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Genetic strategies to access activated neurons

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Abstract

A major goal of modern neuroscience is to understand how ensembles of neurons participate in neural circuits underlying behavior. The recent explosion of genetically-encoded circuit analysis tools has allowed neuroscientists to characterize molecularly-defined neuronal types with unprecedented detail. However, since neurons defined by molecular expression can be functionally heterogeneous, targeting circuit analysis tools to neurons based on their activity is critical to elucidating the neural basis of behavior. Here we review genetic strategies to access activated neurons and characterize their functional properties, molecular profiles, connectivity, and causal roles in sensory-coding, memory, and valence-encoding. We also discuss future possibilities for improving these strategies and using them to screen brain-wide activity patterns underlying adaptive and maladaptive behaviors.

Introduction

A major challenge in cellular and systems neuroscience is to understand how activity in specific neuronal populations produces perception and behavior. Traditional methods including metabolic imaging, pharmacological and physical lesions, and physiological recordings advanced our understanding of how neural activity underlies behavior. However, these techniques are limited in cell-type specificity. Recent studies in the mouse combined molecularly-defined Cre-driver lines (1–3) with Cre-dependent genetic tools to dissect the connectivity (4, 5), physiological response properties (6, 7), and behavioral roles (8–10) of specific neuronal cell-types. However, functionally heterogeneous neurons can belong to the same molecular type and are often spatially intermingled (11–13). Targeting neurons based on activity-dependent gene expression provides an orthogonal approach to dissect the organization and function of activated neural circuits with unprecedented cellular resolution.

When a neuron becomes depolarized, the transient rise of intracellular Ca^{2+} and downstream second messenger pathways transiently activate the expression of immediate early genes (IEGs) within minutes of neuronal activation (14). Most activity-based genetic strategies rely on the best-characterized IEGs, *Fos* (15) and *Arc* (16). *Fos* is rapidly induced by growth factor stimulation and various patterns of neural activity (15, 17–19) and acts as a transcription factor to regulate expression of many genes (20). *Arc* is also rapidly induced by

neural activity and growth factors; its mRNAs and proteins are enriched in dendrites, which play an important role in synaptic plasticity (21, 22).

The discovery of IEGs enabled functional mapping with cellular resolution throughout the central nervous system (23). Early studies mapped *Fos* expression patterns that were consistent with known physiological responses to the same stimuli (18, 24). catFISH (compartmental analysis of temporal activity by fluorescent in-situ hybridization) enabled comparison of neuronal populations activated by two experiences ~30 min apart in the same animal (25). While these studies paved the way for mapping experience-driven activity patterns with IEGs, labeling IEGs in fixed tissues precludes examination of the physiological properties of recently activated neurons.

IEG promoters driving effectors for characterizing recently activated neurons

Transgenic animals using IEG promoters to drive fluorescent reporters (Fig. 1A) enabled researchers to characterize the physiological properties of recently activated neurons. For example, the *Fos-GFP* transgenic mouse, in which the *Fos* promoter drives a Fos-EGFP fusion protein, enables targeting of GFP+ neurons for electrophysiological recordings hours after an experience (26). In barrel cortex, GFP+ cells delineated an ensemble of synaptically connected neurons that were more likely to be excited during network activity (27) and exhibited larger responses to signals from surrounding whiskers (28). The *Arc* promoter has also been used to drive expression of fluorescent reporters in transgenic animals (29–31). *Arc-GFP* knock-in mice allowed tracking of activated populations in primary visual cortex (V1) over time. With repeated stimulus presentations, many GFP+ neurons adapted save a small group of reliable responders (29). Such studies helped elucidate the history of activity preceding IEG expression.

Fos-LacZ transgenic mice and rats (32, 33) were designed to mark recently activated neurons in fixed tissue. In addition, *LacZ* product beta-galactosidase converts the prodrug Daun02 into Daunorubicin, which inactivates neurons through apopotosis or blockade of voltage-gated calcium channels (34). This strategy revealed that learned associations between environmental cues and drug reward are stored in small neuronal ensembles in nucleus accumbens (34), medial prefrontal cortex (mPFC) (35), and ventrolateral orbitofrontal cortex (36), and that intermingled ensembles in vmPFC exert opposing effects on food-seeking behavior (37). Only 2–12% of neurons were inactivated in each case, highlighting the specificity of activity-dependent manipulations (38).

The *Fos* promoter has also been recently used in viruses to drive optogenetic tools to reversibly manipulate the activity of recently activated neurons, revealing the roles of valence-encoding populations in behavior (39, 40). A caveat of transgenic and viral strategies in which IEG promoters directly drive effectors (Fig. 1A) is that effector expression is usually limited to a few hours after experience (Table 1). The next generation IEG-based tools were modular systems designed to drive longer-term effector expression with additional levels of temporal control and have been widely used to investigate the cellular basis of memory.

IEG promoters driving tTA allows manipulation of previously activated neurons

A long-standing question in the memory field is whether neurons activated during learning participate in the memory trace. To test this, the *Fos-tTA* transgenic mouse was developed, in which a *Fos* promoter fragment drives expression of the doxycycline (Dox)-repressible tetracycline transactivator (tTA). In the absence of Dox, a reporter allele tags activated cells with LacZ (driven by the tetracycline response element, *TRE*), which perdures for several days (Fig. 1B) (41). Many basolateral amygdala (BLA) neurons tagged during fear conditioning were reactivated (immunopositive for both LacZ and another IEG product Zif) during memory recall, suggesting they were part of a stable fear memory trace.

While LacZ can only be detected in fixed tissue, the flexible design of the *Fos-tTA* mouse facilitated subsequent studies in which *Fos-tTA* drove expression of *TRE*-driven chemogenetic and optogenetic tools to establish causal roles of neurons activated during learning in subsequent memory recall (42, 43). This technique revealed that dentate gyrus (DG) ensembles tagged in a neutral context could serve as a conditioned stimulus during contextual fear conditioning (CFC) (44). While tagged DG ensembles encoding a negative experience could switch their valence when artificially reactivated during a positive experience, BLA neurons could not. Switching valence in DG reduced tag/Fos overlap in BLA, suggesting that functional connectivity had changed (45). These studies indicate that DG encodes stable representations of environmental cues, and that DG→BLA links connect sensory representation to behavioral response. Recently, studies using *Fos-tTA* to express optogenetic tools in learning-related CA1 neurons revealed their role in activating downstream ensembles in retrosplenial cortex and nucleus accumbens to support contextual fear memory and social memory, respectively (46–48). Of note, silencing CA1 ensembles impaired memory, suggesting that *Fos-tTA* tagged CFC-related CA1 neurons with high efficiency (46).

Fos-tTA has also been used to map cellular and circuit changes in normal aging and disease states. Aged mice had deficits in freezing to photoactivation of CFC-related neural ensembles in CA1. These deficits were rescued by increasing cellular excitability in CA1, indicating that age-related reduction in neuronal excitability impairs aspects of memory (49). A mouse model of Alzheimer's Disease (AD) exhibited impairments in long-term episodic memory that could be rescued by reactivating DG neurons that were activated during conditioning, suggesting that memory failure in early AD reflects an impairment in retrieval rather than in encoding (50). Interestingly, photoactivating DG cells tagged during a positive experience rescued depressive behaviors, suggesting a therapeutic strategy (51).

While the *Fos-tTA* strategy answered fundamental questions about the cellular basis of learning, memory, age-related disease, and maladaptive states, induction of activity-dependent labeling was modest, ranging from ~2–4-fold above home cage levels. Because the slow metabolism of Dox results in a long labeling window on the order of days, background is likely high. Thus, *Fos-tTA* may only achieve workable signal-to-noise ratio (S/N) in brain regions with very low basal expression of *Fos.* Also, while reporter expression outlasts Fos protein, *Fos-tTA* cannot drive permanent effector expression, so manipulations

are limited to a few days after the experience (Fig. 1B). These limitations motivated the development of activity-reporter mice that allow permanent labeling of previously activated neurons.

IEG promoters driving Cre allow permanent labeling and manipulation of previously activated neurons

In TRAP (Targeted Recombination in Active Populations) lines, CreER^{T2} is knocked-in to the *Fos* or *Arc* translation start sites, yielding FosTRAP and ArcTRAP (52). When a neuron is activated in the presence of tamoxifen, CreER^{T2} translocates to the nucleus to recombine floxed alleles, resulting in permanent effector expression (Fig. 1C). Using the active metabolic form of tamoxifen (4-hydroxytamoxifen) limits the drug-active period to <12 hours (52), which is considerably shorter than the labeling window in *Fos-tTA*. FosTRAP drives reporter expression in sensory cortices and hippocampus following sensory stimulation, but some subcortical regions lack activity-dependent reporter expression. In contrast, ArcTRAP labels subcortical regions, but exhibits tamoxifen-independent labeling in cortico-thalamic projection neurons (52). ArcTRAP has been used to compare neurons across the whole brain that are activated by appetitive and aversive experiences, and allowed identification of molecular signatures of such neurons (40). Though no behavioral deficits were reported (52), it is important to note that CreER replaced the IEG coding sequence and displaced regulatory sequences, essentially creating IEG null alleles. Thus, these experiments were performed in heterozygous *Fos* and *Arc* mice. An advantage of BAC transgenics is that the gene of interest remains functional.

A conceptually similar ArcCreER^{T2} BAC-transgenic mouse (Fig. 1C) was developed to investigate the causal role of hippocampal ensembles in long-term memory. Using these mice to drive inhibitory optogenetic tools revealed the obligatory function of learning-activated hippocampal neurons in long-term memory storage and suggested that ArcCreER^{T2} labels CA1 ensembles with high efficiency (53). The same genetic strategy revealed that cortical amygdala neurons activated by attractive or aversive odors represent distinct ensembles that can bidirectionally control corresponding innate behaviors (54).

In CANE (Capturing and manipulating Activated Neural Ensembles), *2A-dsTVA* is knocked-in to the *Fos* locus after the *Fos* open reading frame, such that a destabilized TVA receptor for EnvA (a coat protein for an avian-specific virus) is expressed from the same mRNA as *Fos*. Companion lentiviruses (LV) pseudotyped with EnvA^{M21}, a mutant with reduced TVA-binding potency, infect dsTVA-expressing neurons to deliver effector proteins. CANE-LV-Cre achieves permanent labeling of previously activated neurons (Fig. 1D). CANE-captured social fear-activated neurons (SFNs) in the ventromedial hypothalamus (VMHvl) bi-directionally controlled social fear. Because SFNs comprise only 3% of VMHvl neurons and specific molecular markers are not available, it would have been difficult to target them without activity-based methods (55).

While these strategies support permanent access to previously activated neurons, the CreER^{T2}-based methods have variable S/N in different parts of the brain in part because, compared with Cre, CreER^{T2} has lower recombination efficiency even in the presence of

tamoxifen. Commensurate with *Fos-tTA*, salient experiences induced ~2–4-fold increases in labeling in the hippocampus and amygdala of TRAP and ArcCreER^{T2} mice. CANE may have an improved S/N, but requires virus injections immediately following the experience of interest, which could confound some behaviors. In addition, these strategies may not allow access to all cell-types due to inherent cell-type specificities of endogenous IEGs. Viral strategies based on synthetic promoters feature higher S/N and broader applicability across the cell-type spectrum.

Viral strategies using synthetic promoters improve signal-to-noise

E-SARE is a synthetic promoter in which five repeats of a synaptic-activity responsive element (SARE) (56) that regulates induction of Arc are fused with an *Arc* minimal promoter. *AAV-E-SARE-GFP* boasted much greater reporter induction than *AAV-Fos-GFP in vivo*. E-SARE-labeled populations were further validated by recording their physiological responses. E-SARE driving CreER^{T2} affords temporal control and permanent labeling of activated neurons (Fig. 1E) (57). RAM (Robust Activity Marking) is based on a synthetic enhancer module in which the AP-1 site and Npas4 binding sites are inserted into an element whose secondary structure is favorable for transcription activation. A small synthetic promoter (PRAM) consists of four synthetic enhancers upstream of a minimal *Fos* promoter. PRAM reported a higher induction ratio than E-SARE in culture. Higher than *Fos-tTA* and *ArcCreER^{T2}*, *AAV-PRAM-d2tTA* (a destabilized tTA, Fig. 1F) drove ~7-fold induction of *TRE*-dependent effectors in DG, and a ~37-fold induction in CA3 following CFC. As has been reported for Npas4 (58), these increases were learning-dependent. Although d2tTA had improved fold regulation over tTA, like *Fos-tTA,* RAM-driven effector expression was highest when animals were off Dox for 3 days (43). A Cre-dependent version of RAM (CRAM) effectively labels GABAergic neurons. Since the enhancer sequences are highly conserved, RAM may also be applicable to other species, as was shown in flies and rats (59).

These viral strategies bypass the need for transgenic mice and thus can be used in other species to access activated neurons and can be more conveniently used in mice (e.g., in combination with other genetic manipulations). However, a caveat viral methods share with CANE is the limited spatial reach of viral transductions. Only transgenic strategies such as TRAP, when combined with transgenic reporters, can identify activated neurons throughout the brain and thus can be used for unbiased mapping of whole-brain activity patterns.

Whole-brain imaging of activated neurons

By combining new imaging technologies with genetic strategies to label activated neurons, it is possible to map the brain-wide projections of activated neurons and screen brain-wide activity patterns following an experience. For example, the brain-clearing method CLARITY and light-sheet microscopy (60) have been used in combination with a viral *Fos* promoter-driven CreER^{T2} (Fig. 1C) to map the brain-wide projections of prefrontal cortical neurons activated by appetitive and aversive experiences (40). Similarly, *Fos-GFP* was used in combination with serial two-photon tomography to screen sex-specific activation patterns during social recognition and sex discrimination (61). Other studies used CLARITY (60), CUBIC (62), and iDISCO+ (63) to clear brains for whole-mount imaging of IEG reporter

mice or Fos immunostaining to characterize patterns of neurons activated by specific experiences. Such combinations of powerful technologies will generate new hypotheses about how neuronal activity underlies behavior, and what abnormalities in spatial patterns of activity may be associated with pathological conditions.

Ca^{2+}-based methods

Fluorescent reporters of intracellular Ca^{2+} concentration such as GCaMP are powerful tools for monitoring real-time neural activity *in vivo* (64). CaMPARI (calcium-modulated photoactivatable ratiometric integrator) is a GCaMP derivative that labels neurons activated during user-defined epochs (65). Composed of the photoactivatable protein mEOS2 fused to a calmodulin domain and M13 peptide, CaMPARI fluorescence tracks Ca^{2+} levels and photoconverts from green to red when UV illumination occurs coincidentally with Ca^{2+} influx (Fig. 1G), allowing subsequent characterization of previously activated neurons by tracking red fluorescence. As proof of principle, V1 neurons with the highest red-to-green fluorescence ratio were specifically tuned to the stimulus presented during photoconverting light pulses. CaMPARI also exhibits experience-specific labeling in zebrafish and *Drosophila* (65).

A complementary method available in *Drosophila* called TRIC (Transcriptional Reporter of Intracellular Ca^{2+}) captures brain-wide changes in neural activity over many hours and can drive effector gene expression for subsequent manipulation. In TRIC, calmodulin (CaM) and its target peptide MKII are fused to the transcriptional activation domain and the DNA binding domain of a transcription factor, respectively. When Ca^{2+} enters the cell, it brings the binding and activation domains together to drive transcription of effector genes (Fig. 1H). TRIC is particularly useful for monitoring and manipulating long-term changes in activity underlying physiological states in *Drosophila* (66).

These two Ca^{2+}-based strategies support different applications. While CaMPARI does not allow subsequent manipulation of activated neurons, it can label neurons during very brief epochs on the order of seconds. CaMPARI has low background, but the signal is limited by the rate of CaMPARI protein turnover. Being transcription based, TRIC has temporal resolution on the order of hours. However, as TRIC can drive effector expression it can be used to determine causal roles of activated populations in behavior. Though it has not been demonstrated in TRIC, both systems can theoretically be applied to multiple species, serving as alternatives to transgenic IEG-based tools.

Summary and Future Perspectives

Methods that provide genetic access to activated neurons allow users to specifically target behaviorally-relevant neurons in spatially intermingled, functionally heterogeneous populations. Many groups have applied these tools to elucidate the cellular basis of sensory coding, memory, and emotional valence, and to reveal how these processes are disrupted in aging or disease. An exciting new direction is to combine activity-based genetic strategies with whole-brain imaging and sequencing methods for unbiased mapping and profiling of activated populations.

With many options now available, it is critical to consider the advantages and caveats of each genetic strategy (Table 1) and choose the activity reporter best suited to each specific experimental question. Since different IEGs have unique induction profiles in different cell-types (38, 67, 68), it is critical to characterize IEG expression profiles for brain regions of interest before beginning experiments. With IEG promoters driving reporters to visualize activated neurons, the design of the transgene may dictate how faithfully the reporter follows activity. In tTA- and CreER^{T2}-based strategies, transgene expression and drug metabolism will determine labeling window. New viral strategies using synthetic promoters offer higher S/N and experimental flexibility, with the caveat that RAM labeling may be learning-dependent in hippocampus and amygdala. RAM, CaMPARI, and likely E-SARE and TRIC support labeling in other species, and CaMPARI boasts the shortest temporal window for labeling though it does not allow subsequent manipulation of active neurons.

The field should continue to explore how labeling by existing methods reflects neuronal activity and to improve labeling specificity. These efforts can direct development of new strategies that fill gaps in the existing toolset. For instance, new tools could specifically access neurons based on subthreshold depolarization of membrane potential (69). Several groups rigorously characterized the physiological response properties of labeled neurons (57, 65). Performing similar experiments using the other genetic strategies will elucidate the nature of labeled populations. For example, pairing chronic Ca^{2+} imaging with IEG-based reporters will reveal the cellular activity patterns preceding reporter labeling. Redesigning targeting alleles for existing tools could improve the spatial and temporal profiles of reporter expression throughout the brain. Furthermore, new reporters that allow users to genetically access multiple experiences in the same brain could directly compare two different experiences and help subtract away non-task relevant background cells. When characterizing new genetic strategies, side-by-side comparisons with existing technologies are particularly informative for potential users (59, 66). Developing methods that combine the advantages of existing tools (Table 1), such as the temporal precision of Ca^{2+}-based methods with genetic accessibility, will enhance our ability to interrogate the causal relationships between active populations, circuit function, and animal behavior.

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Highlights

- IEG promoters driving reporters allow targeting of recently active neurons *in vivo*.
- *Fos-tTA* mice drive effectors in previously active neurons for several days.
- IEG promoters drive CreER for permanent effector expression in once active neurons.
- Viruses with synthetic promoters drive effectors in active neurons with high S/N.
- New technologies allow whole-brain mapping of active populations.

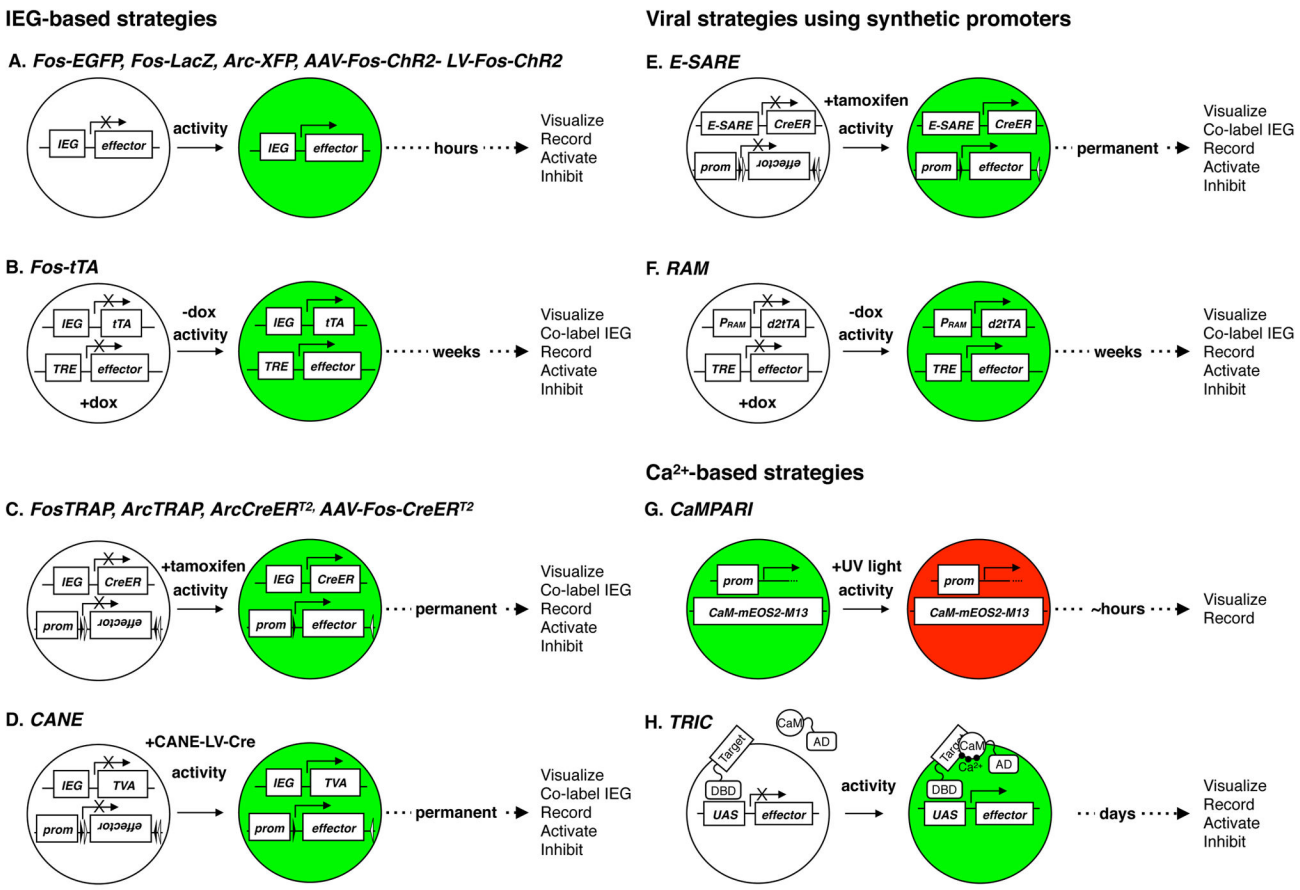

Figure 1.

Summary of genetic strategies to visualize or manipulate previously activated neurons. (A) *Fos* and *Arc* promoters drive fluorescent proteins or optogenetic tools in both transgenic animals and viruses. Typically, peak labeling occurs a few hours after an experience and effector proteins last less than a day. (B) In *Fos-tTA* transgenic mice, neural activity in the absence of Dox leads to expression of *TRE*-conditional effectors including LacZ, chemogenetic tools, and ontogenetic tools for days following an experience. (C) In TRAP and *ArcCreER*^{T2} transgenic mice, *Fos* and *Arc* promoters drive CreER^{T2} to achieve permanent expression of Cre-dependent effector proteins in previously activated neurons. (D) In CANE, *Fos* drives TVA. Injection of EnvA-pseudotyped lentivirus delivers Cre to recently activated neurons, resulting in permanent expression of Cre-dependent effector genes. (E) In E-SARE viruses, a synthetic promoter drives effectors, or CreER^{T2} to achieve permanent expression of Cre-dependent effector proteins in transiently activated neurons with high S/N. (F) In RAM viruses, a synthetic promoter drives destabilized tTA (d2tTA) which can drive expression of *TRE*-dependent effectors when activity occurs in the absence of Dox. (G) CaMPARI integrates a green fluorescent Ca^{2+} indicator with a photoconvertible protein. With the intersection of neural activity and user-delivered UV light, one can visualize recently activated neurons in red until the photoconverted protein is turned over. (H) TRIC uses Ca^{2+} to bring together DNA-binding (DBD) and activation (AD) domains of a transcription activator to drive effector genes to visualize or manipulate previously

activated neurons. TRIC operates on a slower timescale, optimal for measuring slow changes in physiological states.

Table 1

Summary and comparisons of different genetic strategies

Method	Construct	Delivery[1]	Window[2]	Perdurance[3]	Strengths	Caveats	Reference
IEG-based strategies							
Transgenic Mice							
Fos-LacZ	*Fos>FosLacZ*	Tg	< 6hrs	<1d	Daun02 inactivation	Requires fixed tissue to vis.	Smeyne, 1992
Fos-GFP	*Fos>FosEGFP*	Tg	< 6hrs	<1d	Visualize in living tissue, brain-wide	Impermanent, no manipulation	Barth, 2004
Arc-GFP	*Arc>d2EGFP*	KI	< 6hrs	<1d			Wang, 2006
Arc-dVenus	*Arc>dVenus*	Tg	< 6hrs	<1d			Eguchi, 2009
Arc-GFP	*Arc>EGFP*	BAC	< 6hrs	<1d			Gong, 2003
Fos-tTA	*Fos>tTA*	Tg	1–3 days	Weeks	Long-term, brain-wide	Background, long window	Reijmers, 2007
FosTRAP	*Fos>CreER^{T2}*	KI	<12 hrs	Permanent	Permanent	~Long window	Guenthner, 2013
ArcTRAP	*Arc>CreER^{T2}*	KI	<12 hrs	Permanent		Background, ~long window	
ArcCreER^{T2}	*Arc>CreER^{T2}*	BAC	~24h	Permanent		Not knock-in, ~long window	Denny, 2014
CANE	*Fos>Fos-2A-TVA*	KI	< 6hrs	Permanent	Permanent together with Cre virus	Limited temporal control; spatially limited	Sakurai, 2016
Transgenic Rats							
Fos-LacZ	*Fos>FosLacZ*	Tg	< 6hrs	<1d	Daun02 inactivation	Requires fixed tissue to vis.	Kasof, 1995
Viruses							
LV-Fos-ChR2	*LV-Fos>ChR2*	LV	< 6hrs	<1d	No transgene	Impermanent	Gore, 2015
AAV-Fos-ChR2	*AAV-Fos>ChR2*	AAV	< 6hrs	<1d	No transgene	Impermanent	Ye, 2016
E-SARE	*AAV-E-SARE>ER^{T2}CreER^{T2} -PEST*	AAV	< 6hrs	Permanent	High S/N, permanent	Spatially limited	Kawashima, 2013
RAM	*AAV-PRAM>mKate2*	AAV	1–3 days	Weeks	High S/N	Spatially limited, long window	Sorensen, 2016
AAV-Fos-CreER^{T2}	*AAV-Fos>ER^{T2}-CreER^{T2}-PEST*	AAV	< 6hrs	Permanent	No transgene, Permanent	Spatially limited	Ye, 2016
Calcium-based strategies							
CaMPARI	*promoter>CaM-mEOS2-M13*	AAV	> seconds	<1d	Short window	Impermanent	Fosque, 2015
TRIC	*promoter>CaM-AD; promoter>GAL4 DBD-CaM target; UAS>effector*	Tg (*Drosophi la*)	Hours	Days	Drives effectors	Long labeling window, background	Gao, 2015

[1]Abbreviation: Tg, random transgenic integration; KI, knock-in; BAC, bacterial artificial chromosome-mediated random transgenic integration, LV, lentivirus; AAV, adeno-associated virus.

[2]Refers to the window during which activity of neurons is captured.

[3]Refers to the period during which transiently active neurons can be observed and/or manipulated.