

## How to use Tetbow

An overview of Tetbow plasmids for stochastic multicolor labeling.

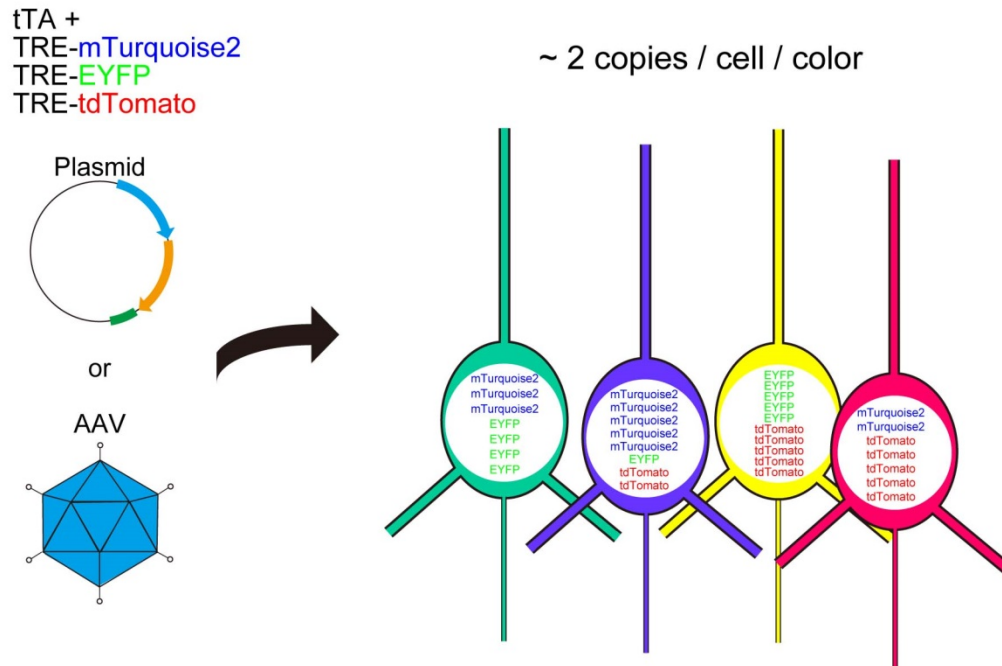


Figure 1 | Vector-mediated stochastic multicolor labeling.

### In utero electroporation of Tetbow plasmids (fluorescent proteins) (Figure 2A)

1. Introduce four plasmids (Addgene #104102 - #104105) into the brain using *in utero* electroporation. Optimum concentrations of vectors were 0.1  $\mu\text{g}/\mu\text{L}$  for pCAG-tTA (#104102) and 0.25  $\mu\text{g}/\mu\text{L}$  each for pBS-TRE-XFP-WPRE (#104103-104105) in the mouse cerebral cortex.
2. Sacrifice animals at your preferred time point and fix the samples with 4% paraformaldehyde in PBS. Section the brain (0.2-1 mm thick slices) with a vibratome.
3. Clear the brain slices with SeeDB2 (Ke & Imai, 2018).

### Tetbow AAVs (fluorescent proteins)

1. Prepare four AAVs from Addgene #104109-104112. Each AAV should be prepared separately. See an Addgene blogpost for more details: <https://blog.addgene.org/tips-for-a-1st-time-aav-user-by-a-rookie-aav-user>
2. Inject of AAVs to your preferred brain area.  
Optimum ranges of AAV titers:  $3 \times 10^7 \sim 4 \times 10^8$  gc/mL (pAAV-SynI-tTA)  
 $1 \times 10^{10} \sim 1 \times 10^{11}$  gc/mL (pAAV-TRE-XFP-WPRE).

Note: Optimum titer may be different for different cell types. We typically inject 100-1,000 nL of AAV solution. The optimum titer may be different for different injection volumes too.

- Sacrifice animals at the appropriate time and fix samples.  
Note: The timing of the sacrifice is critical. Typically, it takes a few weeks for the optimum expression of XFPs. After that, neurons may start to show morphological abnormalities due to the excessive amount of XFPs expressed. However, the optimum time is different for different cell types.
- Clear the brain tissues or slices with SeeDB2 (Figure 2B).

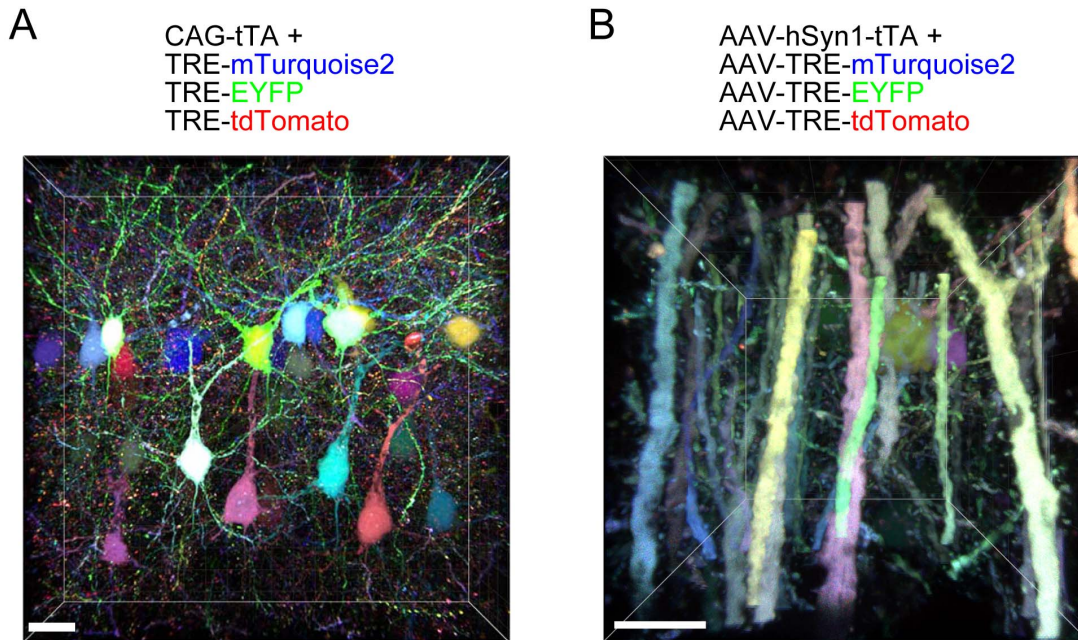
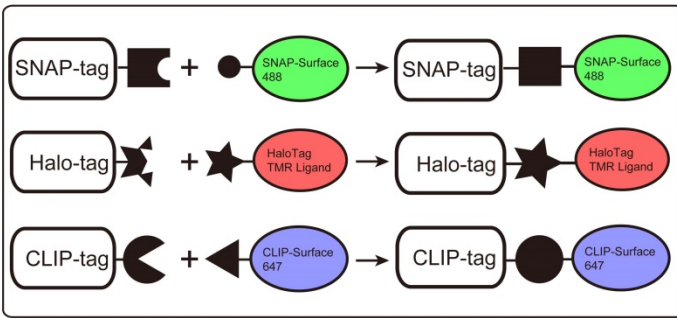


Figure 2 | (A) Multicolor labeling using Tetbow. Layer 2/3 cortical pyramidal neurons were labeled with Tetbow (*in utero* electroporation) (P70). (B) Layer 5 pyramidal neurons in the cerebral cortex were labeled with Tetbow AAVs. Scale bars are 20  $\mu\text{m}$  (A) and 10  $\mu\text{m}$  (B).

#### Tetbow with chemical tags

- Introduce four plasmids (Addgene #104102, #104106 - #104108) to the brain using *in utero* electroporation.
- Sacrifice the animals at your preferred timing and fix samples.
- Incubate the brain slices (up to  $\sim 1$  mm) in the chemical tag substrate solution for a minimum of several hours to overnight (Figure 3A, Kohl et al., 2014).
- Tissue clearing with SeeDB2 or other clearing methods (e.g., 3DISCO or TDE) (Figure 3B).

**A****B**

CAG-tTA +  
 TRE-SNAPf      SNAP-Surface 488  
 TRE-HaloTag +    HaloTag TMR Ligand  
 TRE-CLIPf      CLIP-Surface 647

3DISCO

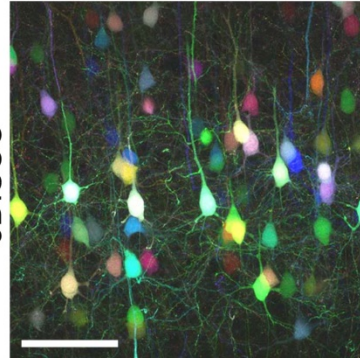


Figure 3 | (A) Chemical tags form covalent bonds with their substrates that have chemical fluorophores attached. Chemical tags were expressed using the Tetbow system and the brain slices were stained with the required substrate solution. (B) Layer 2/3 cortical pyramidal neurons labeled with SNAP, Halo, and CLIP tags were visualized with SNAP-Surface 488, HaloTag TMR Ligand, and CLIP-Surface 647, respectively (P21). The brain slice was cleared with 3DISCO (right). The scale bar represents 50  $\mu\text{m}$ .