

# Anatomical tracing techniques



Lecture 8

Anita Autry, Ph.D.

# **THEORY**

When you know everything,  
but nothing works.

# **PRACTICE**

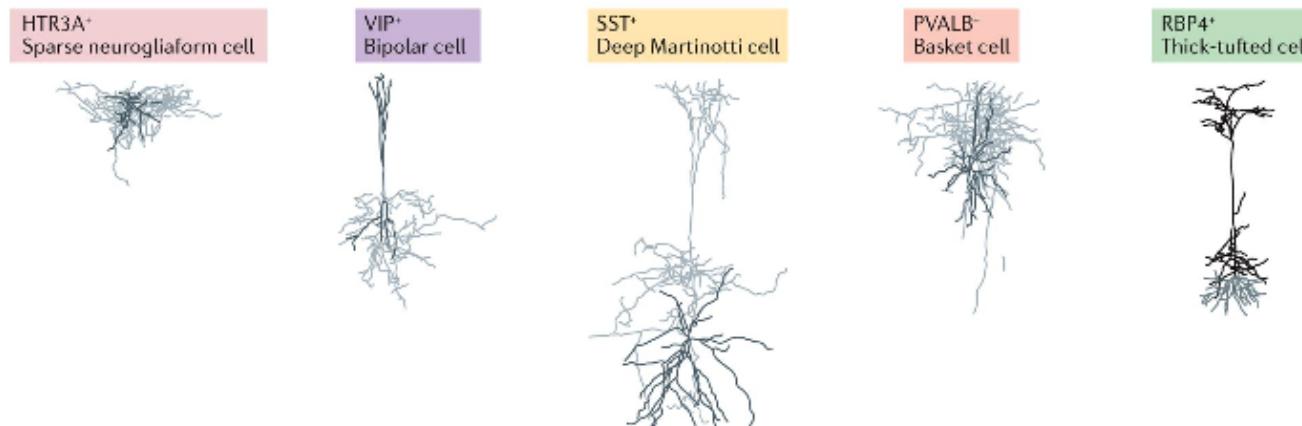
When everything works, but  
no one knows why.

# **OUR LAB**

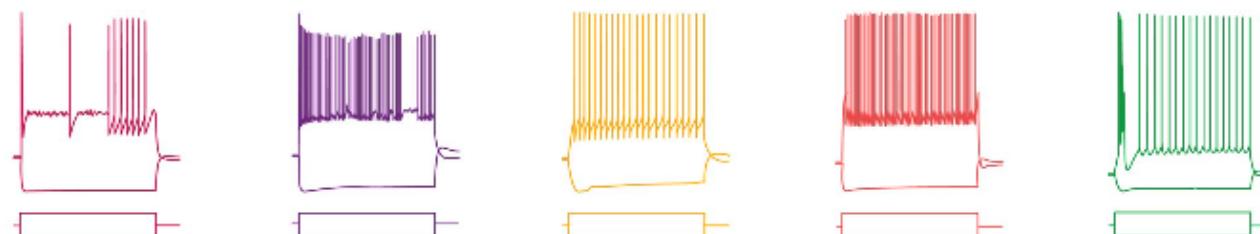
We combine theory and  
practice. Nothing works,  
and no one knows why.

# How might a neuronal cell type be defined?

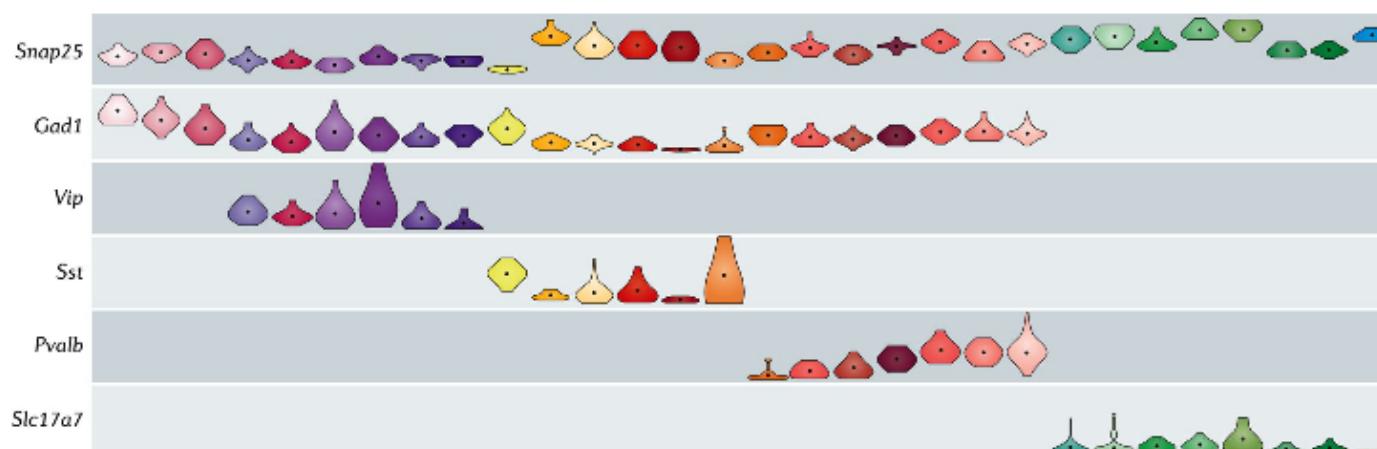
## a Morphology



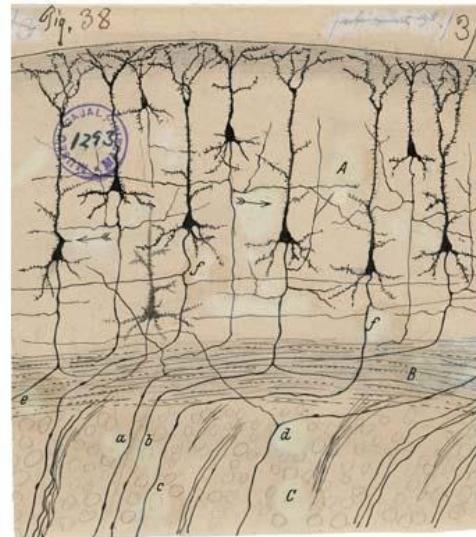
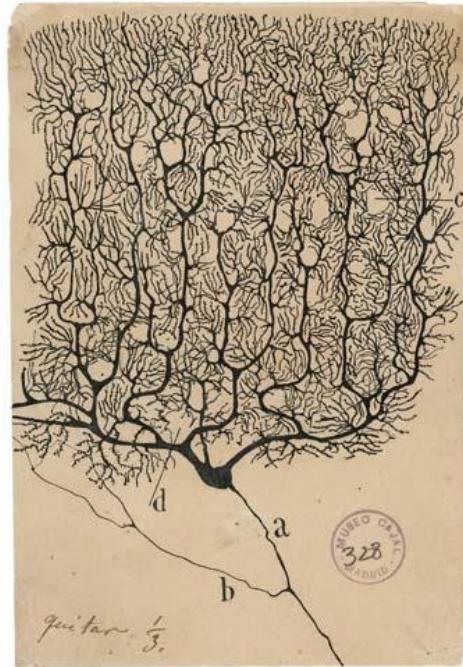
## b Physiology



## c Molecular signature

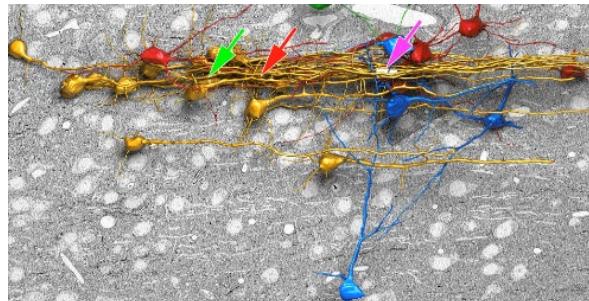


# Power of anatomy: uncovering the synapse

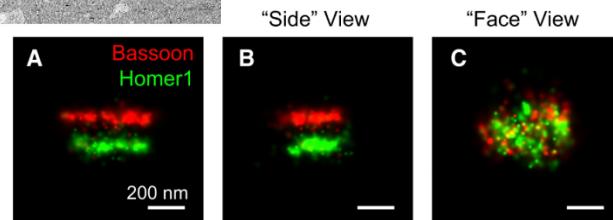


- Santiago Ramon y Cajal (1852-1934)
- Neuron Doctrine

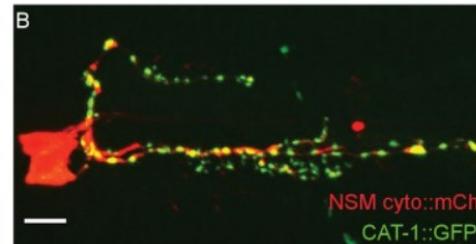
# Connectomics



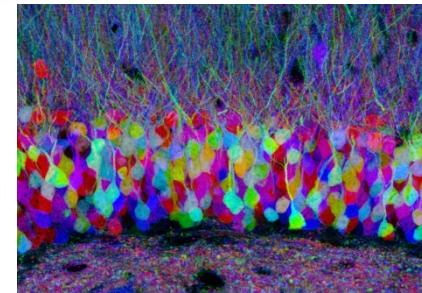
Kasthuri, Cell, 2015.



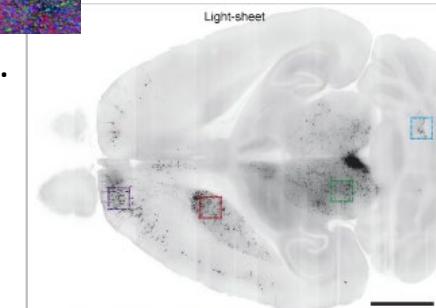
Dani, Neuron, 2010.



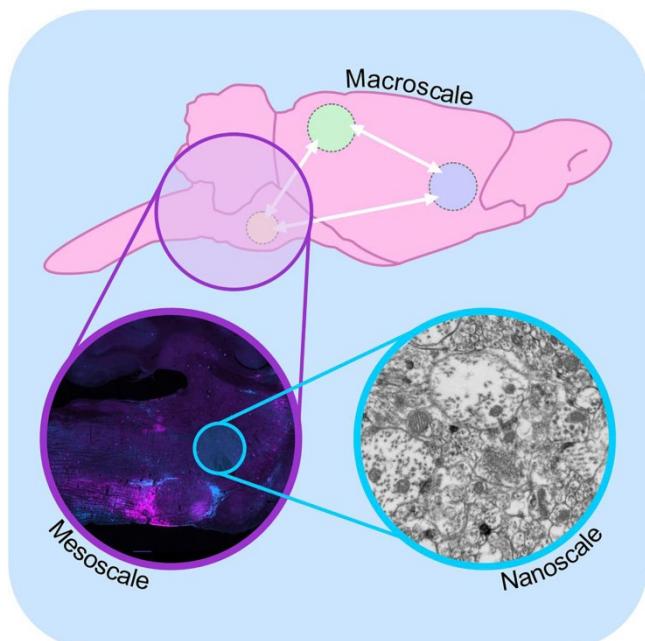
Xuan, eLife, 2017.



Livet, Nature, 2007.

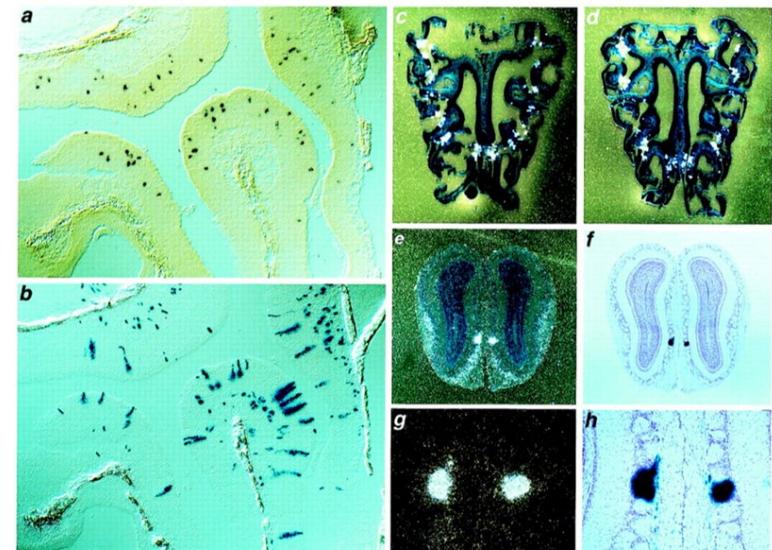


Menegas, eLife, 2015.

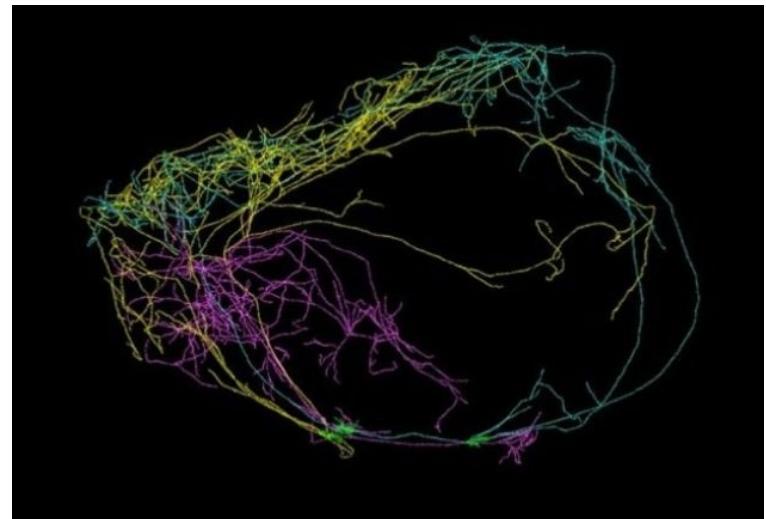


# Tracing techniques

- Stains and dyes
- Antibody labeling
- Viral labeling

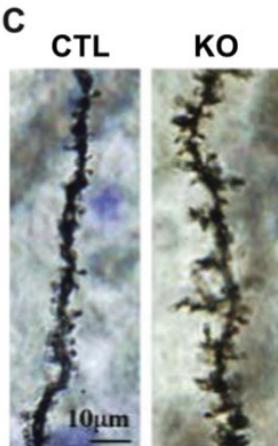
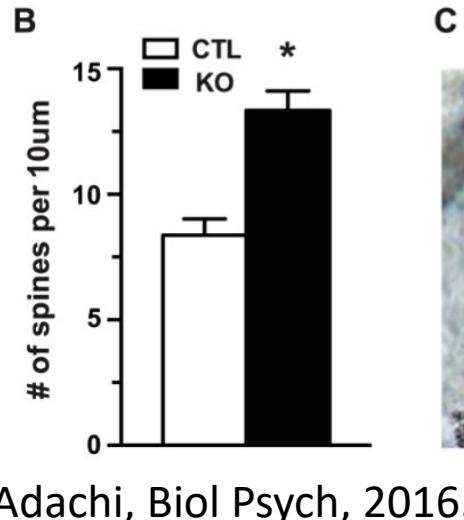
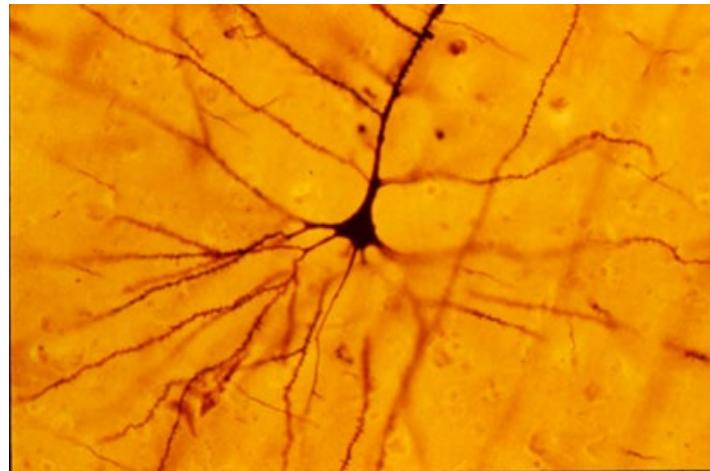


Mombaerts et al., Cell, 1996.

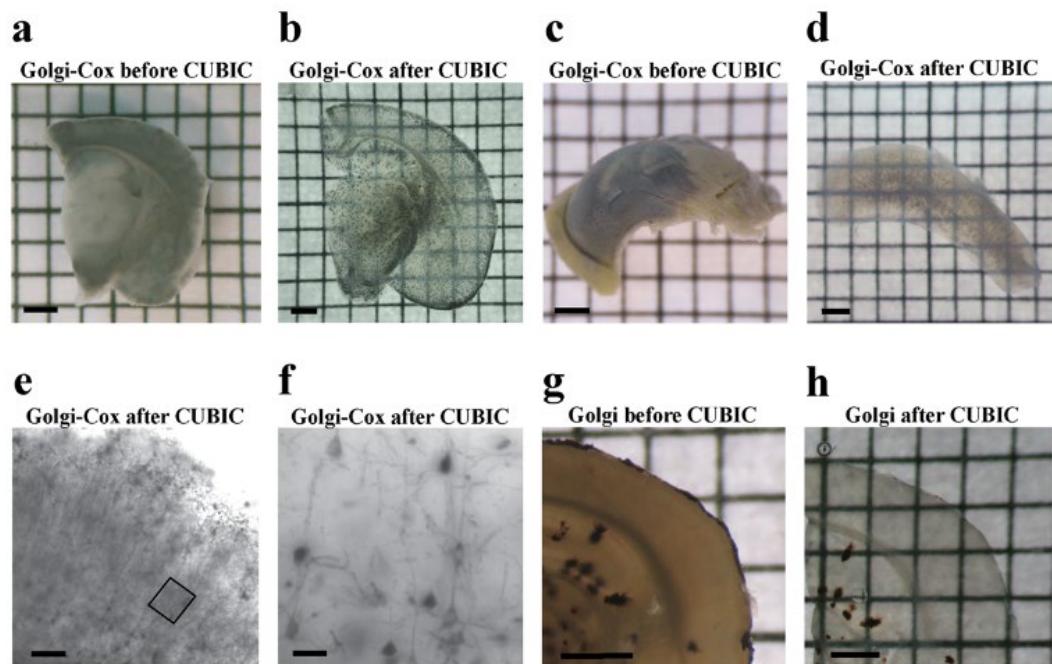


Koch, Allen Brain Institute.

# Golgi staining



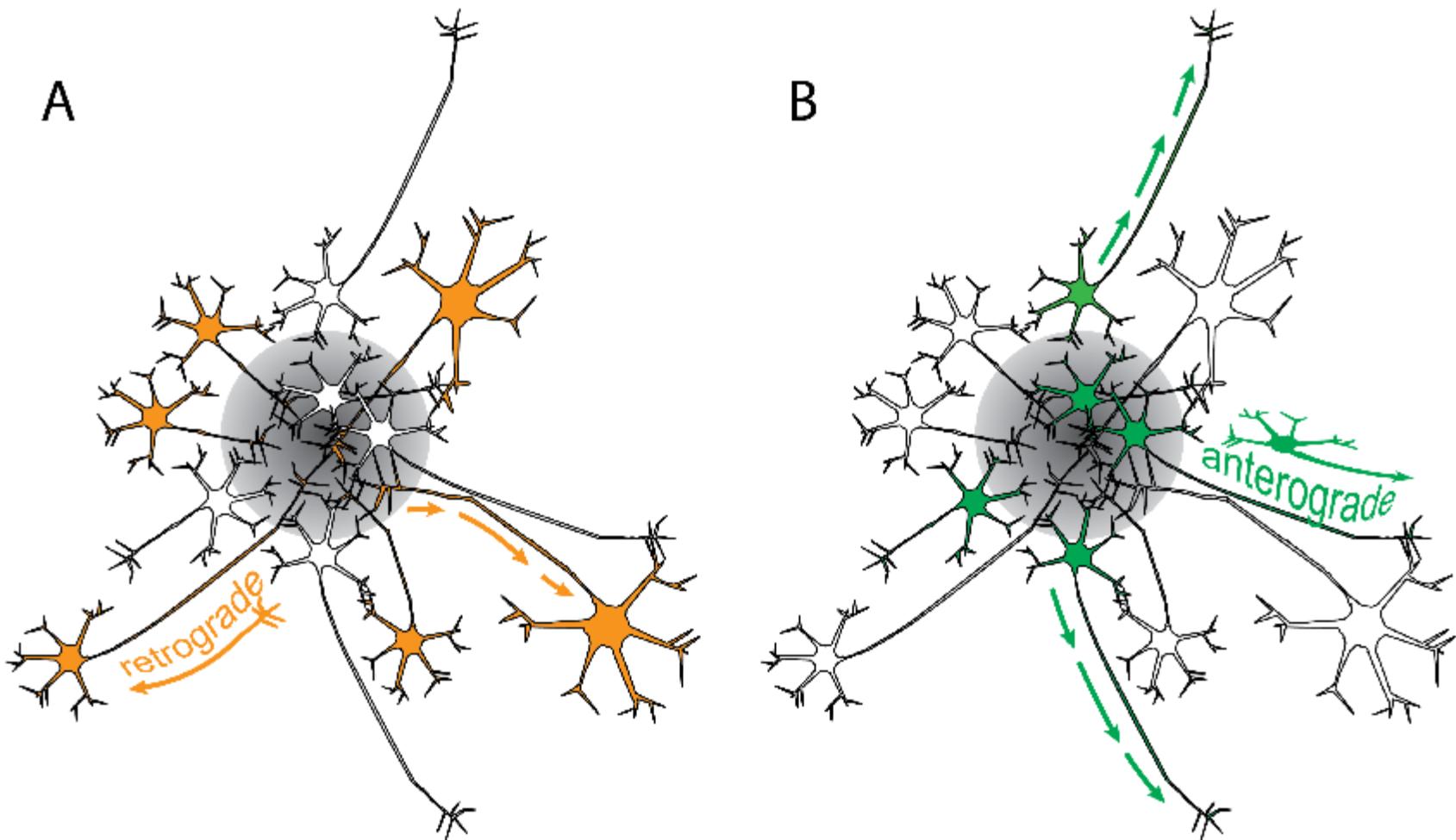
- Developed by Camillo Golgi (1843-1926)
- Dense silver nitrate impregnation of single cells
- Sparse labeling in tissue block



Adachi, Biol Psych, 2016.

Vints, Sci. Reports, 2019.

# Tracing: anterograde vs retrograde



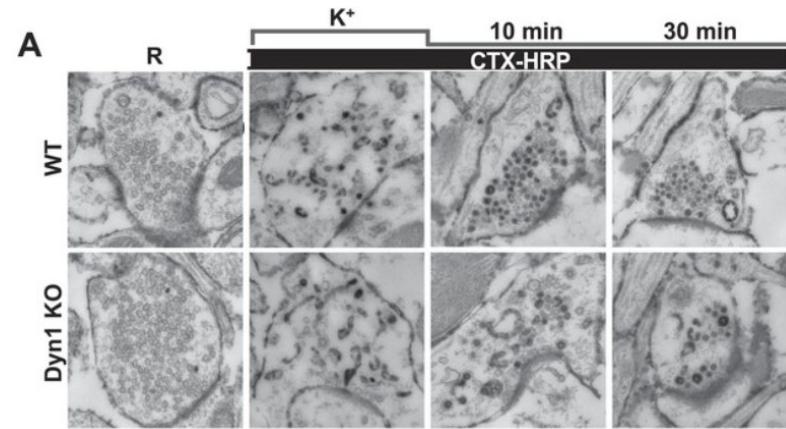
# Dyes

Family	Examples	Dir	Spd	Fill?	Reference
Small proteins	Horseradish peroxidase (HRP) albumin	R/A	F	N	Kristensson and Olsson (1971) LaVail and LaVail (1972)
Inorganic fluorescent molecules	Fast Blue (FB) Diamidino yellow (DY) Fluoro-gold (FG)	R	M	N	Kuypers et al. (1979) Bentivoglio et al. (1980) Schmued and Fallon (1986)
D dextrans	Fluoro-Ruby (FR) Biotinylated dextran amine (BDA)	A/R A/R	M	N Y	Glover et al. (1986) Nance and Burns (1990) Veenman et al. (1992)
Lectins	Wheat germ agglutinin (WGA; WGA-HRP) <i>Phaseolus vulgaris</i> -leucoagglutinin (PHA-L)	R/A	F	N	Schwab et al. (1978) Gonatas et al. (1979) Gerfen and Sawchenko (1984)
Beads	Latex microspheres	R	F	N	Katz et al. (1984) Katz and Iarocci (1990)
Bacterial toxins	Tetanus cholera (B fragment)	R R/A	F	N	Stoeckel et al. (1977) Schwab and Agid (1979) Trojanowski et al. (1981)
Trophins	Nerve growth factor (NGF) brain-derived neurotrophic factor (BDNF)	R	F	N	Hendry et al. (1974) Stoeckel and Thoenen (1975)
Amino acids	<sup>3</sup> H-leucine <sup>3</sup> H-proline biocytin	A	F/S	N Y	Cowan et al. (1972) Hendrickson (1982) King et al. (1989)
Carbocyanine dyes	Dil DiO	A/R	S	N	Honig and Hume (1986) Honig and Hume (1989)

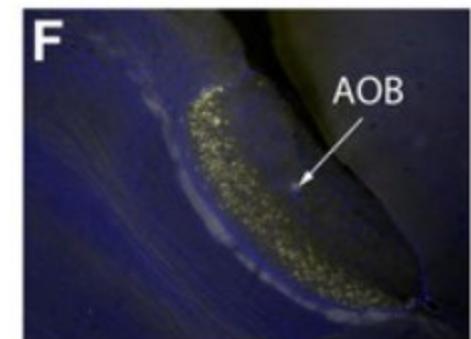
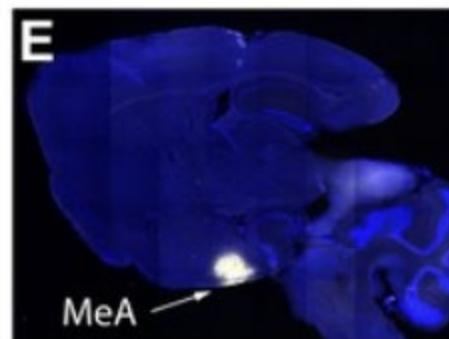
Abbreviations: "Dir", direction; "A", anterograde; "R", retrograde (for bi-directionally transported substances, bold-face indicates predominant direction or most common usage); "Spd", speed; "F", fast; "S", slow; "M", moderate; "Fill?", Does the tracer produce Golgi-like fills of neuronal cell bodies?; "Y", yes; "N", no.

# Method of cell entry

- Passive transport
  - HRP
- Injury site
  - Dextran amines
- Binding mediated
  - CTBs (GM1 ganglioside: mostly axonal localization)
  - WGA/PHA-L (N-acetylgalactosamine/sialic acid: membrane)
- Endocytosis
  - Retrobeads
- Iontophoresis
  - Fluorogold



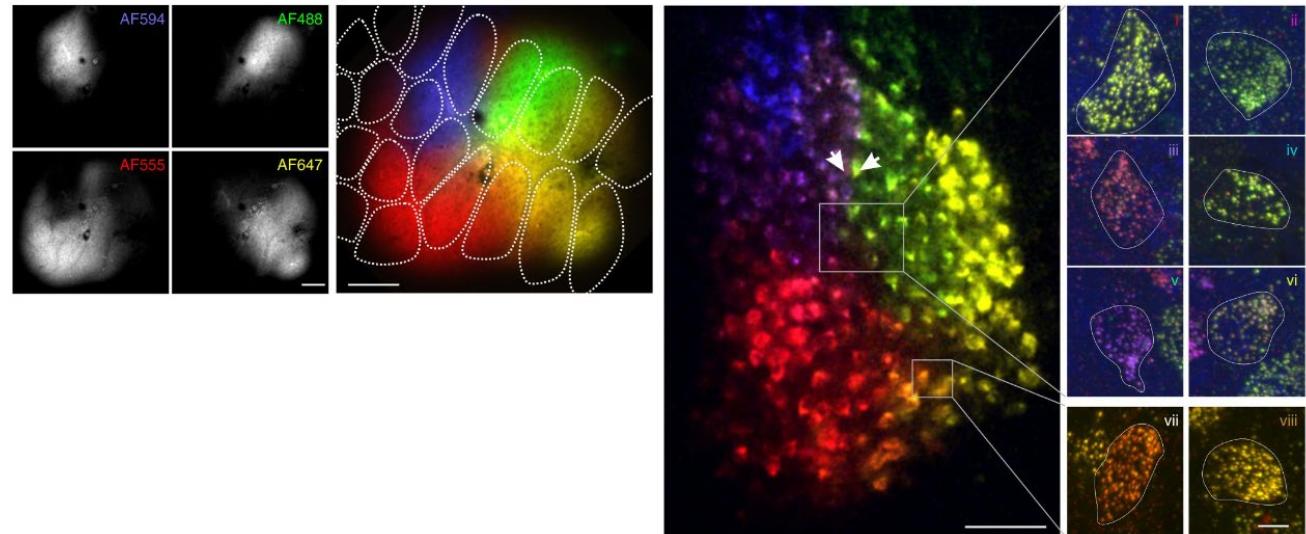
Wu et al., eLife, 2014.



Bergan et al., eLife, 2014.

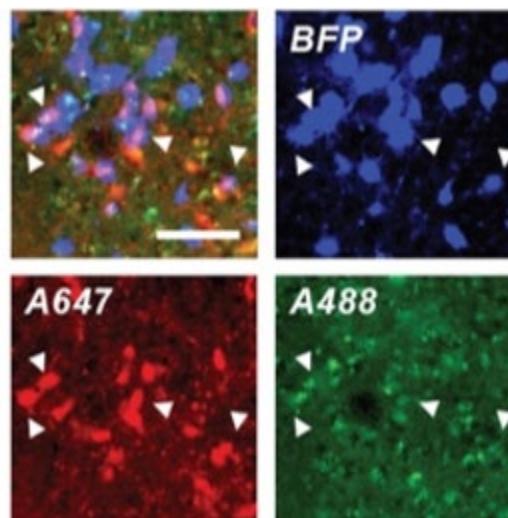
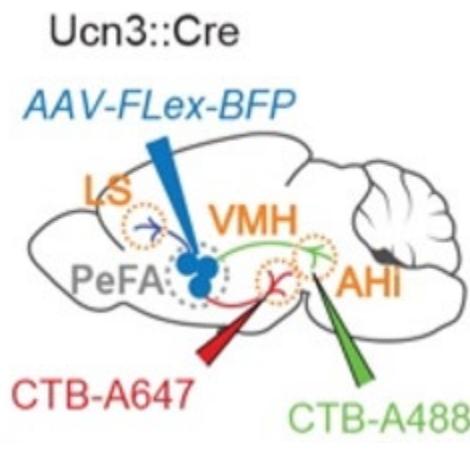
# Applications

- WGA for anterograde tracing



Tsuriel, Nat. Methods, 2015.

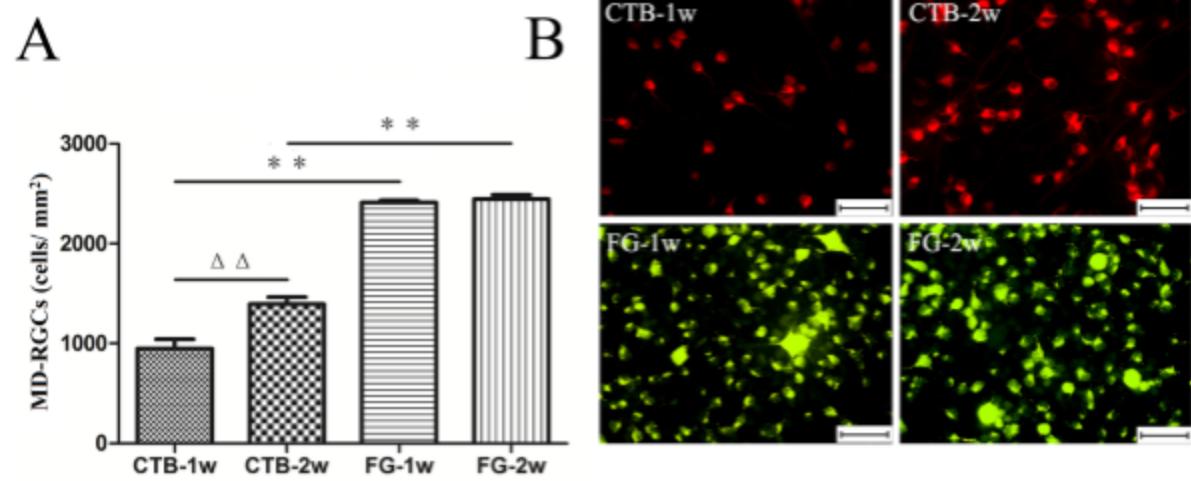
- CTB for retrograde



Autry, bioRxiv, 2019.

# Dye tracing in practice

- Time
- Concentration
- Volume

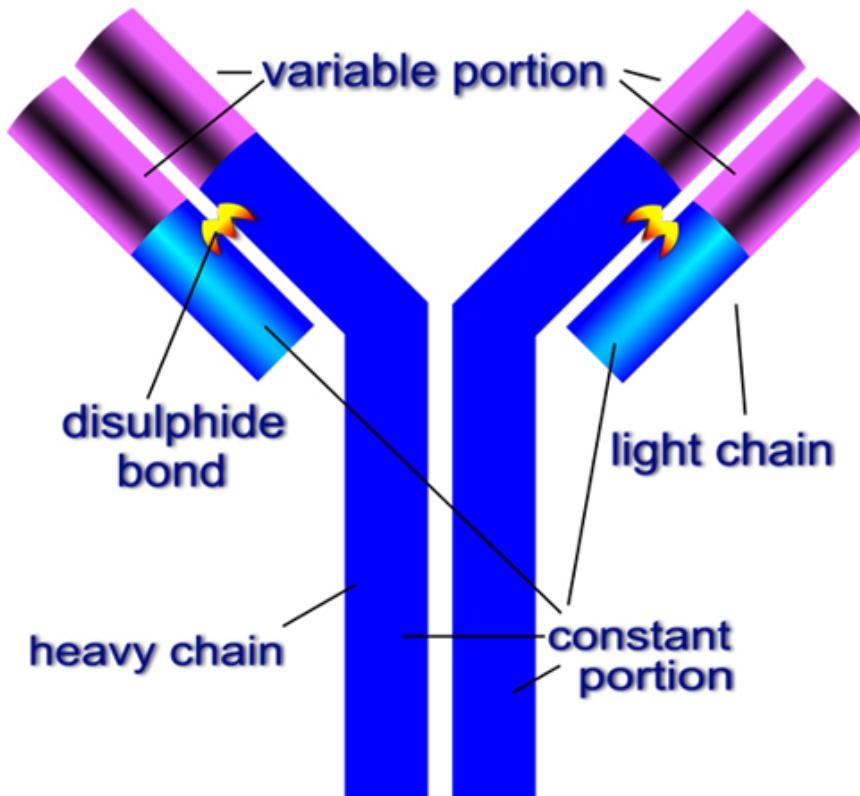


Yao, PLOS One, 2018.

# Immunohistochemistry

- Antibodies
  - Polyclonal
  - monoclonal
- Amplification
  - DAB
  - NBT/BCIP
  - Dark product
  - Avidin/Biotin
  - TSA

# Antibody structure



**Figure 2.** Diagram of antibody structure. The protein comprises two heavy and two light chains arranged in a Y-shaped complex. Both chains have variable domains at the tips of the arms of the Y where antigen binds. Each variable domain contains three complementarity-determining regions (CDRs), which come into direct contact with the bound antigen. Reproduced from Wikimedia Commons under the Creative Commons Attribution-Share Alike 3.0 Unported license.

# Not all antibodies are equivalent

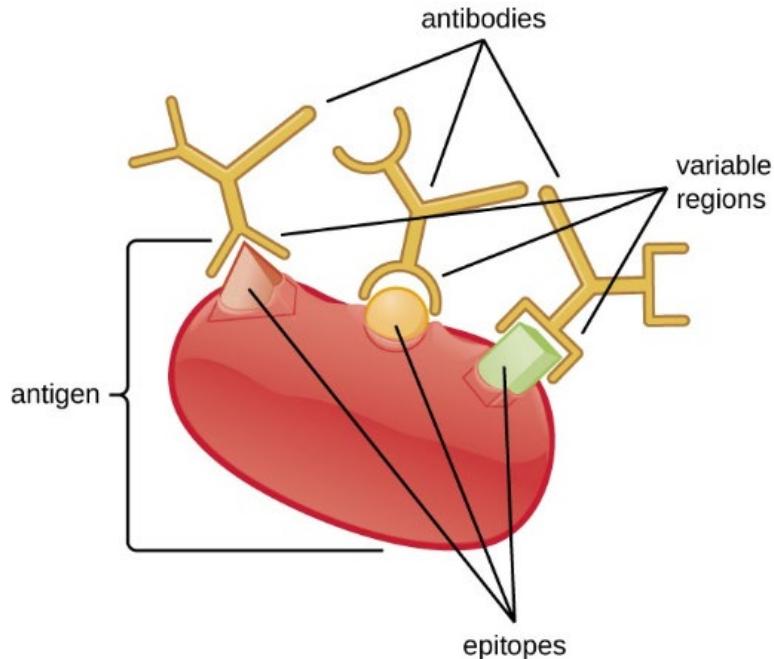


Figure 1. An antibody binds to a specific region on an antigen called an epitope. A single antigen can have multiple epitopes for different, specific antibodies.

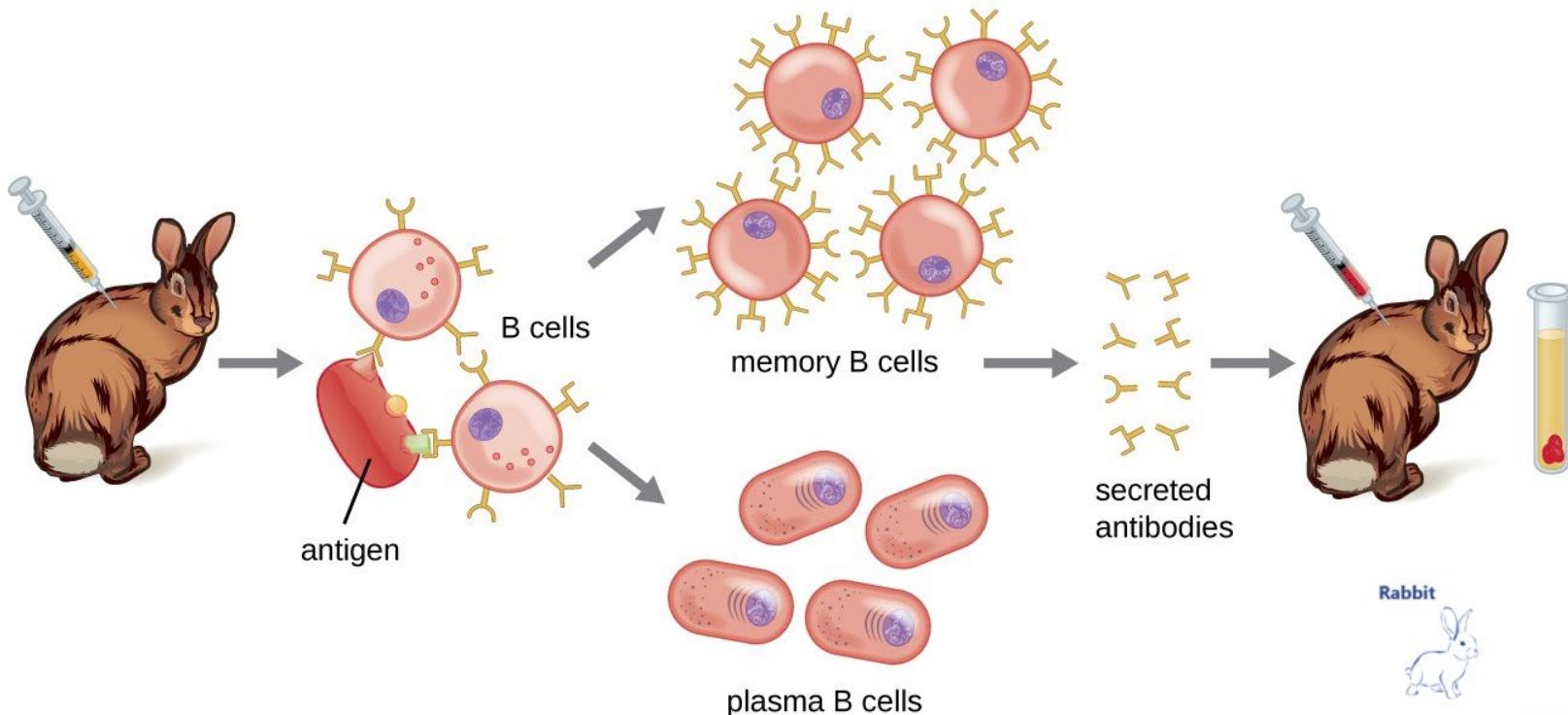
# Polyclonal antibodies

1 Inject antigen into rabbit.

2 Antigen activates B cells.

3 Plasma B cells produce polyclonal antibodies.

4 Obtain antiserum from rabbit containing polyclonal antibodies.



Rabbit



Chicken



Rodents



Goat

Polyclonal Antibodies

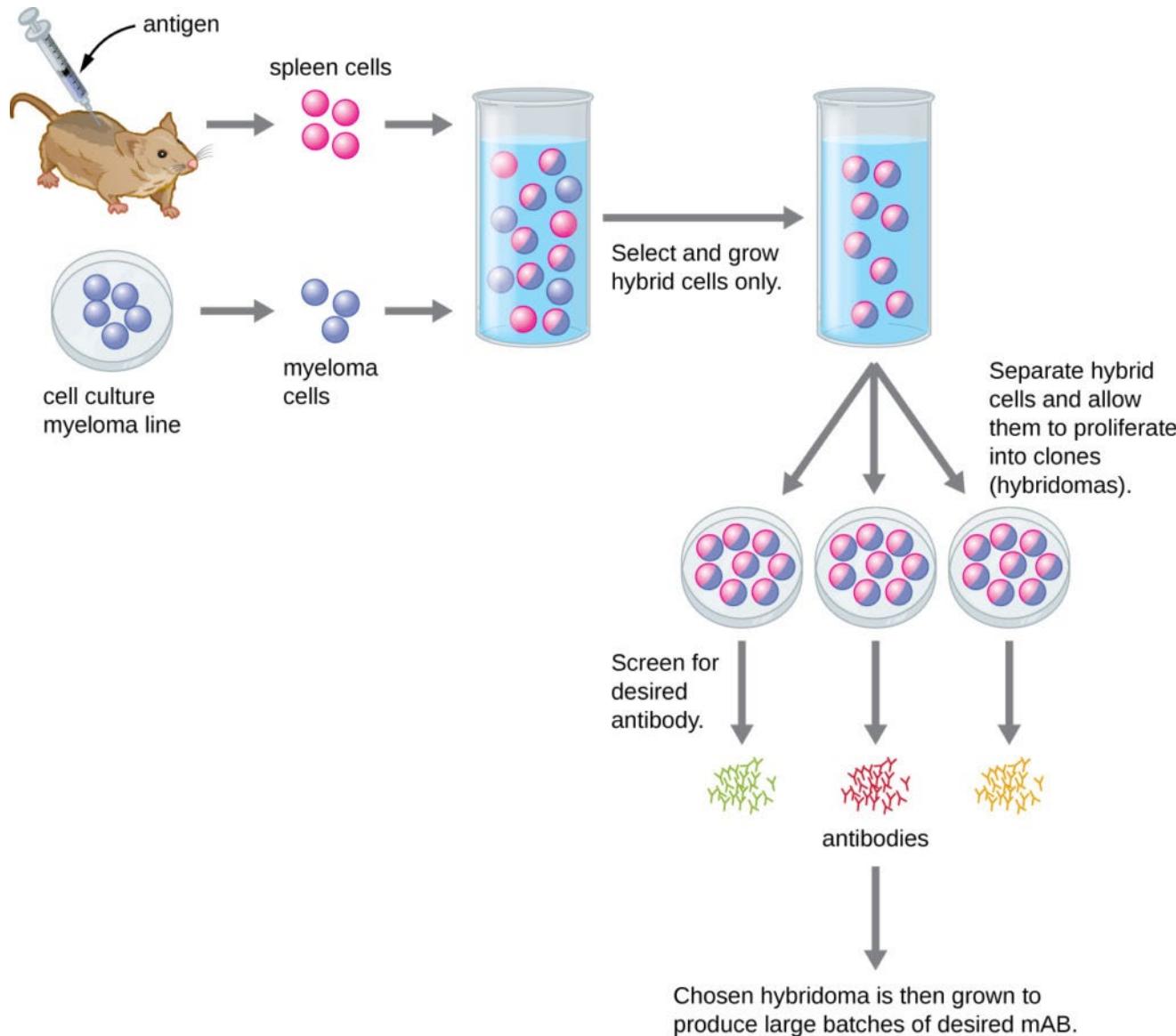


Sheep



Camelids

# Monoclonal antibodies



# Mono vs poly

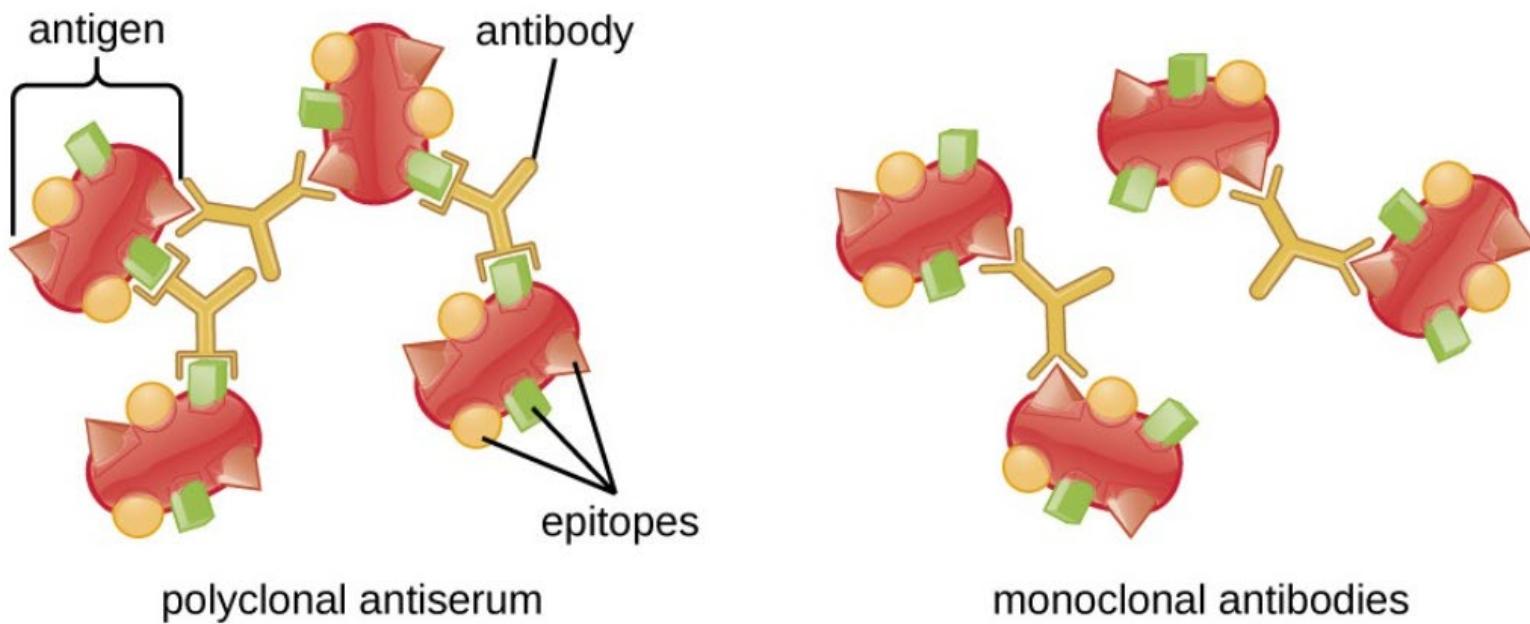
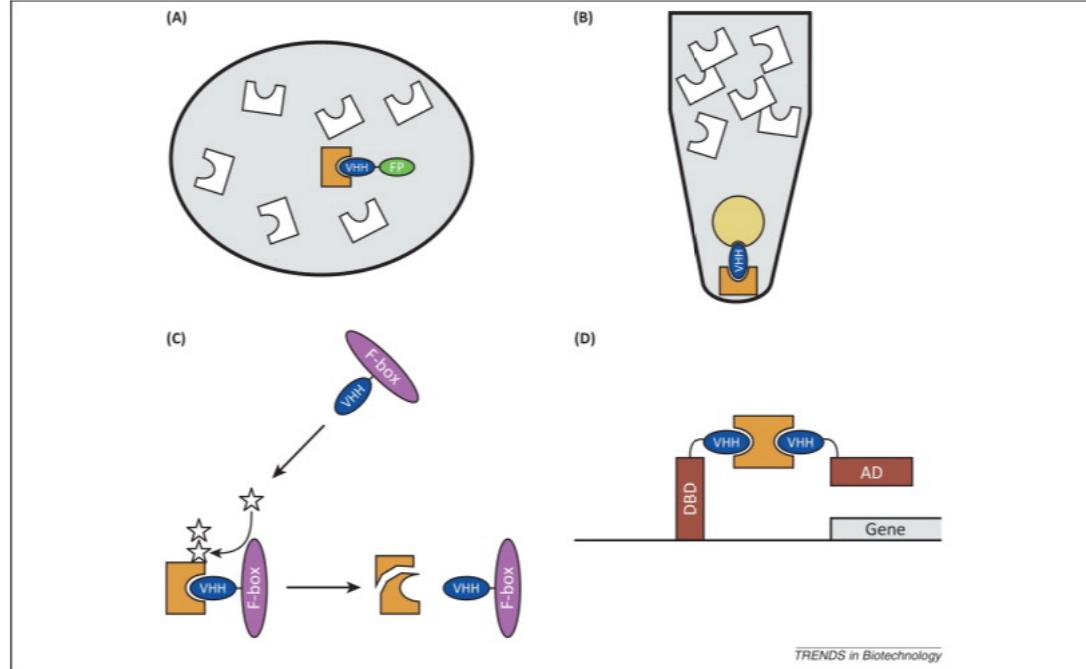
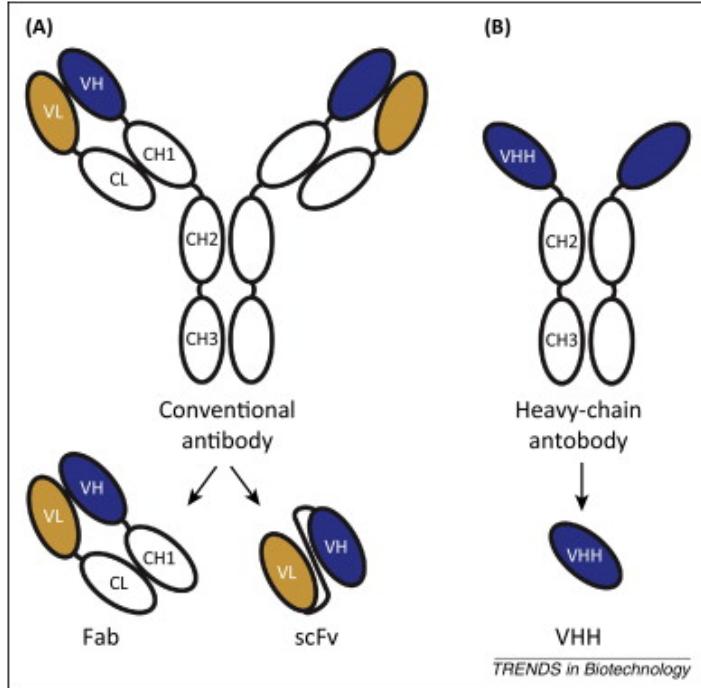


Figure 1. Polyclonal antiserum binds to multiple epitopes on an antigen, leading to lattice formation that results in a visible precipitin. Monoclonal antibodies can only bind to a single epitope; therefore, less binding occurs and lattice formation generally does not occur.

# Camelid nanobodies

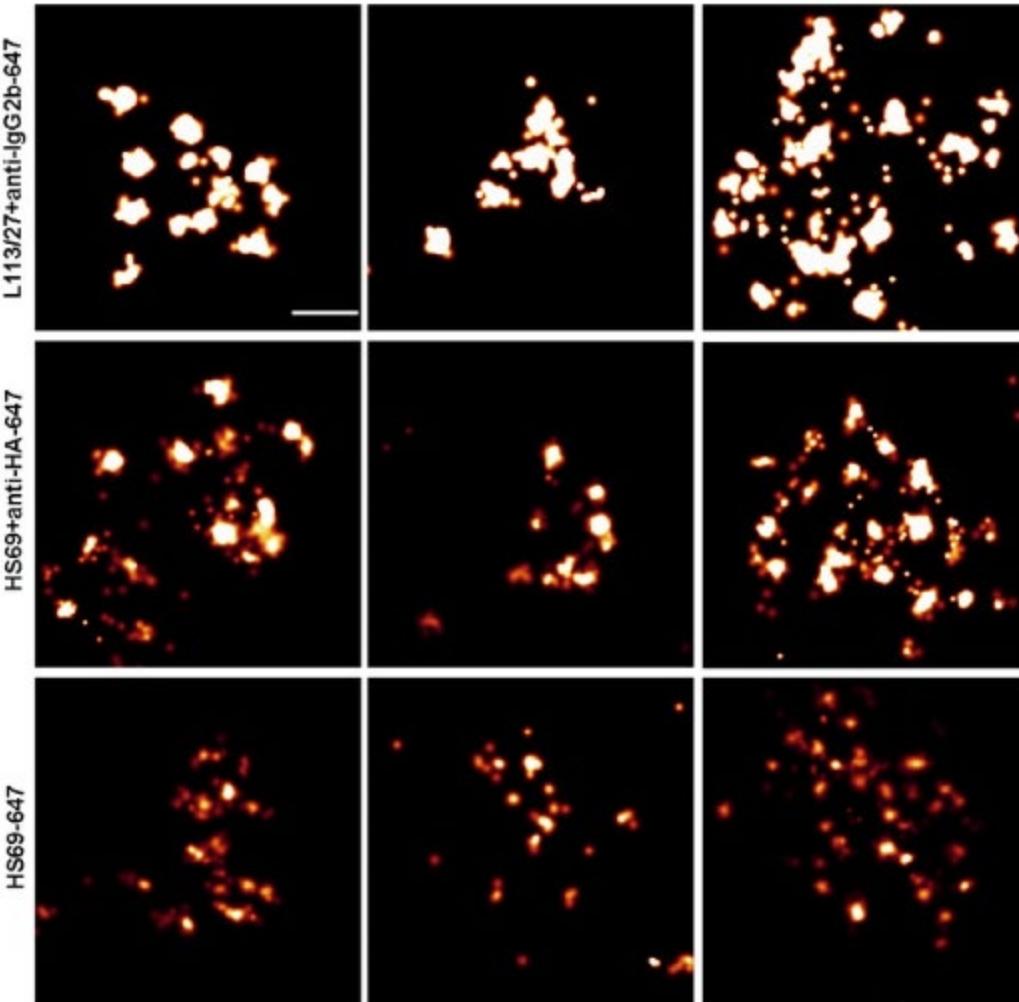


- Single domain antibodies/V<sub>H</sub>H
- Lack a light chain (heavy chain-HCabs)



# Camelid nanobodies

B



- mAb
- Nanobody primary
- Conjugated nanobody

# Antibodies in practice

Lot Number: RD2182776

Product Data Sheet

## MSH2 Antibody

Tested Species Reactivity	
Human (Hu)	
Tested Applications	Dilution *
Immunohistochemistry (Paraffin) (IHC (P))	1:100

\* Suggested working dilutions are given as a guide only. It is recommended that the user titrates the product for use in their own experiment using appropriate negative and positive controls.

Did you  
read the  
datasheet?

Details	
Catalog Number:	PA5-32507
Size:	500 µL
Class:	Polyclonal
Type:	Antibody
Clone:	
Host / Isotype:	Rabbit / IgG
Immunogen:	Synthetic peptide corresponding to internal region of human MSH2.

Form Information	
Form:	Liquid
Purification:	Antigen affinity chromatography
Storage Buffer:	PBS, pH 7.6, with 1% BSA
Preservative:	<0.1% sodium azide
Storage Conditions:	4° C, do not freeze

## Product Specific Information

Heat-mediated antigen retrieval is recommended prior to staining, using a 10mM citrate buffer, pH 6.0, for 10 minutes followed by cooling at room temperature for 20 min. Following antigen retrieval, incubate samples with primary antibody for 30 min at room temperature. A suggested positive control is colon carcinoma.

*For Research Use Only. Not for use in diagnostic procedures. Not for resale without express authorization.*

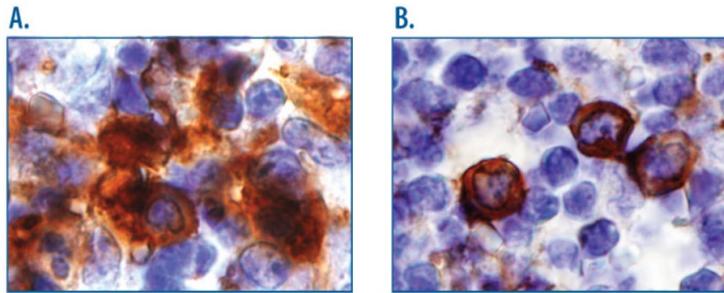
## General Information

MSH2 is involved in DNA repair as a mismatch repair protein, and mutations of MSH2 are found in approximately 50% of inherited non polyposis colorectal carcinoma (HNPCC) (Lynch syndrome) cases. HNPCC is an autosomal, dominantly inherited disease associated with marked increase in cancer susceptibility. It is characterized by a familial predisposition to early onset colorectal carcinoma and extra-colonic cancers of the gastrointestinal, urological and female reproductive tracts. HNPCC is reported to be the most common form of inherited colorectal cancer in the western world.



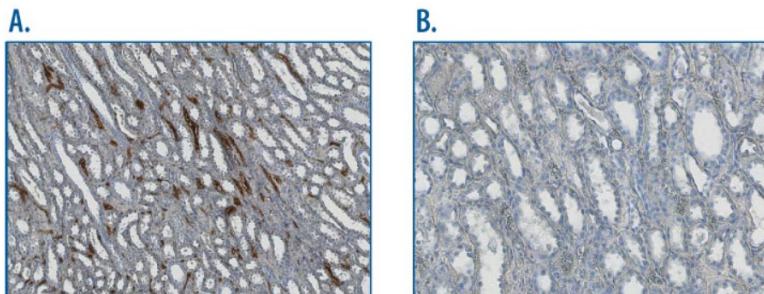
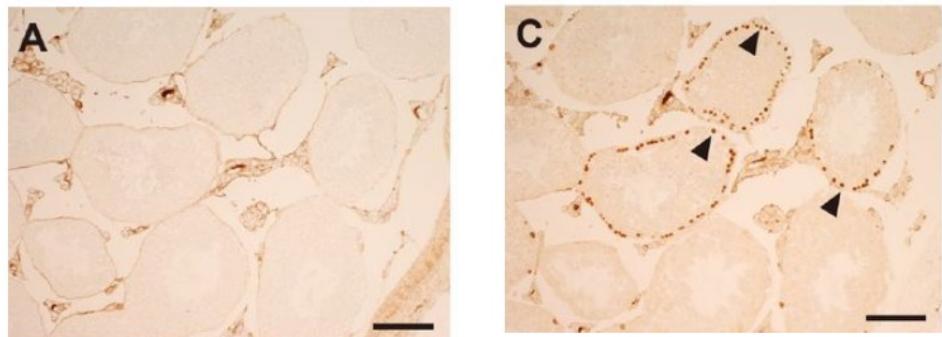
# Can I skip this step?

- Blocking



**Blocking Non-specific Binding with Serum.** A. CD14 was detected in paraffin-embedded human tonsil tissue using anti-human CD14 biotinylated affinity-purified polyclonal antibody (Catalog # BAF383). Tissue was subjected to antigen retrieval and stained using high sensitivity streptavidin conjugated to HRP (HSS-HRP) and DAB, and counterstained with hematoxylin (blue). B. Non-specific background staining is markedly reduced in a parallel experiment which included a blocking step using animal serum for 15 minutes at room temperature prior to incubation with the primary antibody. (Data provided by R&D Systems)

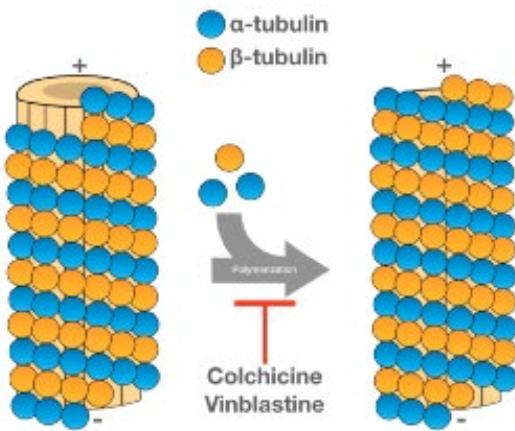
- Antigen retrieval



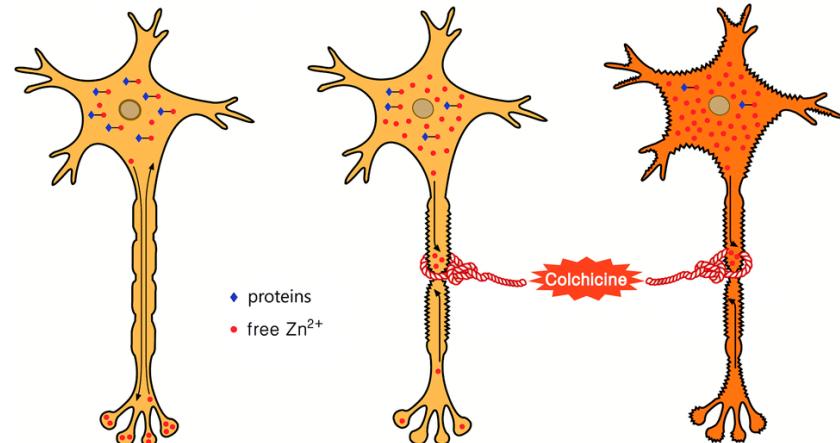
**Quenching Endogenous Peroxidase Activity.** A. Failing to quench endogenous peroxidase prior to staining produced a false positive signal in sections of human kidney. Tissue was stained using the anti-goat HRP-DAB Cell & Tissue Staining Kit (Catalog # CTS008; brown). B. Endogenous peroxidase activity was quenched in the same tissue using 3% H<sub>2</sub>O<sub>2</sub> in methanol for 15 minutes at room temperature prior to staining.

- Peroxide

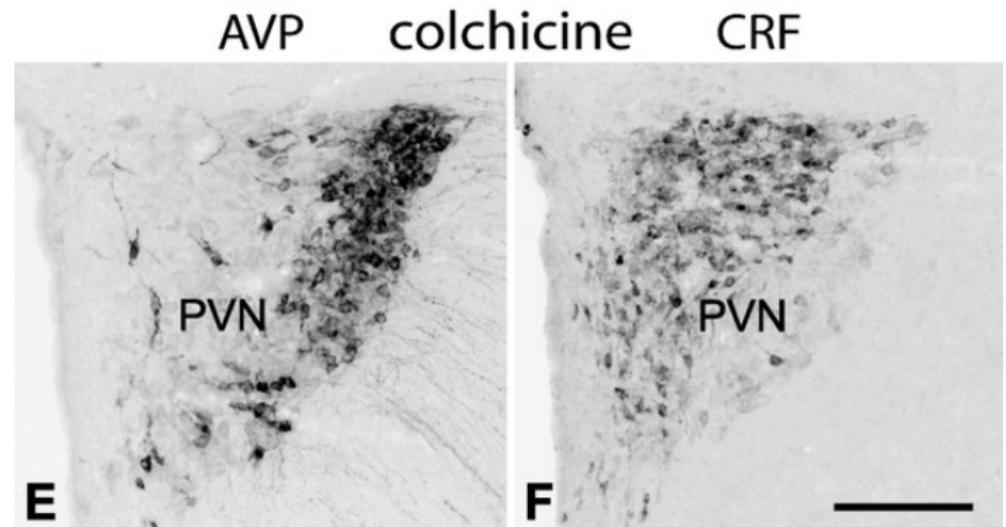
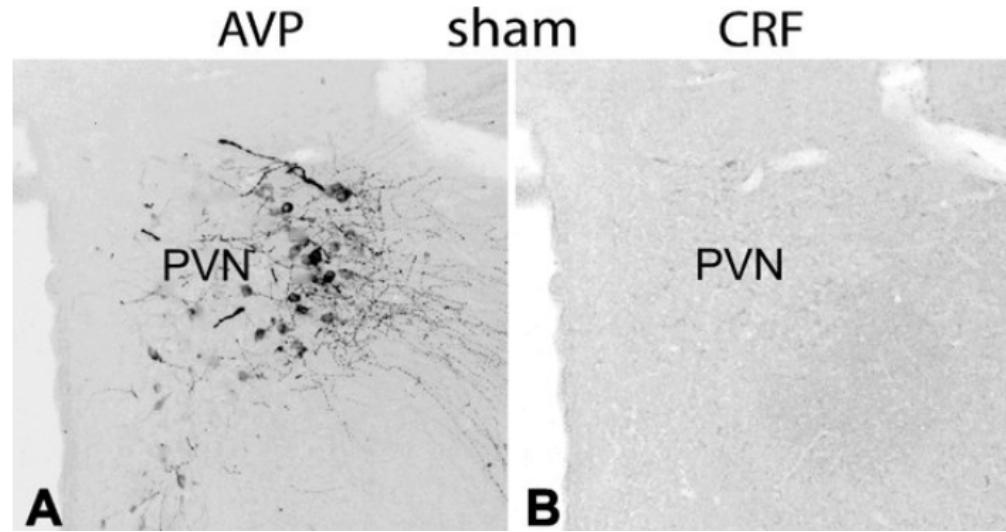
# Colchicine



A. Normal axonal flow    B. Blocked axonal flow by colchicine    C. Zinc accumulation & death



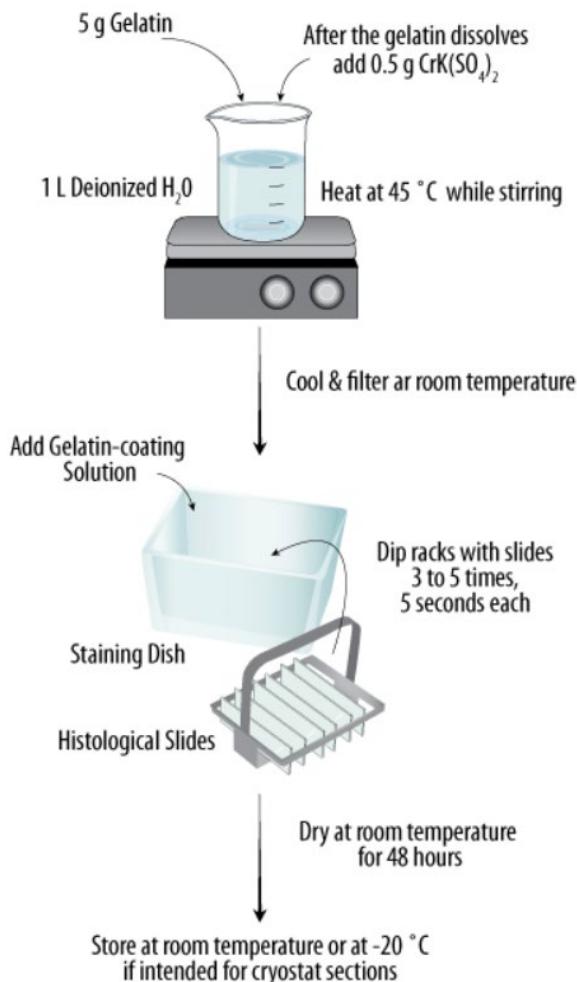
Choi et al., Metallomics, 2014.



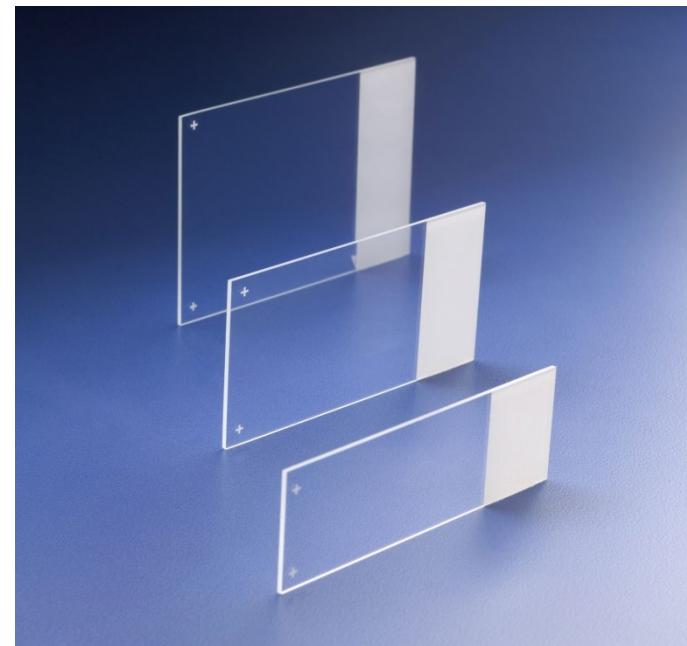
Gonzalez-Hernandez et al., J. Histochem. and Cytochem., 2006.

# How to keep tissue on slides for a whole staining process

Gel-subbed slides



Charged slides

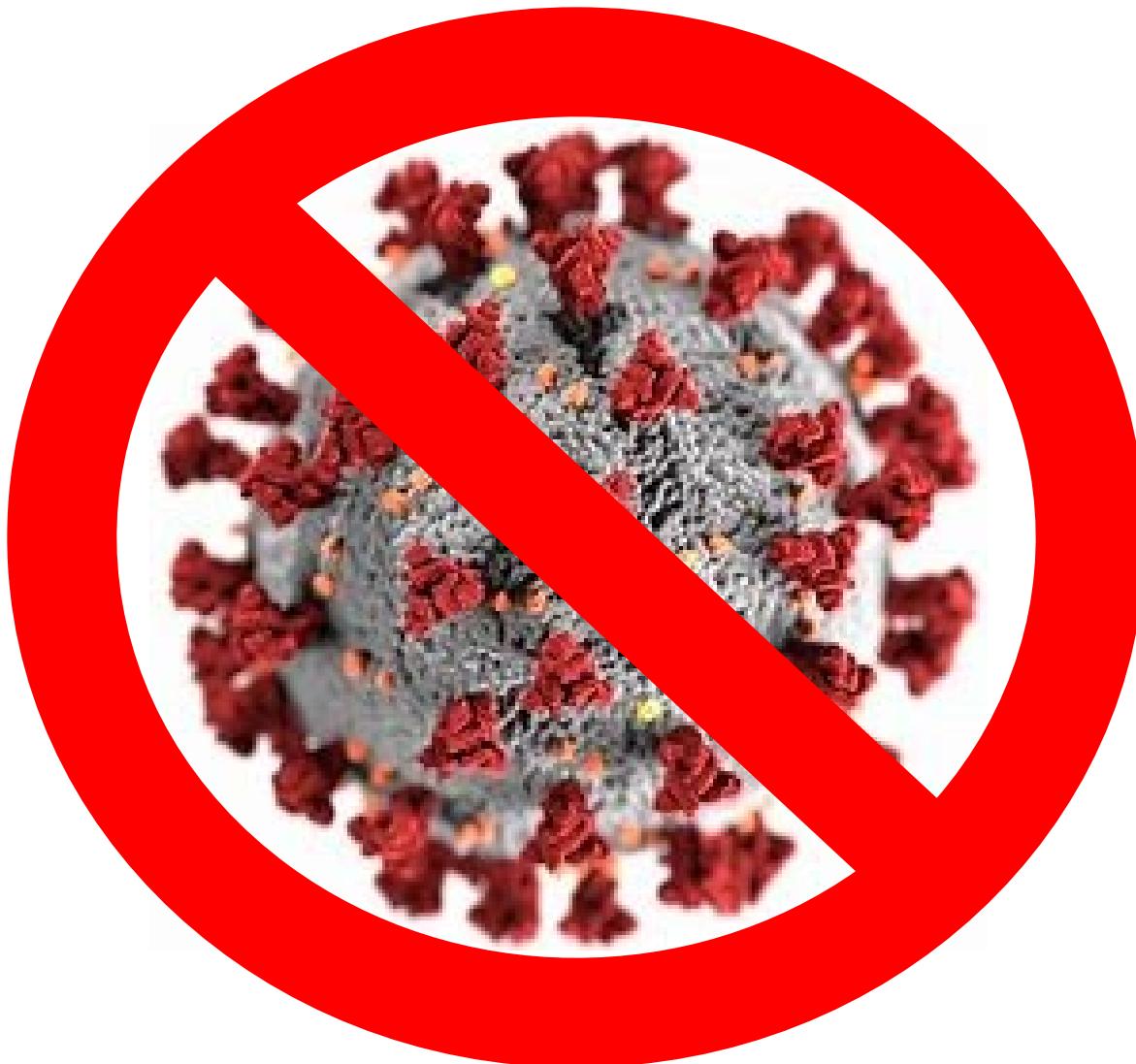


\*Tissue sections carry a slightly negative charge when immersed in neutral pH solutions

# Mounting media



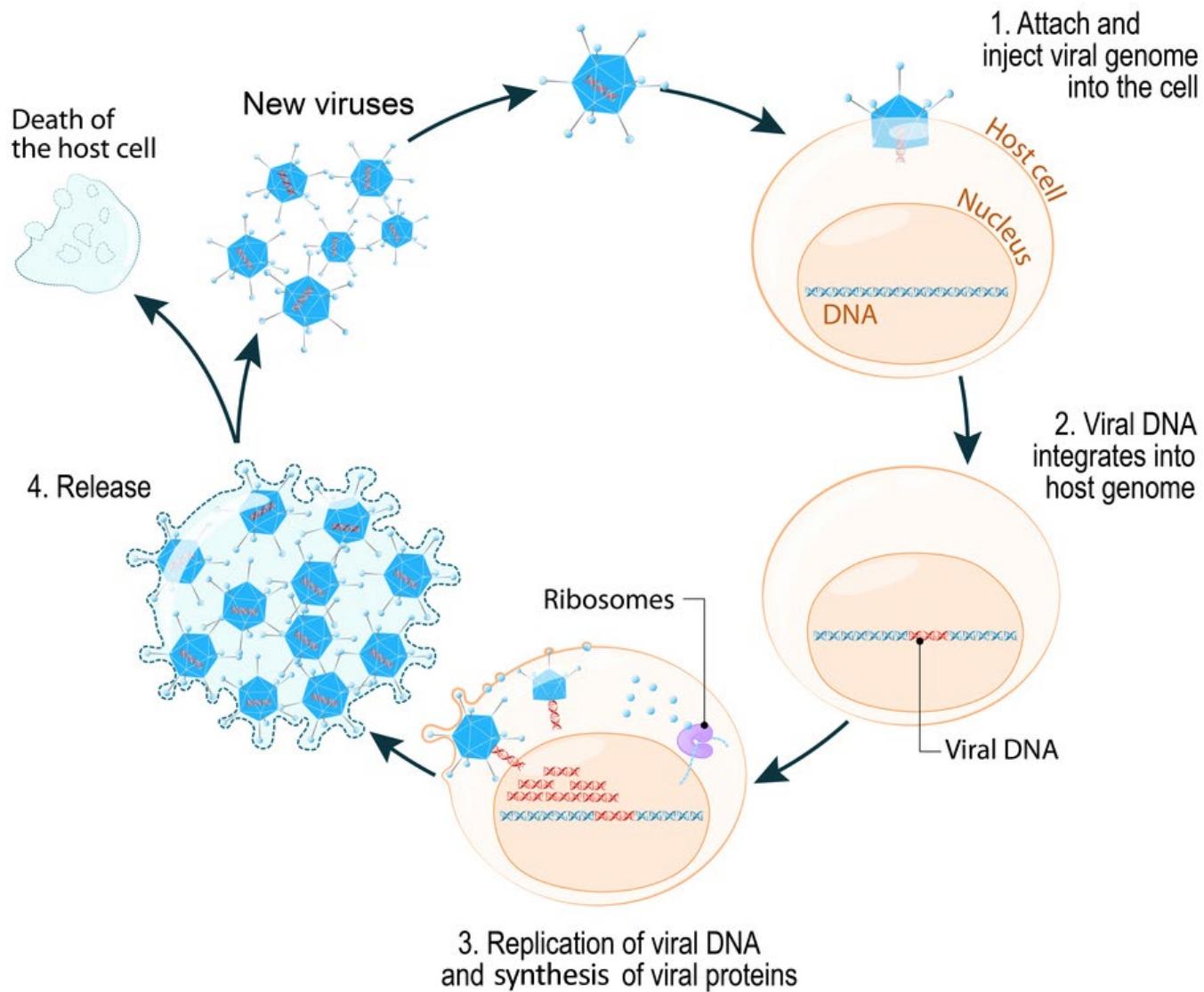
# Viruses: Intermission



# Using Viruses: things you need to know

- Viruses are chosen as “backbones” for their tropism and toxicity
- Viruses enter cells through a receptor-mediated process which will affect tropism of specific types of viruses/serotypes of a virus (AAV)
- Virulence factors are typically removed from the backbone

# LIFE CYCLE OF VIRUSES

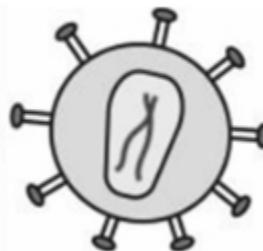


# Viruses used in neuroscience research

- Adeno-associated viruses
- Lentivirus
- Retrovirus
- Herpes-simplex virus
- Pseudorabies\*
- Pseudo-typed rabies\*
- Canine adenovirus

# Pros and cons

	HSV	AAV	Retro	Lenti
Genomic integration	Episomal (100%)	Episomal (90%)	Integrated	Integrated
Cloning capacity	150 kb (40 kb)	3.5-4 kb	7-8 kb	7-8 kb
Tropism	Broad	Broad	Dividing cells	Broad
Expression duration	Days (months)	2.5-6 months	Days-months	Years
Expression onset	12 hours	7-10 days		
Disadvantages	Cytotoxicity	Low payload	Mutagenic	Mutagenic
Biosafety Level	2	1	2	2



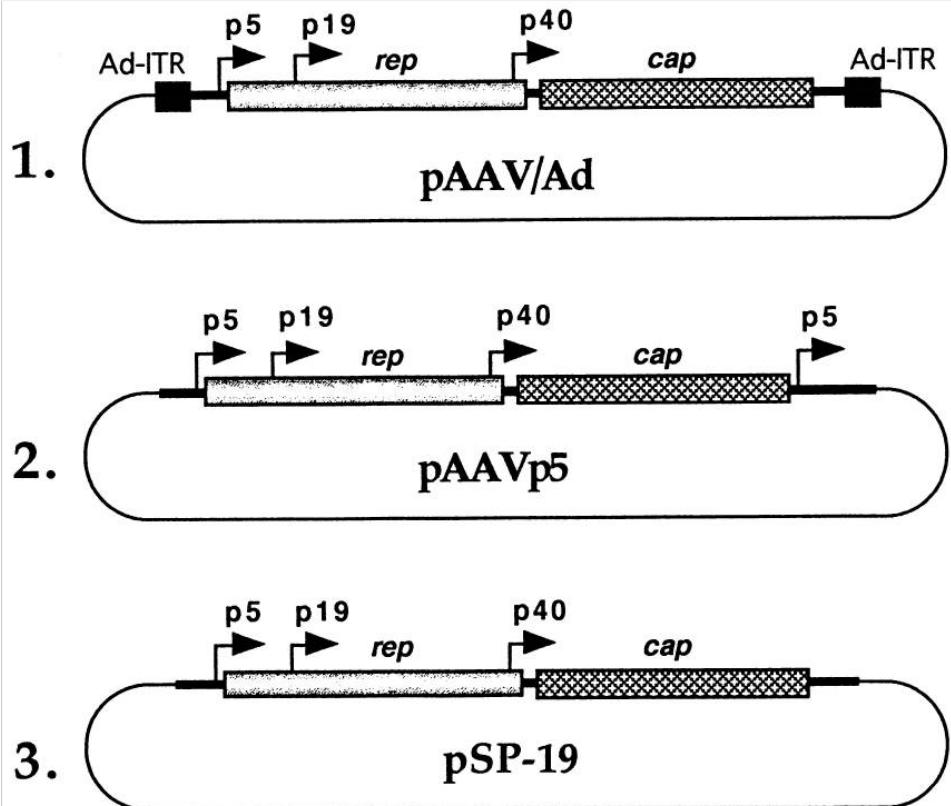
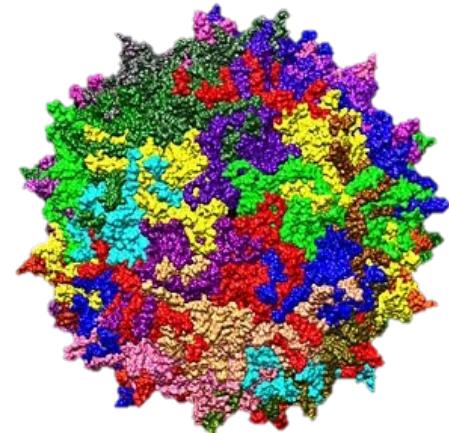
Adapted from Neve, Curr. Prot. In Neurosci., 2012.

# How big is your payload?

- **Your gene of interest (depends)**
  - ChR2 (1662 bp)
  - GCaMP (2160 bp)
  - Cre recombinase (1029 bp)
- **Reporter/tag**
  - Fluorescent (typical size: ~700-1400)
  - HA tag (typical size: 27 bp)
- **Your desired promoter**
  - Broadly active (CAG: 278 bp)
  - Specific (hSynapsin: 485 bp; GFAP: 2207 bp)
- **Control and enhancer elements**
  - P2A (18-22 bp)
  - IRES internal ribosomal entry site (500 bp)
  - WPRE woodchuck hepatitis virus posttranscriptional regulatory element (588 bp)
  - hGH-polyA human growth hormone eukaryotic termination signal (479 bp)
  - loxP (34 bp each)



# Wild type AAV



- ORF1 = Rep: contains the replication elements
- ORF2 = Cap: contains capsid protein sequences
- ITR: inverted terminal repeat
- Total capacity: 4.8 kb
- Single stranded but uses DNA polymerase in host cell to make complementary strand
- Prefers to incorporate into human Ch 19 at the AAVS1 site which contains a Rep binding element
- Integration into host genome requires one of the following: Co-infection with Adenovirus or HSV, or genotoxicity including UV radiation or hydroxyurea

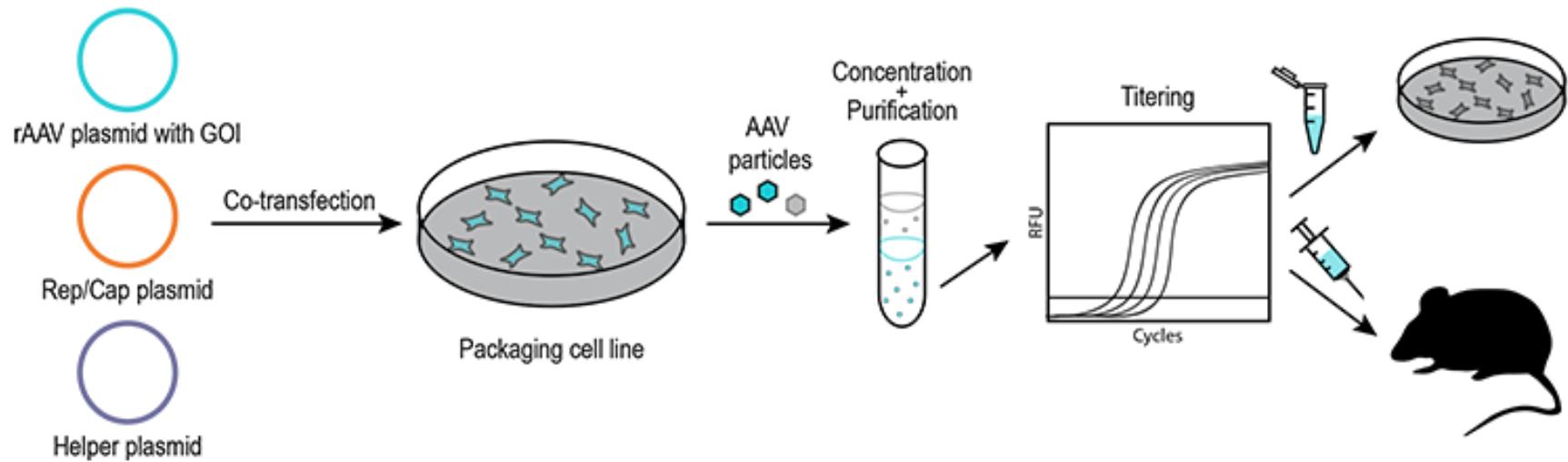
Wang, J. Virol., 1998.

# Why were AAVs developed for research purposes?

- **Non-pathogenic\***: no disease, low or no immune response
- Non-replicative



# Pseudotyping AAVs



\*ITRs are the only element that must be supplies in cis

# AAV variant: scAAV

- Self-complementary AAV
- Disadvantage: cuts the payload capacity in half (4.8 kb down to 2.4 kb)

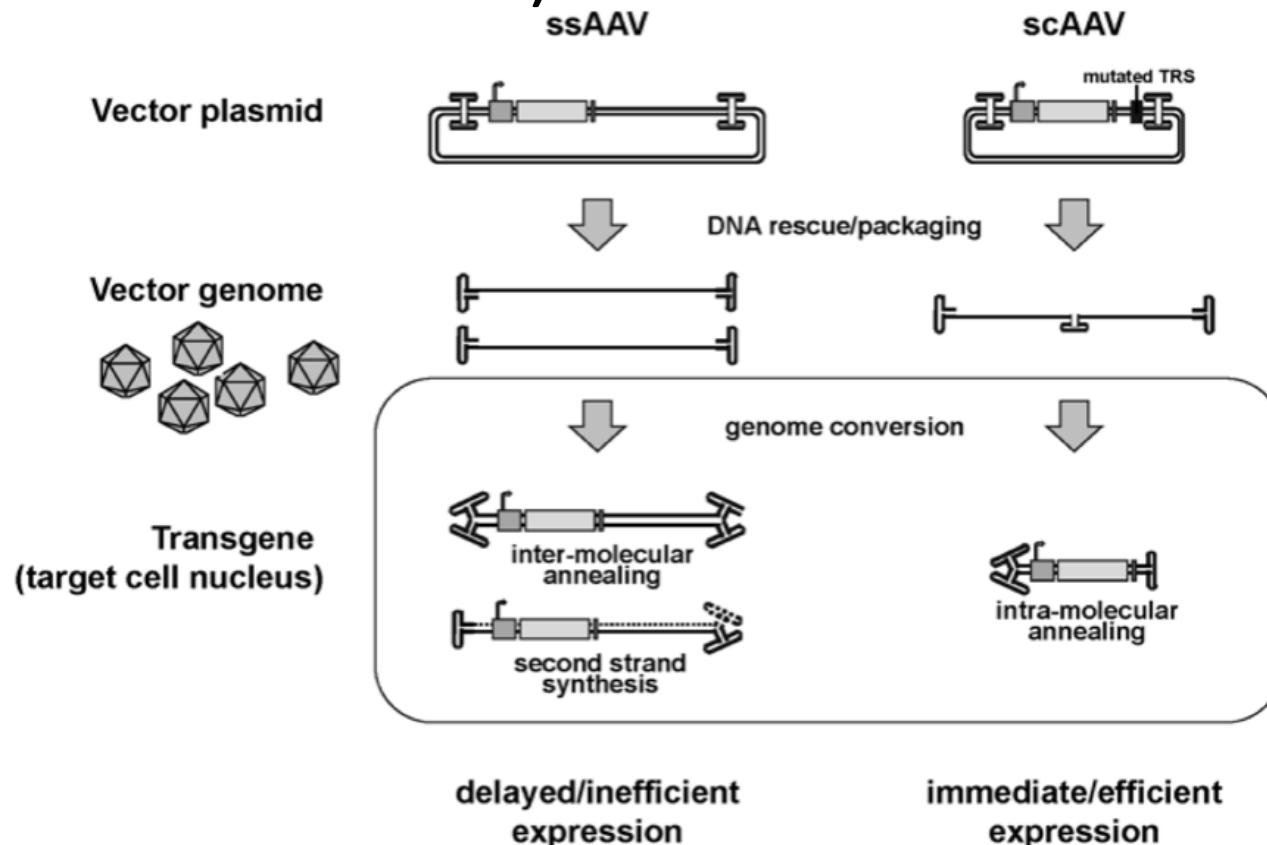


Figure 1. Comparison of ssAAV and scAAV genomes, packaging and transduction. From Takashi Okada (2013). Efficient AAV Vector Production System: Towards Gene Therapy For Duchenne Muscular Dystrophy, Gene Therapy - Tools and Potential Applications, Dr. Francisco Martin (Ed.), InTech, DOI: 10.5772/53023.

# AAV serotype tropism

Tissue	Optimal Serotype
CNS	AAV1, AAV2, AAV4, AAV5, AAV8, AAV9
Heart	AAV1, AAV8, AAV9
Kidney	AAV2
Liver	AAV7, AAV8, AAV9
Lung	AAV4, AAV5, AAV6, AAV9
Pancreas	AAV8
Photoreceptor Cells	AAV2, AAV5, AAV8
RPE (Retinal Pigment Epithelium)	AAV1, AAV2, AAV4, AAV5, AAV8
Skeletal Muscle	AAV1, AAV6, AAV7, AAV8, AAV9

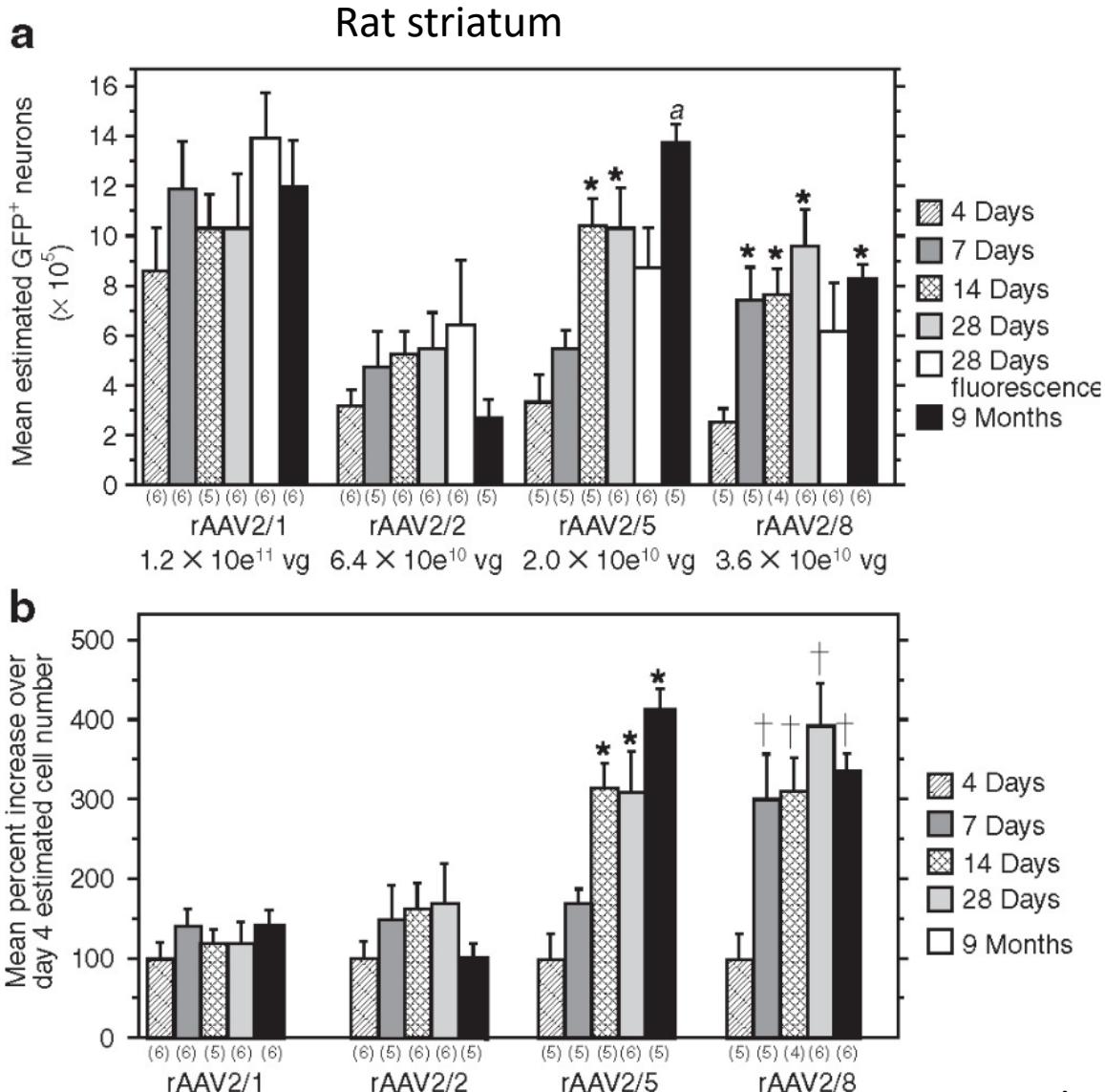
AAV-DJ

AAV-DJ8

**AAV-retro**

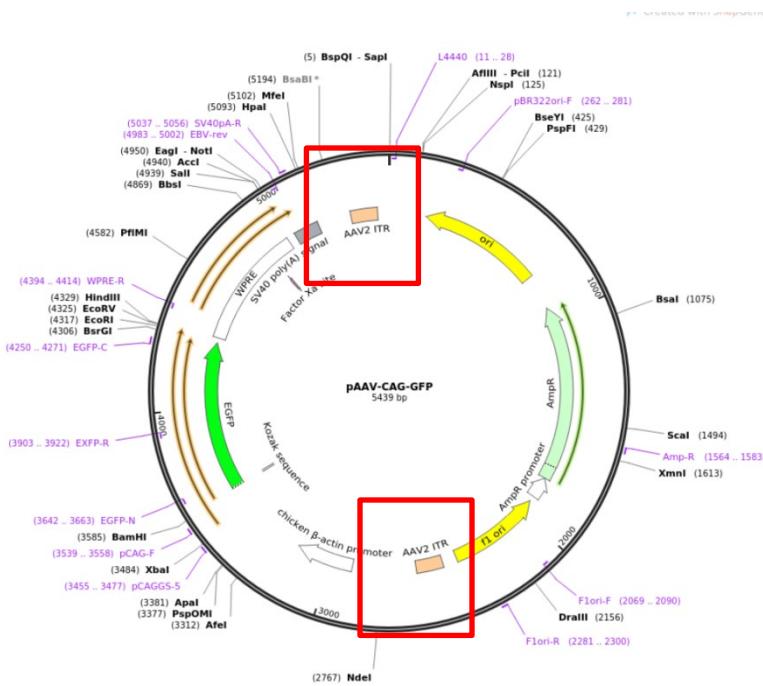
**AAV-PHP.eB**

# AAV serotype expression



# Serotypes used in Neuroscience

- Most all of the Addgene.org viruses are hybrids: all use the genomic backbone of AAV2 but borrow capsid proteins from other AAVs...hence the nomenclature AAV2/5 or AAV2.5



rAAV Serotype Family Properties

Serotype	Transport Direction		Expression by Brain Region			Cell-Type Expression		
	Anterograde	Retrograde	Striatum	Hippocampus	Cortex	Neurons	Astrocytes	Microglia
rAAV1	+	+	*	*	*	***	*	**
rAAV2	+	+	*	*	*	*	*	
rAAV5	+		***	***	*	**	*	
rAAV6	-	+	*	*	**	**		
rAAV7	+	+	**	**	***	**	**	**
rAAV9	+	+	**	*	***	***		

The number of \* represent relative rAAV expression levels by brain region or cell-type. \* represents the lowest level of expression, \*\* represents general expression levels, and \*\*\* represents highest expression levels in comparison to other rAAV serotypes.

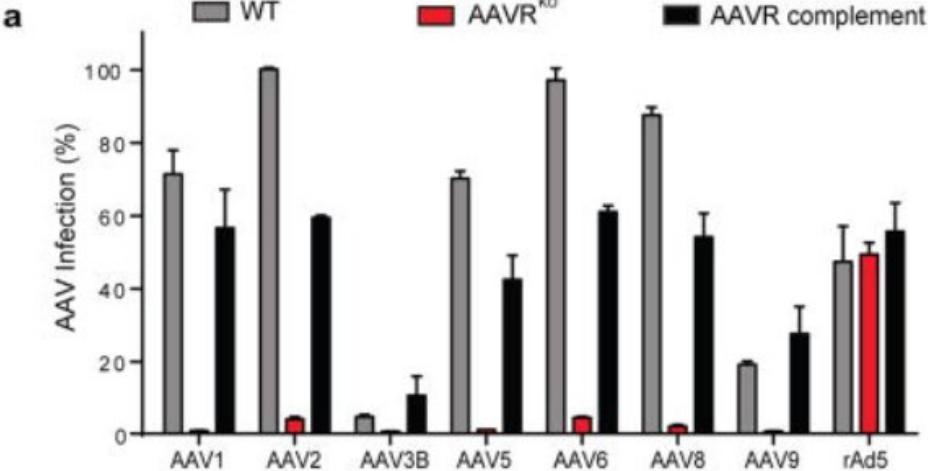
This table was developed with information based on the following citations: [161](#), [162](#), [163](#), [164](#), [165](#), [166](#).

# AAV Receptors as of 2006

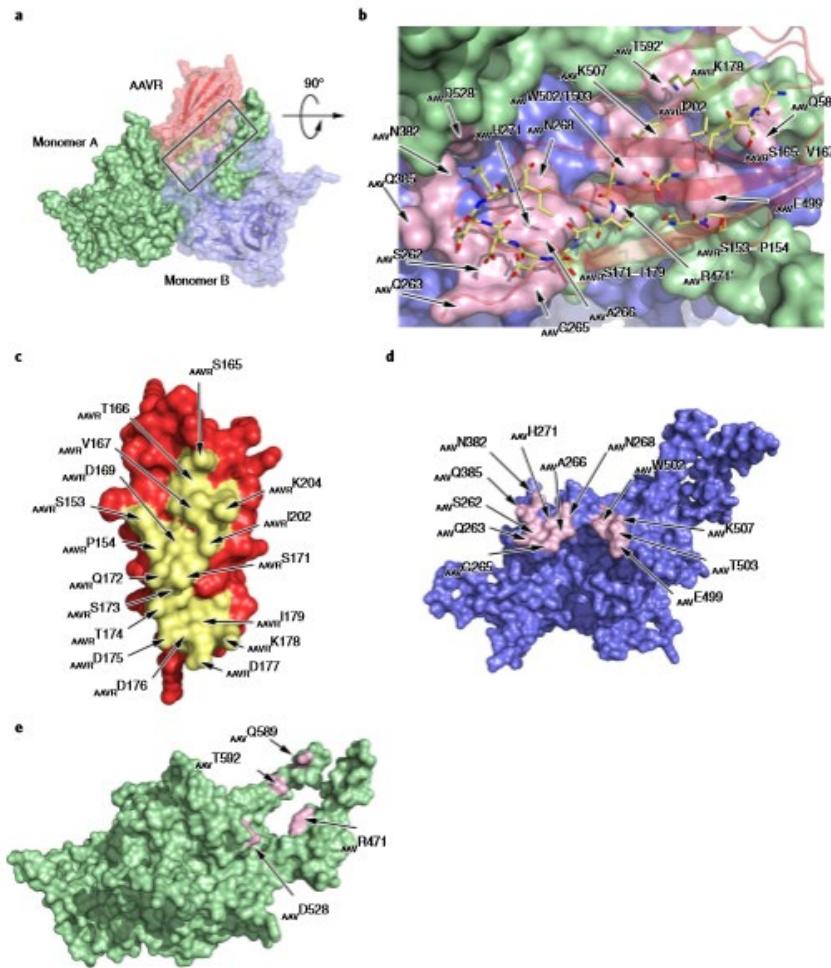
<b><i>Serotype</i></b>	<b><i>Attachment receptor</i></b>	<b><i>Coreceptor</i></b>
AAV1, 6, 7, 8	Unknown	Unknown
AAV2	HSPG <sup>39</sup>	$\alpha 5\beta 1$ integrin; <sup>50</sup> hFGFR1 <sup>53</sup>
AAV3	HSPG <sup>18, 43</sup>	Unknown
AAV4	O-linked sialic acid <sup>45</sup>	Unknown
AAV5	N-linked sialic acid <sup>45, 46</sup>	PDGFR <sup>54</sup>

Ding, Gene Therapy, 2005.

# AAV Receptor (AAVR) identified



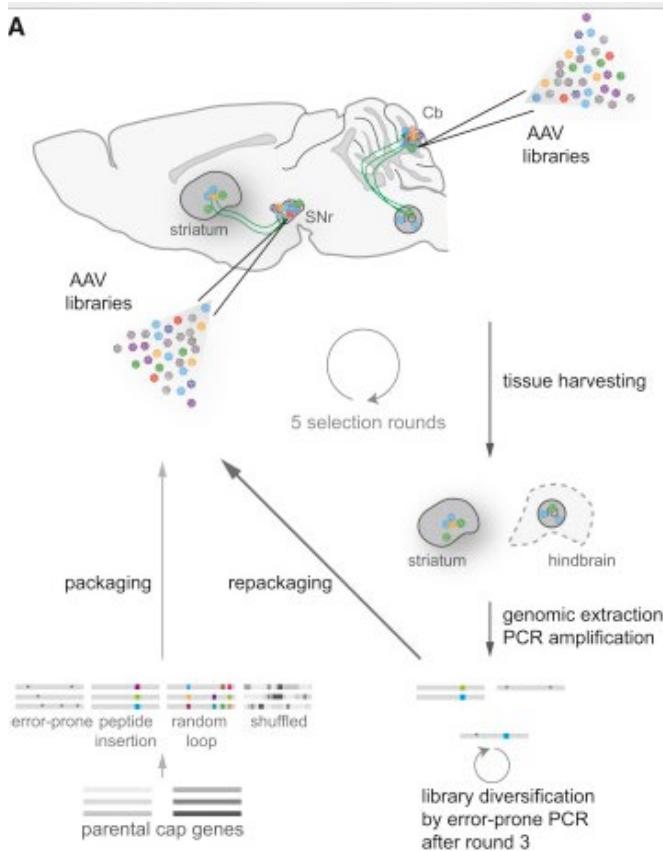
Pillay, Nature, 2016.



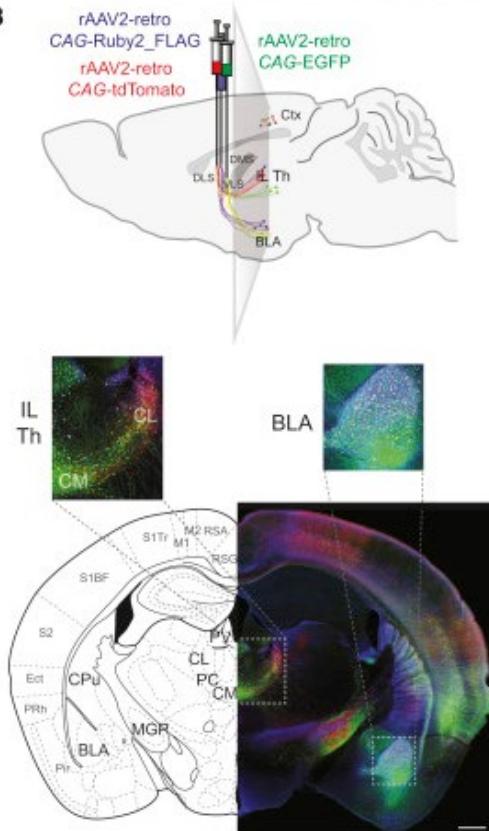
Zhang, Nat. Microbiol., 2019.

# AAV retro

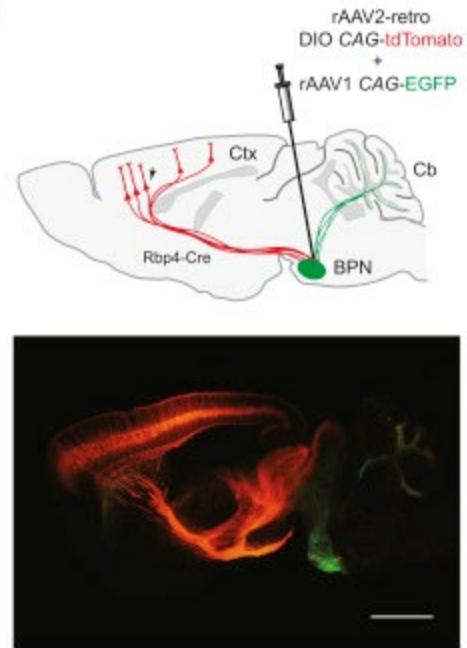
A



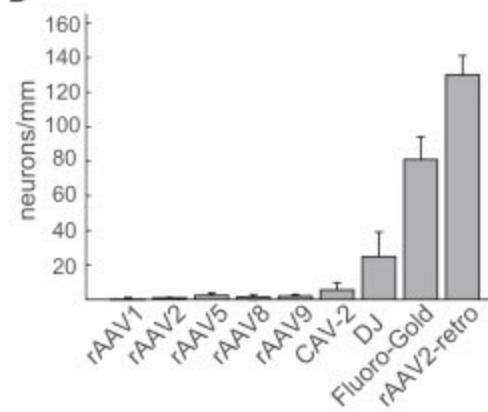
B



A



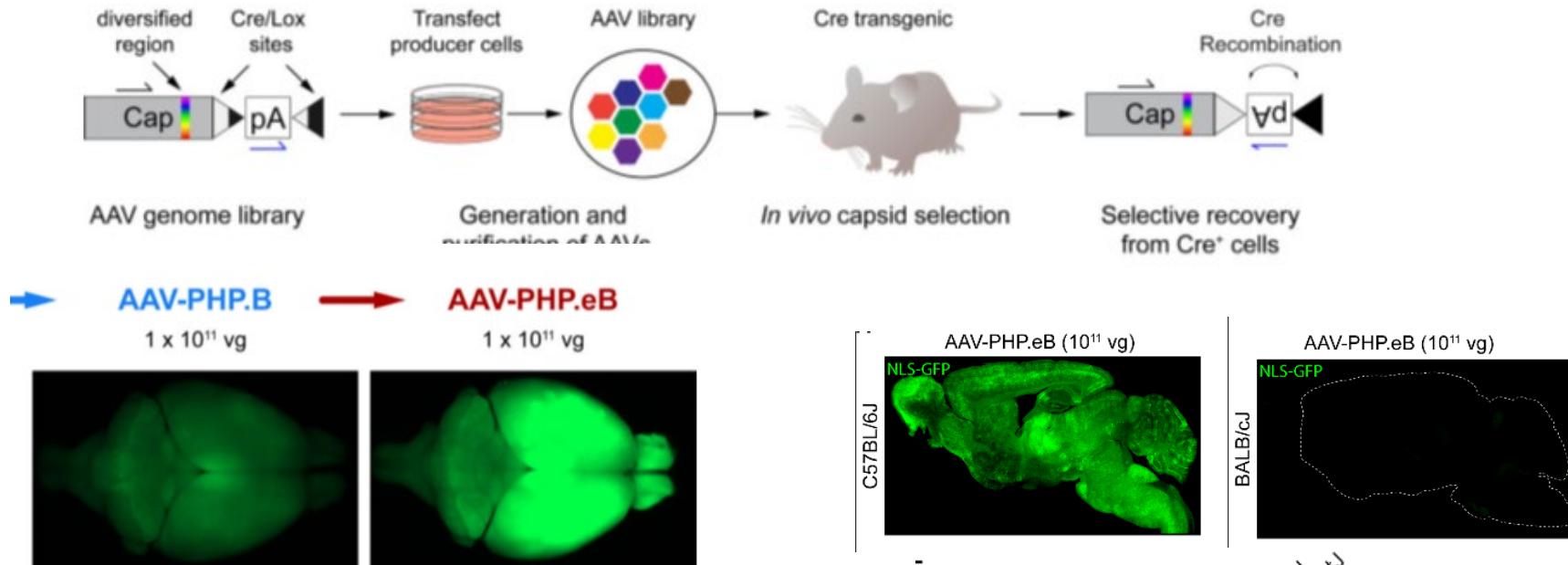
D



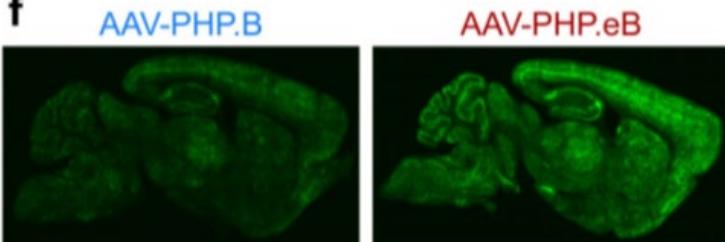
# AAV PHP.eB

**a**

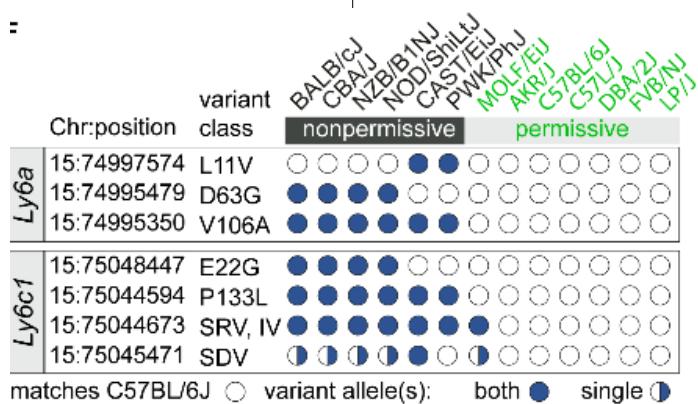
Cre-dependent recovery of AAV capsid sequences



**f**

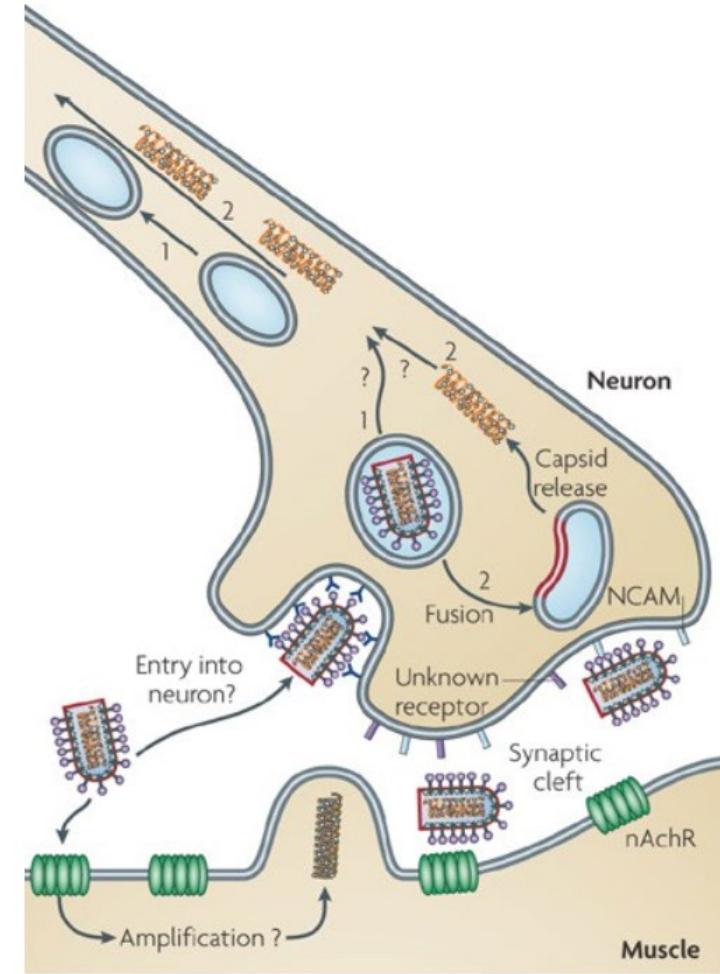
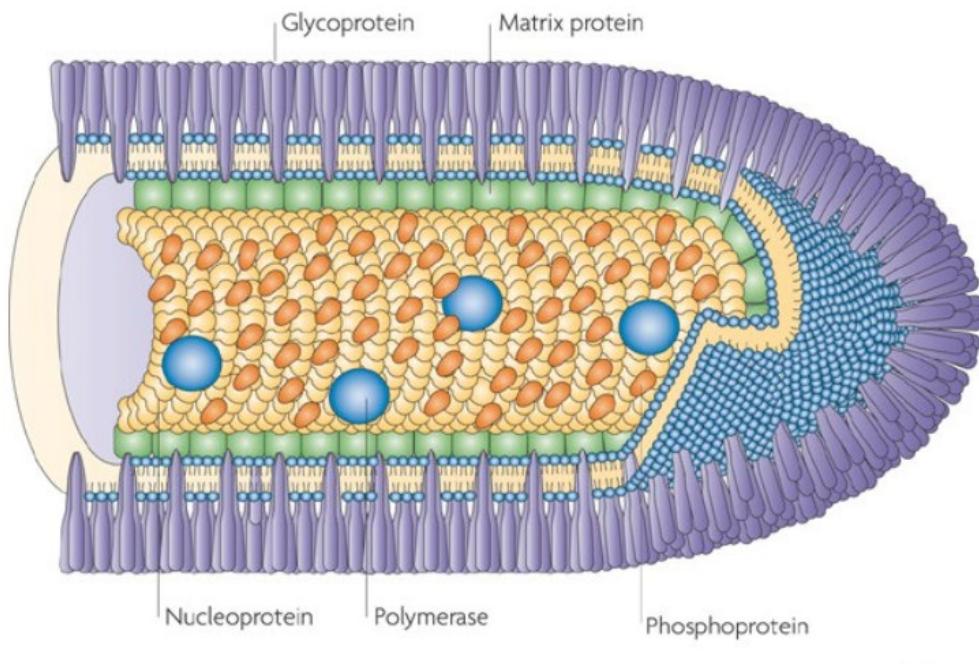


Chan, Nat. Neurosci., 2017.



Huang, PLoS One, 2019.

# Rabies virus (RV)

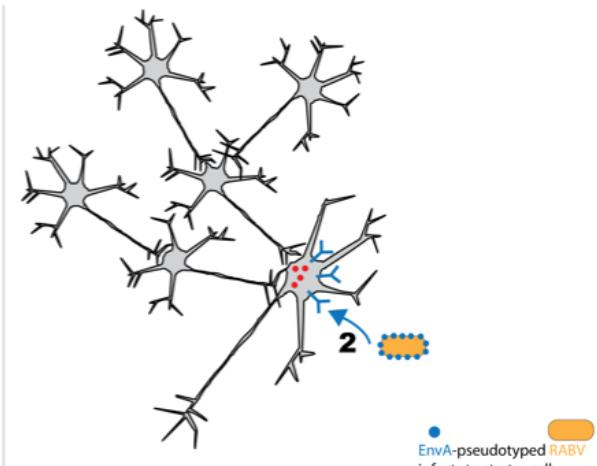
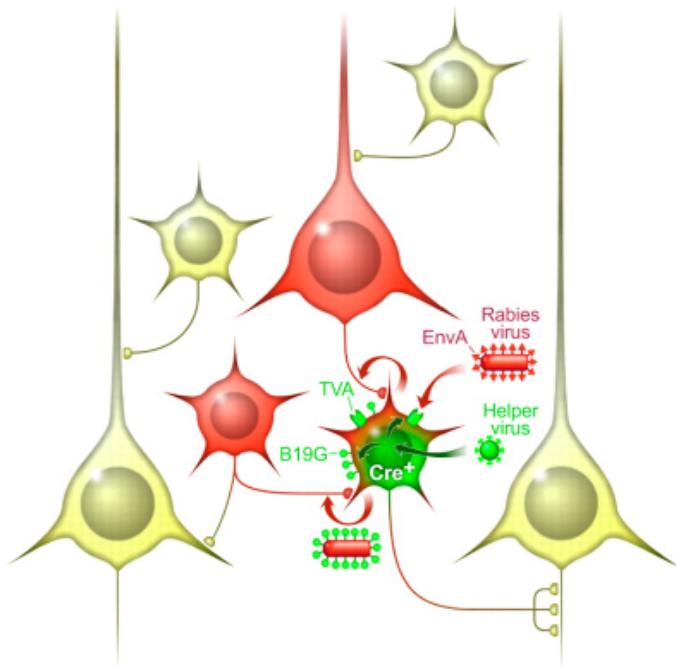


- Envelope
- Glycoprotein
- Genetic material

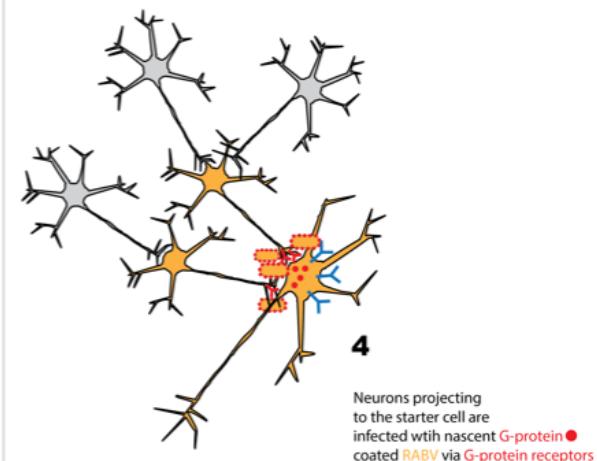
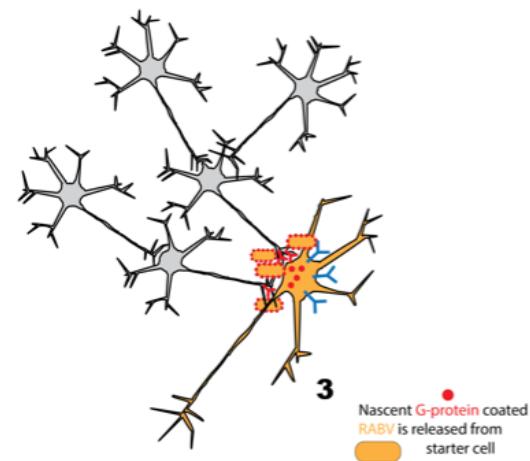
Schnell, Nat. Rev. Micro., 2010.

# Pseudo-typed Rabies

A

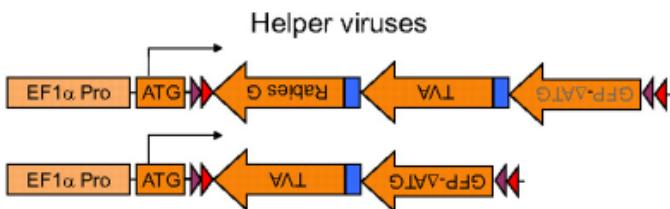


EnvA-pseudotyped RABV  
infects to starter cell

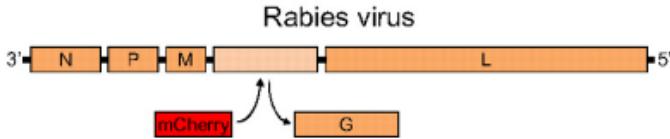


Neurons projecting  
to the starter cell are  
infected wtih nascent G-protein  
coated RABV via G-protein receptors

B

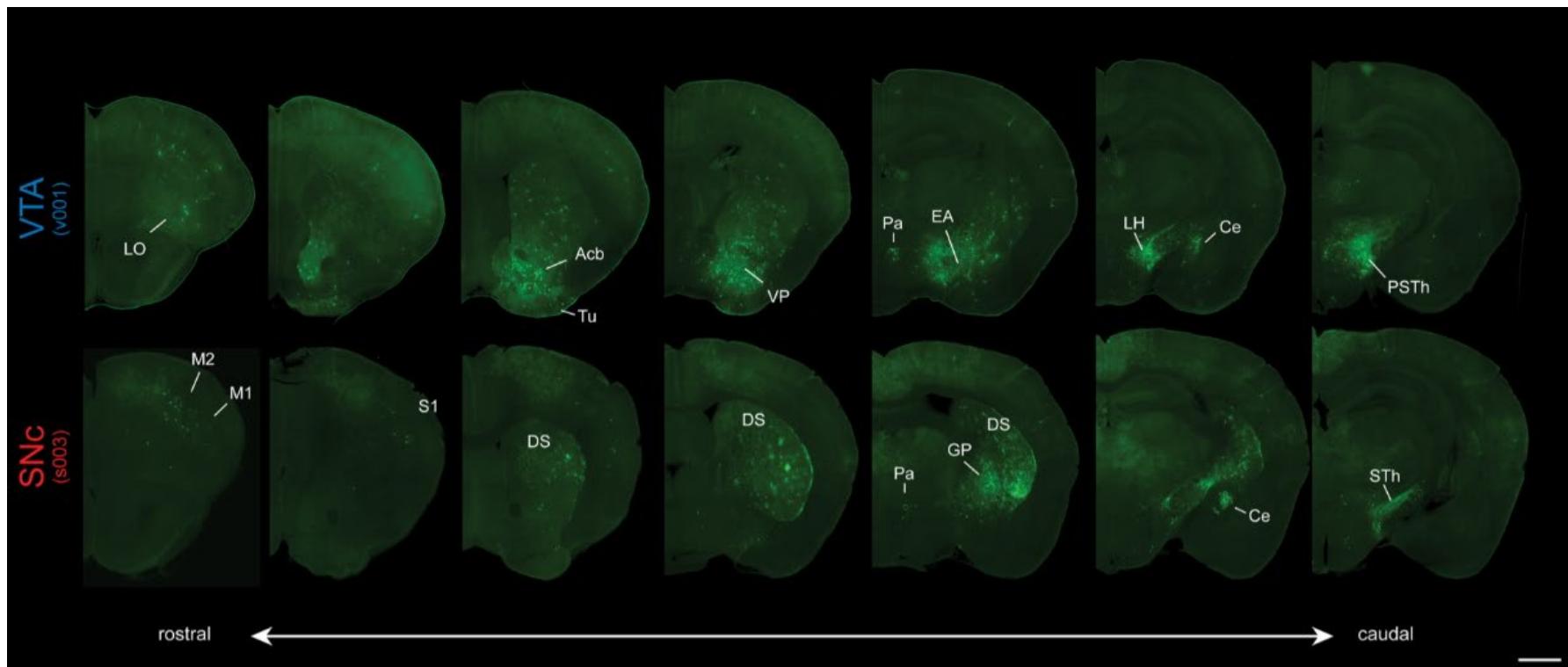
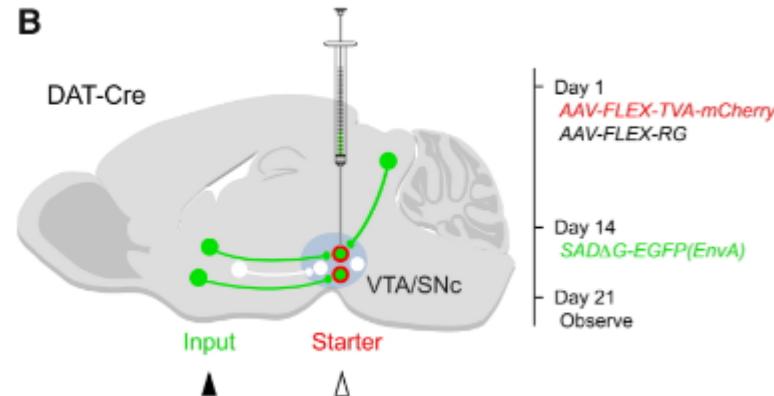


C



# Pseudo-typed Rabies in practice

B

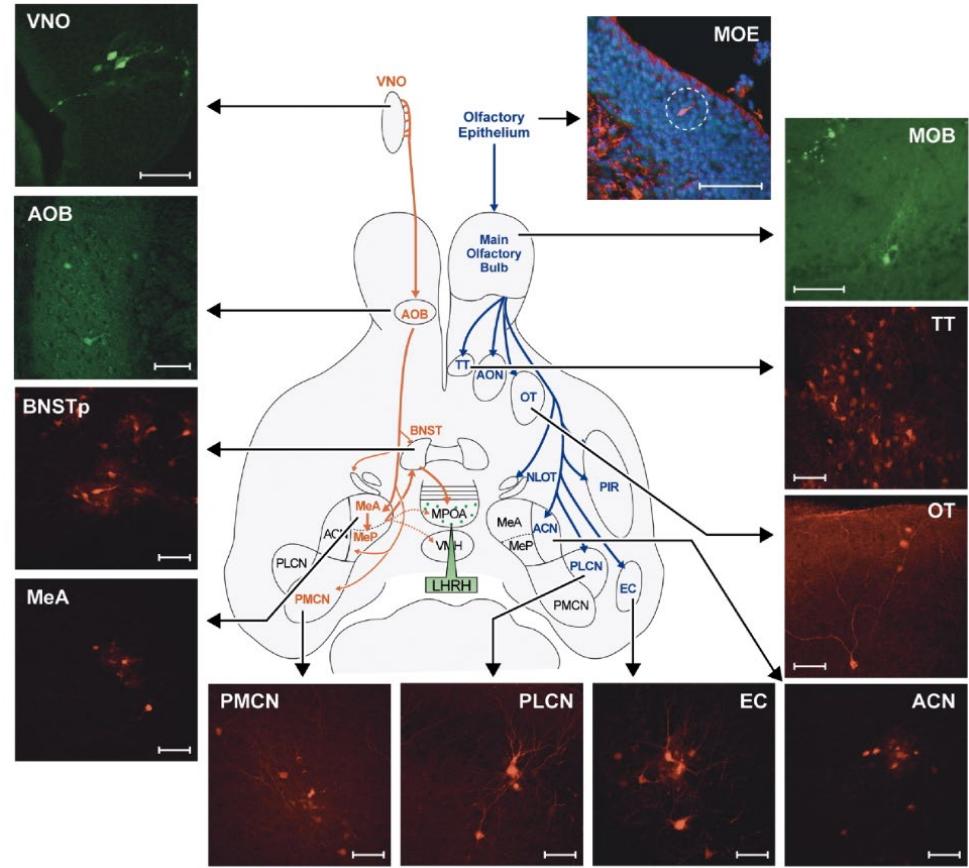
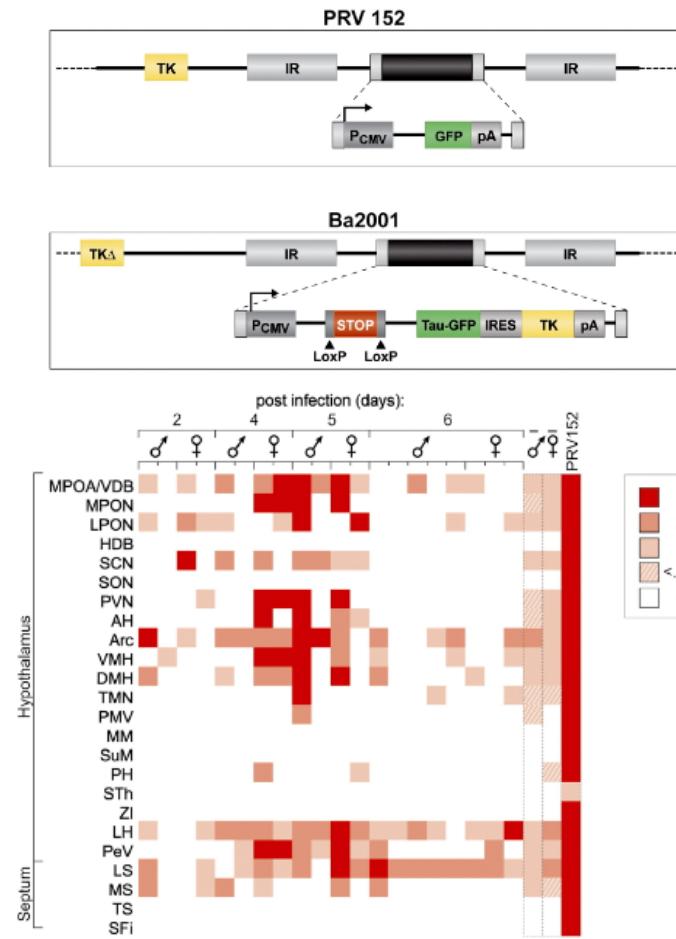


# Pseudorabies (PRV)

## Pseudorabies

From Wikipedia, the free encyclopedia

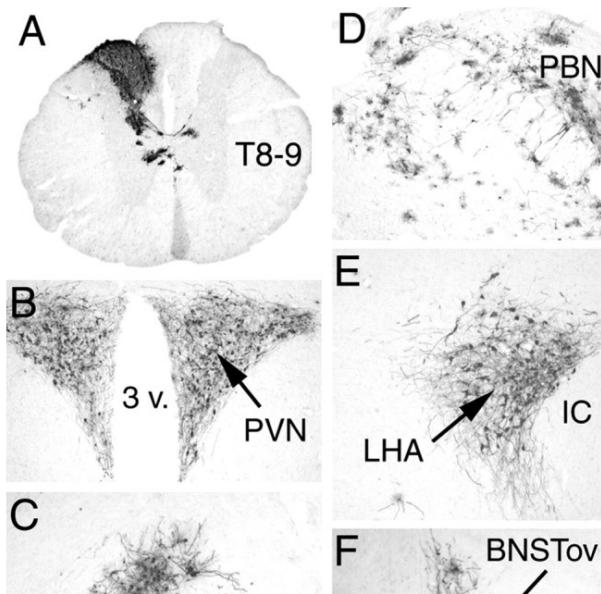
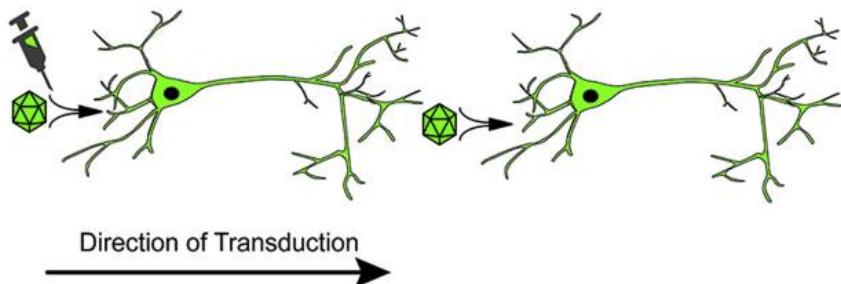
*Not to be confused with rabies or rabies virus.*



Yoon et al., Cell, 2005.

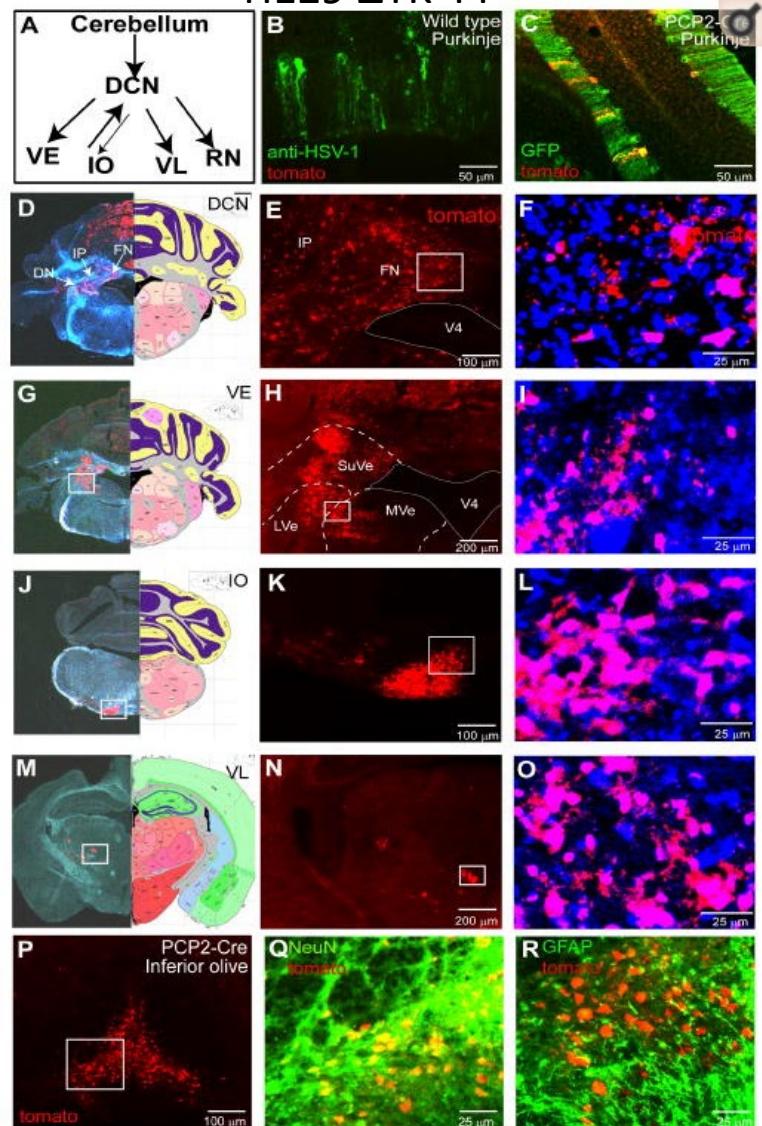
# HSV-H129

## Trans-synaptic Transport



Rinaman and Schwartz, J. Neurosci, 2004.

## H129 ΔTK-TT



Lo et al., Neuron, 2011.

# Viral repositories

- Addgene (has all UPenn's viruses)



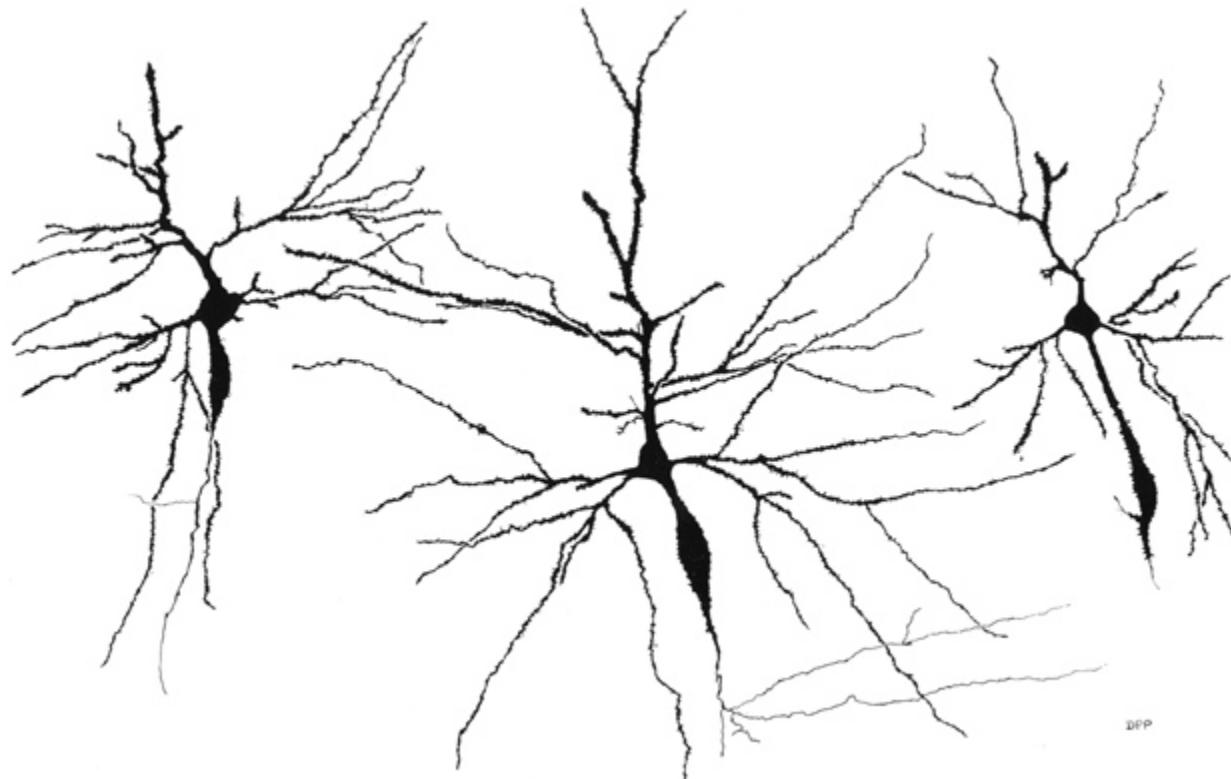
- UNC vector Core



- Salk Viral Vector Core



# Anatomical tracing techniques: addendum

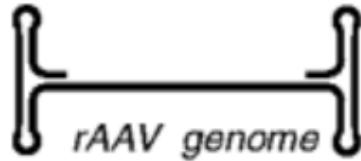


Lecture 8

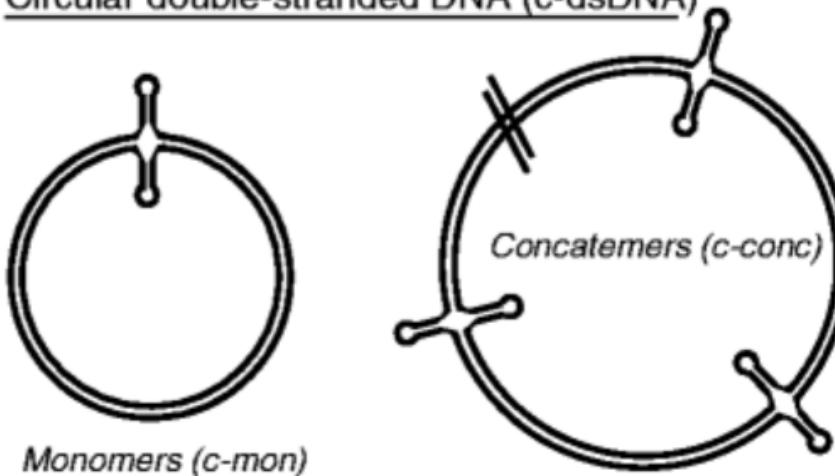
Anita Autry, Ph.D.

# AAV episomal monomers(concatemers

Single-stranded DNA (ssDNA)



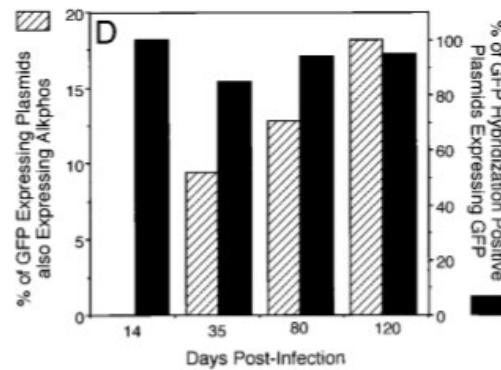
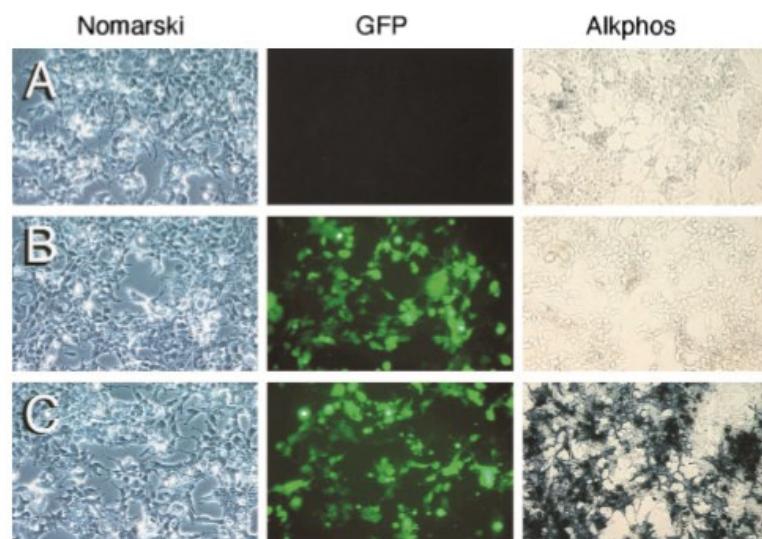
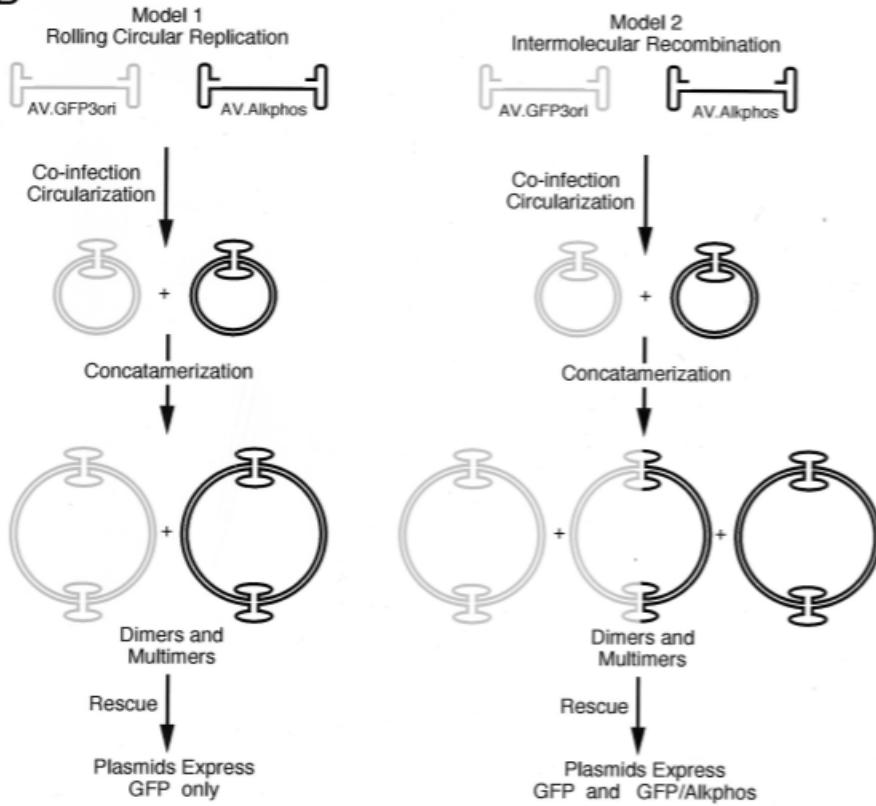
Circular double-stranded DNA (c-dsDNA)



*Adeno-associated virus p. 239-258 (2011)*

# Hybrid concatamerization

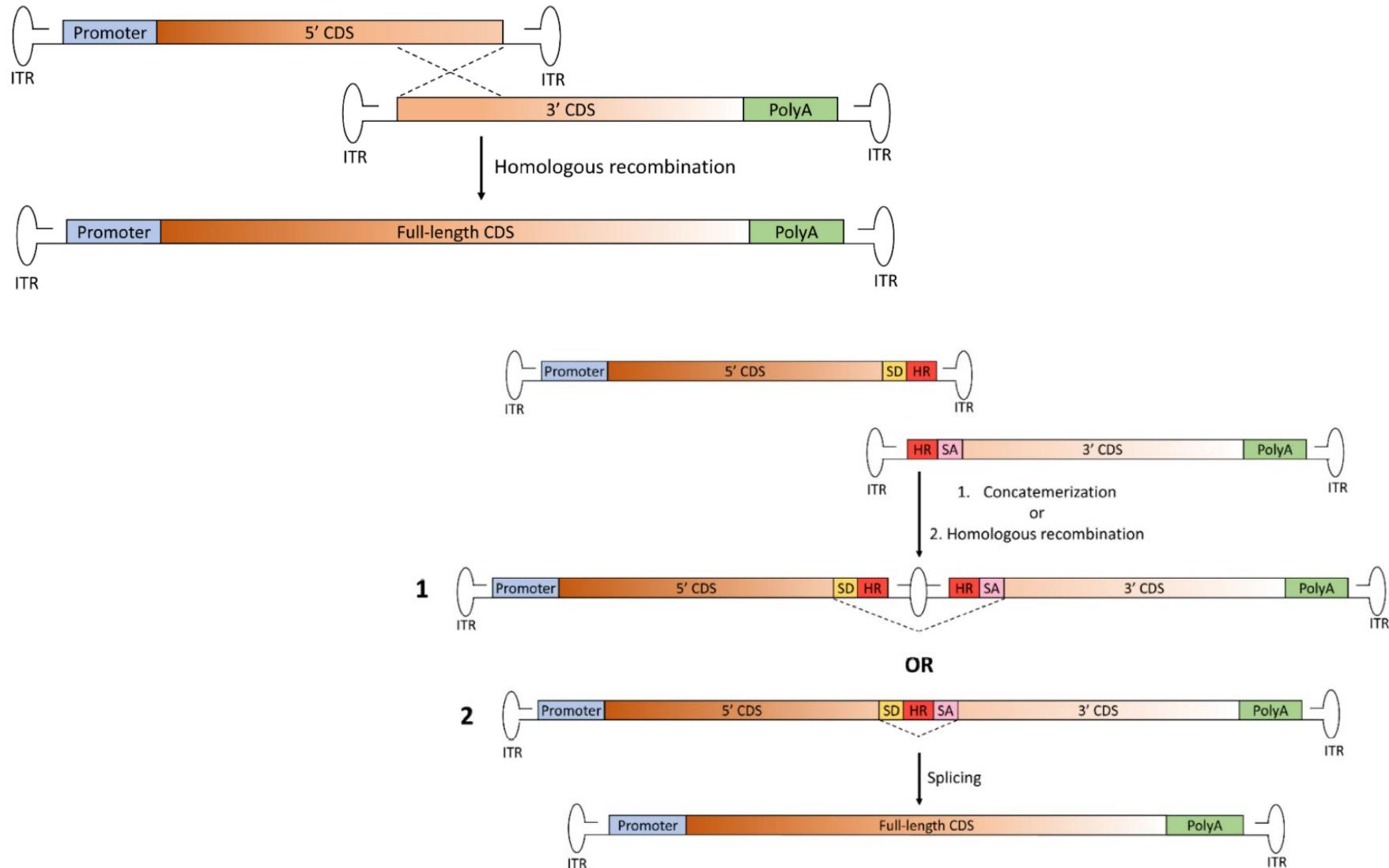
B



Yang et al., J. Virol., 1999.

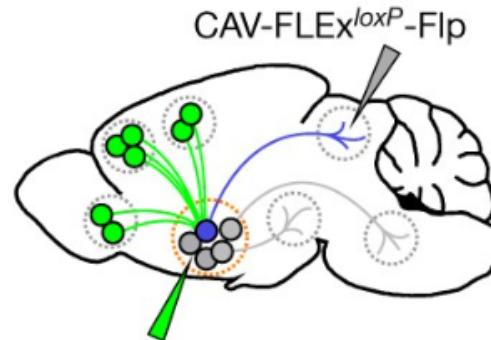
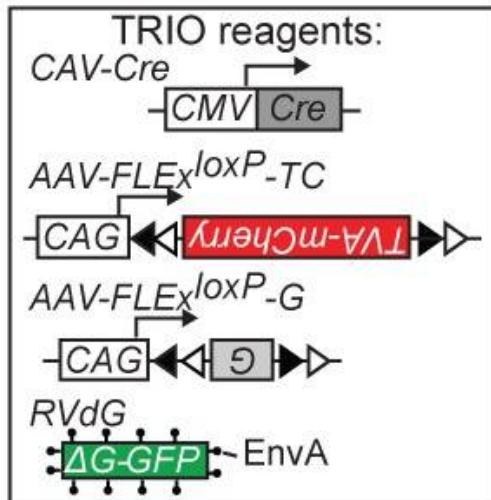
Concatemerization through ITR (or free DNA ends see Nakai et al., Mol. Ther., 2003.)

# Multi-AAV strategies

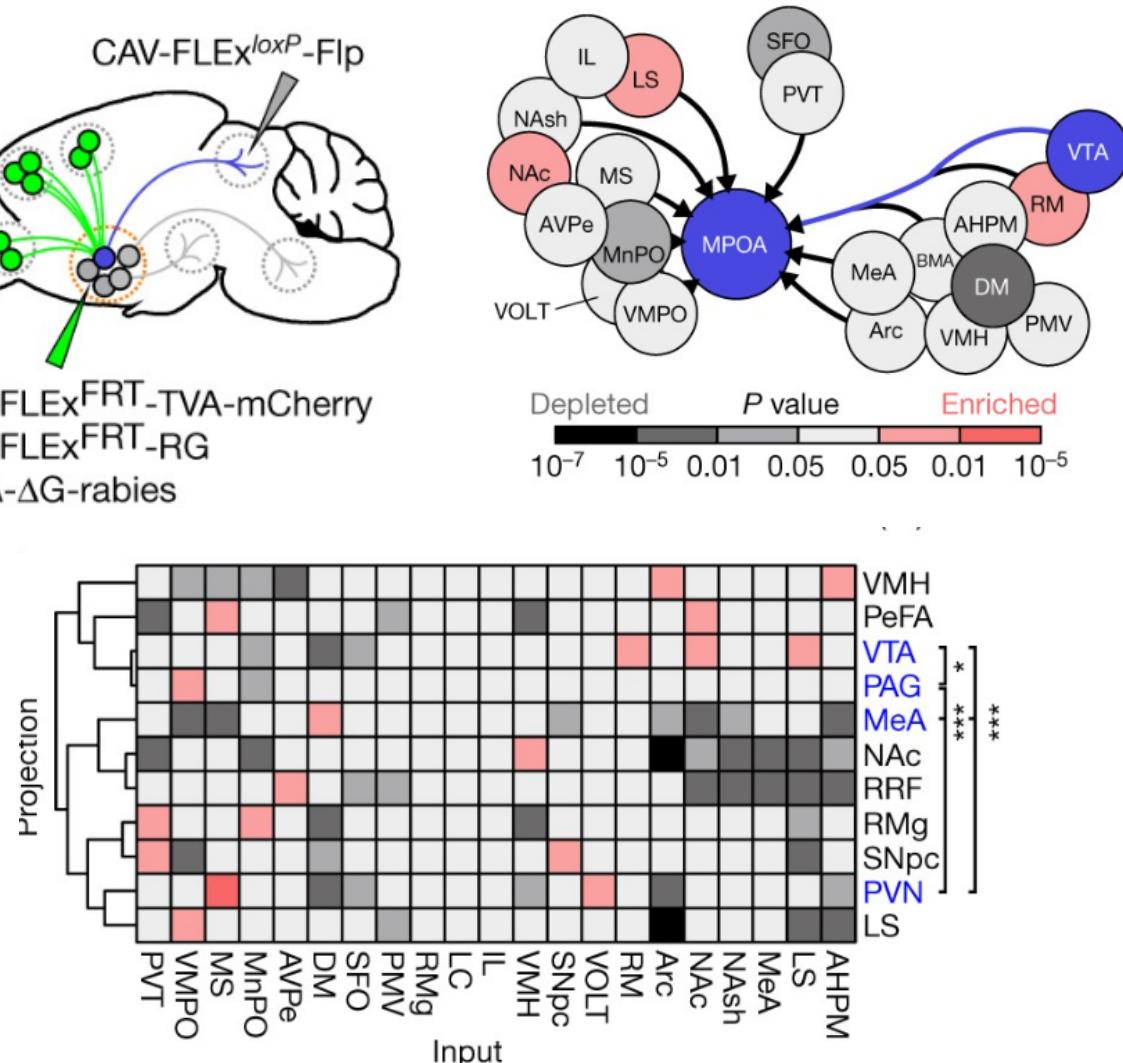
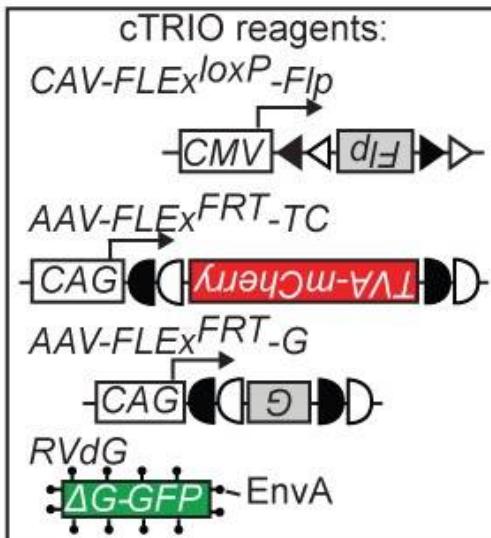


# TRIO/cTRIO

Cell-type specific {Tracing Relationship of Input and Output}



AAV-FLEX<sup>FRT</sup>-TVA-mCherry  
 AAV-FLEX<sup>FRT</sup>-RG  
 EnvA-ΔG-rabies



Schwarz et al., Nature, 2015.

Kohl et al., Nature, 2018.