



Advanced methods in memory research

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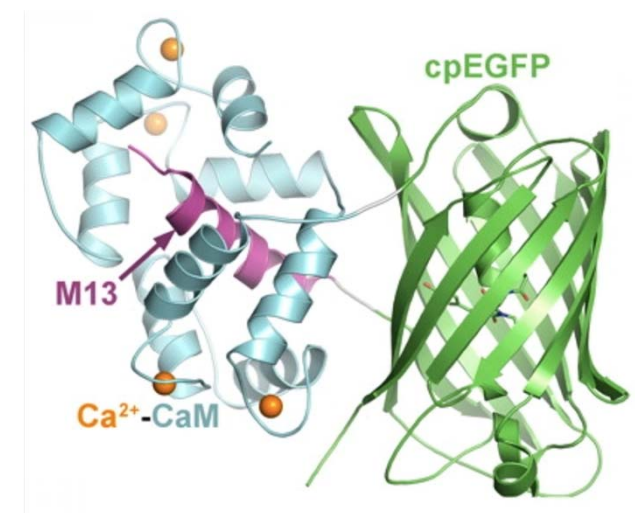
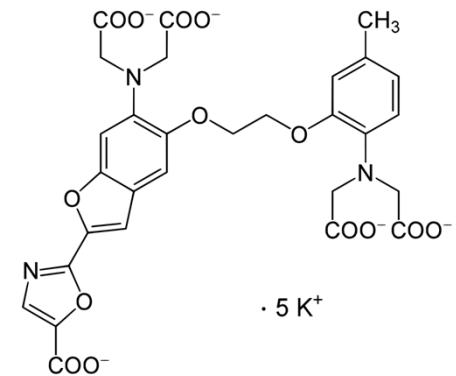


Outline

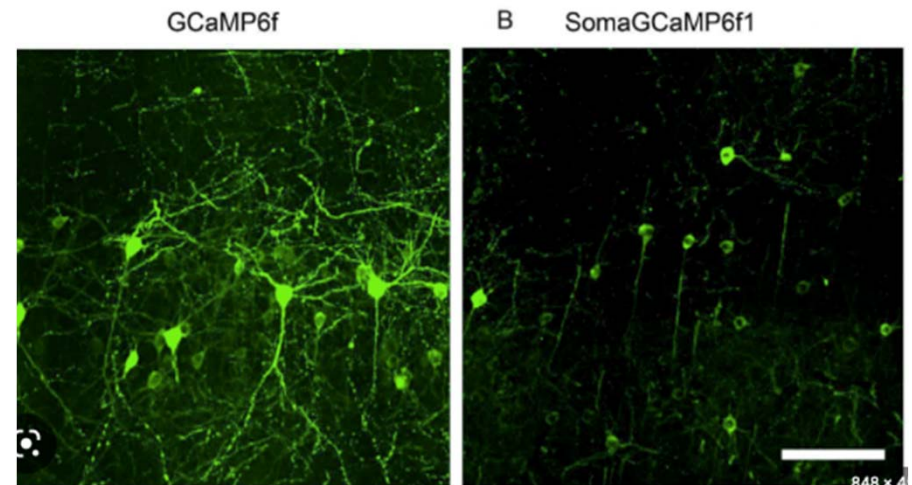
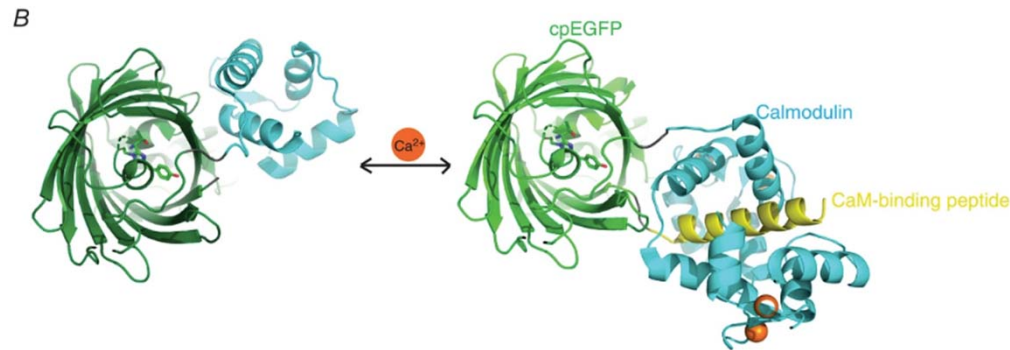
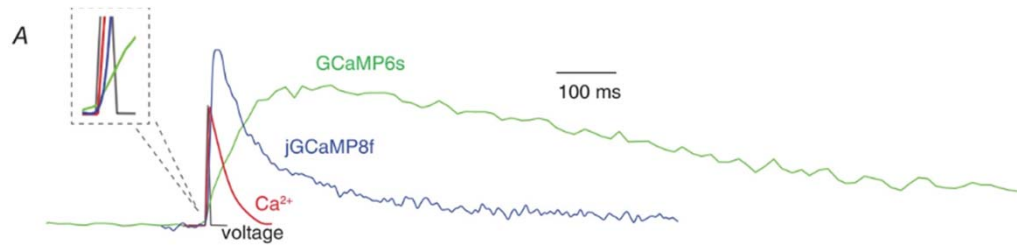
- Visualization of neuronal activity
- Traditional methods
 - Calcium imaging
 - IEG immunostaining
 - catFISH
- Memory engram technologies
 - TetTag
 - TRAP
 - CANE
 - CaMPARI
 - Cal-light
 - FLARE
 - FLiCRE
 - Expression recording island
 - Other methods

Visualization of neuronal activity

- Calcium imaging
 - Eg. Fura-2: (Grynkiewicz et al., 1985) excitation with Ca^{2+} : 339nm; without Ca^{2+} 376nm; emission always 510nm
- Genetically encoded calcium indicators (GECIs)
 - GCaMP - a synthetic fusion of green fluorescent protein (GFP), calmodulin (CaM), and M13 peptide
 - Bound to Ca^{2+} excitation 480nm emission 510nm
- Voltage imaging
 - electrochromic dyes and photo-induced electron transfer (PeT)
- Genetically encoded voltage indicators (GEVIs)
 - fusions of fluorescent proteins to voltage-sensing domains, opsins, and hybrid opsin – 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)

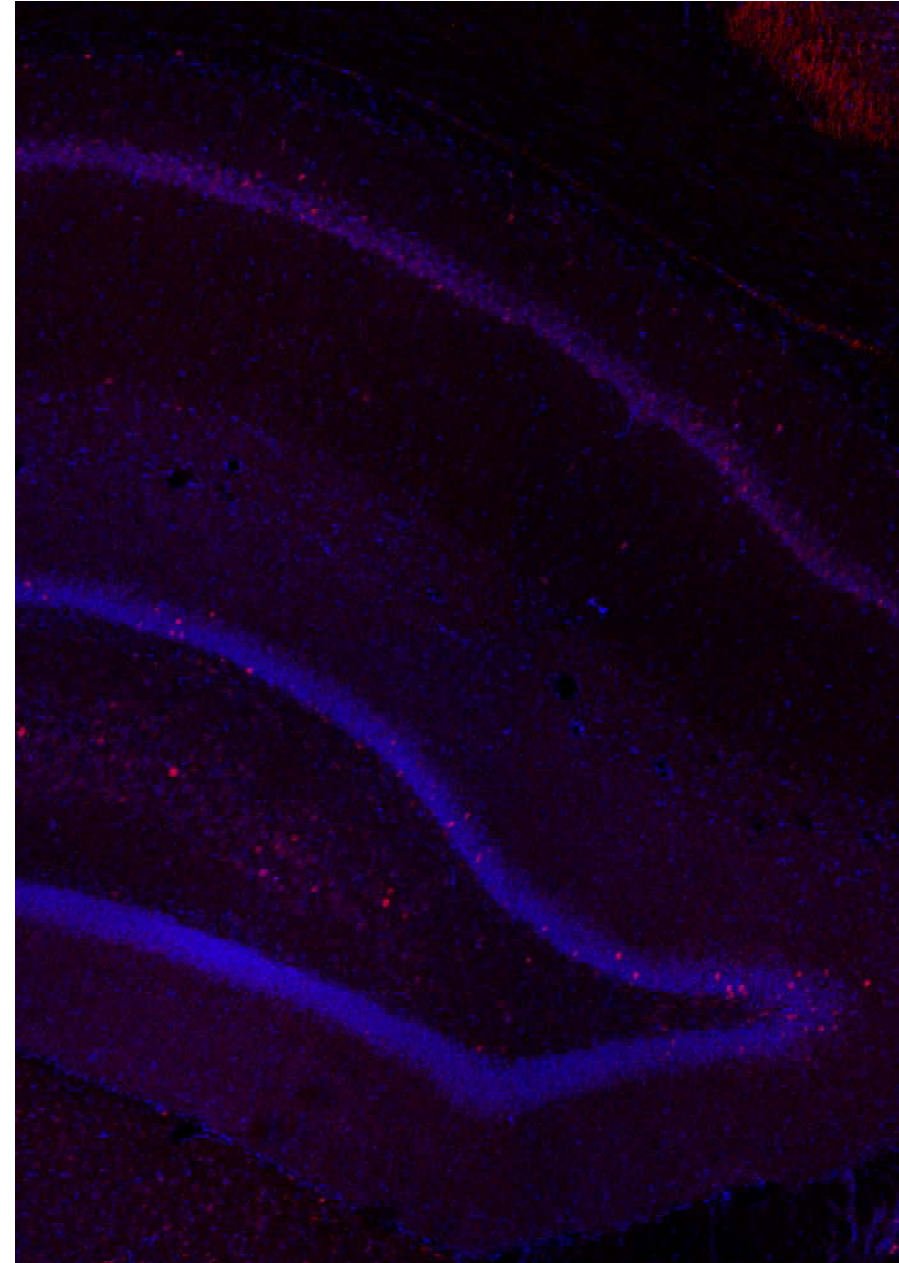


jGCaMP8 (Zhang et al., 2023), GCaMP-X (Yang et al., 2018), and soma targeted GCaMP6f1 (Shemesh et al., 2020)



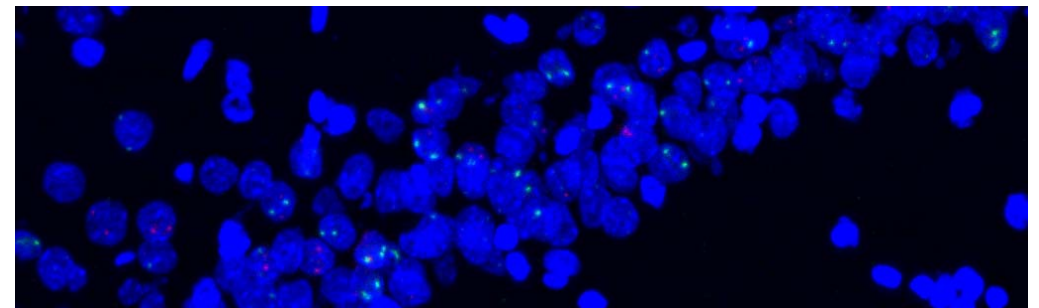
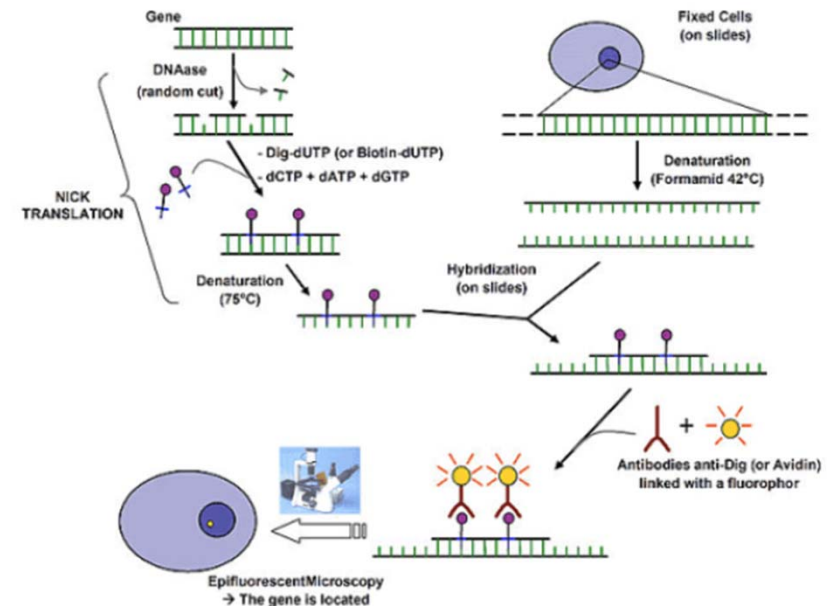
cFos protein staining to map neuronal activity

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



FISH and catFISH

- Nuclear or cytosolic signal of mRNA of IEGs (cFos, Arc) is detected
- More complicated than 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
- Fluorescent precipitate forms around peroxidase



Memory engram technologies

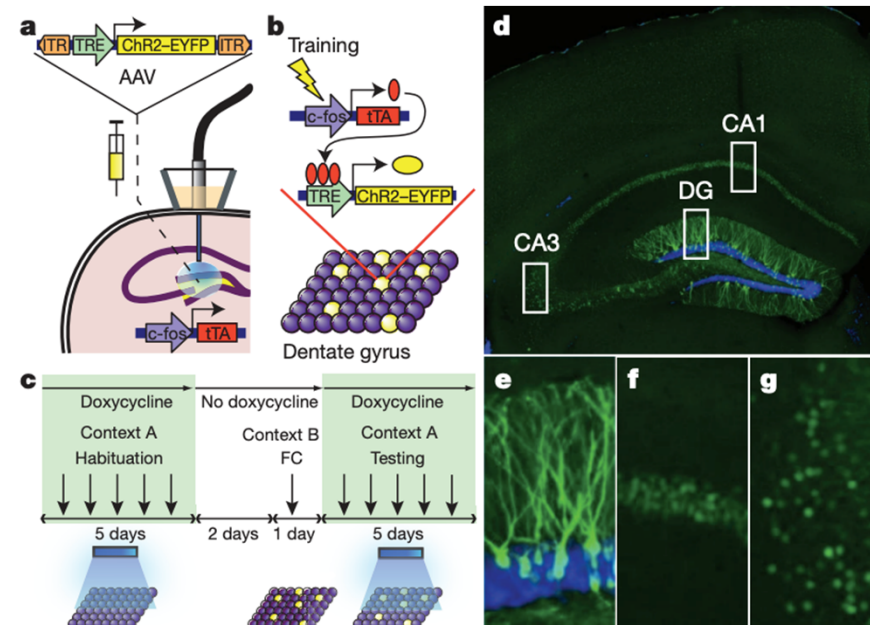
- Rely on endogenous markers of neuronal activity, such as IEGs (c-fos, Arc) or calcium levels to identify and manipulate neurons that were activated in response to a particular experience
- IEG expression is low in non-active neurons but can be induced rapidly and transiently by external stimuli
- Neurons 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

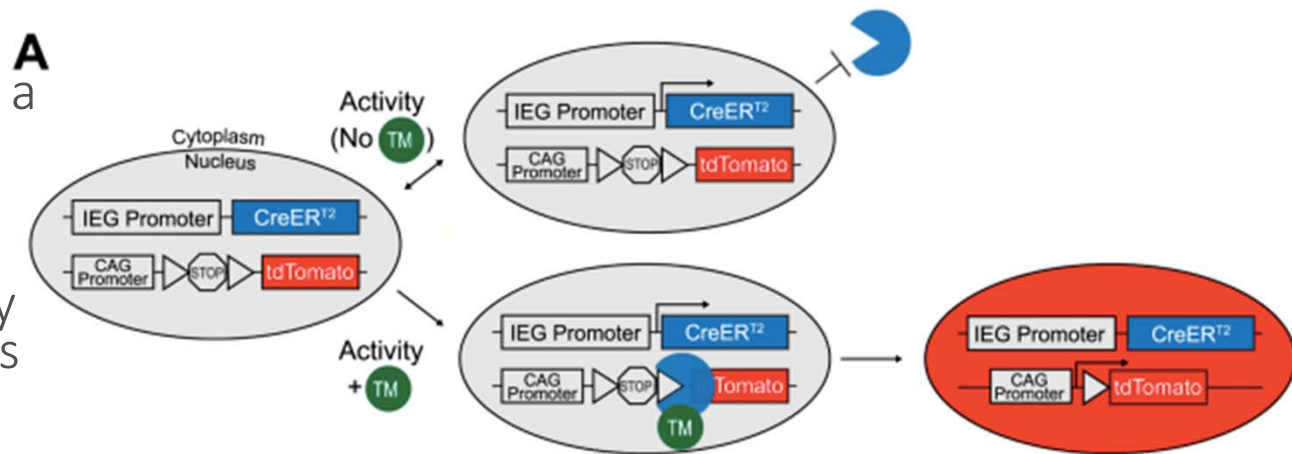


Cre recombinase

- 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)

- Guenther 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-iCreER} (TRAP2) mice (JAX: 030323)



Tamoxifen

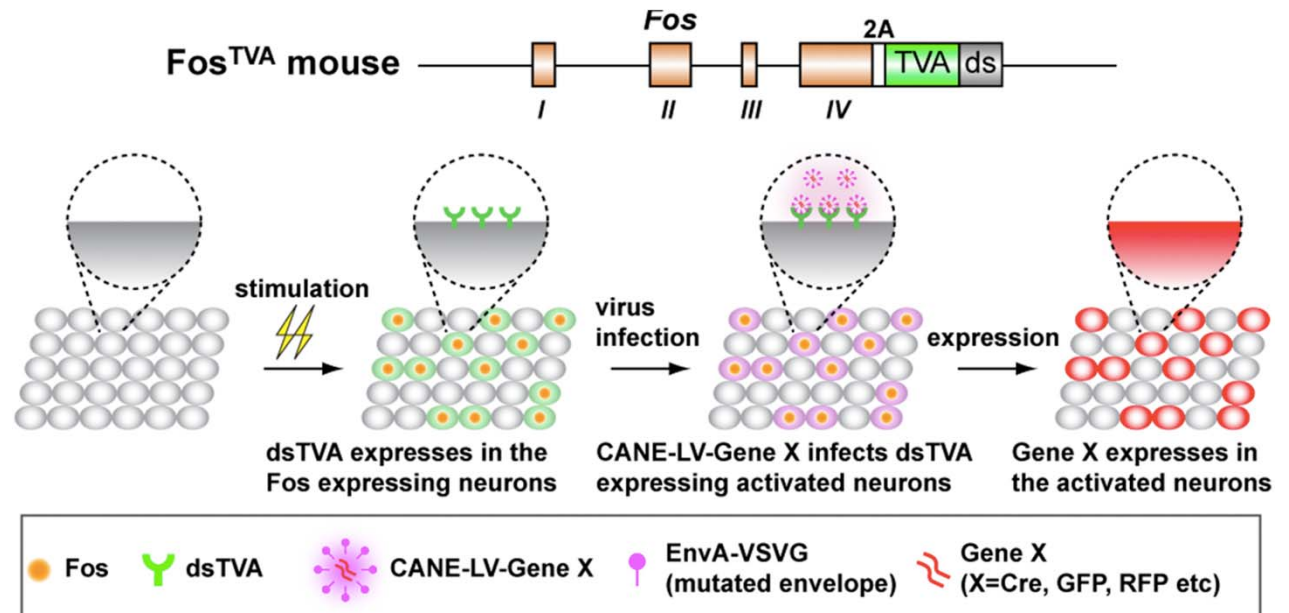
- acts as a selective estrogen receptor modulator/partial agonist of the estrogen receptors
- Used in breast cancer treatment
- In engram technology tamoxifen application must co-occur with cFos/Arc expression to label active neurons
- Due to its pharmacokinetics, tamoxifen results in cca 12 hour of 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 transcriptomics
- 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

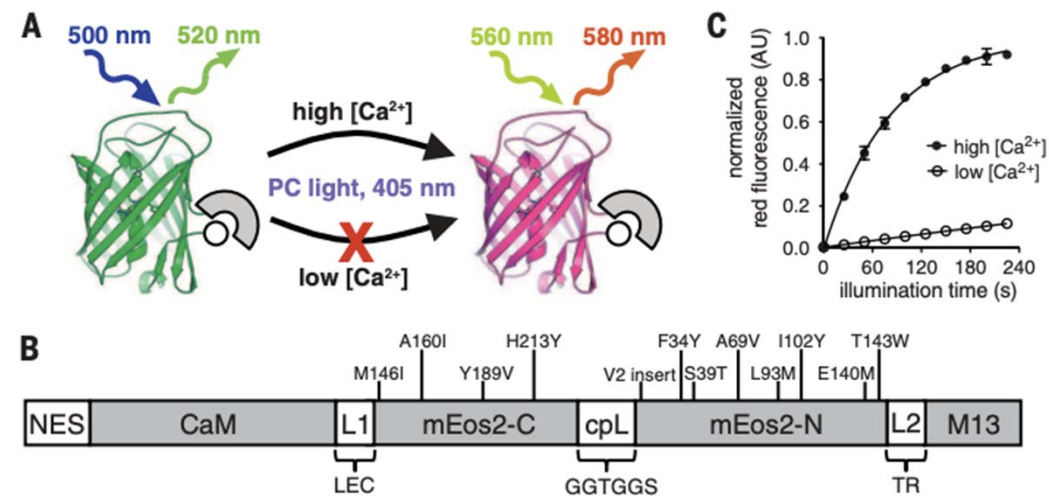
Capturing and Manipulating Activated Neuronal Ensembles with CANE

- Sakurai et al., 2016
- Addresses 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)



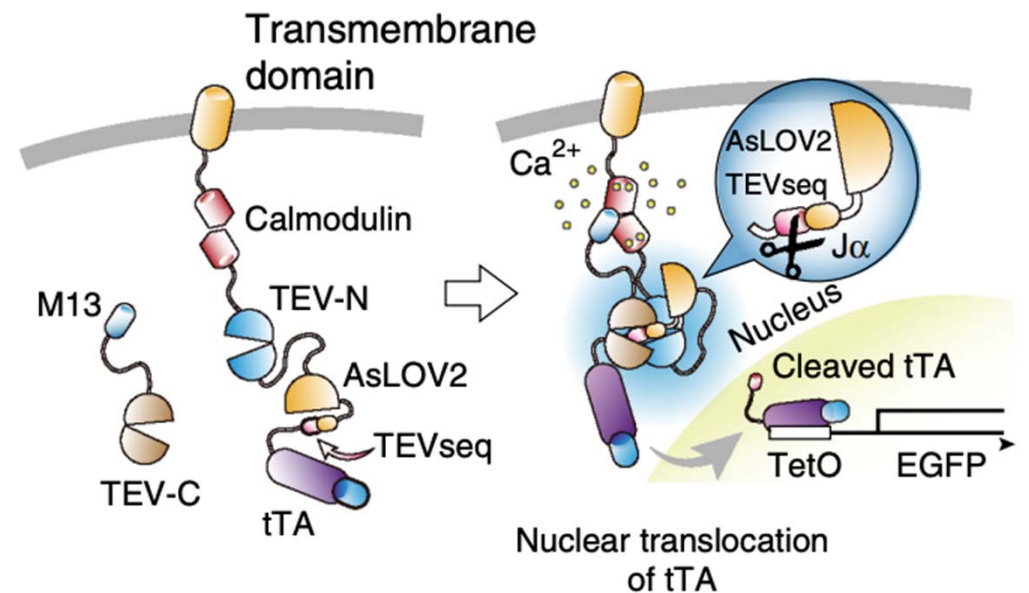
CaMPARI (calcium-modulated photoactivatable ratio-metric integrator).

- 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 Ca^{2+} 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)



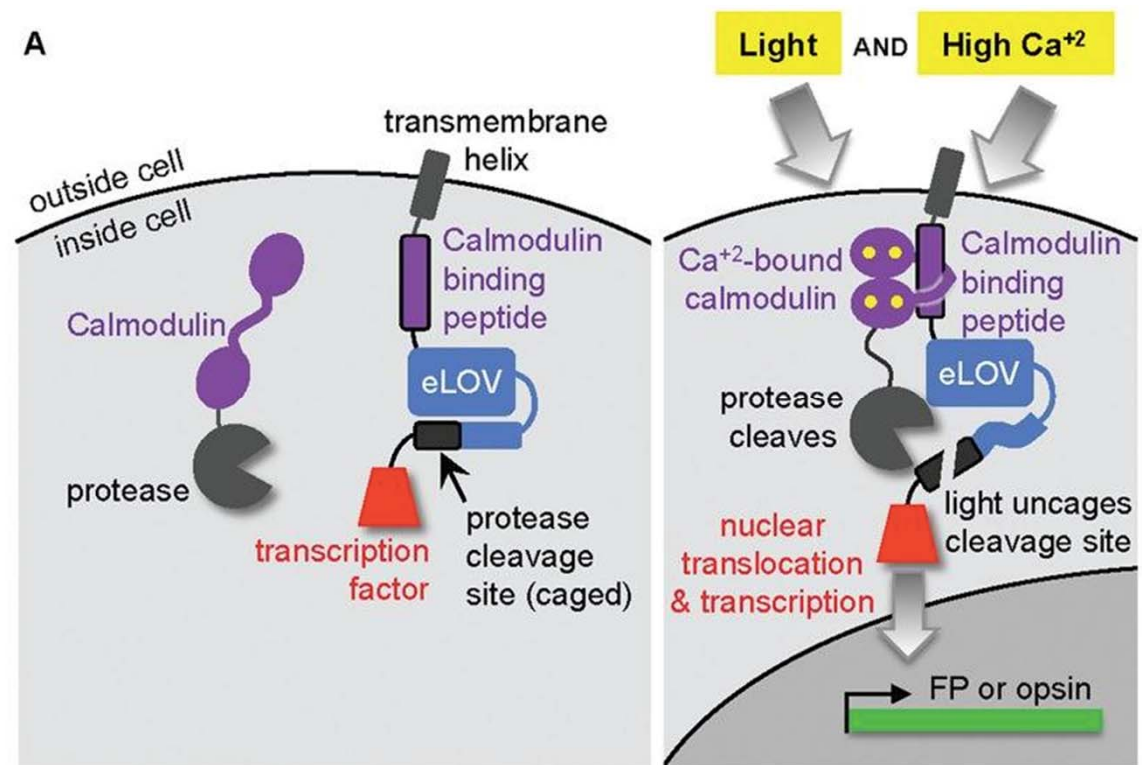
A calcium- and light-gated switch to induce gene expression in activated neurons (Cal-Light)

- 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
- TEV-N and TEV-C come together during high Ca²⁺ conditions while TEVseq is unmasked only with light (asLOV2)
- 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
- (Plasmid unpublished)



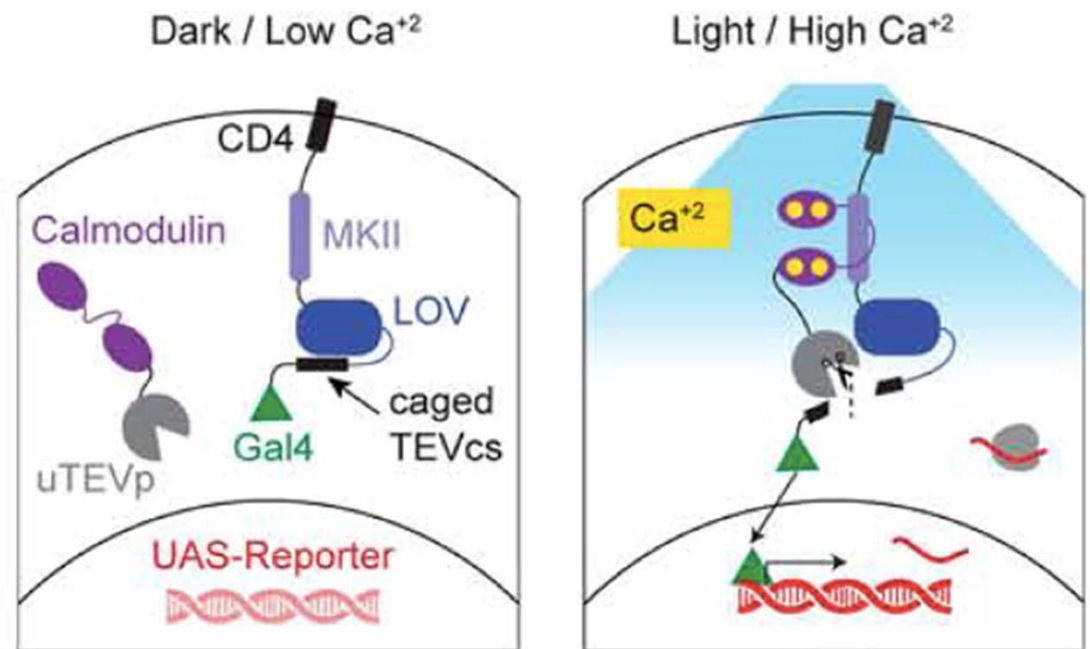
A light- and calcium-gated transcription factor for imaging and manipulating activated neurons (FLARE)

- 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) (Addgene, plasmid #92213)
- Ca-FLARE (protease) (Addgene, plasmid #92214)
- TRE-mCherry (Addgene, plasmid #92202)



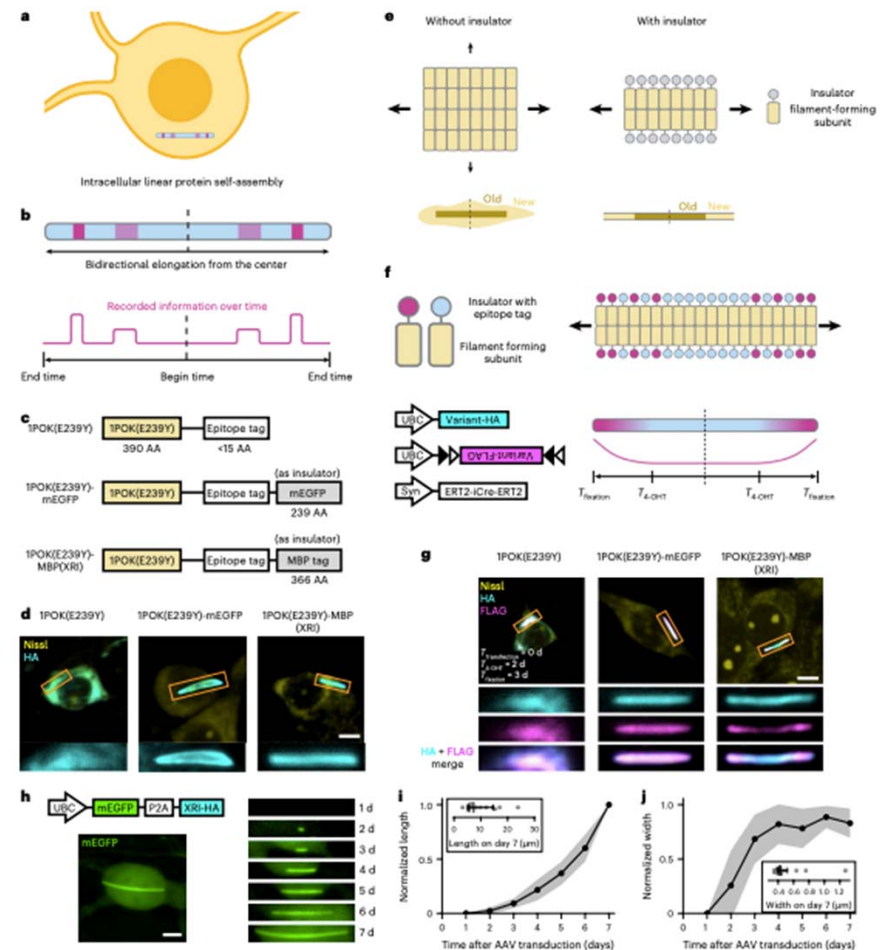
FLiCRE (Fast Light and Calcium-Regulated Expression)

- Kim et al., 2020
- Very similar to Cal-Light and FLARE
- Part attached to membrane: Nrnx3b-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)



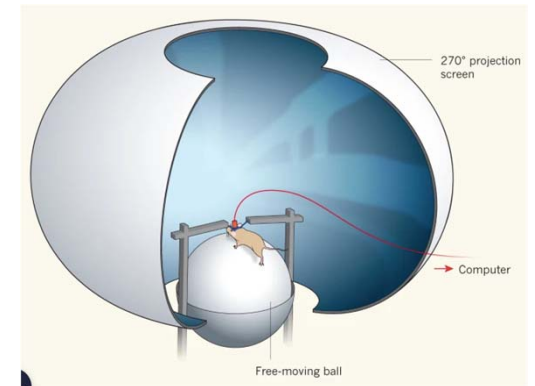
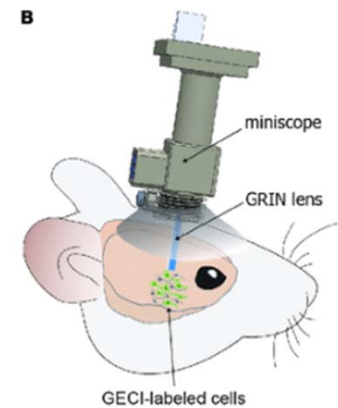
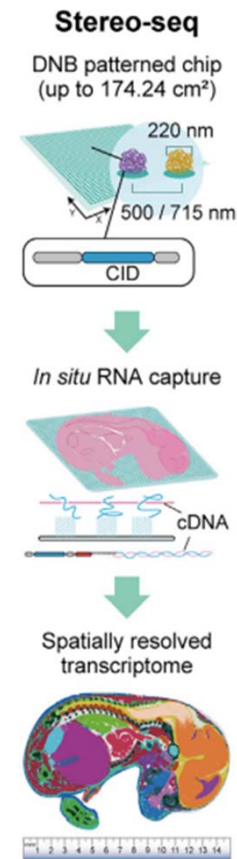
Recording of cellular physiological histories along optically readable self-assembling protein chains

- 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 promoter
- Other protein is expressed under cFos promoter 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!