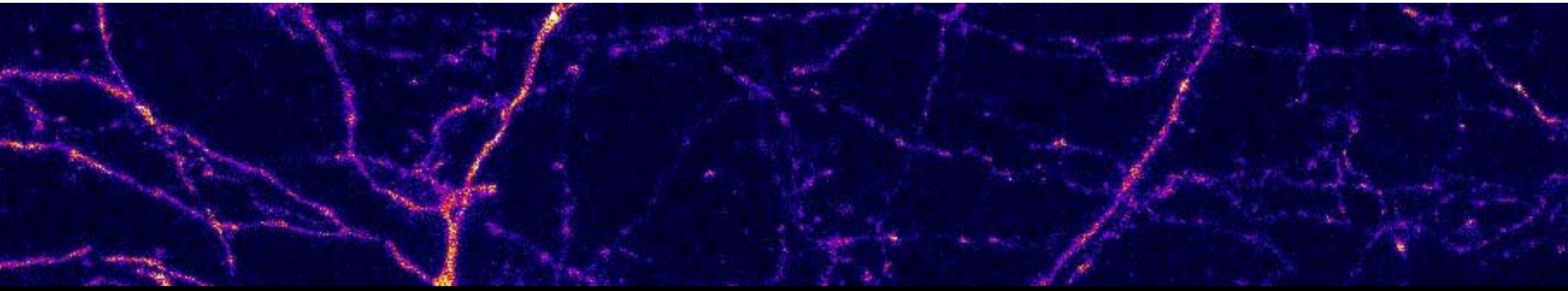
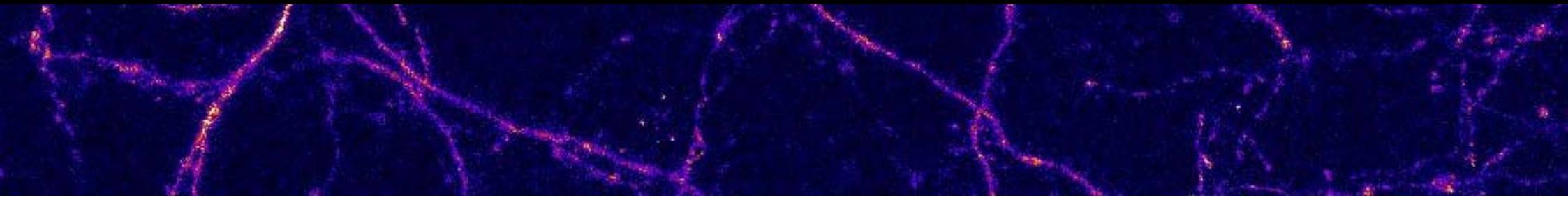


# Advanced methods in memory research

By Hana Brozka

2023

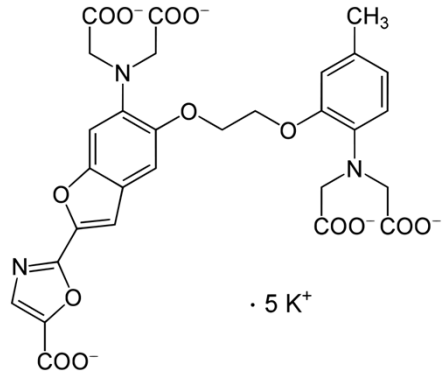


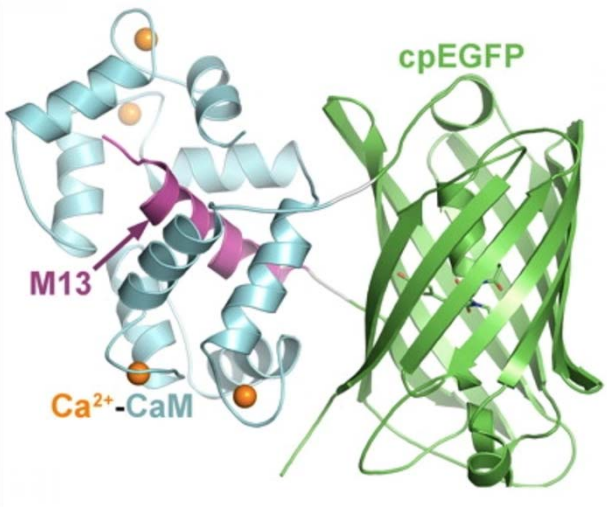
# Outline

- Visualization of neuronal activity - traditional methods
  - Calcium imaging
  - IEG immunostaining
  - catFISH
- Memory engram technlogies
  - TetTag
  - TRAP
  - CANE
  - CaMPARI
  - Cal-light
  - FLARE
  - FLiCRE
  - Expression recording island
  - Other methods

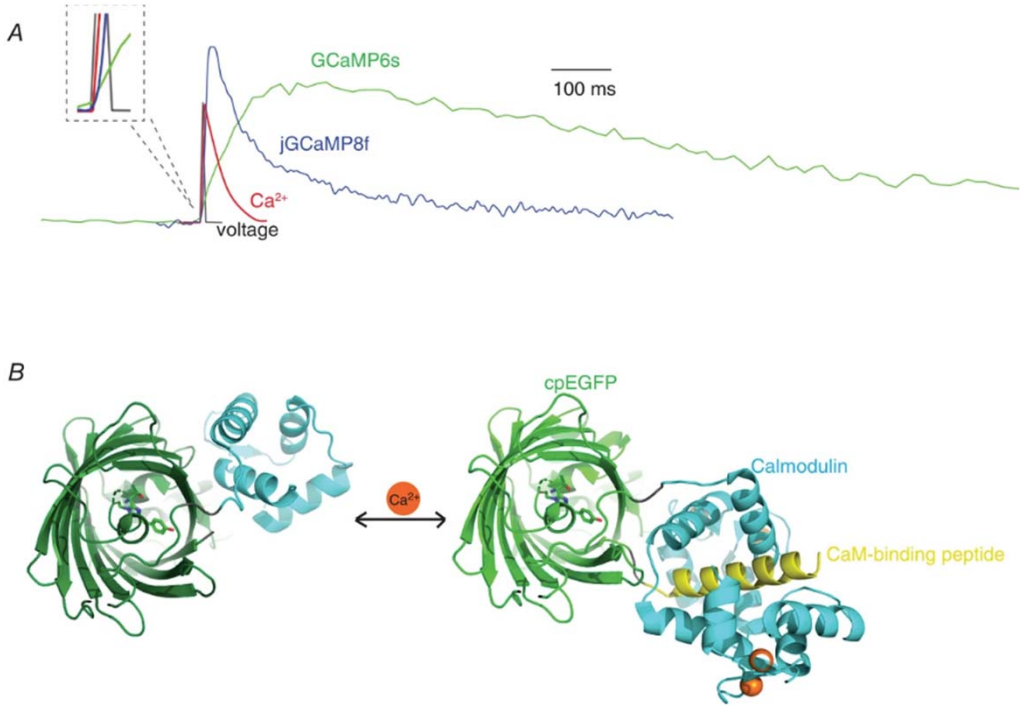
# Visualization of neuronal activity

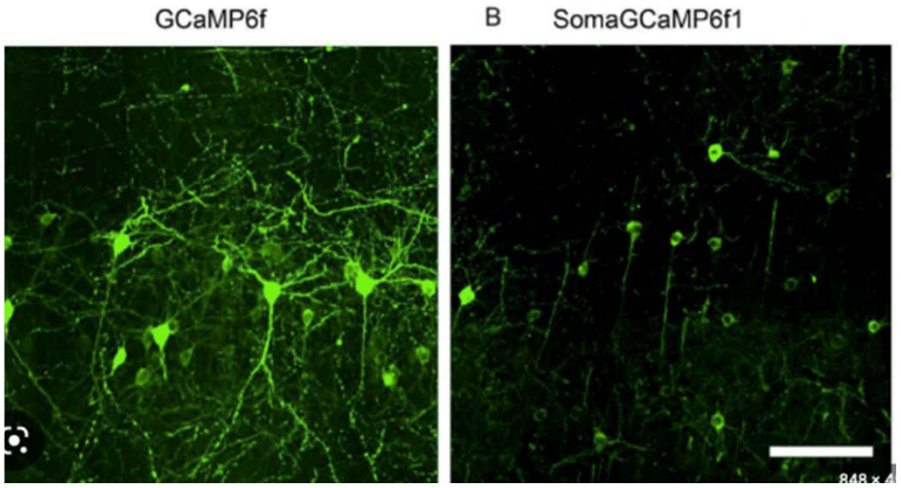
- Calcium imaging
  - Eg. Fura-2: (Grynkiewicz et al., 1985) excitation with Ca2+ : 339nm; without Ca2+ 376nm; emission always 510nm
- Geneticaly encoded calcium indicators (GECIs)
  - GCaMP - a synthetic fusion of green fluorescent protein (GFP), calmodulin (CaM), and M13 peptide
  - Bound to Ca2+ excitation 480nm emission 510nm
- Voltage imaging
  - electrochromic dyes and photo-induced electron transfer (PeT)
- Geneticaly encoded voltage indicators (GEVIs)
  - fusions of fluorescent proteins to voltage-sensing domains– fluorescent protein pairs
- Fluorescent neurotransmitter indicators (Glutamate, Ach, GABA)
- Fos-GFP mice (Barth et al., 2004)
- catFISH (Cellular compartment analysis of temporal activity by fluorescence in situ hybridization) (Guzowski et al., 2001)
- Immunostaining (cFos)



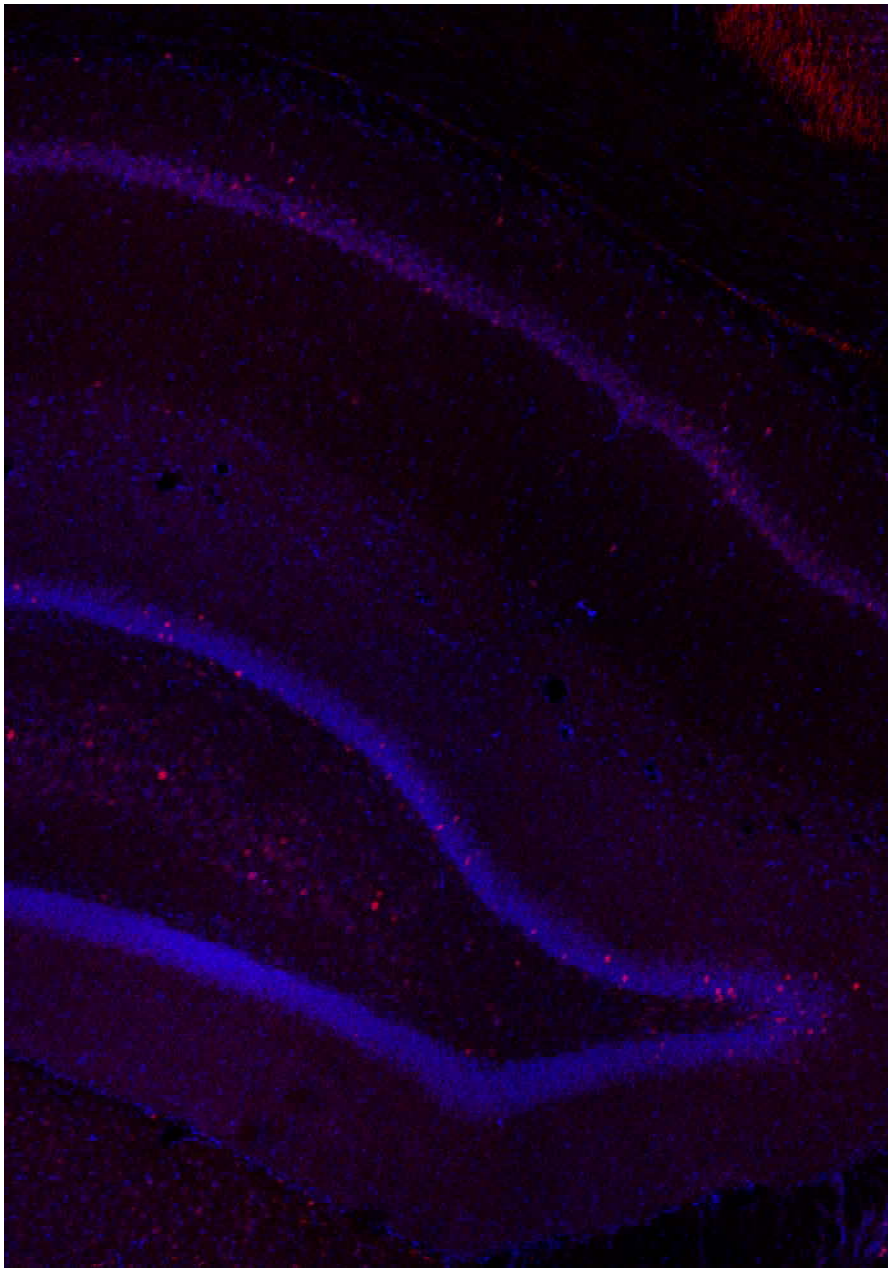
# jGCaMP8 (Zhang et al., 2023), GCaMP-X (Yang et al., 2018),and soma targeted GCaMP6f1 (Shemesh et al., 2020)



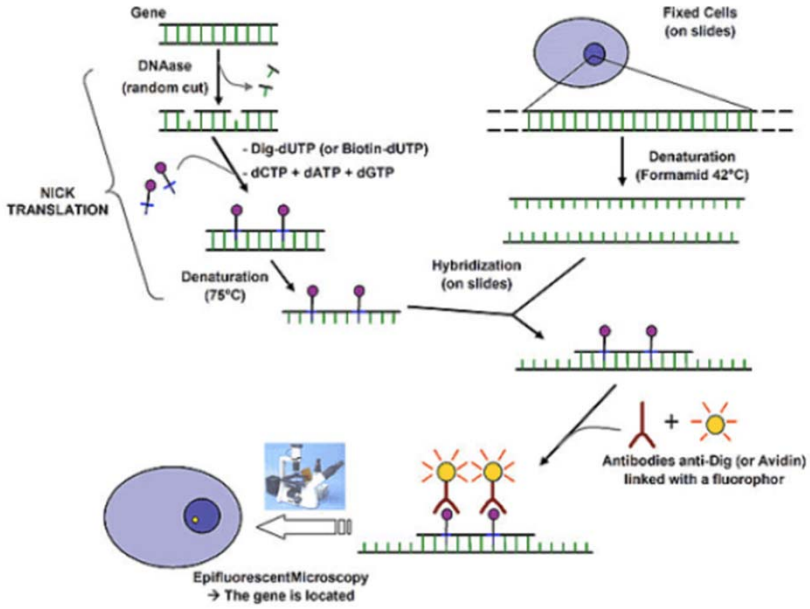
# cFos protein staining to map neuronal activity

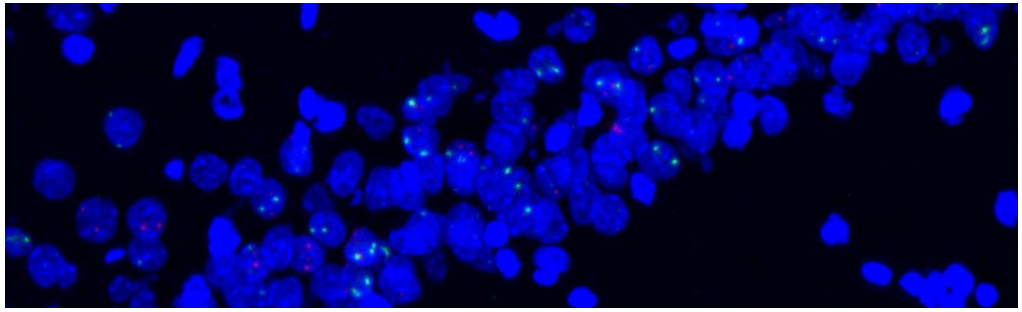
- Sufficient in many cases
- Robust expression
- Can be visualized using classical imunohistochemistry (such as DAB percipitation) or florescent labeling
- Peak expression 90-120 min after the stimulus



# FISH and catFISH

- Flourescent in situ hybridization
- compartment analysis of temporal activity fluorescent in situ hybridization
- Nuclear or cytosolic signal of mRNA of IEGs (cFos, Arc) is detected
- More complicated that protein staining
- Environment has to be kept RNAse free
- Haptene labelled probes label RNA of IEG
- Primary antibody against haptene conjugated to peroxidase is used to amplify the signal
- Further amplified by tyramide amplification system
- Flourescent precipitate forms around peroxidase



# Memory engram technologies

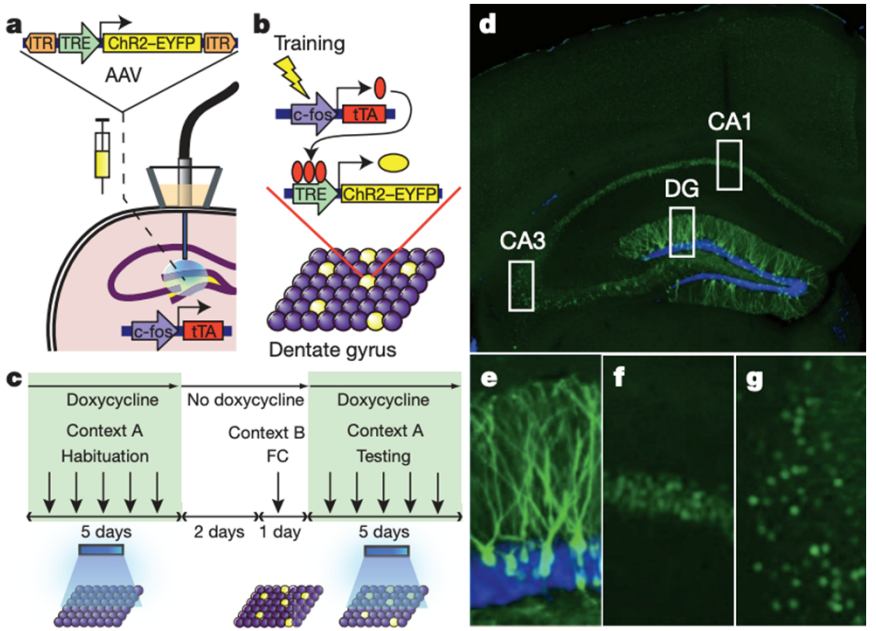
- Rely on endogenous markers of neuronal activity, such as IEGs (c-fos, Arc) or calcium levels to identify and manipulate neurons that were activated in response to a particular experience
- IEG expression is low in non-active neurons but can be induced rapidly and transiently by external stimuli
- Neurons expressing IEGs are believed to be involved in forming memory engrams
- Calcium levels are more temporally precise marker of neuronal activity
- Calcium levels do not have to correlate with plastic changes
- Neurons can be labeled, traced, recorded, and/or manipulated

# Memory engram technologies

- Tetracycline transactivator controlled genetic Tagging of active neural circuits (TetTag) (Reijmers et al., 2009)
- Targeted Recombination in Active Populations (TRAP)(Guenthner et al., 2013)
- Capturing activated neuronal ensembles (CANE) (Sakurai et al., 2016)
- Calcium-modulated photoactivatable ratio-metric integrator (CaMPARI) (Fosque et al., 2017)
- Cal-light (Lee et al., 2017)
- Fast Light– and Activity-Regulated Expression (FLARE) (Wang et al., 2017)
- Fast Light and Calcium-Regulated Expression (FLiCRE) (Kim et al., 2020)
- Expression recording island (Linghu et al., 2010)

# Optogenetic stimulation of a hippocampal engram activates fear memory recall

- Liu et al., 2012 – the first use of engram technology to activate engram
- Used c-fos-tTA transgenic mice (Rejmirez et al., 2007)
- DG transfected with AAV9-TRE-ChR2-EYFP virus + optic fibre
- Dox inhibits c-fos-promoter-driven-tetracycline trans activator (tTA) from binding to its target tetracycline-responsive element (TRE)
- When off DOX cFos expression can drive tTA expression
- tTA binds to TRE  and drives expression of ChR2-EYFP
- Time window of labelling is wide >24h
- Lasts 5-30 days

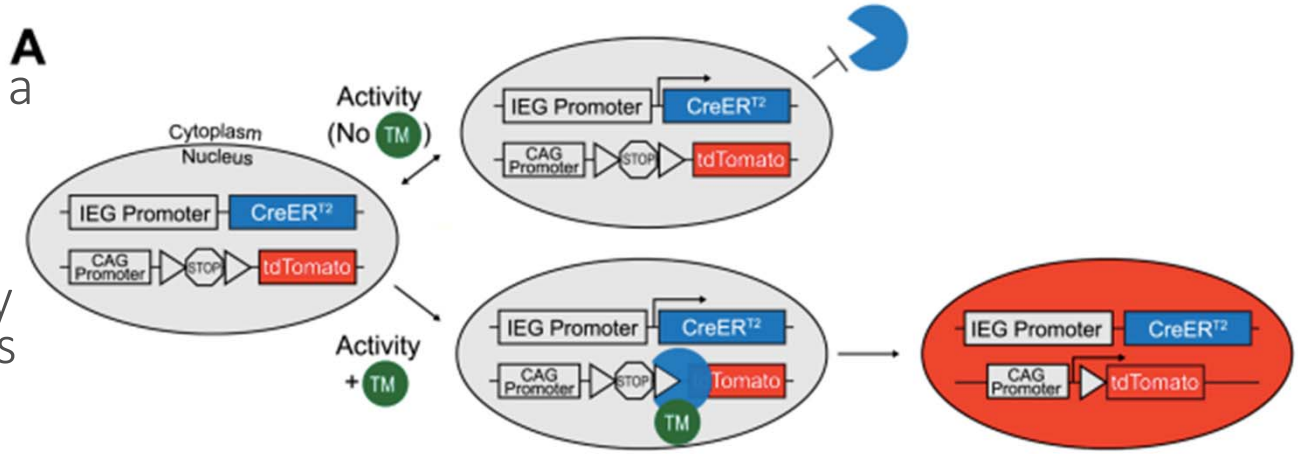


# Cre recombinase

- Type I topoisomerase from bacteriophage P1 that catalyzes the site-specific recombination of DNA between two loxP sites
- floxed-stop or floxed-inverse (FLEX) cassettes
- Can either cut-out STOP sequence flip target sequence
- https://old.abmgood.com/marketing/knowledge_base/Cre-Lox_Recombination.php

# Targeted Recombination in Active Populations (TRAP)

- Guenthner et al., 2013
- Uses a fusion protein containing a mutated estrogenic receptor + Cre recombinase (Cre-ER$^{T2}$)
- Cre-ER$^{T2}$ is sequestered in the cytoplasm after neuronal activity until tamoxifen presence induces Cre-ER$^{T2}$ translocation to the nuclei leading to recombination of the desired transgene.
- Results in the permanent expression of recombined gene
- Fos$^{2A\text{-}iCreER}$ (TRAP2) mice (JAX: 030323)



# Tamoxifen

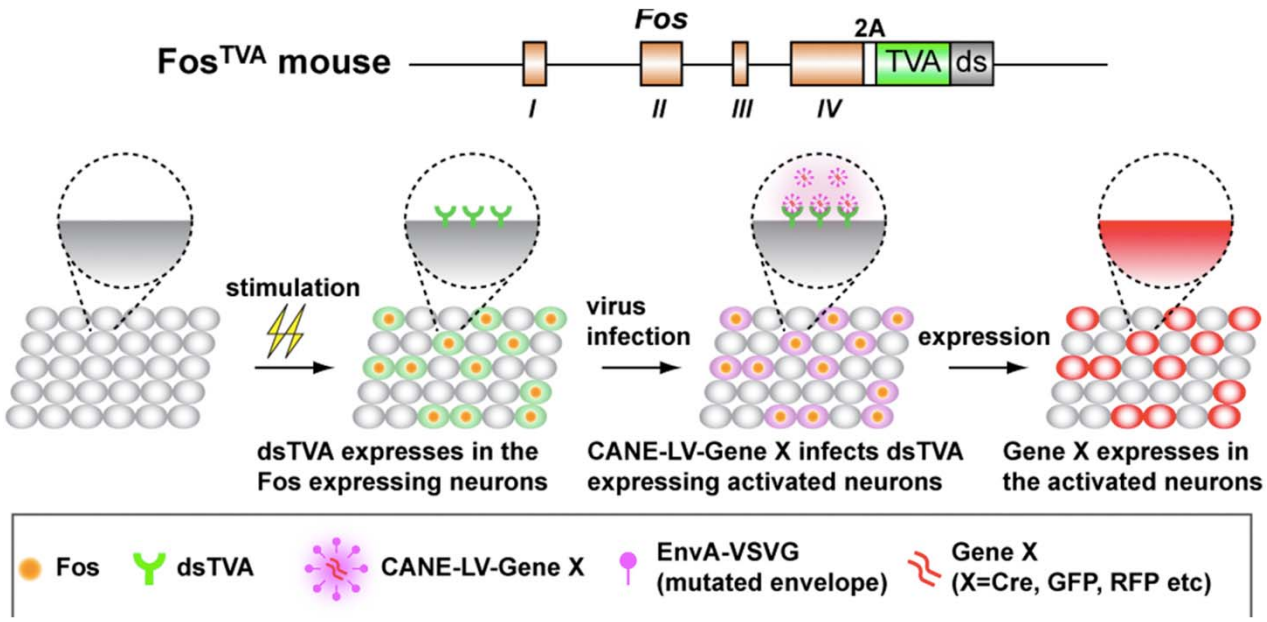
- acts as a selective estrogen receptor modulator/partial agonist of the estrogen receptors
- Used in breast cancer treatment
- In engram technology tamoxifen application must co-occur with cFos/Arc expression to label  active neurons
- Due to its pharmacokinetics, tamoxifen results in cca 12 hour of activity labelling (half life 11.5h)
- 4-hydroxytamoxifen (4-OHT) labels shorter time window: 3h before event and 3 h post event. Injection is given right after the behavior in question (half life 6h)

# Persistent transcriptional programmes are associated with remote memory

- Chen et al., 2020
- What are long term changes in gene expression in engram neurons?
- TRAP + single cell trascriptomics
- identified membrane-fusion genes that could have important roles in the maintenance of remote memory
- Unexpectedly, astrocytes and microglia also acquired persistent gene expression signatures that were associated with remote memory, suggesting that they actively contribute to memory circuits

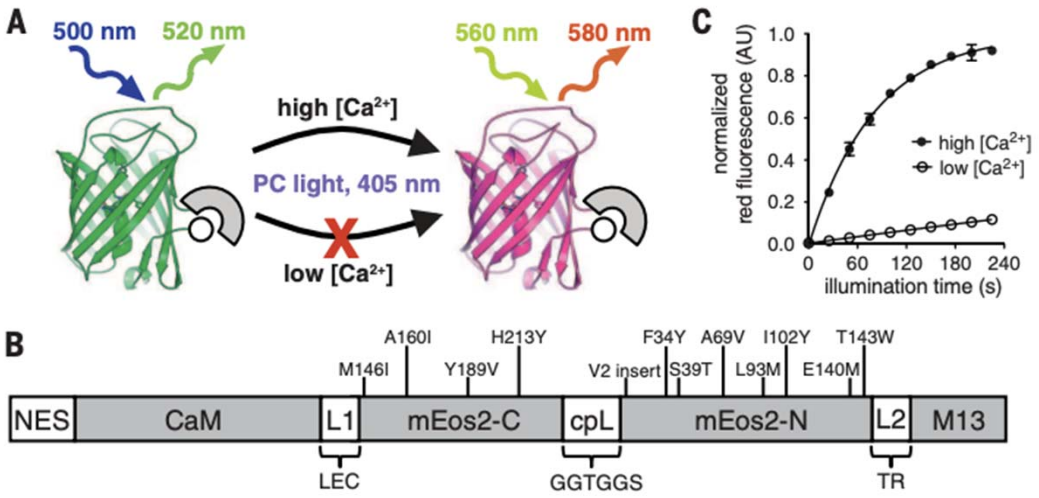
# Capturing and Manipulating Activated Neuronal Ensembles with CANE

- Sakurai et al., 2016
- Adresses shortcomings of TetTag and TRAP (long temporal window of tagging)
- TVA is a specific receptor for the envelope protein of the avian sarcoma and leukosis virus (EnvA)
- Injection of desired gene in pCAGGS/ES-M21EnvA-VSVg-WPRE LV envelope is done 1.5h after behavior (stereotaxic)
- 10 days to 3 weeks to reach peak expression
- $Fos^{TVA}$ mice: B6;129-Fostm1.1Fawa/J mice (JAX)



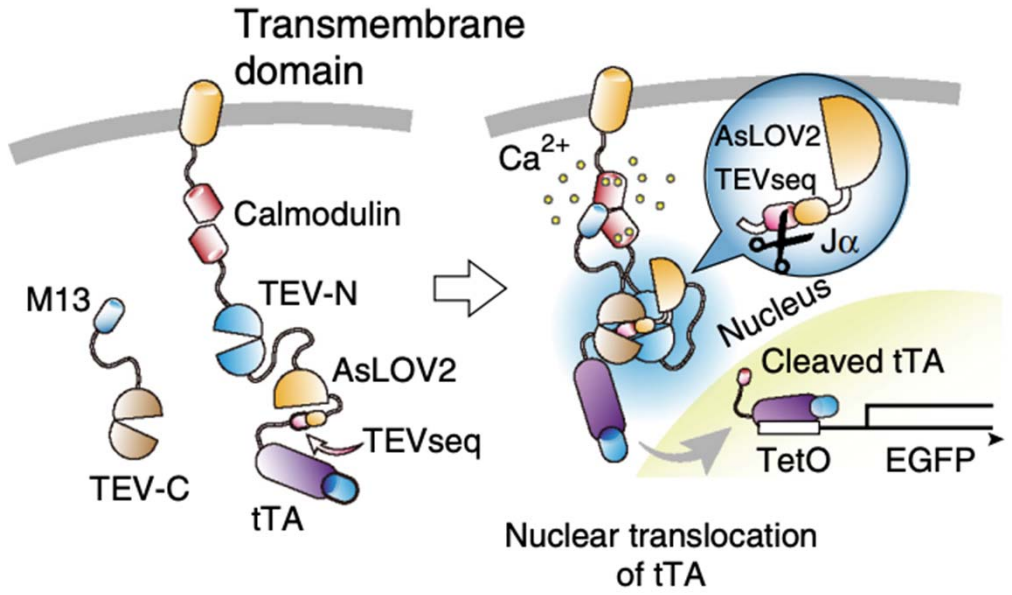
# CaMPARI (calcium-modulated photoactivatable ratio-metric integrator).

- Fosque et al., 2017
- temporally precise “activity snapshot” of a large tissue volume
- CaMPARI protein undergoes efficient and irreversible green-to-red conversion only when elevated intracellular Ca2+ and experimenter-controlled illumination (UV) coincide
- Developed by fusing calmodulin (CaM) and M13 peptide with photoconvertible mEos2 fluorescent protein
- Original Eos protein converted only with UV, calcium had no effect
- New protein, CaMPARI, converts 3x faster in $Ca^{2+}$-bound state to red and 1/7 less likely in $Ca^{2+}$-free state than original protein
- pAAV.hSyn.CaMPARI.WPRE.SV40 (Addgene, plasmid #100832)
- R26-CAG-lsl-CaMPARI2 mice (JAX:034240)



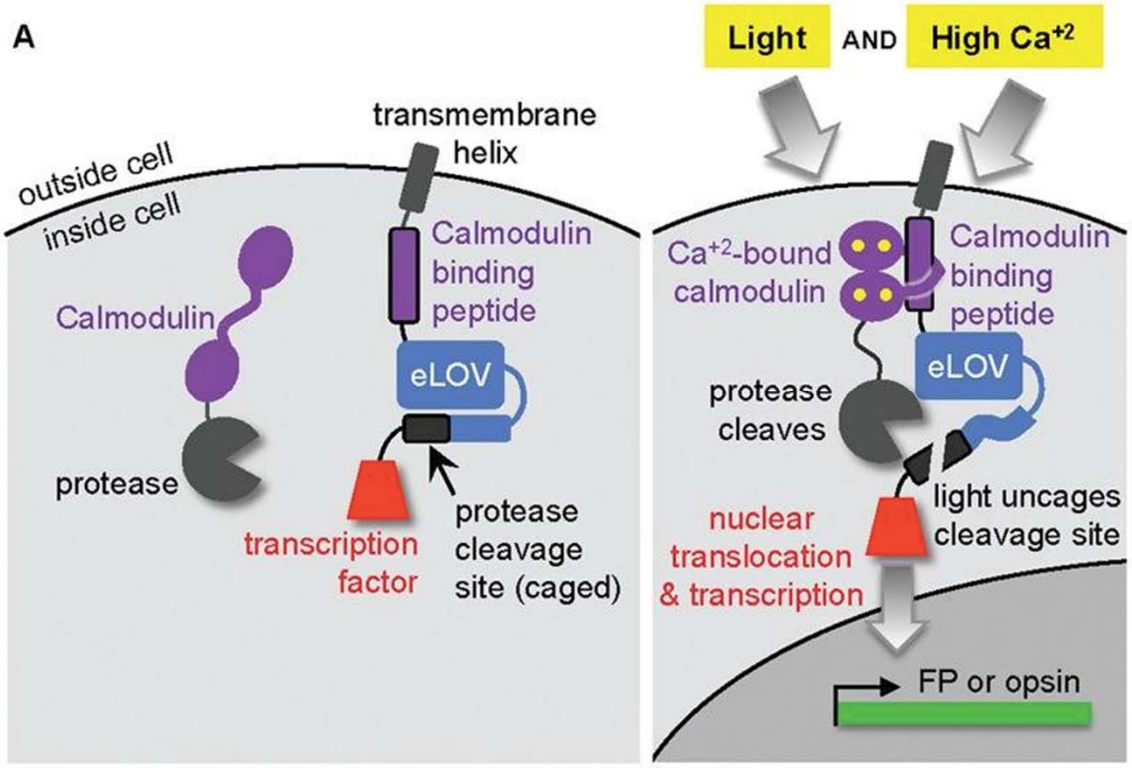
# A calcium- and light-gated switch to induce gene expression in activated neurons (Cal-Light)

- Lee et al., 2017
- Cal-Light consists of two synthetic proteins
- 1st protein has 5 domains: CaM, TEV- N, TEVseq inserted into a truncated form of AsLOV2, and tTA
- 2nd protein is fusion between M13 and TEV-C
- TEV-N, TEV-C make up together TEV protease that cleaves TEVseq site (Tobacco Etch Virus protease)
- TEV-N and TEV-C come together during high $Ca^{2+}$ conditions while TEVseq is unmasked only with light (asLOV2 - LOV2 domain of *Avena Sativa* phototropin 1)
- Complete TEV protease cleaves tTA off the membrane
- Therefore, tTA translocate to nucleus only in the presence of both blue light and calcium
- tTA drives gene expression when attaching to TRE sequence enabling downstream transcription



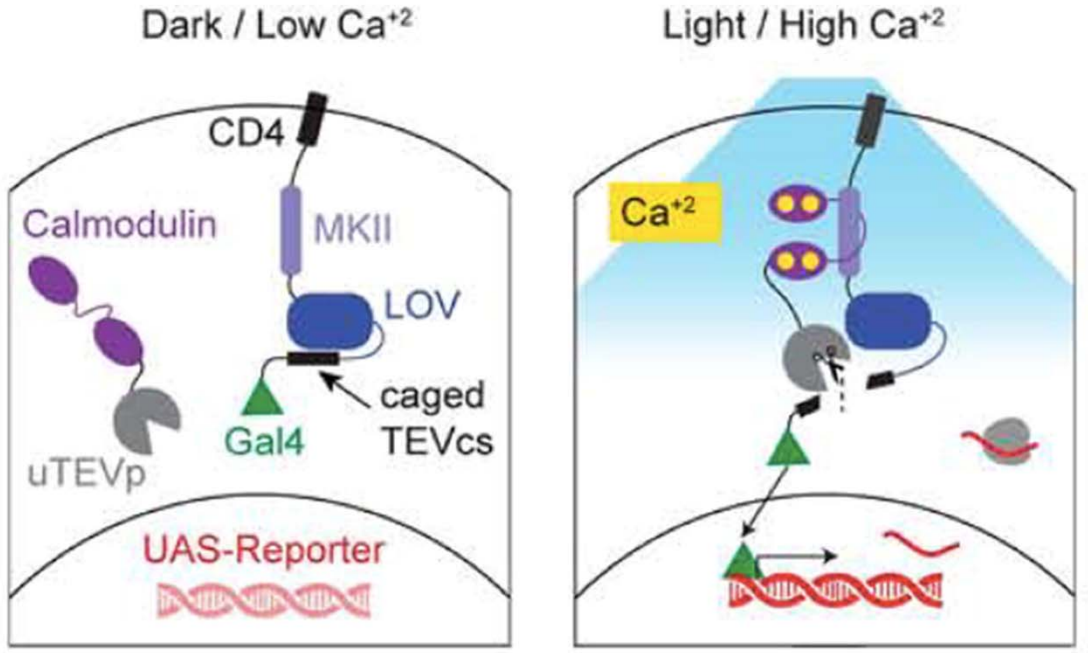
# A light- and calcium-gated transcription factor for imaging and manipulating activated neurons (FLARE)

- Wang et al., 2017
- Fast Light– and Activity-Regulated Expression (FLARE)
- minute-scale temporal resolution, and minimal dark-state leak
- Same TEV protease as in Cal-Light
- In neurons they used tTA as a transcription factor (but also used other transcription factors)
- Ca-FLARE (TF component) (Addgene, plasmid #92213)
- Ca-FLARE (protease) (Addgene, plasmid #92214)
- TRE-mCherry (Addgene, plasmid #92202)



# FLiCRE (Fast Light and Calcium-Regulated Expression)

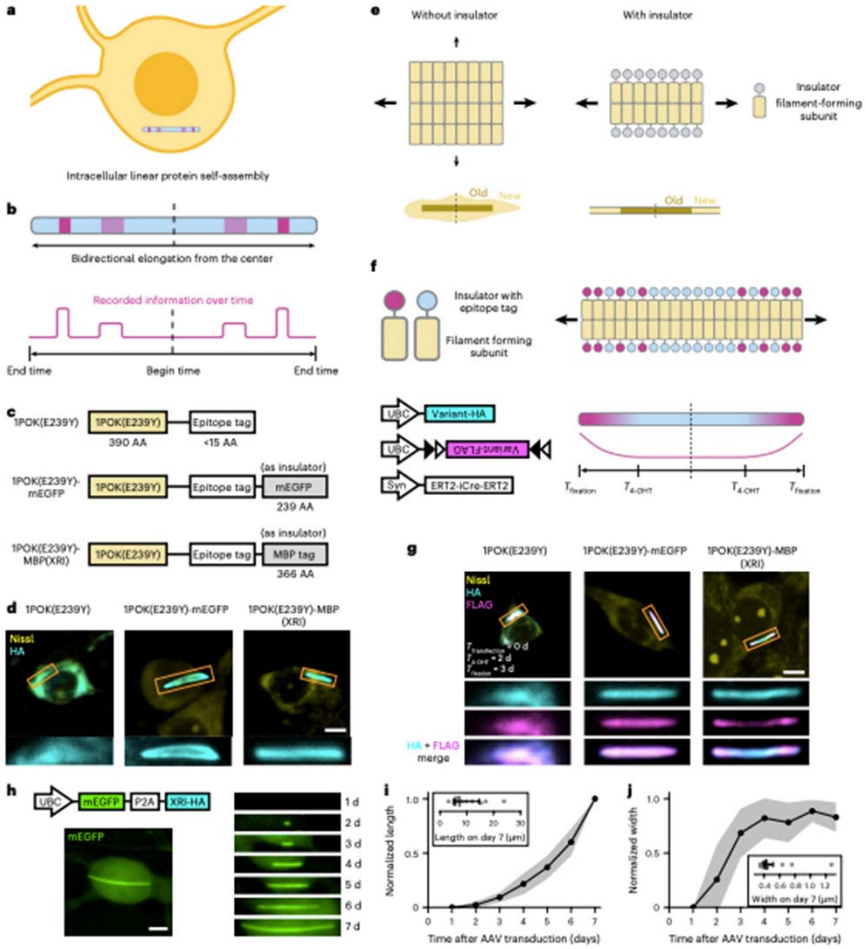
- Kim et al., 2020
- Very similar to Cal-Light and FLARE
- Part attached to membrane: Nrxn3b-Nav1.6-MKII-f-hLOV1-TEVcs(ENLYFQ/M)-tTA-VP16 (Addgene, plasmid #163031)
- Cytosolic part: GFP-CaM-uTEVp (Addgene, plasmid #163032)
- TRE:GFP (Addgene, plasmid #163036)



# Recording of cellular physiological histories along optically readable self-assembling protein chains

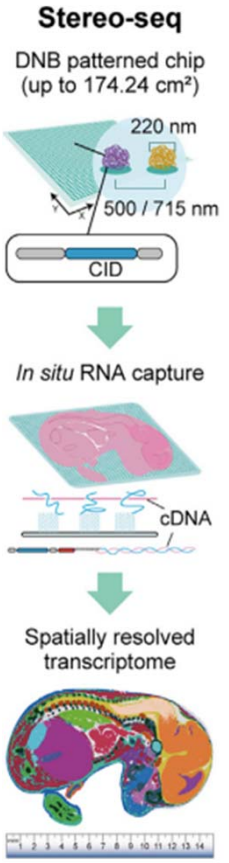
- Linghu et al.,2023
- continual digital recording of biological information within cells and subsequent high-throughput readout in fixed cells
- intracellular protein chains made of self-assembling subunits, human-designed filament-forming proteins bearing different epitope tags that each correspond to a different cellular state or function
- One protein is continually expressed under UBC promotor
- Other protein is expressed under cFos promotor to mark periods of neuronal activity

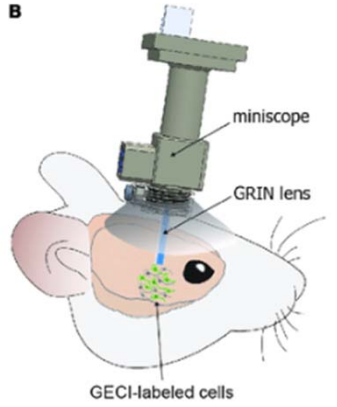
- pAAV-UBC-XRI-HA (Addgene, plasmid #178056)
- pAAV-cFos-XRI-V5 (Addgene, plasmid #178058)

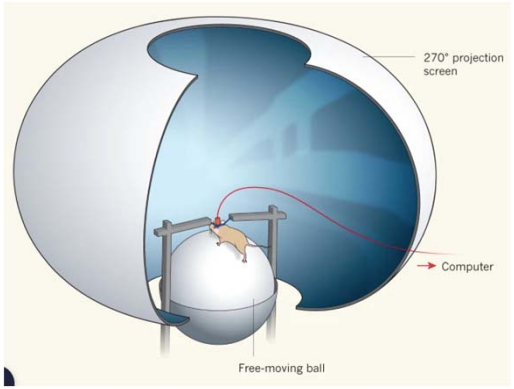


# Other new techniques in learning and memory research

- Single-cell and in-situ transcriptomics
- GRIN lenses and miniscopes (https://www.youtube.com/watch?v=xUf7HHiazEI)
- 2 and 3 photon Calcium imaging in awake mice
- Virtual reality for rodents (https://www.youtube.com/shorts/kzs070xmOrU)



Thank you for your attention!