



Two-Photon Imaging Applications In Brain Tissue

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nature
biotechnology 21: 1369-1377, 2003

Nonlinear magic: multiphoton microscopy in the biosciences

Warren R Zipfel, Rebecca M Williams & Watt W Webb

- “Multiphoton Microscopy” is a non-linear, optical imaging technique - **small increases in illumination intensity yield large increases in fluorescence**
- “Two-Photon Microscopy” is one type - invented in 1990 by Winfried Denk and colleagues at Cornell Univ. (School of Applied & Engineering Physics)
- Through 2003, it was **used primarily by physicists** studying biological phenomena
- It is similar to laser-scanning confocal microscopy; it is **the preferred method for imaging living cells in intact tissue**

Two-Photon Fluorescence Excitation

Theory of Operation:

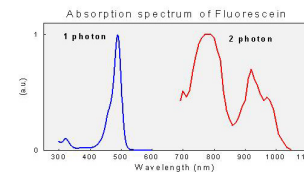
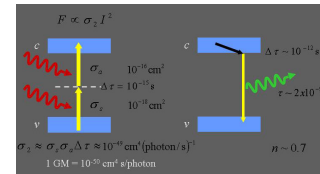
If a fluorophore requires energy **X** to be excited to fluoresce, then it can be excited with 2 photons with $\frac{1}{2} X$ if the photons are absorbed nearly simultaneously

Low probability event;
(Göppert-Mayer, 1931).
requires high photon density
(i.e., high power infrared laser

= \$110K – \$165K)

2P Excitation

1P Emission



2P spectrum is not always 2x the 1P spectrum

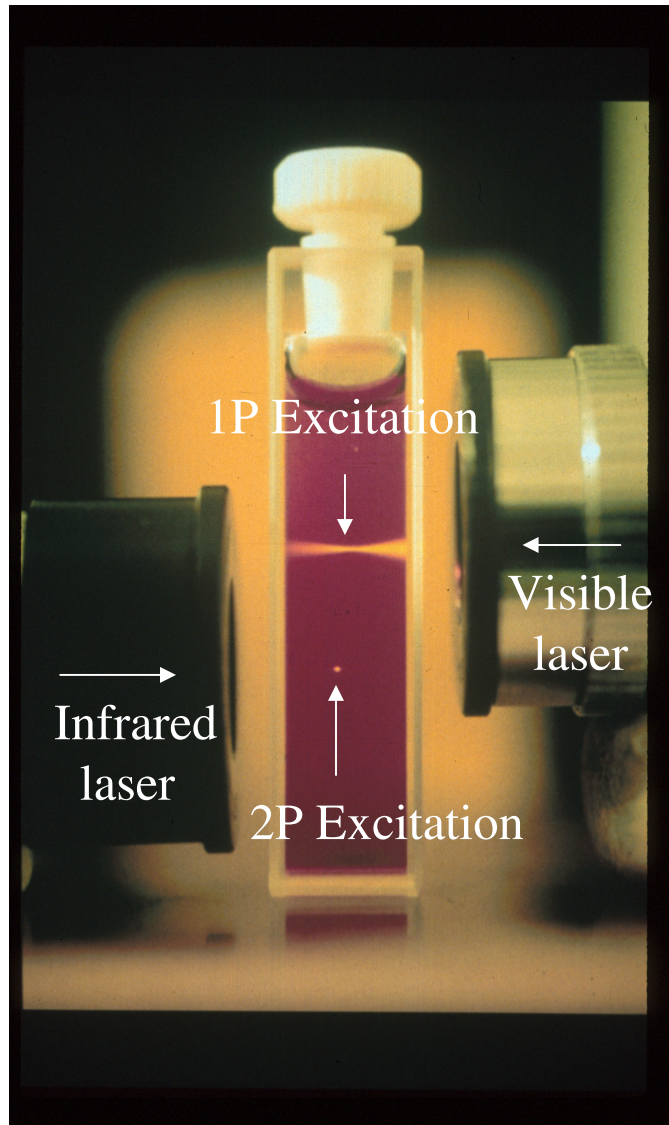
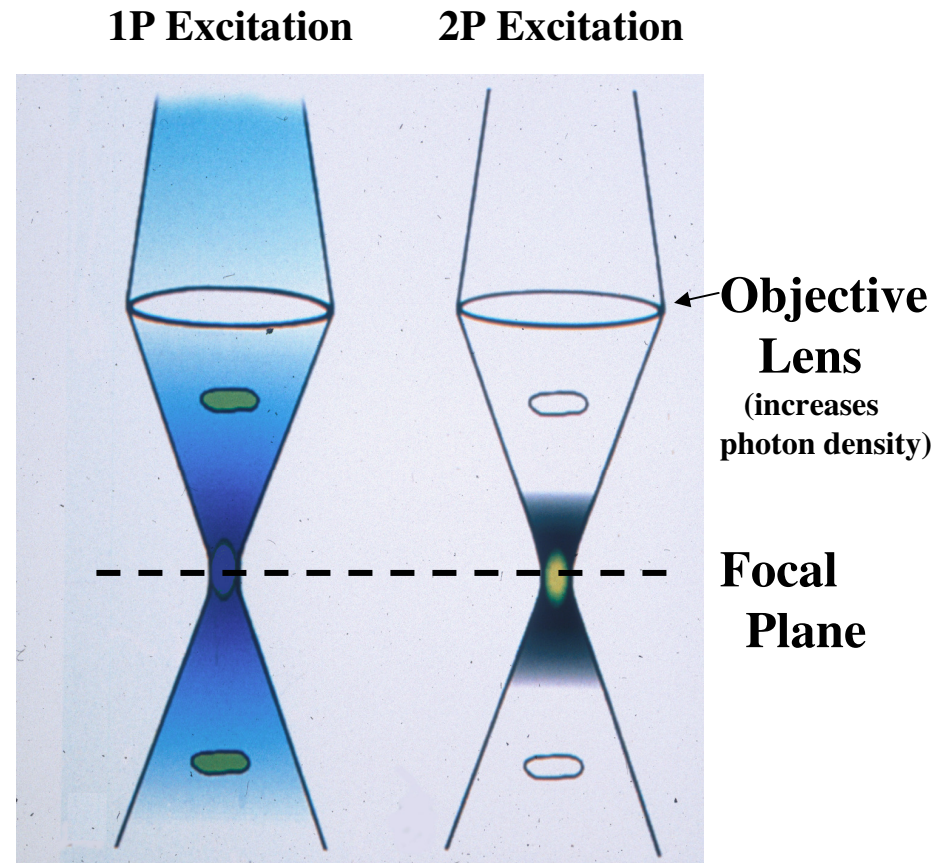


Photo by Prof. BRAD AMOS, Cambridge U.



Advantages of 2P Excitation

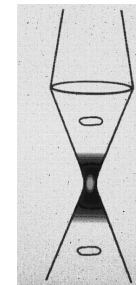
Localized ($\sim 1 \mu\text{m}^3$)

Deeper penetration ($\sim 400 \mu\text{m}$)

Enhanced collection efficiency

Advantages of 2P Microscopy

- Enables imaging of cells *in situ*
- Enables parfocal excitation of two or more dyes
- Reduces photobleaching of fluorophores
- Reduces phototoxicity of cells
- Extends the duration of imaging living cells and subcellular compartments - e.g., dendrites, spines, mitochondria



Excitation
(focal)
volume



Multiphoton Imaging Core

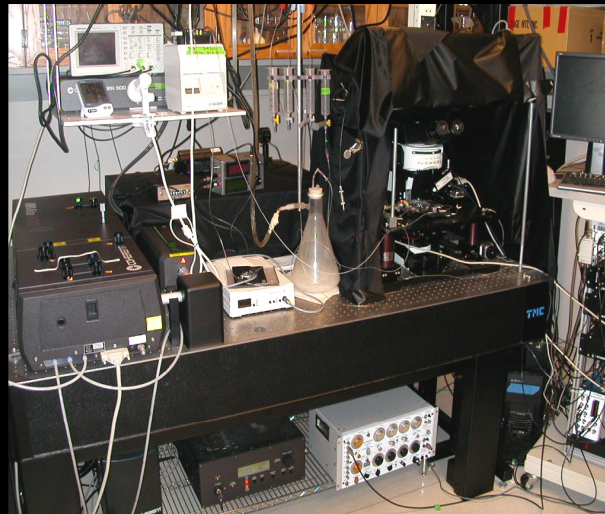
2P Research Projects

- | | |
|--|--|
| <ul style="list-style-type: none">• Cell Migration• Ca_i Dynamics• ROS_i Dynamics• Mitochondrial Function | <ul style="list-style-type: none">• Dendritic Physiology• Synaptic Plasticity• In Situ Imaging• In Vivo Imaging |
|--|--|

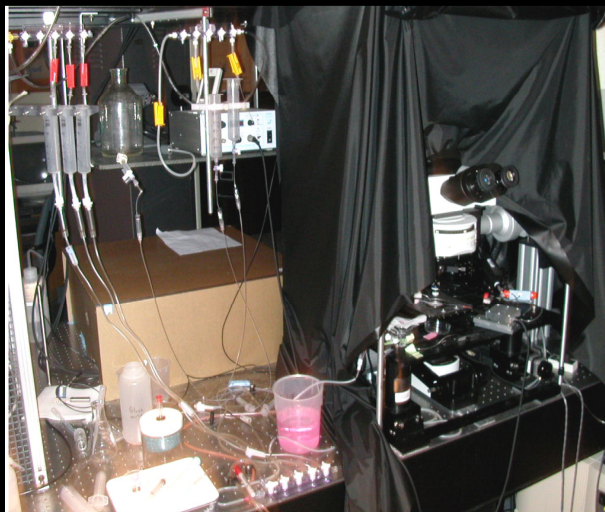
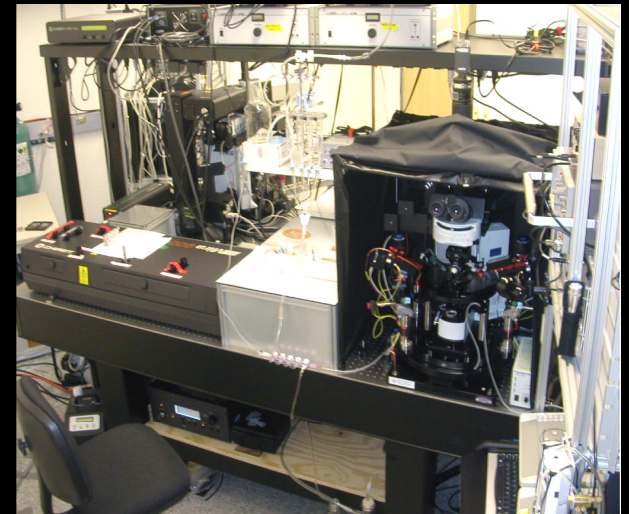
Yu



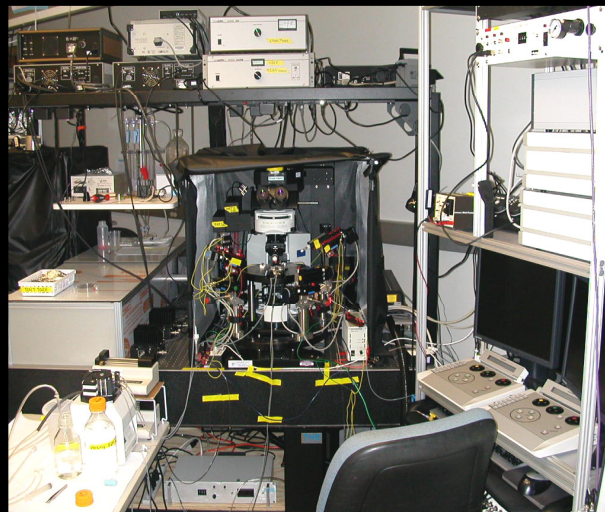
Hockberger



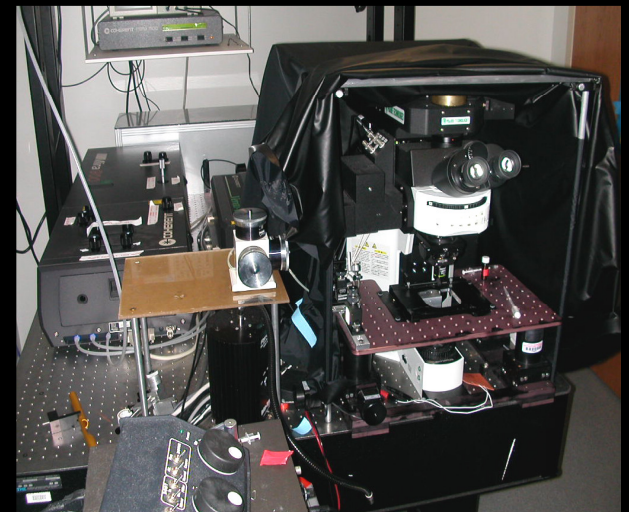
Day



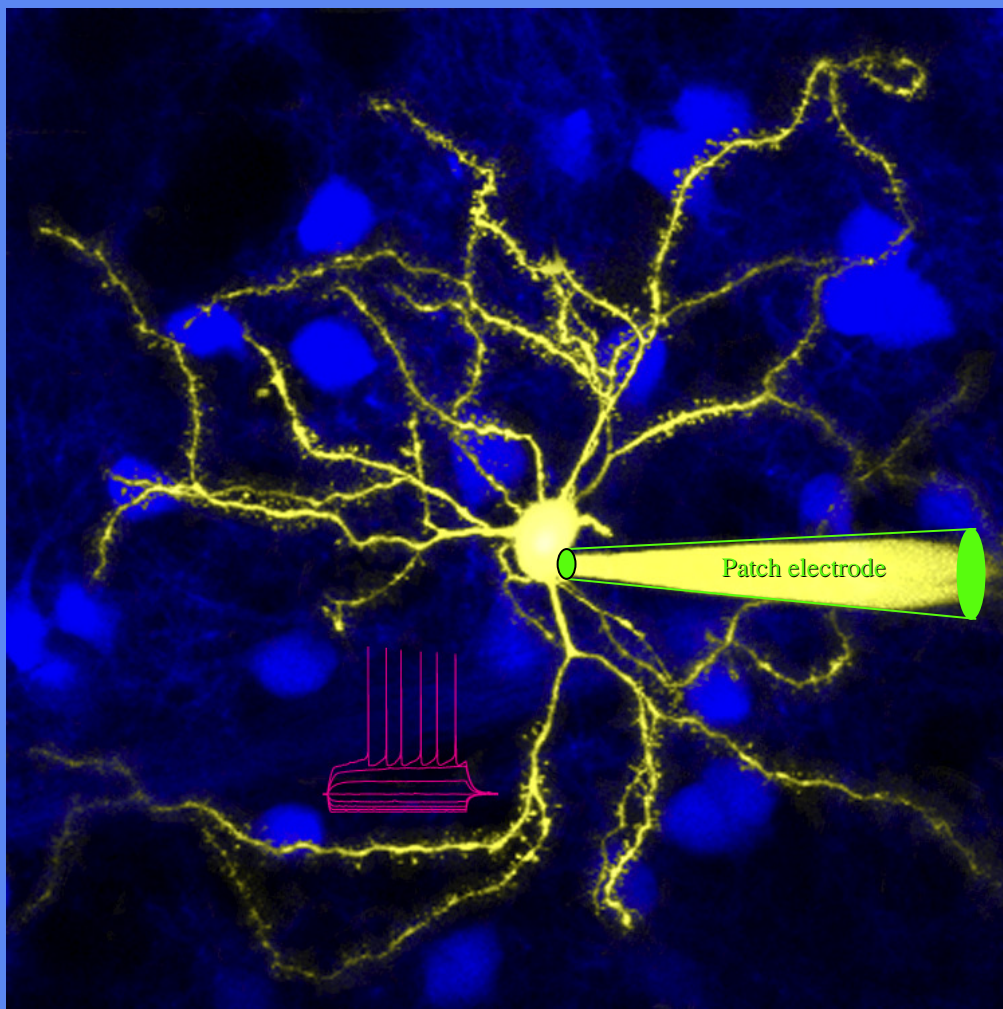
Wokosin



Bevan



Grutzendler

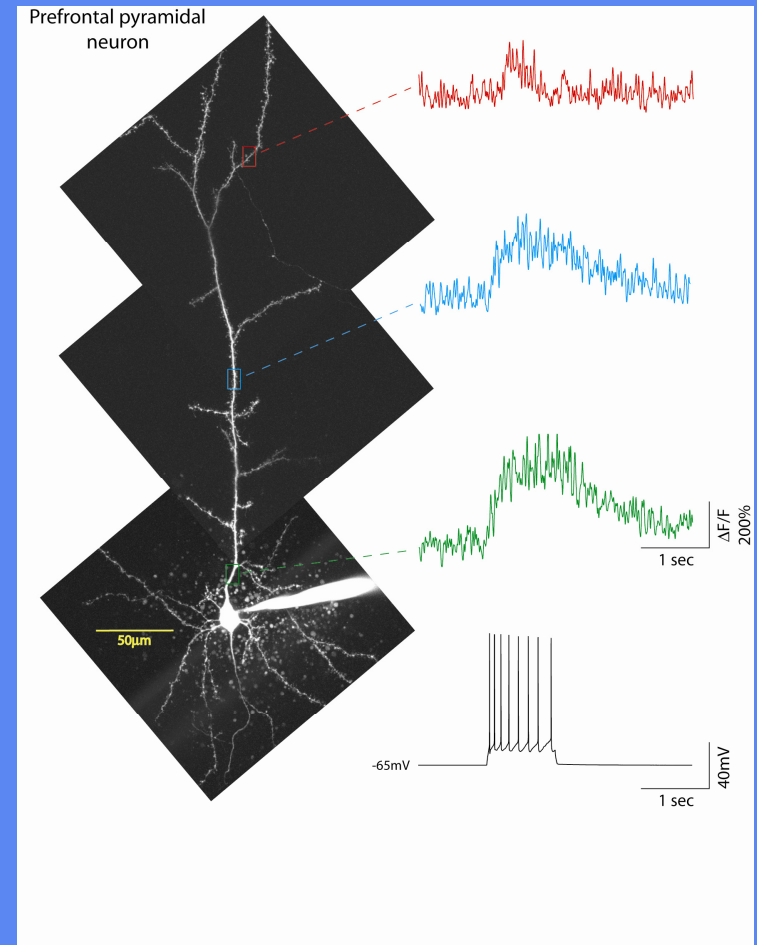
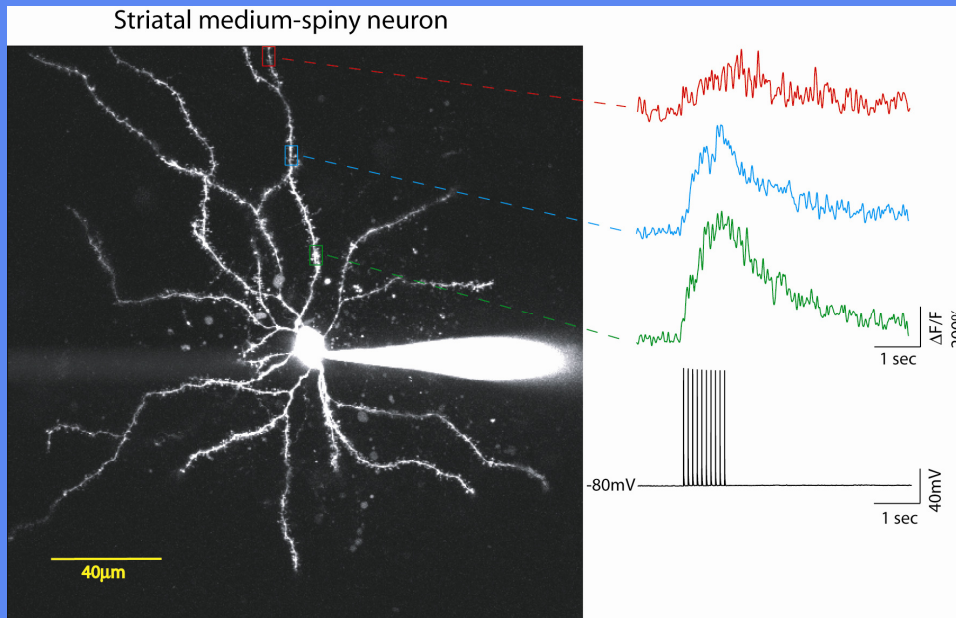


**Integrating Ca²⁺ Imaging,
Electrophysiology and
Molecular Uncaging
using the Prairie "Ultima"
2P System for Studying
Dendritic Physiology
*in Situ***



Dr. Michelle Day

Ca²⁺ Imaging: a tool for measuring dendritic properties



2P imaging of Ca²⁺ demonstrates that APs “back propagate” from cell bodies into dendrites of CNS neurons

Dyes:

Fluo-3 / Alexa 594

Objective:

60x / 0.9 na

Ex. Wavelength:

810 nm

Em. Wavelength:

490 - 560 nm

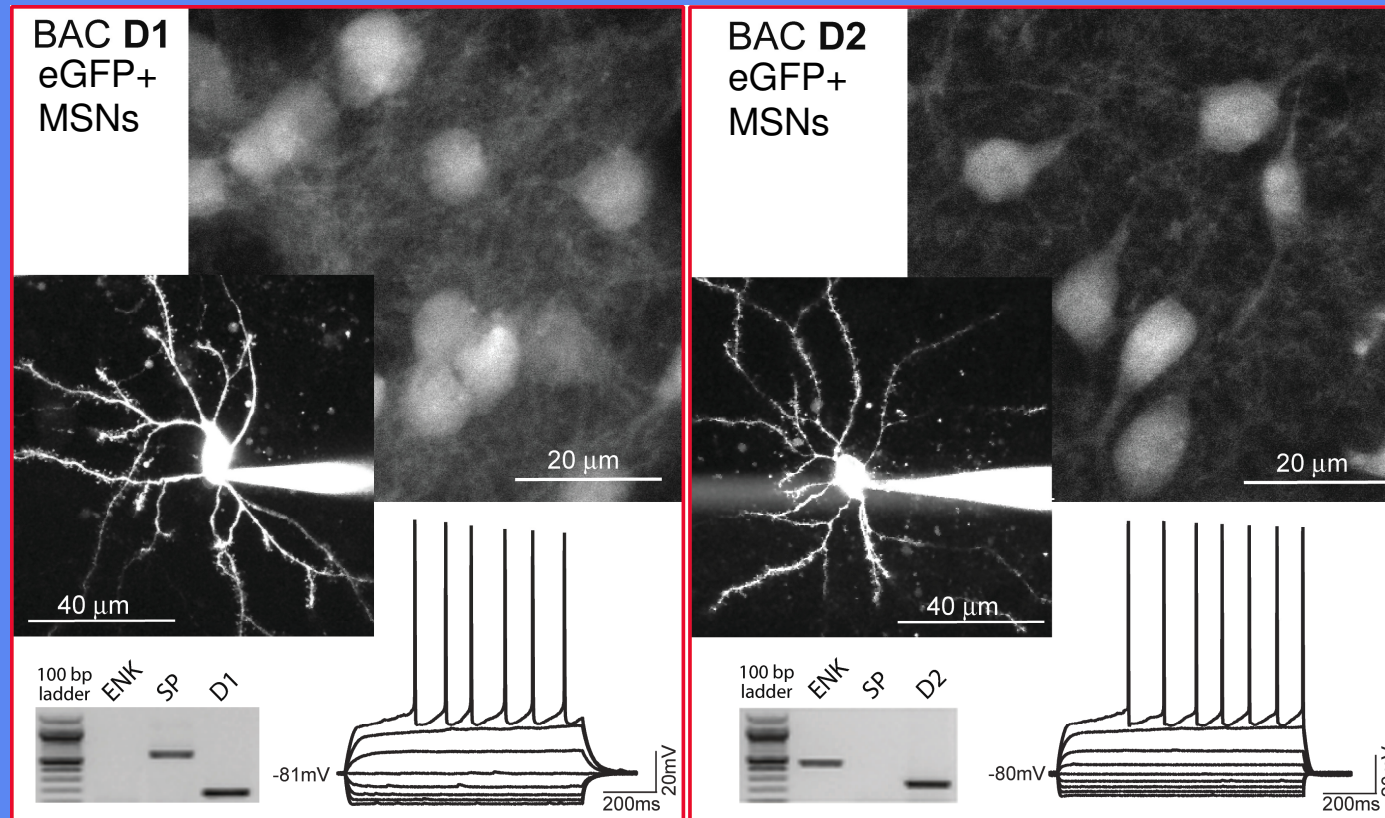
Pixel Dwell time:

10.5 µs (Line scans)

Pixel size:
2.4X

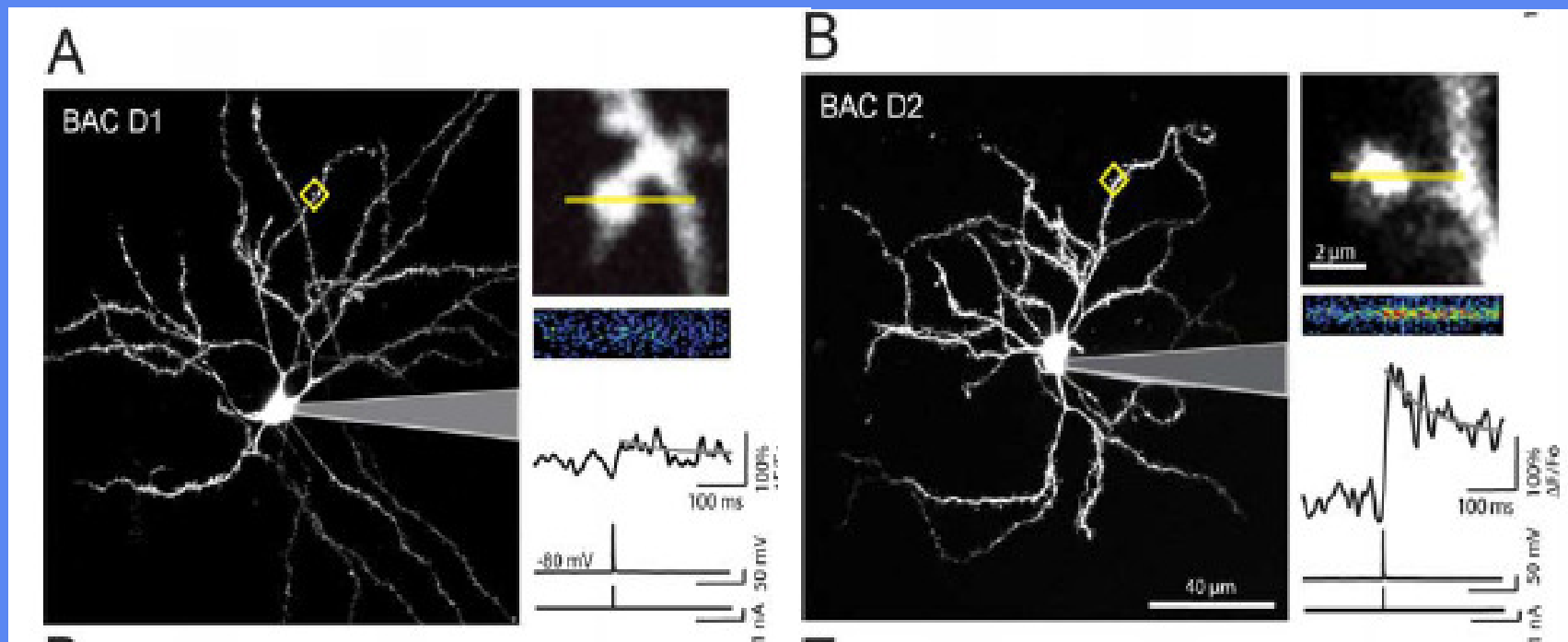
0.17 µm (zoom =

Identification of Striatonigral Neurons using Transgenic Mice



- Somatic whole-cell patch recordings exhibit no differences between D1 and D2 Neurons
- “BAK” mice express eGFP in DA neurons under the control of D1 or D2 receptor
- D1 Neurons in BAK mice coexpress SP and D1 receptor mRNA
- D2 Neurons in BAK mice coexpress ENK and D2 receptor mRNA
- Using BAK mice is a convenient way to identify D1 and D2 neurons

Distal Dendrites of D2 Cells Display Ca Elevation in Response to Back-Propagating Action Potential



Day et al. J. Neuroscience 28: 11603-14, 2008

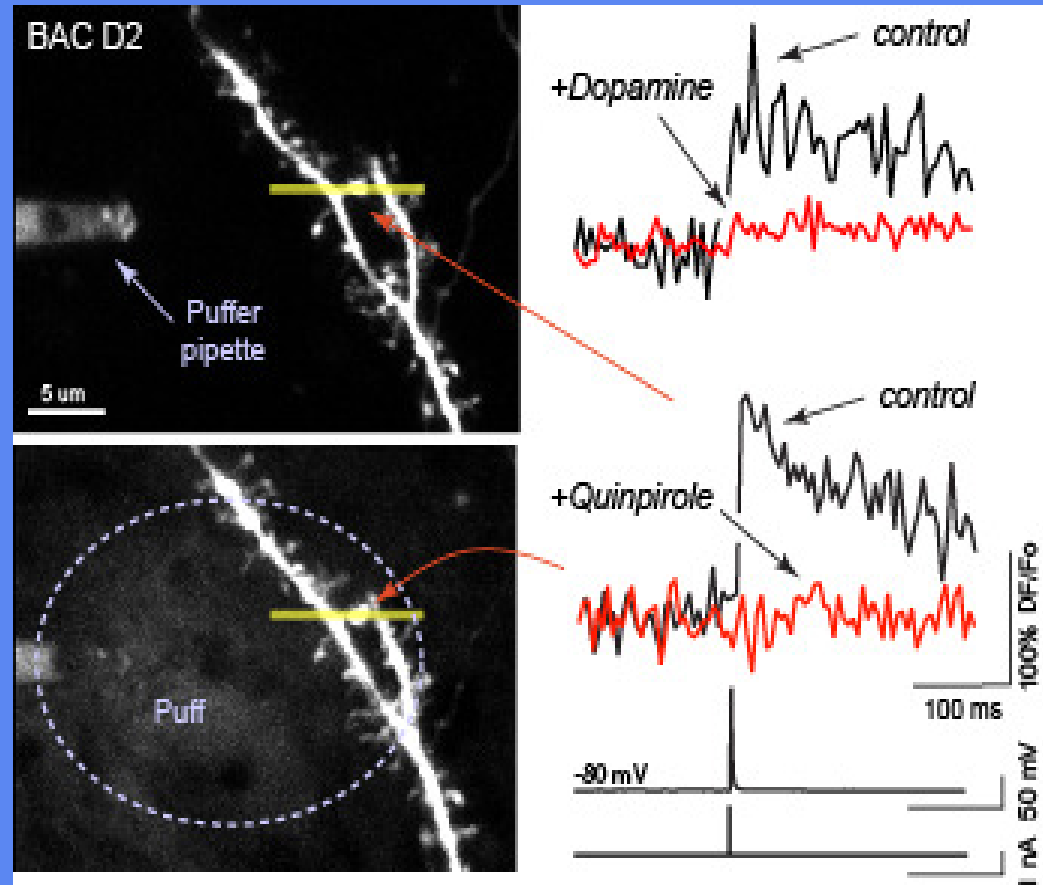
D2 receptor stimulation suppresses spine Ca^{2+} transients evoked by bp-APs

Striatopallidal MSN in *BAC D2* transgenic mouse

10-100 μM Dopamine or “Quinpirole” (D2 agonist) applied focally via puffer pipet to distal dendrites suppressed the Ca^{2+} transient induced by a single bp-AP

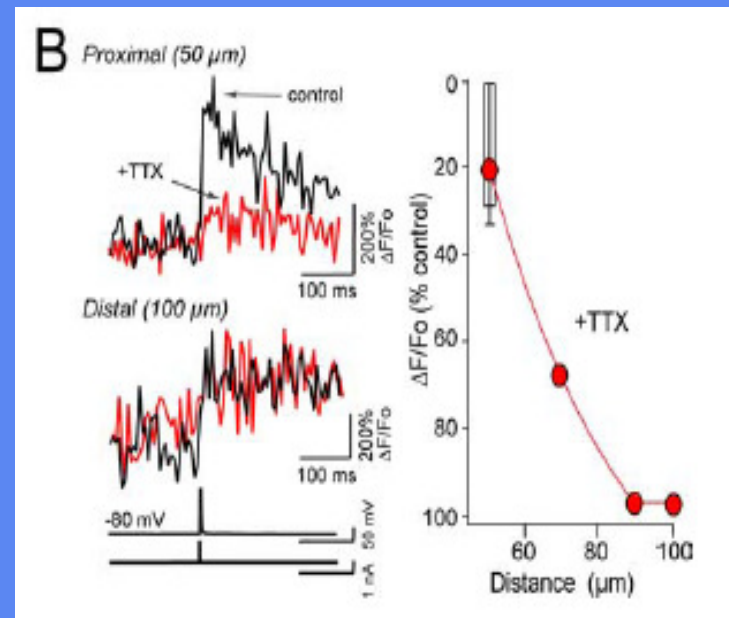
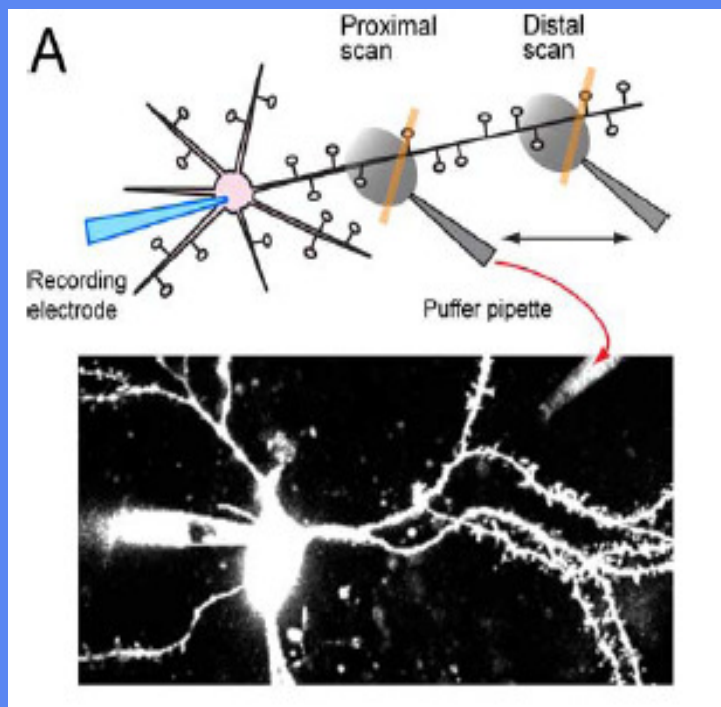
Puffer pipet also contained 50 μM Alexa 594 to monitor diffusion

No effect detected in *D1* MSNs

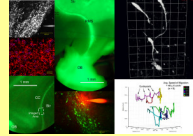


Day et al. *J. Neuroscience* 28: 11603-14, 2008

Back-Propagating Action Potential in D2 Dendrites Induced Ca Elevation in the Presence of TTX



Day et al. J. Neuroscience 28: 11603-14, 2008



Time-Lapse Video Microscopy of Neural Stem Cells *in Situ*

Yongsoo Kim ¹, Sang Nam ^{2,3}, Francis Szele ¹ Philip Hockberger ²

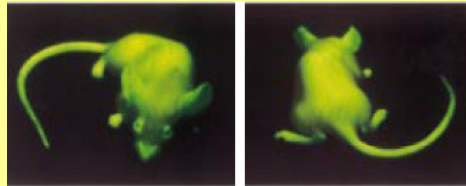
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Department of Physiology ², Feinberg School of Medicine, Northwestern University, Chicago, Illinois USA

Department of Physiology ³, Chonnam National University Medical School, Gwanju, Republic of Korea

Live Cell Fluorescence Microscopy – new developments:

- **green fluorescent protein (GFP)** expressed in *E. coli* and *C. elegans*
Chalfie et al. Science 263: 802-805, 1994
- “enhanced” green fluorescent protein (eGFP) expressed in mice
Hadjantonakis et al. Mechanisms of Development 76: 79-90, 1998



- 2-photon microscopy reduced photobleaching of fluorophores
Denk, Strickler & Webb Science 248: 73-76, 1990

General Methods

2 transgenic mouse lines (ages P10 - P99): eGFP/GAD and eGFP/Nestin
Acute parasagittal brain slices (300 μ m thick) imaged at 35 C in oxygenated Artificial CSF
Two-photon video microscopy: XYZ stacks (40-60 planes) collected every 7 min for

No. Slices	2	12	11	9
Hours	0 - 5	5.5 - 10	10.5 - 15	15.5 - 18

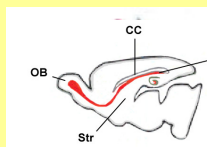
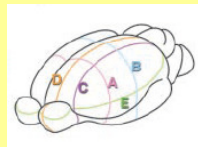
= 5,760 images

Epifluorescence microscopy:

digital images of slices after 2P imaging

Confocal microscopy:

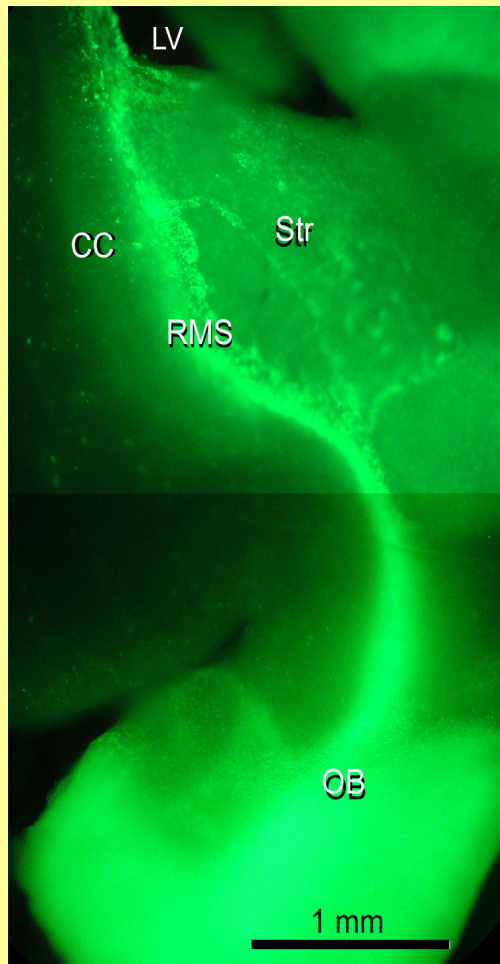
whole mount immunostaining of slices after 2P imaging



Rostral Migratory Stream (RMS)
contains neural stem cells that
produce neuroblasts that migrate
to the olfactory bulb (OB)

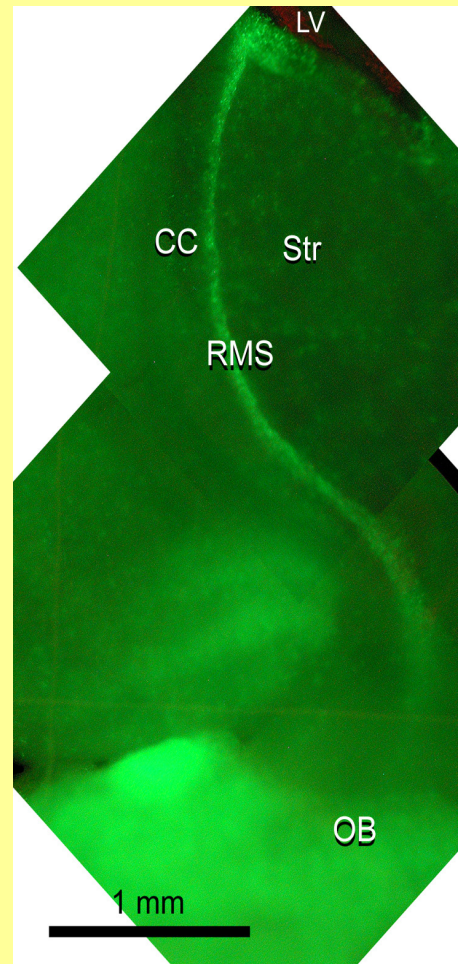
RMS – OB Pathway

Epifluorescent images (low magnification)



eGFP/GAD
P39 mouse
parasagittal
brain slice

Migratory “neuroblasts”



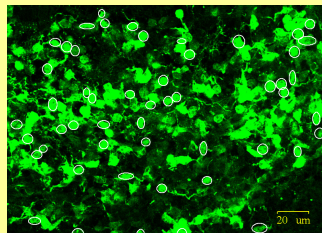
eGFP/Nestin
P10 mouse
parasagittal
brain slice

Stationary “stem” cells

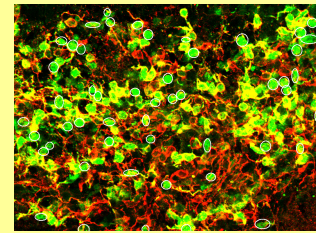
RMS – OB Pathway

single confocal image (P16 brain slice)

eGFP/GAD



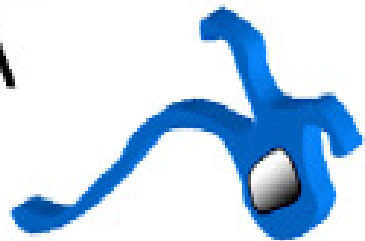
Cy3/anti-Dcx



- ~ 70% of GAD cells in RMS were neuroblasts (Dcx-positive)
- ~ 30% of GAD cells in RMS were not neuroblasts (circled cells)
- + 10% of cells in RMS were Dcx-positive and GAD-negative (arrows)

Types of Cells in Mouse SVZ

A



GFAP+ stem cells



Transit amplifying progenitors



Neuroblasts

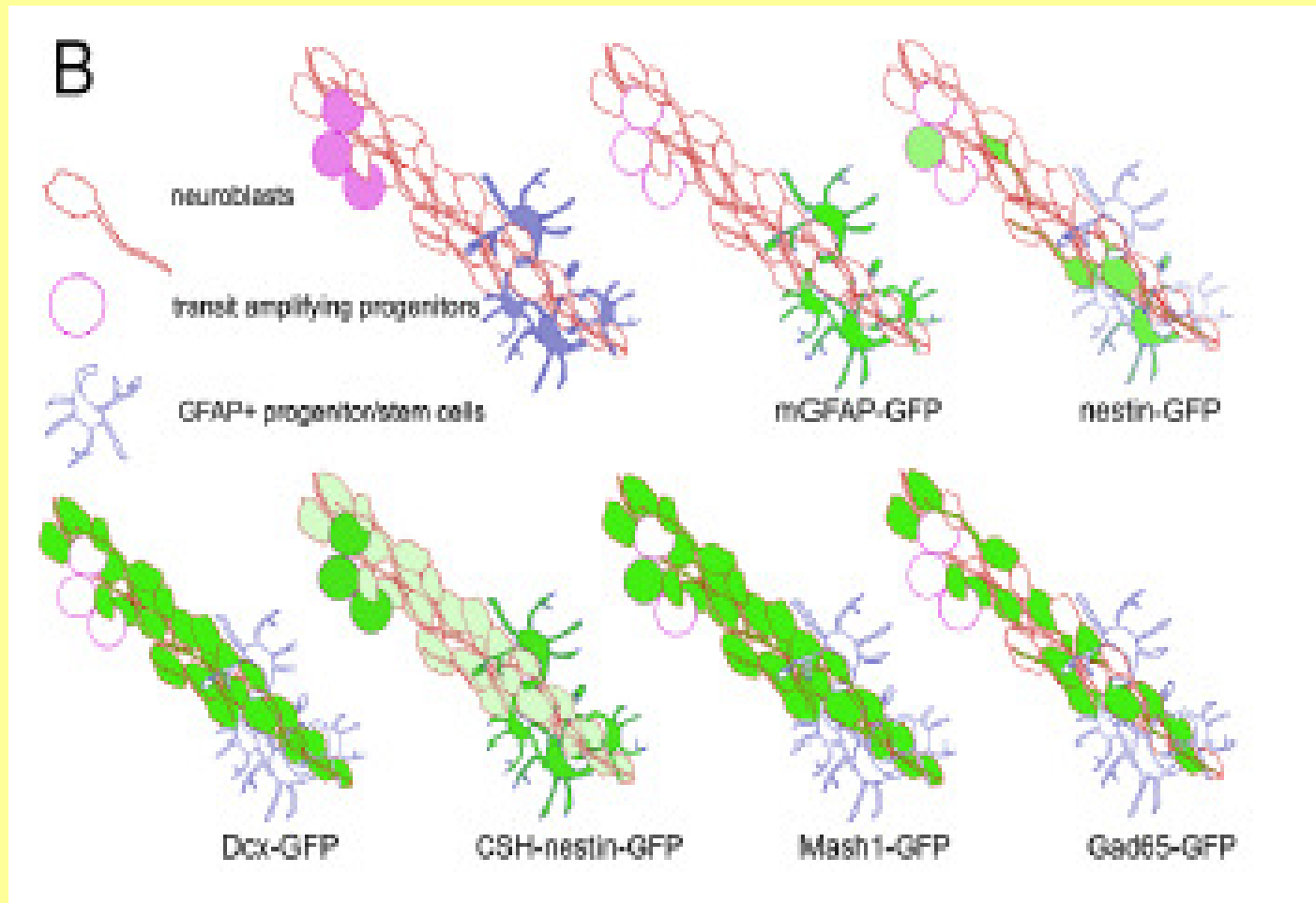
GFAP

EGFr

Mash1

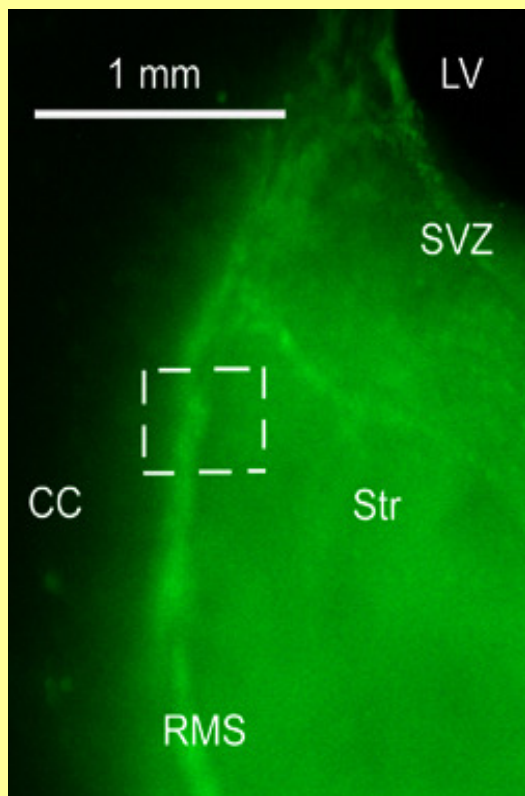
β III-tubulin/Dcx/PSA-NCAM

Anatomical Relationship Among Mouse SVZ Cells

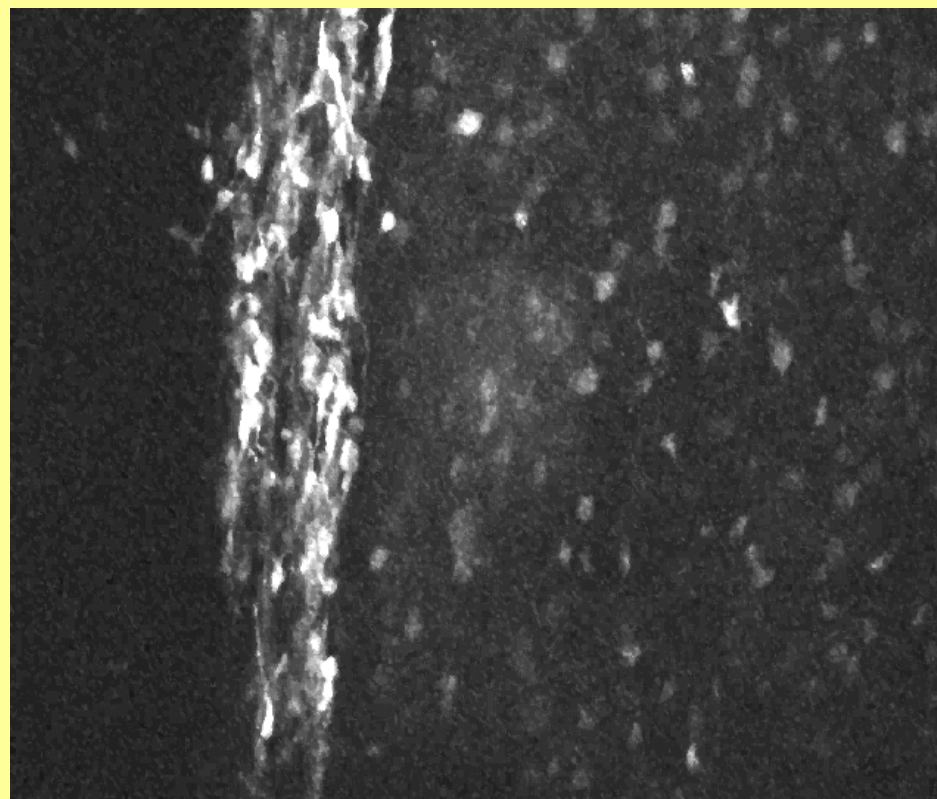


GAD Cell Migration

Nam et al. J. Comp. Neurol. 505: 190-208, 2007

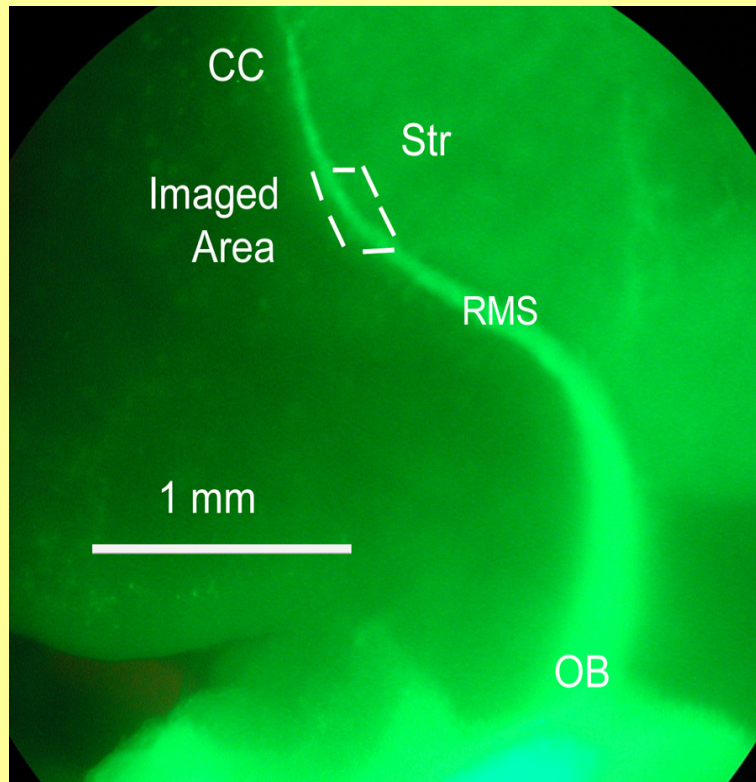


**P19 brain slice at low magnification
hrs
(epifluorescent image)
planes/frame)**



**2P microscopy of boxed area for 17
(movie: XYZ stack = 40**

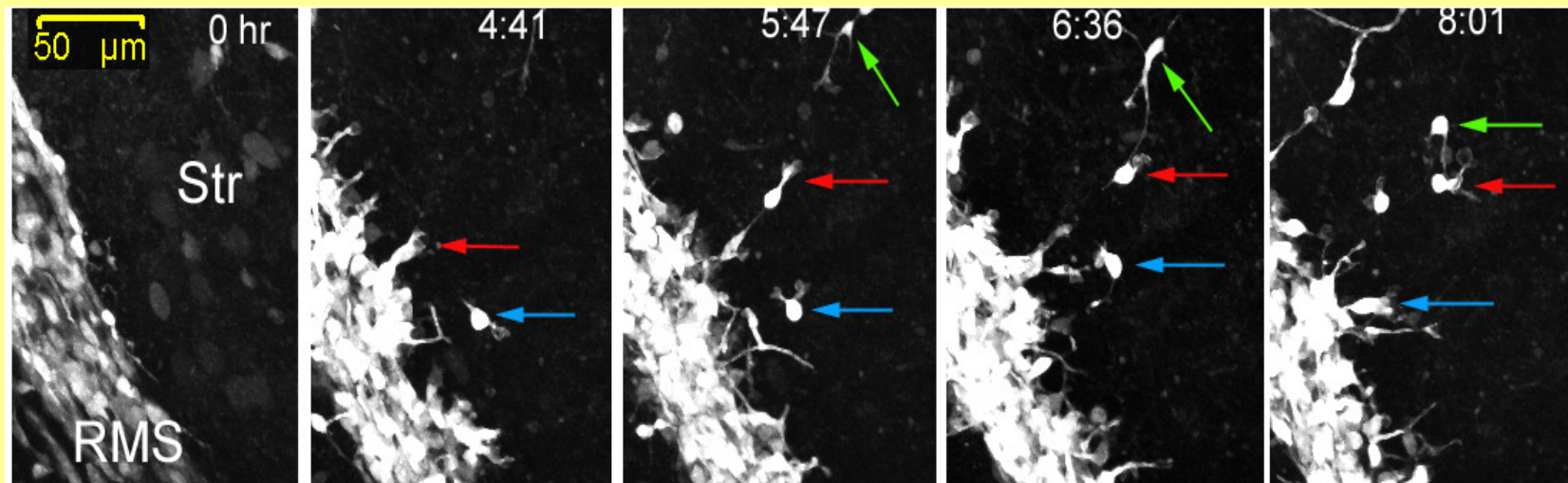
GAD Cell Migration



**P14 brain slice at low magnification
(epifluorescent image)**

**2P microscopy of imaged area for 8 hrs
(movie: XYZ stack = 40 planes/frame)**

GAD Cells – sequential images from previous movie at higher magnification revealing microglia-like activity (red + blue arrows)

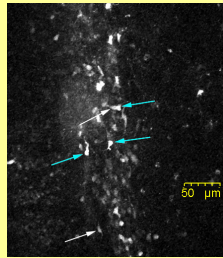


Two cells (red + green arrows) touched at 6:36 and remained touching for 1.5 hrs

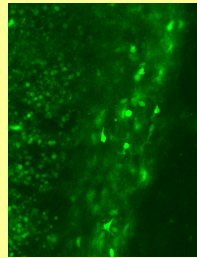
suggesting prolonged intercellular communication

Nestin “Exploratory” Cells are NOT Neuroblasts

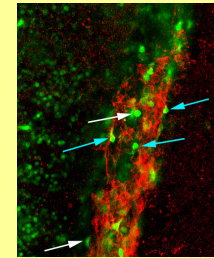
A



B



C



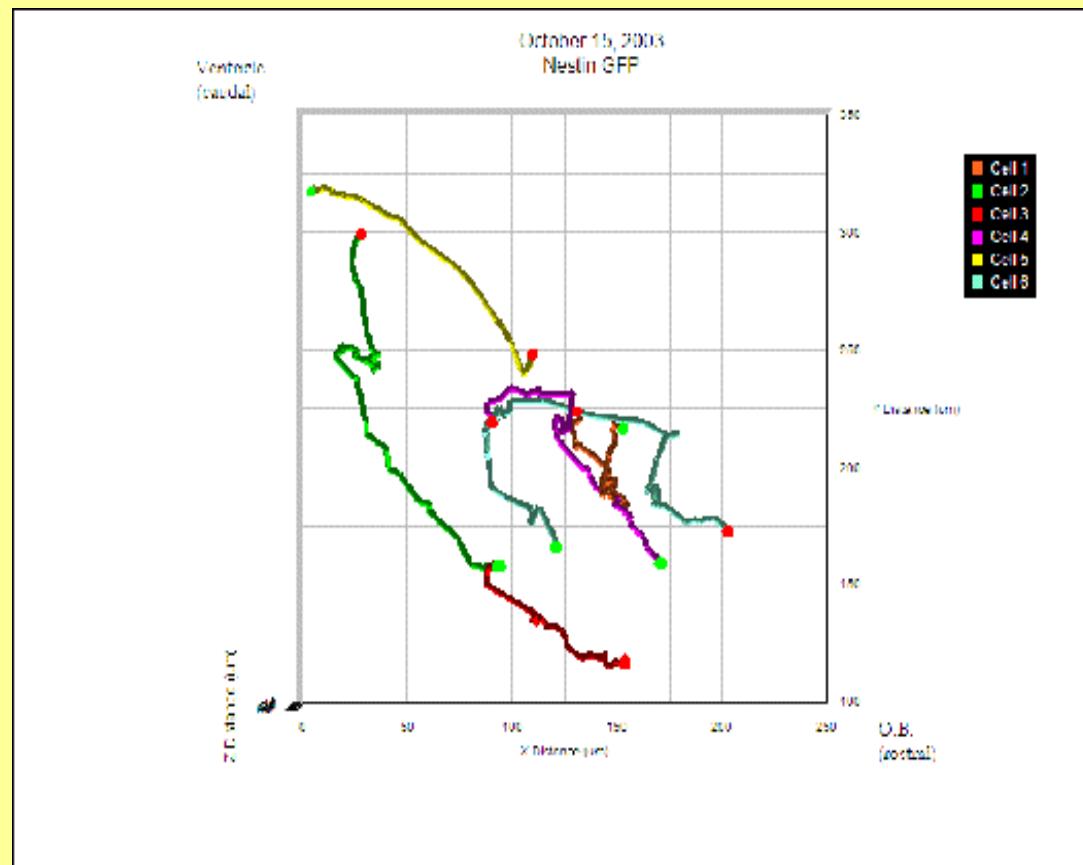
A = last frame of movie from previous slide (after 8 hrs) showing final positions of exploratory

cells (arrows); collapsed XYZ stack (50 slices)

B = confocal image of same area exciting eGFP/Nestin; XYZ stack (4 slices)

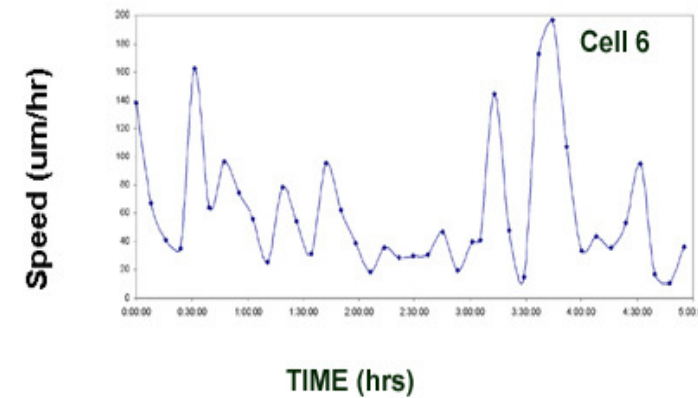
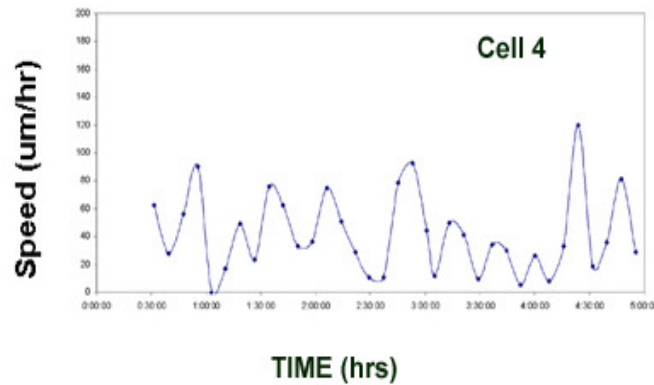
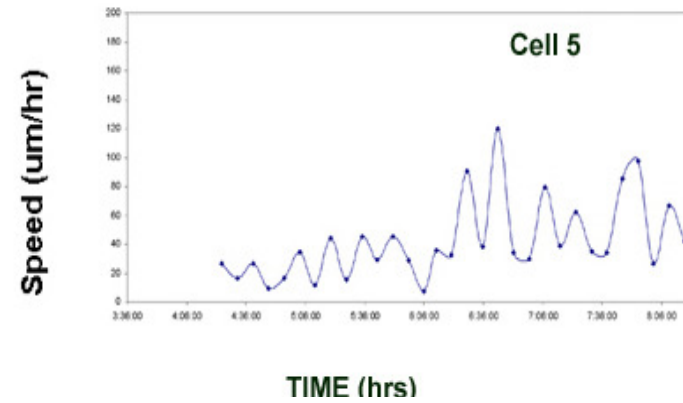
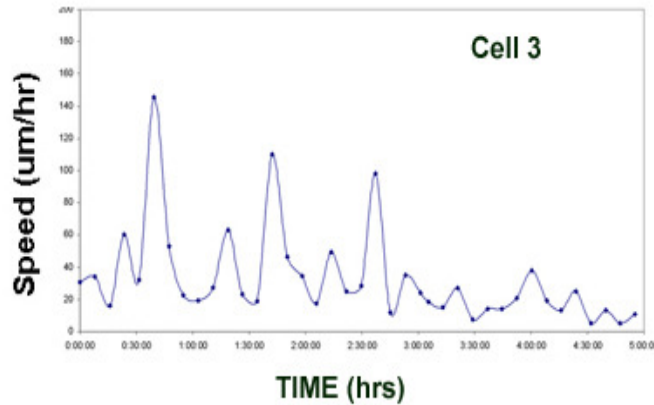
C = confocal image of same area exciting eGFP/Nestin and Cy3/Dcx; XYZ stack (4 slices)

4D Analysis of Nestin “Exploratory” Cells – steps 3 & 4



**Cells 4 & 6 displayed abrupt changes in direction
(colored traces: cell body position over time)**

4D Analysis of Nestin “Exploratory” Cells



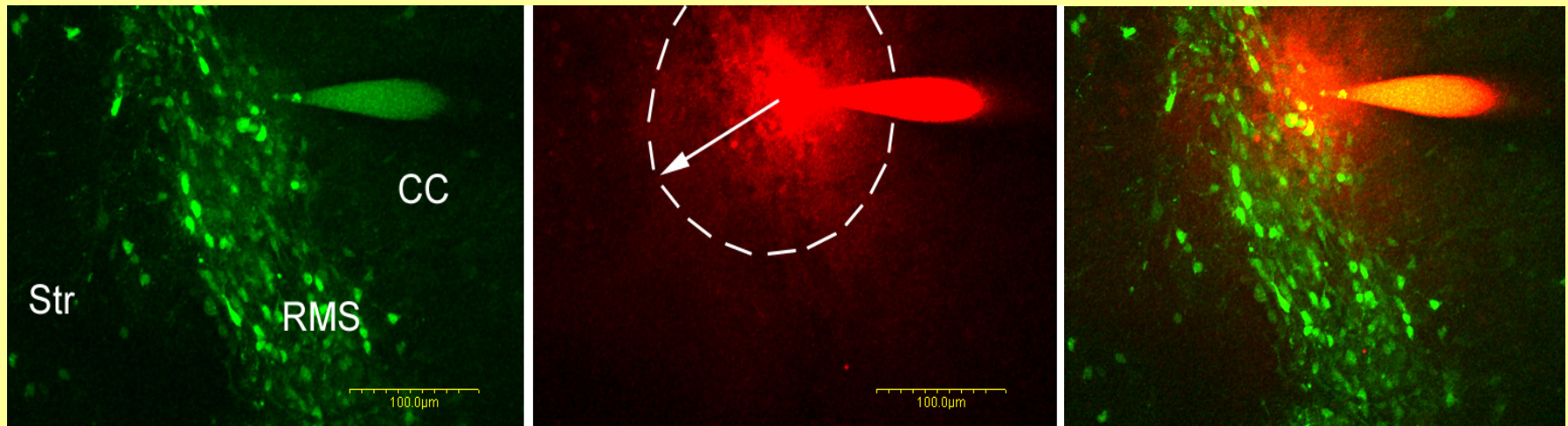
Migration speed of the cell bodies fluctuated over time

Future Studies: Local Application of Growth Factors

eGFP/Nestin

EGF + Texas Red dextran

Merge (movie)



P10 brain slice imaged with 2P microscopy for 2.5 hrs during continuous application of EGF (diffusion radius is for dye)

EGFr is Correlated with Differences in Motility

Kim et al. PLoS One 4: e8122, 2009

