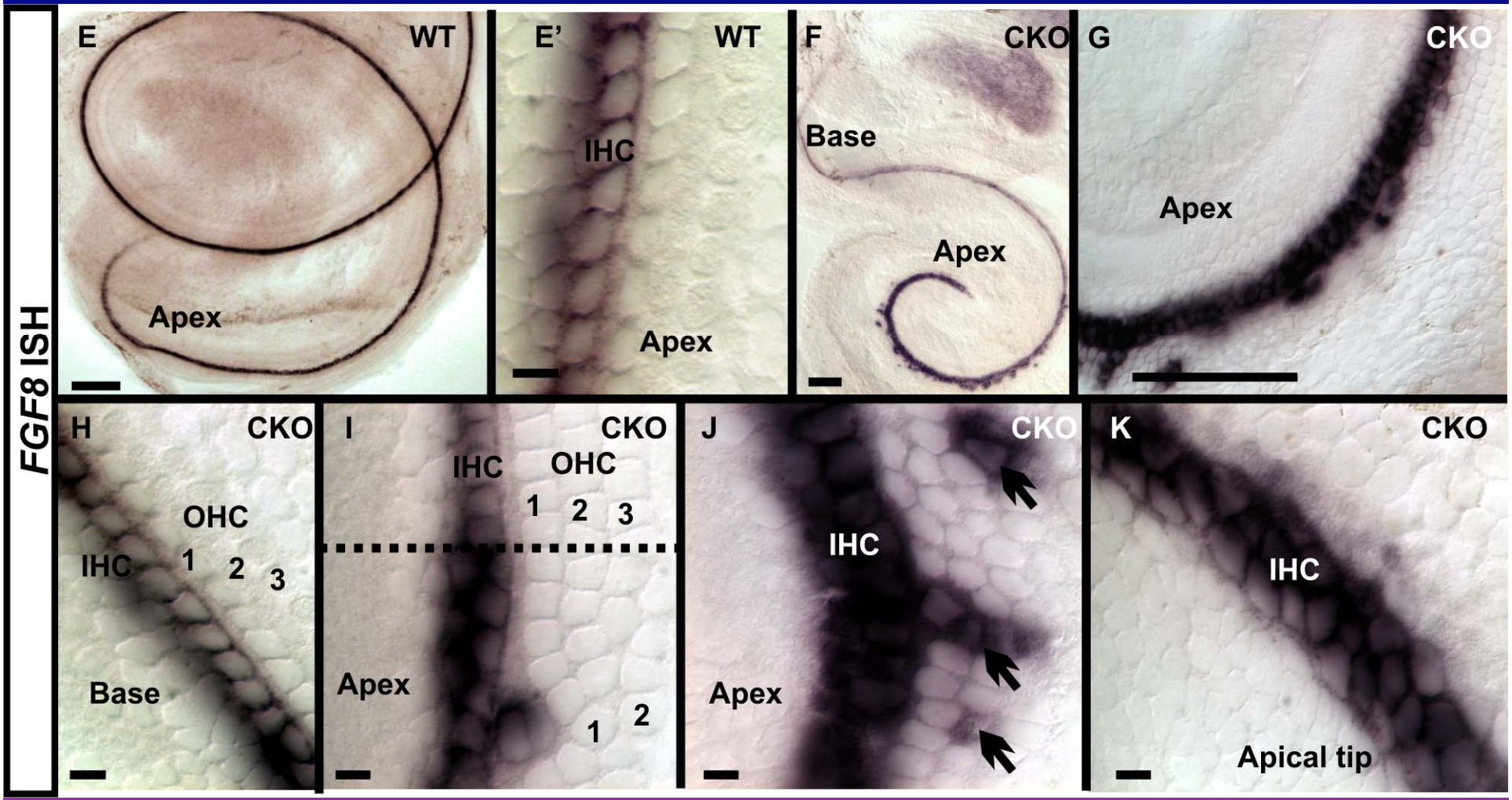
Neurod1 null mice develop unusual OHC innervation



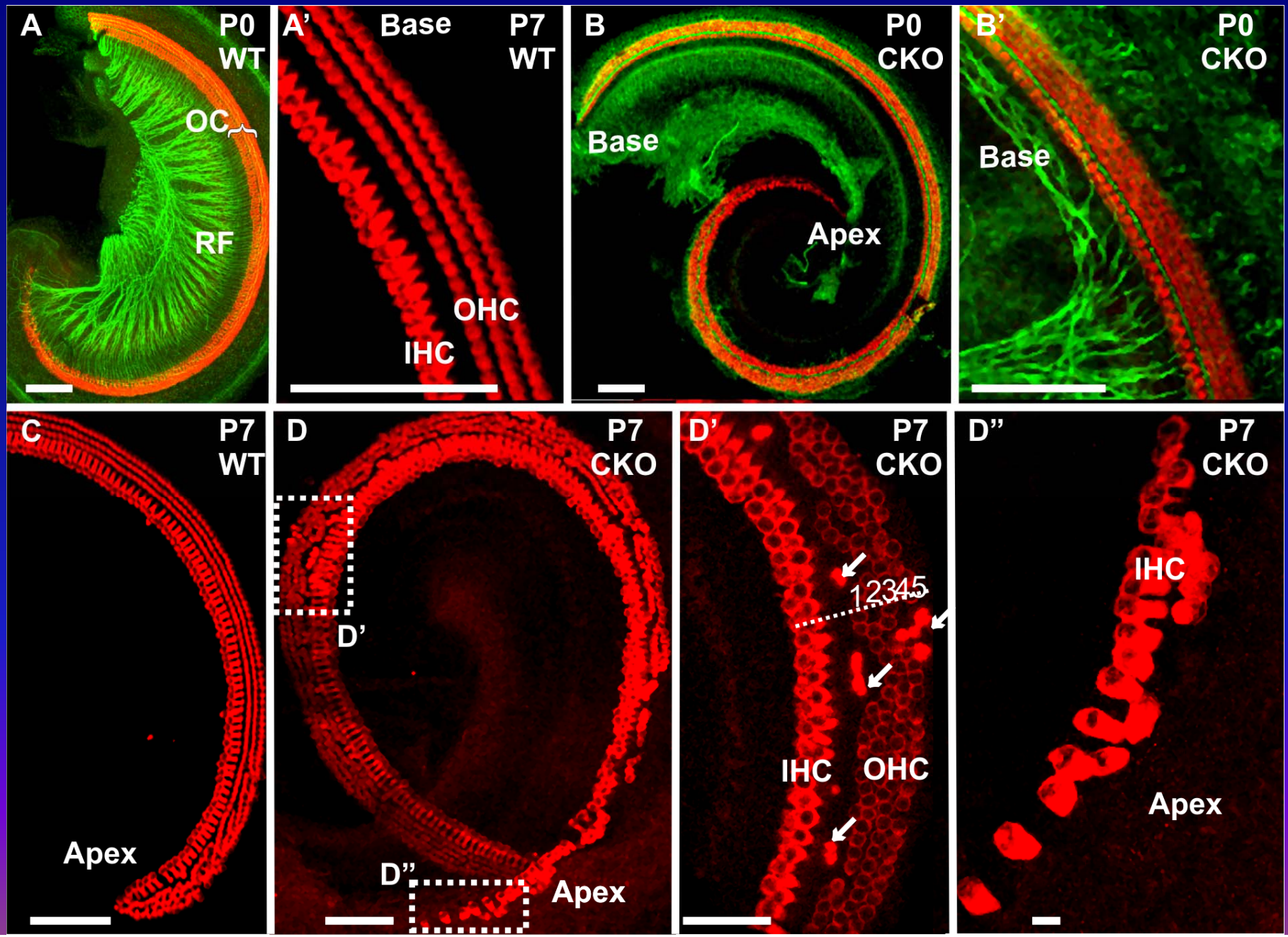


Jahan et al., 2010

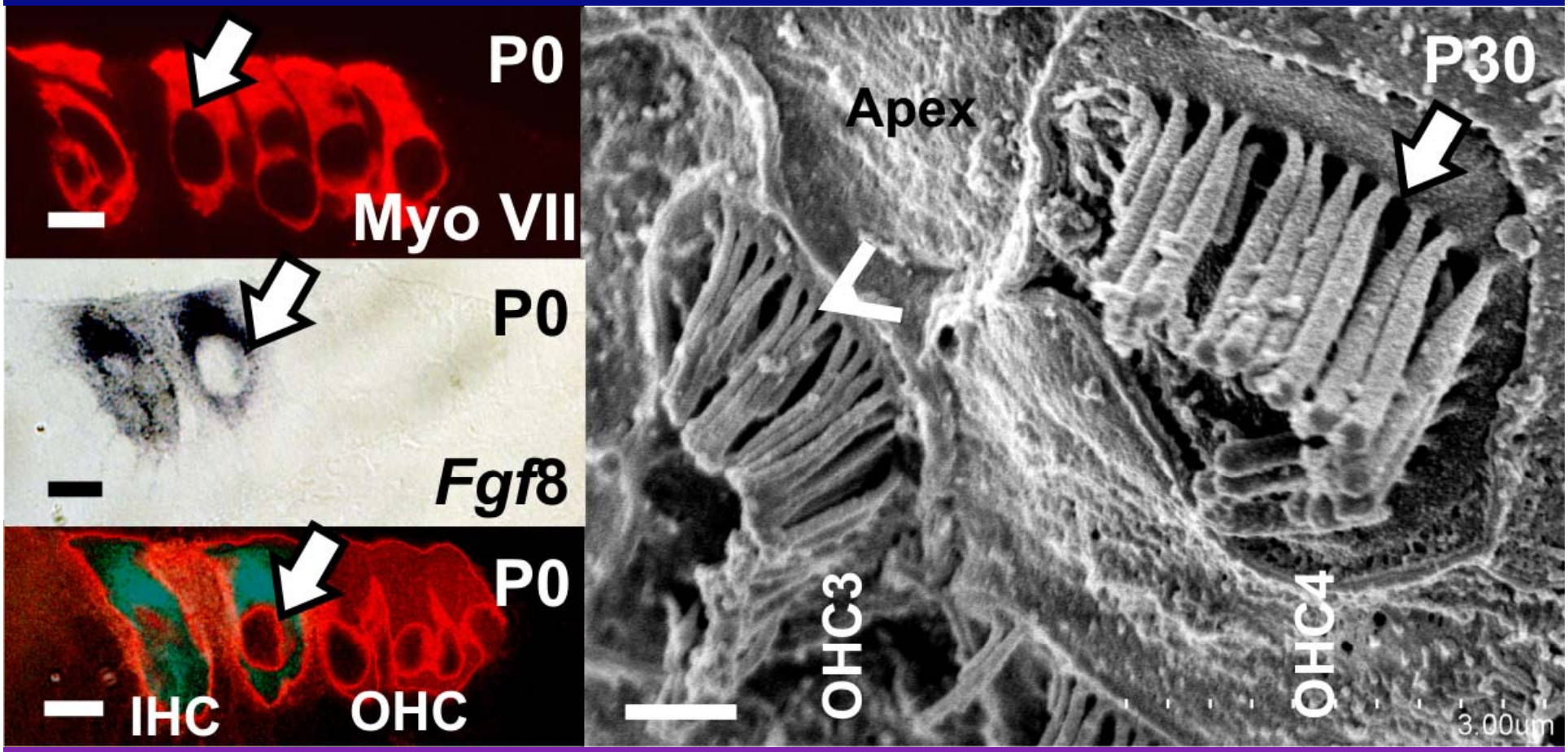
Absence of NeuroD1 results in altered expression of Fgf8 in OHC's



Lack of Neurod1 converts OHC into IHC

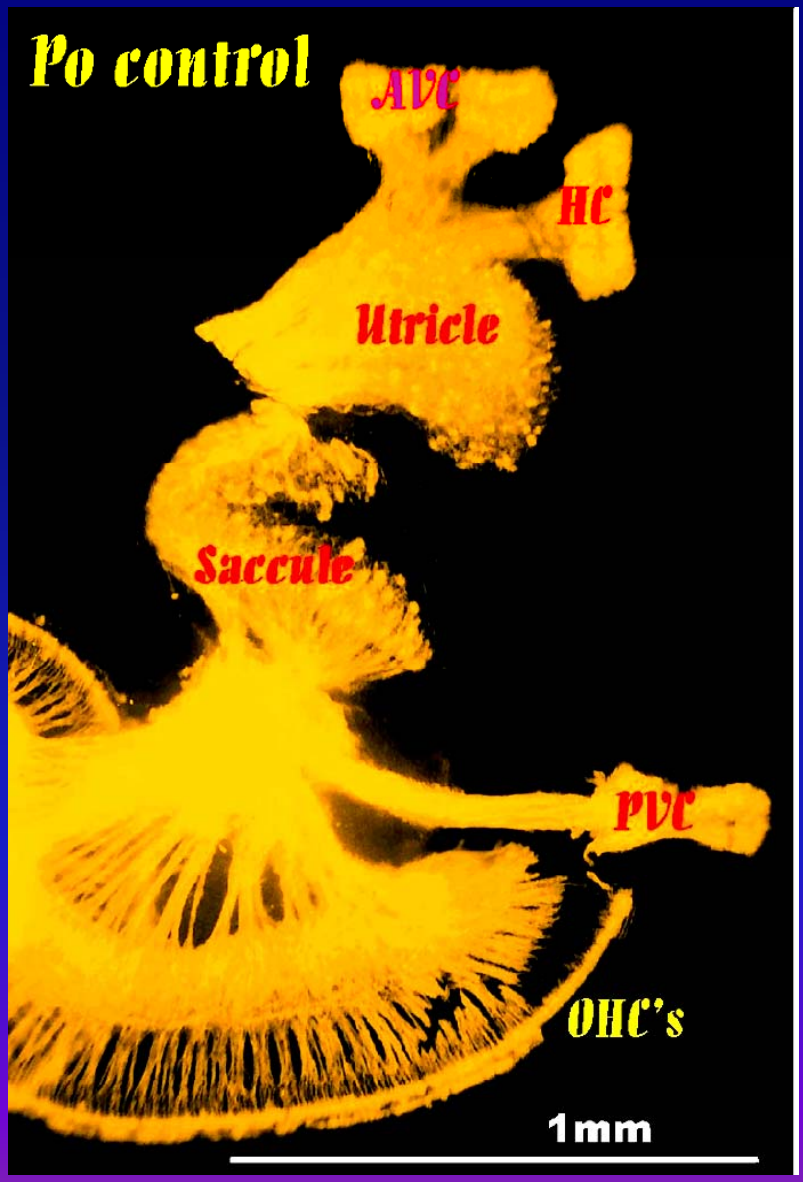


After lipophilic dye tracing, tissue can be used for in situ hybridization followed by immuno or SEM.

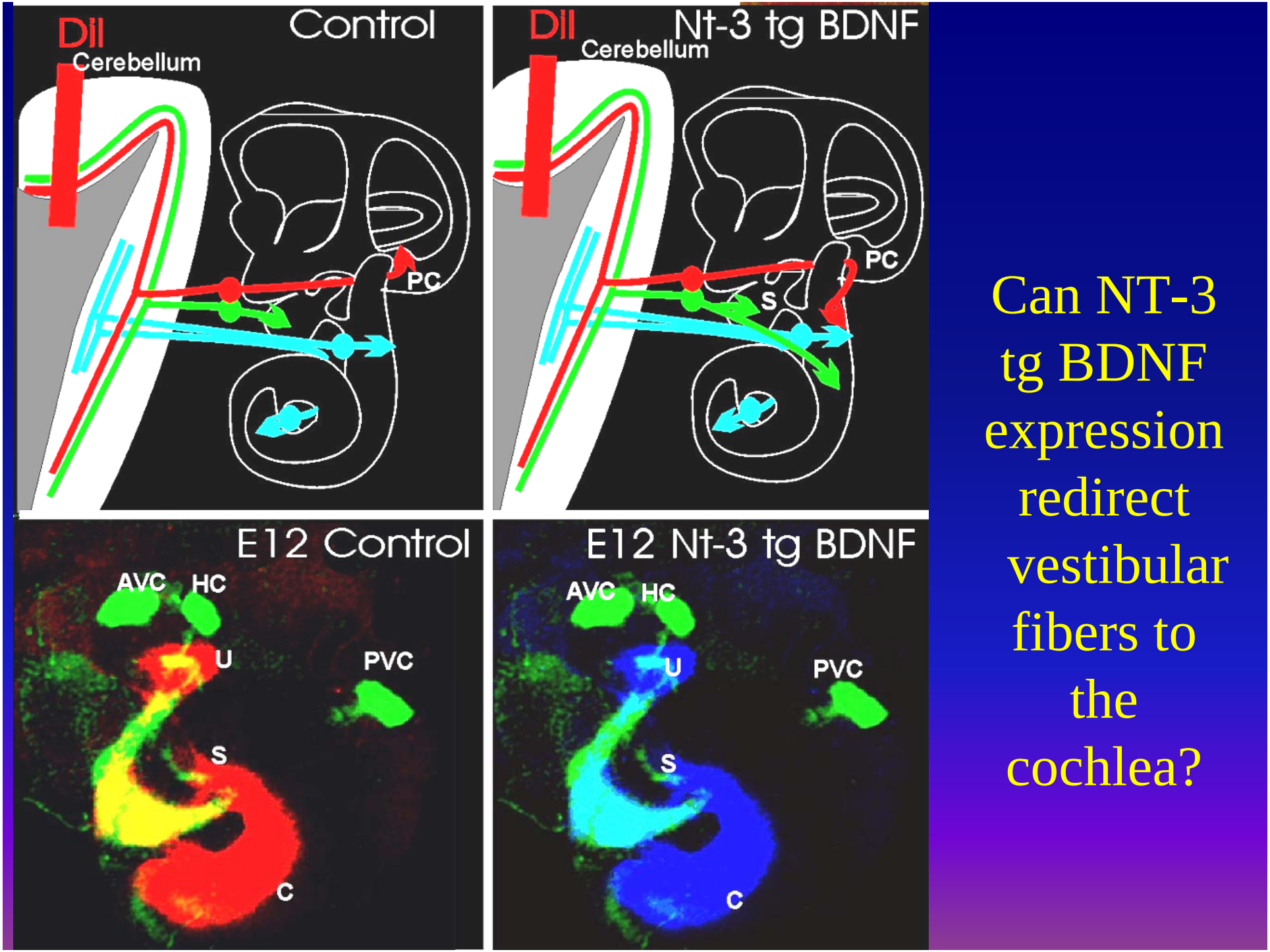


Only two NT' s/Trk's are important for ear development

P0 trkB/trkC mutant
P0 BDNF/NT-3 mutant

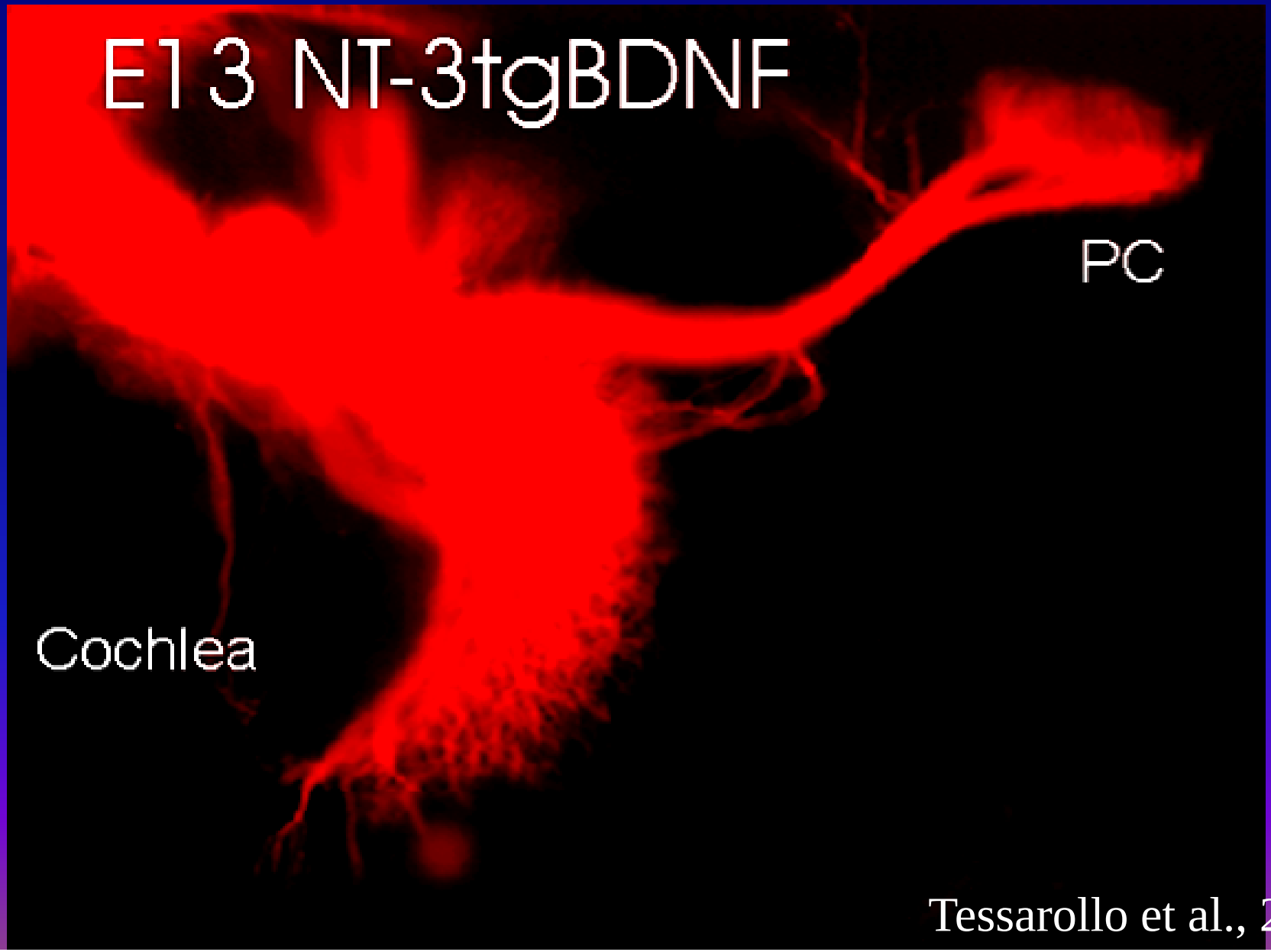


Silos-Santiago et al., (1997) Eur. J. Neurosci., 9:2045; Ernfors et al., (1995) Neuron 14: 1153



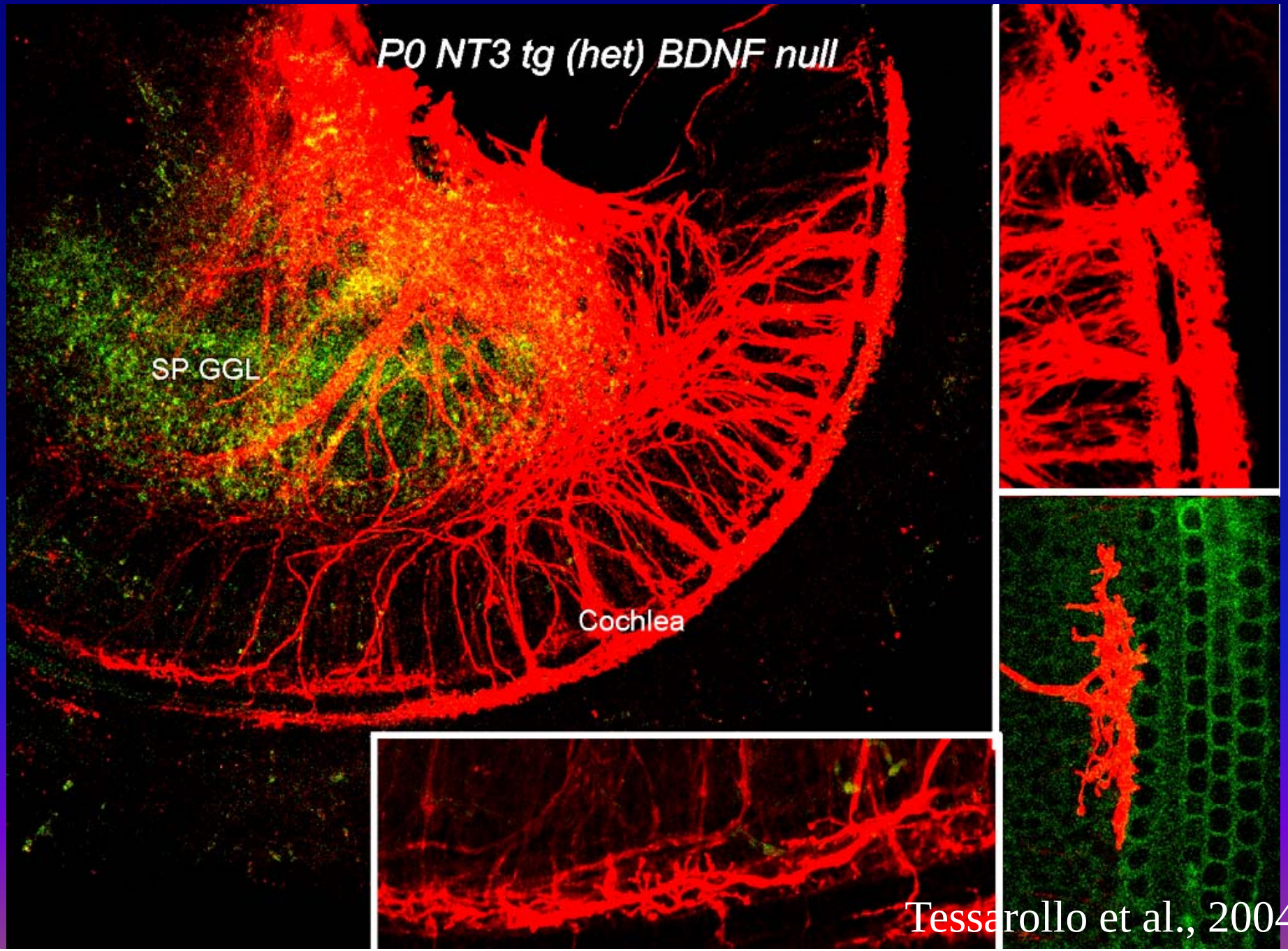
Can NT-3 tg BDNF expression redirect vestibular fibers to the cochlea?

BDNF mis-expression redirects vestibular fibers



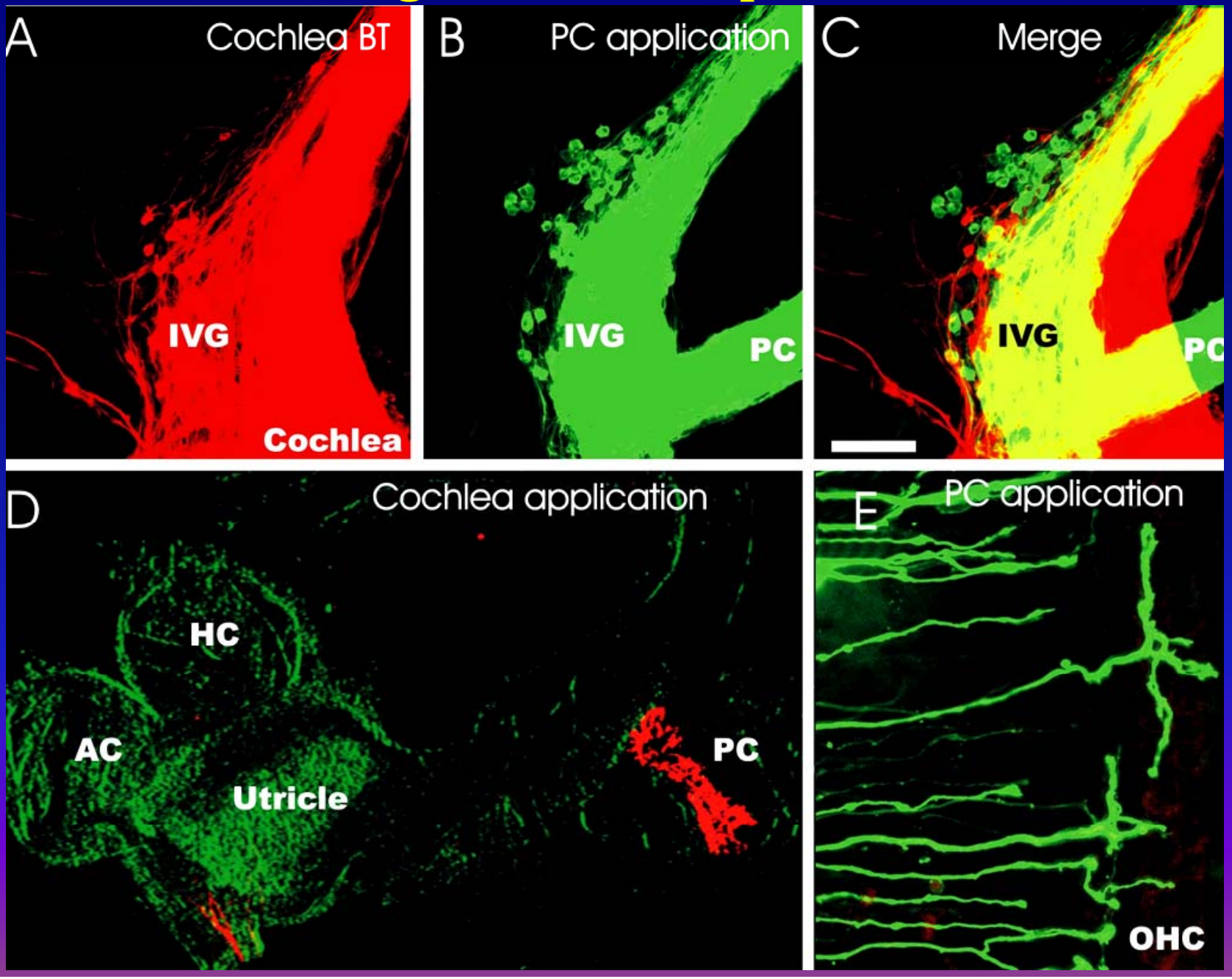
Tessarollo et al., 2004

All additional cochlear fibers are redirected vestibular fibers



Tessarollo et al., 2004

Double labeling verifies reciprocal connections



Conclusion

- Up to six differently colored lipophilic dyes can be used to trace neuronal connections.
- Lipophilic dye tracing can be combined with gene expression labeling such as GFP and LacZ reporters.
- LacZ reaction product (BCI) can be converted into a fluorescent product using 720-730 nm 2P excitation.
- Lipophilic dye traced tissue can be used for in situ hybridization followed by immunocytochemistry and Epoxy resin embedding.

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  - *House Ear Institute, LA; Pax-Cre line*
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