[*This is a protocol for inclusion in CPMB. There are 3 figures and 1 Table]

SPARK: a transcriptional assay for recording protein-protein interactions in a defined time window

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ABSTRACT

Protein-protein interactions (PPIs) are ubiquitously involved in cellular processes such as gene expression, enzymatic catalysis, and signal transduction. To study dynamic PPIs, real-time methods such as Förster resonance energy transfer and bioluminescence resonance energy transfer can provide high temporal resolution, but they only allow PPI detection in a limited area at a time and do not permit post-PPI analysis or manipulation of the cells. Integration methods such as the yeast two-hybrid system and split protein systems integrate PPI signals over time and allow subsequent analyses, but they lose information on dynamics. To address some of these limitations, an assay named SPARK (Specific Protein Association tool giving transcriptional Readout with rapid Kinetics) has recently been published. Similar to many existing integrators, SPARK converts PPIs into a transcriptional signal. SPARK, however, also adds blue light as a co-stimulus to achieve temporal gating; SPARK only records PPIs during light stimulation. Here, we describe the procedures for using SPARK assays to study a dynamic PPI of interest, including designing DNA constructs and optimization in HEK293T/17 cell cultures. These protocols are generally applicable to various PPI partners and can be used in different biological contexts.

Basic Protocol 1: Designing DNA constructs for SPARK

Basic Protocol 2: Performing the SPARK assay in HEK293T/17 cell cultures

Support Protocol 1: Lentivirus preparation

Support Protocol 2: Immunostaining of SPARK components

Keywords

protein-protein interaction, transcriptional assay, optogenetics, temporal gating

INTRODUCTION

Protein-protein interactions (PPIs) play fundamental roles in regulating cellular processes and biological functions, including signal transduction, enzyme activities, metabolism, cell cycle, and cellular structure formation (Braun & Gingras, 2012). The majority of cellular processes are controlled by transient PPIs, and detecting these interactions is of tremendous biological interest. A genetic assay named SPARK (Kim et al., 2017) that converts transient, binary PPIs into gene expression has recently been reported. In SPARK, one interacting partner is fused to a tobacco etch virus protease (TEVp), and the other partner, which is normally a transmembrane protein or is tethered to a transmembrane domain, is fused to a transcription factor (TF) (Figure 1). The TF component is designed to include a photoswitchable TEVp cleavage site (TEVcs), and the photoswitch makes SPARK gated by externally-applied blue light. As such, the TF can only be released from the plasma membrane when both light (which uncages TEVcs) and PPI (which brings TEVp in close proximity to TEVcs) are present. By using light for temporal control, SPARK allows the detection of PPIs in a user-defined period, a feature distinct from other available transcriptional assays such as yeast two-hybrid (Fields & Song, 1989) and TANGO (Barnea et al., 2008). In addition, SPARK shows higher signal-to-noise ratio

(Kim et al., 2017) than a conceptually similar temporally-gated transcriptional assay, iTANGO (Lee et al., 2017).

Here, we describe the design and optimization of a SPARK assay for detecting a PPI of interest. Basic Protocol 1 details the design of the three SPARK components: TEVp, TF, and the reporter gene. Basic Protocol 2 illustrates how to perform a SPARK assay in a HEK293T/17 cell culture. Further, we describe the procedures for preparing lentivirus for efficiently transducing HEK293T/17 cells with the SPARK components (Support Protocol 1), and the procedures for performing immunostaining on HEK293T/17 cells to check the expression of the SPARK components (Support Protocol 2). We present representative results from two PPI pairs with sub-nanomolar and low single-digit micromolar affinities, respectively. Of note, the interaction between both PPI pairs described here are induced by a chemical (referred to as 'stimulant' in the protocol steps); PPI pairs that do not require induction have not yet been tested by SPARK.

[*Figure 1 near here]

BASIC PROTOCOL 1: DESIGNING DNA CONSTRUCTS FOR SPARK

This protocol details the design of the three SPARK components (Figure 1): a TEVp fused to a cytosolic PPI interacting partner (called the "SPARK TEVp component"), a TF fused to the membrane-bound PPI interacting partner (either a transmembrane protein itself or tethered to a transmembrane domain) via a photoswitchable TEVcs (called the "SPARK TF component"), and a reporter gene component. The design of each component is presented in the order of N-terminus to C-terminus. Note that this protocol is just a discussion on how to *design* the constructs, but not a step-by-step protocol on how to perform the actual cloning. For cloning protocols, readers are directed to (Kramer & Coen, 2006; Lohman, Tabor, & Nichols, 2011).

Supplementary Information (Supplementary Text 1, 2, and 3) lists commonly used amino acid and DNA sequences for the SPARK TEVp component, TF component, and reporter gene component, respectively. All recommended designs are optimized for testing in HEK293T/17 cells using lentiviral transduction. For applications in neurons, readers are directed to (Kim et al., 2017; Wang et al., 2017).

TEVp and TF components need to be cloned into the pLX208 lentiviral vector backbone, and the reporter component needs to be cloned into the pFPGW lentiviral vector backbone. Both vector backbones are available from Addgene. To replace the open reading frame in the pLX208 vector, users can digest the plasmid with BamHI and MluI restriction enzymes. To replace the open reading frame in the pFPGW vector, users can digest the plasmid with AgeI and AscI restriction enzymes. Standard cloning techniques such as polymerase chain reaction (Kramer & Coen, 2006), ligation reaction (Lohman et al., 2011), and Gibson assembly can be used to clone in new open reading frames. For first-time users, we recommend using commercially synthesized gene fragments, such as gBlocks gene fragments from Integrated DNA Technologies or gene fragments from Twist Bioscience, to avoid complex cloning. It is recommended to include unique restriction sites in appropriate places of each construct to allow further modifications.

Materials

pLX208 vector backbone for the SPARK TF and TEVp components (Addgene, plasmid #104846, RRID:Addgene_104846, a gift from Alice Ting)

pFPGW vector backbone for the SPARK reporter gene component (Addgene, plasmid #125232, RRID:Addgene_125232, a gift from Alice Ting)

BamHI-HF restriction enzyme (New England BioLabs, cat. no. R3136S)

MluI-HF restriction enzyme (New England BioLabs, cat. no. R3198S)

AgeI-HF restriction enzyme (New England BioLabs, cat. no. R3552S)

AscI restriction enzyme (New England BioLabs, cat. no. R0558S)

Protocol Steps

Designing the DNA Construct for the SPARK TEVp Component

1. Select a fluorescent protein as an expression marker and place it at the very N-terminus of the SPARK TEVp component. Immediately adjacent to the C-terminus of the fluorescent protein, place a self-cleaving peptide P2A.

   We highly recommend using fluorescence imaging as the detection method when using a new pair of PPI partners in SPARK, as it allows detection of all three SPARK components simultaneously and expedites troubleshooting. The fluorescent protein on the SPARK TEVp component will help validate expression of this DNA construct. If the user decides to use a non-fluorescence-based method, the fluorescent protein and P2A will be unnecessary, though we advise against such design during assay optimization. Fluorescent proteins of any color can be used as long as they are compatible with your microscope, are bright, have good photostability, and do not overlap with the fluorescence (including immunofluorescence) from the SPARK TF and reporter gene components. We routinely use mTurquoise2, EGFP, or mCherry as cyan, green, or red fluorescent protein in this component, respectively. Note that mTurquoise2 is not compatible with the nuclear stain DAPI, which may be used together with immunostaining (Support Protocol 2) to determine the transduction efficiency of the lentiviruses. P2A induces ribosomal skipping during translation, separating the fluorescent protein from the rest of this component. This reduces the chance of steric

hinderance between the SPARK TEVp component and the TF component when PPI occurs.

2. Design the cytosolic PPI partner (partner "A" in Figure 1) fused to TEVp. After P2A, first fuse a nuclear export signal. Then, add the cytosolic PPI partner, a linker, and a truncated TEVp (1-219) with S153N and S219V mutations. When establishing the assay for the first time, test both fusing the PPI partner before and after the TEVp, and screen different linker lengths (e.g., 10 and 20 amino acids with GGGS repeats) between the PPI partner and the TEVp to find the configuration that gives the highest signal-to-noise ratio. For commonly used sequences, see Supplementary Text 1 in the Supplementary Information. For an example, see Supplementary Text 4 in the Supplementary Information.

The nuclear export signal keeps the protein in the cytosol, increasing the chance of its interaction with the membrane-bound PPI partner. If needed, an epitope tag (we routinely use HA tag) can be added to the N-terminus of the nuclear localization signal. This allows checking the expression level and localization of the protein through immunostaining, though, in most cases, the expression level of the fluorescent protein before P2A suffices. If both PPI partners are normally cytosolic, we recommend testing both the SPARK system with either of the PPI partner on the SPARK TEVp component. The fusion order of the PPI partner and TEVp can be a determining factor to achieve optimal geometry for PPI-induced protease cleavage in SPARK. Therefore, a new SPARK assay must test both configurations. The length of the linker between the PPI partner and TEVp is also important. We recommend testing linkers with 10 and 20 amino acids in length to ensure there is enough flexibility for the TEVp to reach the TEVcs

when the PPI takes place. The S219V mutation in the truncated TEVp (1-219) prevents autolysis of TEVp (Kapust et al., 2001)*. The S153N mutation enhances the initial turnover rate by 5.4-fold and could increase SPARK signal by >10-fold* (Sanchez & Ting, 2020)*. Truncated TEVp (1-219) without the S153N mutation can also be used in SPARK (Supplementary Text 1* in the Supplementary Information*), though it gives lower signal. We do not recommend using full-length TEVp (1-242) as it gives high background.*

Designing the DNA Construct for the SPARK TF Component

3. Select the transmembrane protein domain in the SPARK TF component. If one of the PPI partners is normally membrane-bound (e.g., G protein-coupled receptors, receptor tyrosine kinases), use it for the SPARK TF component. Otherwise, select a transmembrane protein domain with good plasma membrane trafficking/localization, and fuse a PPI interacting partner to the C-terminus of the transmembrane protein domain. We recommend using the transmembrane domain from CD4 (Feinberg et al., 2008). For commonly used sequences, see Supplementary Text 2 in the Supplementary Information. For an example, see Supplementary Text 4 in the Supplementary Information.

A transmembrane protein domain ensures that the TF component is excluded from the nucleus until the light- and PPI-dependent protease cleavage takes place. We advise against using CAAX motifs as they have been found to give high background. If both PPI partners are normally cytosolic, test both SPARK systems with either of the PPI partners fused to the transmembrane domain. Surface trafficking signal peptides, such as amino acids 1-20 from the mouse Ig kappa chain V-III region MOPC 63 protein (UniProt P01661), should be placed at the very beginning of the N-terminus of the TF construct

for effective plasma membrane trafficking/localization (Supplementary Text 2 in the Supplementary Information*). For GPCRs, we recommend fusing a nuclear export signal (e.g., amino acid sequence ELAEKLAGLDIN) to the immediate C-terminus. For CD4, we recommend fusing a modified N-terminal sequence of amino acids 1-171 (CIB1N, later also known as CIBN)* (Liu et al., 2008) *from the Arabidopsis cryptochrome-interacting basic-helix-loop-helix protein (CIB1, UniProt A0A178V495) to the C-terminus to enhance membrane trafficking* (Wang et al., 2017)*. A flexible linker (e.g., 5, 10, 15, 20, 25 amino acids with GGGS repeats) should be added both after the C-terminus of the PPI partner and, if applicable, between the membrane anchor and the PPI partner. The specific linker length depends on the geometry of the PPI partners; we recommend starting with 10 amino acids and screening other lengths should this not be optimal.*

4. Select a light, oxygen, voltage sensing domain (LOV) with TEVcs. When establishing a SPARK assay for the first time, test both eLOV and hLOV1 (Kim et al., 2017), two variants of the second LOV domain from *Avena sativa* phototropin 1. Immediately after the C-terminus of eLOV or hLOV1, fuse the optimal TEVcs sequence for SPARK, ENLYFQM. After the TEVcs, add a flexible linker with at least four amino acids (e.g., GGGS) to facilitate cleavage by TEVp. For commonly used sequences, see Supplementary Text 2 in the Supplementary Information. For an example, see Supplementary Text 4 in the Supplementary Information.

In general, hLOV1 gives better caging than eLOV, and will produce both lower signal and lower background. eLOV is recommended when the light window is short (< 5 min) or the expression level of the SPARK components is low. Conversely, hLOV1 is recommended when the light window is long (> 5 min) or when the expression level of

the SPARK components is high. The activity of the TEVcs sequence ENLYFQ/M (cleaved between Q and M) has been found to be optimal for SPARK assays (Kim et al., 2017)*.*

5. Select a TF. For testing in HEK293T/17 cultures, we recommend using the yeast Gal4 which binds to an upstream activating sequence (UAS) in the SPARK reporter gene component.

   Theoretically, other transcription factors not naturally found in HEK293T/17 cells can also be used as the TF. However, we have found that the use of stronger TF such as VP16 results in high background. We use a 260-amino-acid Gal4 that includes both the transactivation domain and the DNA-binding domain (Supplementary Text 2 in the Supplementary Information*).*

6. Select an epitope tag. Include an epitope tag at the C-terminus of the SPARK TF component.

   An epitope tag allows checking the expression and membrane trafficking/localization of the SPARK TF component through immunostaining. We routinely use V5 tag (amino acid sequence GKPIPNPLLGLDST), but other epitope tags such as FLAG (amino acid sequence DYKDDDDK) and HA (amino acid sequence YPYDVPDYA) should also work.

Designing the DNA Construct for the SPARK Reporter Gene Component

7. Place a UAS promoter upstream to the reporter gene for Gal4 to bind and initiate gene transcription. We recommend keeping the UAS in the SPARK reporter gene plasmid template (Addgene plasmid #125232).

8. Select a reporter gene. Users can use any reporter gene to identify or manipulate SPARK-positive cells for their final applications, including fluorescent proteins for fluorescent imaging and fluorescence-activated cell sorting, luciferase for luminescence assays, β-galactosidase for colorimetric assays, and rhodopsins or DREADDs for optogenetic and chemogenetic applications, respectively. When establishing a SPARK assay for the first time, we highly recommend choosing a fluorescent protein as the reporter gene. We routinely use citrine and mCherry. For commonly used sequences, see Supplementary Text 3 in the Supplementary Information. For an example, see Supplementary Text 4 in the Supplementary Information.

   Fluorescence imaging allows detection of all three SPARK components simultaneously and helps with troubleshooting. We, therefore, recommend using fluorescence imaging to first establish a new PPI partner in SPARK, and then switching the reporter gene for the final application if necessary. The color of the fluorescent reporter must not overlap with the fluorescence (including immunostaining fluorescence) from the SPARK TEVp or TF components.

Using Basic Protocol 1, users should be able to design and clone the three SPARK components for any PPI of interest. An example of the overall design of the open reading frame (ORF) of

three SPARK components (with rat β-arrestin2 as the cytosolic PPI partner, β_2-adrenergic receptor as the membrane-bound PPI partner, and UAS-mCherry as the reporter gene component) is shown in Supplementary Text 4 in the Supplementary Information.

BASIC PROTOCOL 2: PERFORMING THE SPARK ASSAY IN HEK293T/17 CELL CULTURES

This protocol describes the steps for performing SPARK assays in HEK293T/17 cell cultures, including seeding cells, lentiviral transduction, stimulation, imaging, and data analysis. Due to the complexity of SPARK, we highly recommend performing the initial optimization in HEK293T/17 cells and using fluorescence microscopy, as such assay has high transduction efficiency and has the advantage of allowing visualization of all SPARK components simultaneously. Before performing this protocol, users must have designed the three SPARK components according to Basic Protocol 1, cloned all SPARK components, and have produced lentiviruses of the three components according to Support Protocol 1.

All steps should be performed in a biosafety level 2 (BSL-2) laboratory following standard regulatory procedures. All solutions and equipment in contact with cells must be sterile. All culture incubations should be performed in a humidified 37 °C, 5% CO_2 incubator unless otherwise specified. For illustrative purposes, we describe procedures for performing SPARK experiments in a 48-well plate with a surface area of 1 cm^2/well. For well plates with different surface areas, volumes of all reagents and number of cells should be adjusted accordingly, unless otherwise specified.

Materials

24- or 48-well plate (24-well glass: Cellvis, cat. no. P24-1.5H-N; 48-well plastic: Greiner, cat. no. 677102)

Human fibronectin (HFN, MilliporeSigma, cat. no. FC010). *Dissolve and aliquot HFN to 0.5 mg/mL in ultrapure water. Dissolved HFN can be stored in -20 °C for at least three years. Before use, dilute HFN to 25 μg/mL in ultrapure water. Diluted HFN can be stored at 4 °C for at least half a year.*

Ultrapure water (Invitrogen, cat. no. 10977)

HEK 293T/17 cells (ATCC, cat. no. CRL-11268)

Complete cell culture media (see Reagents and Solutions)

Lentiviruses of three SPARK components (prepared according to Support Protocol 1)

Aluminum foil

Humidified 37 °C, 5% CO_2 incubator (Eppendorf, cat. no. Galaxy 170 S)

Hemocytometer (Hausser Scientific, cat. no. 3100), to be used together with 0.4% Trypan blue solution (Gibco, cat. no. 15250061)

37 °C water bath (Thermo Scientific, cat. no. TSGP10)

White or blue LED light source or ambient room light source with at least 0.5 mW/cm^2 of light power

Programmable digital timer outlet (Nearpow, Togoal, or similar brand)

Nikon inverted confocal microscope equipped with 10× air, 20× air, and 60× oil-immersion objectives, Yokogawa CSU-X1 5000RPM spinning disk confocal head, Ti2-ND-P perfect focus system 4, an ORCA-Flash 4.9 LT+sCMOS camera, and a compact 4-line laser source: 405 nm (100 mW), 488 nM (100 mW), 561 nM (100 mW), and 640 nm (75 mW); or fluorescence microscopes with similar capacities.

Protocol Steps

1. One day before lentiviral transduction, passage HEK293T/17 cells to 40% confluence in complete cell culture media (see "Reagents and Solutions"). One T25 (25 cm^2) is sufficient for all wells in a 48-well plate (1 cm^2/well).

   40% confluent HEK293T/17 cells will grow to 80-90% confluence overnight. We have found 80-90% cells to be most healthy for lentiviral transduction.

2. Coat a 48-well plate with 200 µL per well of 25 µg/mL human fibronectin (HFN) in ultrapure water. For each set of SPARK experiment, prepare two plates, for light and dark conditions. For each plate, coat at least two wells, for with-stimulant and no-stimulant conditions. Let the plates sit in the incubator for at least 10 min. Aspirate HFN.

   We routinely use plastic 48-well plates for imaging purposes, but glass-bottom 24-well plates can be used for obtaining higher quality images. HFN helps cell attachment and ensures good cell health after lentiviral transduction.

3. Resuspend the 80-90% confluent cells from Step 1 in pre-warmed complete cell culture media, count cells with a hemocytometer, dilute to 500,000 cells/mL and seed 200 µL of cells per well. Shake the plates gently back and forth, and then left and right for a couple times to ensure cells distribute evenly. Avoid circular motion. Let the plate sit in the incubator for 1-5 hours until most cells are settled.

   Users can proceed to the next step when most cells attach to the wells, which usually happens within 2 hours. The initial cell confluence is around 50%. For optimal cell

health, we recommend using fresh aliquots of complete cell culture media (that has not been previously subjected to warm-cool cycles) to perform this step.

4. Thaw lentiviruses (prepared according to Support Protocol 1) in a 37 °C water bath. Immediately after thawing, prepare a viral master mix for all wells to be transduced, by mixing the lentiviruses for all three SPARK components. For each well in a 48-well plate, mix 100 µL of each virus (i.e. for each well, prepare a ~300 µL viral master mix, containing 100 µL of each virus).

   Viral master mix will ensure all wells receive the same amount of lentiviruses. Once thawed, lentiviruses must be mixed and added to wells immediately to prevent loss of activity.

5. Add 280 µL of the viral master mix to each well. Shake the plates gently back and forth, and then left and right for a couple times to ensure viruses distribute evenly.

   . Following the lentivirus preparation protocol detailed in Support Protocol 1, we routinely get close to 100% transduction efficiency. To check transduction efficiency, perform immunostaining and nuclear staining as described in Support Protocol 2.

6. Wrap both light and dark plates in aluminum foil. Make sure to leave some space between the plates and the aluminum foil to ensure sufficient air flow. Incubate for 40-56 hours (2 days).

Light should be avoided for both plates during incubation by wrapping plates with aluminum foil, as extended light exposure opens up the LOV domain and can lead to high background in the absence of PPI. If spraying ethanol for disinfection, wrap the plates with aluminum foil first before spraying the outside of the wrap. For lentiviral transduction, proteins should be well expressed in 2 days. Users can check the level of protein expression by using the fluorescent protein on the SPARK TEVp component.

7. Unwrap both plates in a dark room with only a red light source that is just sufficient to allow viewing the plates (dim lights from computer monitors will not stimulate SPARK). For no-stimulant wells, aspirate and replace media with 200 µL of pre-warmed complete cell culture media. For with-stimulant wells, aspirate media and replace with 200 µL of pre-warmed complete cell culture media that contains the stimulant. Wrap the dark plate in aluminum foil and shine light on the light plate. After stimulation for the desired length of time, wash the with-stimulant wells three times with 200 µL of pre-warmed complete cell culture media (each time in the dark room), wrap both plates with aluminum foil, and incubate for 18-30 h (overnight).

Aspirating lentivirus from all wells and replacing with fresh cell culture media can enhance cell health. To avoid disturbing the cells, use a manual pipette to slowly draw media from the bottom edge of each well, and add media by slowly dispensing to the side wall of each well. Do not draw or dispense too quickly, or drop media directly on top of cells. LOV domain requires only < 0.5 mW/cm^2 of light power to be uncaged (Pudasaini & Zoltowski, 2013)*, and it has been previously found that light stimulation with ordinary room light source has the same effect of a blue LED array* (Kim et al.,

2017). *In our hands, cells can be stimulated continuously for at least 30 min without observing phototoxicity, but users are encouraged to test with their own light source. If longer light stimulation is desired, we recommend doing "5 seconds on, 20 seconds off" light intervals using a timer switch. When establishing a SPARK assay for a PPI pair for the first time, we recommend using a high dose of the stimulant*.

8. Perform fluorescence imaging. For each well, acquire 6-12 fields of view using 10× or 20× objective with a region of interest (ROI) of 11 mm × 11 mm. For each field of view, acquire images using two different channels: acquire one image with the SPARK TEVp component fluorescent protein, and another with the SPARK reporter gene component fluorescent protein.

   Proper image acquisition techniques are key to the success of SPARK. For novice users, we recommend consulting your fluorescence microscope manufacturer for advice and training. Avoid the edge of the wells, as cell density tends to be drastically different from the rest of the well. During imaging, find the optimal focal plane for each field of view (using autofocusing if available) using the fluorescent protein on the SPARK TEVp component, and re-focus when changing field of view. Adjust laser power and exposure time so that signal is distinct from the background.

9. If applicable, perform immunostaining according to Support Protocol 2, and re-image your samples.

Immunostaining is not required for SPARK. However, if your first experiment does not work, it will provide valuable information about the expression and localization of the SPARK TF component. See the Troubleshooting section for details.

10. If quantitative analysis is desired, calculate signal-to-noise ratio. Apply a mask or threshold to the images with SPARK reporter gene component such that it is just above the background. Calculate the total fluorescence intensity above the threshold from each field of view by multiplying mean fluorescence intensity with total object area. Then, subtract total fluorescence intensity with total background intensity (calculated by multiplying the mean intensity value of a no-reporter-expression area with the total object area). For each of the four SPARK conditions (+light+stimulant, +light-stimulant, -light+stimulant, -light-stimulant), calculate the background-subtracted total fluorescence intensity from all field of views collected, and display as a dot plot.

Consult your fluorescence microscope manufacturer for available image analysis programs. We use General Analysis 3 from the Nikon NIS-Elements analysis module. For quantitative analysis, we recommend one technical replicate (one well per SPARK condition) and two biological replicates.

Representative fluorescence microscopy images of two SPARK assays with different PPI affinities are shown in Figure 2.

[*Figure 2 near here]

SUPPORT PROTOCOL 1: LENTIVIRUS PREPARATION

This protocol describes the procedures for preparing second-generation lentiviruses of the three SPARK components (Basic Protocol 1) for HEK293T/17 cell transduction (Basic Protocol 2). We find this protocol to robustly produce high-quality lentiviruses for SPARK assays. If users prefer to use third-generation lentiviruses, they are directed to (Gill & Denham, 2020) for a detailed protocol. For illustrative purposes, we will describe the procedures using a T25 cell culture flask. For preparing lentiviruses in flasks with different sizes, volumes of all reagents and number of cells should be adjusted accordingly, unless otherwise specified.

Materials

HEK293T/17 cells (ATCC, cat. no. CRL-11268)

6-well plate (Greiner, cat. no. 647160), T25 culture flask (Alkali Scientific, cat. no. TVN0025), or T75 culture flask (Alkali Scientific, cat. no. TVN0075).

Human fibronectin (MilliporeSigma, cat. no. FC010)

Ultrapure water (Invitrogen, cat. no. 10977)

Complete cell culture media (see Reagents and Solutions)

Dulbecco's Modified Eagle Medium (DMEM, Gibco, cat. no. 11885084)

pVSVG lentiviral envelope plasmid (This material is not publicly available. Please request from authors)

Δ8.9 lentiviral packaging (helper) plasmid (This material is not publicly available. Please request from authors)

Plasmids of the three SPARK components (designed according to Basic Protocol 1)

HiSpeed Plasmid Maxi Kit (Qiagen, cat. no. 12662 or 12663)

Polyethylenimine hydrochloride (PEI MAX, Polysciences, cat. no. 24765-100). *Dissolve PEI MAX to 1 mg/mL in ultrapure water, adjust pH to 7.0. Dissolved PEI MAX can be stored at -20 °C for at least 3 years. Thawed PEI MAX can be stored at 4 °C for at least a month.*

Liquid nitrogen

Humidified 37 °C, 5% CO_2 incubator (Eppendorf, cat. no. Galaxy 170 S)

Centrifuge (Eppendorf, cat. no. 5810R)

Protocol Steps

1. On the day before lentivirus preparation, passage HEK293T/17 cells to 40% confluence in complete cell culture media. One T25 cell culture flask will produce 6 mL of lentivirus, enough for lentiviral transduction for 60 wells of cells growing in 48-well plates (1 cm^2/well).

   40% confluent HEK293T/17 cells will grow to 80-90% confluence overnight. We have found 80-90% cells to be most healthy for lentivirus preparation.

2. Coat a T25 cell culture flask with 1 mL of 25 µg/mL HFN in ultrapure water. Let the plate sit in the incubator for at least 10 min. Aspirate HFN.

   HFN helps cell attachment and ensures good cell health for transfection.

3. Resuspend the 80-90% confluent cells from Step 1 in pre-warmed complete cell culture media, count cells with a hemocytometer, dilute to 700,000 cells/mL and seed 1 mL of cells per T25 flask. Shake the flask gently back and forth, and then left and right for a

couple times to ensure cells distribute evenly. Avoid circular motion. Let the flask sit in the incubator for 1-5 hours until most cells are settled.

Users can proceed to the next step when most cells attach to the flask, which usually happens within 2 hours. The initial cell confluence will be around 80%. For optimal cell health, we recommend using fresh aliquots of complete cell culture media (that has not previously been subjected to warm-cool cycles) to perform this step.

4. For every T25 flask, prepare a lentiviral plasmid master mix by mixing 250 µL of DMEM (no FBS) with 250 ng of pVSVG lentiviral envelope plasmid and 2250 ng of Δ8.9 helper plasmid.

pVSVG and Δ8.9 are lentiviral envelope plasmid and packaging (helper) plasmid, respectively. To ensure high plasmid DNA quality, pVSVG and Δ8.9 must be prepared using a Maxiprep. We routinely use the Qiagen Plasmid Maxi kit (see Materials) and store plasmid DNA in ultrapure water. Plasmid DNA amplified by Maxiprep and stored in ultrapure water can be stored at 4 °C for at least a year.

5. For every T25 flask, mix 250 µL of the lentiviral plasmid master mix with 2500 ng of the appropriate plasmid DNA, i.e., plasmid DNA of one of the SPARK components. Thoroughly mix by pipetting. Prepare one mix per SPARK component plasmid.

To ensure high plasmid DNA quality, we recommend using freshly-amplified plasmid DNA of the SPARK components within 2 weeks. Viral DNA can be prepared through Miniprep and stored at room temperature or 4 °C for at least 3 years.

6. Add 25 µL of 1 mg/mL PEI MAX to each mix. Thoroughly mix by pipetting. Let the mixture sit at room temperature for at least 10 min.

   All plasmid DNA has to be added and thoroughly mixed before PEI MAX is added. Do not pre-mix PEI MAX with any plasmid DNA.

7. Add 1 mL of pre-warmed complete cell culture media to each mix, and transfer the entire mixture to each T25 flask. Gently shake the flasks back and forth, and then left and right for a couple times to ensure the PEI-plasmid DNA mixture distributes evenly.

8. Incubate for 48-56 hours (2 days).

9. Aliquot lentivirus-containing supernatant into 1.5-mL Eppendorf tubes and discard the attached cells. Flash freeze in liquid nitrogen and then store at -80 °C.

   Cells producing lentivirus will get very unhealthy at this point. Typically, this will not affect the lentivirus quality or subsequent experiments. However, if clumps of cells can be seen lifted by naked eye, users should centrifuge the supernatant at 200 × g for 2 min in a spinning-bucket centrifuge to remove solid particles. Otherwise, filtration through a 0.45 µm syringe filter unit can be performed to remove debris.
   Users should aliquot and freeze lentiviruses as fast as possible to preserve viral quality. Lentiviruses can be stored in -80 °C for at least a year. We do not titer lentiviruses. If desired, users may measure transduction efficiency using immunostaining and nuclear staining (Support Protocol 2).

SUPPORT PROTOCOL 2: IMMUNOSTAINING OF SPARK COMPONENTS

This protocol describes the procedures for immunostaining for the SPARK TEVp component and TF component in HEK293T/17 cells. Immunostaining is not an essential step for SPARK but can provide valuable information about the expression and localization of the SPARK TEVp and TF components. Immunostaining for the SPARK TEVp component is usually unnecessary as the fluorescent protein before P2A is normally a good indication of its expression, unless there is a concern that the protein after P2A may be mistrafficked or degraded. Immunostaining for the SPARK TF component can be used to evaluate the expression and membrane trafficking/localization of this membrane-bound protein. This protocol also describes how to simultaneously perform nuclear staining on the same samples, which can be used to determine the transduction efficiency of lentiviruses. Users can perform immunostaining using samples from post-stimulated SPARK assays (Basic Protocol 2, after Step 9). Alternatively, users can transduce individual components into HEK293T/17 cells and perform immunostaining two days after transduction. Always include an untransduced control. Formaldehyde in the fixative is a health hazard. Users must be properly trained to use formaldehyde, use appropriate personal protective equipment, and perform all related procedures in a fume hood. For illustrative purposes, we describe procedures for performing immunostaining and nuclear staining in a 48-well plate format with a surface area of 1 cm^2/well. For well plates with different surface areas, volumes of all reagents should be adjusted accordingly, unless otherwise specified.

Materials

HEK293T/17 Cells from Basic Protocol 2, after Step 6 or Step 9

Immunostaining fixative with 4% formaldehyde (see Reagents and Solutions)

Phosphate buffered saline (see Reagents and Solutions)

Cold Methanol (-20 °C)

Bovine serum albumin (Dot Scientific, cat. no. DSA30075-100)

Primary antibody – mouse anti-FLAG tag M2 antibody (MilliporeSigma, cat. no. F3165)

Primary antibody – rabbit anti-HA tag antibody (Cell Signaling Technology, cat. no. 3724S)

Primary antibody – mouse anti-V5 tag antibody (Invitrogen, cat. no. R960-25)

DAPI nuclear staining dye (Bio-Rad, cat. no. 1351303) *Dissolve DAPI to a final concentration of 100 ug/mL with ultrapure water. Dissolved DAPI can be stored at 4 °C for at least three years.*

Secondary antibody – goat anti-mouse IgG antibody, Alexa Fluor 488 (Invitrogen, cat. no. A-11001)

Secondary antibody – goat anti-mouse IgG antibody, Alexa Fluor 568 (Invitrogen, cat. no. A-11004)

Secondary antibody – goat anti-mouse IgG antibody, Alexa Fluor 647 (Invitrogen, cat. no. A-21235)

Secondary antibody – goat anti-rabbit IgG antibody, Alexa Fluor 488 (Invitrogen, cat. no. A-11008)

Secondary antibody – goat anti-rabbit IgG antibody, Alexa Fluor 568 (Invitrogen, cat. no. A-11011)

Secondary antibody – goat anti-rabbit IgG antibody, Alexa Fluor 647 (Invitrogen, cat. no. A-21244)

All primary and secondary antibodies in this list can be diluted with PBS to 1 mg/mL and stored at 4 °C for at least a year. For long-term storage, follow manufacturer's instructions.

Plate shaker (ThermoFisher, cat. no. 88861025, or Benchmark Scientific, cat. no. BT3001)

Protocol Steps

1. Aspirate media from wells with HEK293T/17 (from Basic Protocol 2, after Step 6 or Step 9) by using a manual pipette to slowly draw media from the bottom edge of each well.

2. Add 200 µL of fixative gently to the side wall of each well in a 48-well plate. Let it sit at room temperature for 15 min. Avoid shaking.

3. Aspirate fixative. Wash three times with 200 µL of 1× PBS each time by repeating the aspiration and addition steps.

   After washing with PBS, samples may be stored at 4 °C for up to 16 h (overnight) before further processing. For best results, we recommend performing all steps on the same day.

4. Aspirate PBS. Add 200 µL of -20 °C methanol gently to the side wall of each well. Immediately incubate the plate at -20 °C for 5 min. Avoid any shaking, tilting, or disturbance to the sample.

5. Aspirate methanol immediately after incubation. Wash three times with 200 µL of 1× PBS each time.

6. Dilute primary antibodies to final concentrations of 1 µg/mL each in 1× PBS with 0.01 g/mL bovine serum albumin. If also performing nuclear staining, add DAPI to the

antibody mixture to a final concentration of 0.1 µg/mL. Each well in a 48-well plate will require 200 µL of the antibody solution.

1× PBS with 0.01 g/mL bovine serum albumin can be stored at -20 °C for at least a year, and is good for at least three freeze-thaw cycles.

7. Aspirate PBS. Add 200 µL of primary antibody/DAPI mixture to each well. Incubate at room temperature and room light for 30 min with gentle shaking on a tilting or orbital shaker.
8. Aspirate primary antibody/DAPI mixture. Wash each well three times with 200 µL of 1× PBS each time.
9. Dilute secondary antibodies to final concentrations of 1 µg/mL each in 1× PBS with 0.01 g/mL bovine serum albumin. Each well in a 48-well plate will require 200 µL of the antibody solution.
10. Aspirate PBS. Add 200 µL of the secondary antibody mixture to each well. Incubate at room temperature and room light for 20 min with gentle shaking on a tilting or orbital shaker.

    Prolonged incubation with secondary antibody can lead to non-specific binding. Do not over incubate.

11. Aspirate secondary antibody mixture. Wash three times with 200 µL of 1× PBS each time, and leave 200 µL of 1x PBS in each well.

The samples are now ready for fluorescence imaging.

A representative immunofluorescence image of SPARK TF component is shown in Figure 3.

[*Figure 3 near here]

REAGENTS AND SOLUTIONS

Complete cell culture media

500 mL Dulbecco's Modified Eagle Medium (DMEM, Gibco, cat. no. 11885084)

500 mL Minimum Essential Medium (MEM, Gibco, cat. no. 11095080)

100 mL fetal bovine serum (FBS, Biowest, cat. no. S1620)

20 mL 1 M HEPES (Gibco, cat. no. 15630080)

10 mL Pen Strep with 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin (Gibco, cat. no. 15140122)

In a cell culture hood, mix all reagents and filter into a sterile 1 L bottle with a 0.45 µm PES filter (Alkali Scientific, cat. no. VH50045). Complete cell culture media can be stored at 4 °C for at least half a year. We recommend aliquoting into 50 mL sterile tubes to reduce warm-cool cycles.

10× Phosphate buffered saline (PBS)

400 g sodium chloride

10 g potassium chloride

72 g disodium phosphate

12 g monopotassium phosphate

Dissolve in 5 L of deionized water. To get 1× PBS, dilute 10× PBS 10-fold with deionized water.

Immunostaining fixative with 4% formaldehyde

20 mL 10% (w/v) formaldehyde (Macron Fine Chemicals, cat. no. H121-05)

5 mL 10× phosphate buffered saline (PBS)

25 mL autoclaved distilled water

Immunostaining fixative can be stored at room temperature for at least 2 weeks. Formaldehyde is a health hazard. Store fixative in a fume hood.

COMMENTARY

BACKGROUND INFORMATION

PPIs are essential for cell survival. Consequently, many methods have been developed to study PPIs. Of these methods, genetically-encoded techniques have been particularly useful due to their cell-type specificity and ease of implementation in cell cultures, where the tool can be introduced through DNA transfection or viral infection. Genetically-encoded PPI detection methods can be classified into several broad categories: Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), protein-complementation, and transcriptional methods. Both BRET (Pfleger & Eidne, 2006) and FRET (Truong & Ikura, 2001) measure PPI in real-time, offering valuable information on PPI dynamics. However, these techniques often have low fluorescence signal and require extensive optimization in geometry to achieve an optimal energy transfer. Protein-complementation assays, such as split fluorescent protein (Cabantous et al., 2013; Pedelacq, Waldo, & Cabantous, 2019), cannot interrogate PPI dynamics but usually have high signal. Split luciferase assays (Ohmuro-Matsuyama & Ueda, 2019) have high signals and can detect PPIs with temporal resolution but lose the spatial

information of PPIs. Transcriptional assays with temporal controls, such as iTANGO (Lee et al., 2017) and SPARK (Kim et al., 2017), provide both information on protein dynamics, due to their light-gated temporal control, and a high signal due to the transcriptional amplification of the reporter expression. Additionally, transcriptional assays offer versatility in their readout by the ability to change the reporter gene used. For these reasons, temporally-gated transcriptional assays are advantageous for interrogating PPIs when the user needs cell type specificity, dynamic information, and a versatile read-out. Additionally, multiple PPI detection methods are often required to study a particular PPI since each method often have a certain degree of false positive and false negative detections. In this sense, SPARK complements existing systems and provides an alternative approach to study PPIs.

Transcriptional assays were first developed without a temporal control, such as yeast two-hybrid (Fields & Song, 1989) and TANGO (Barnea et al., 2008). Due to the lack of dynamic information, transcriptional assays with a temporal control, namely SPARK and iTANGO, were developed. Temporally-gated assays provide a time-window that can be used to integrate PPIs during a specific time period of interest. Although the tool designs of SPARK and iTANGO are similar, SPARK has lower background than iTANGO. It was found that there was 3- to 26-fold greater background in iTANGO for the negative dark and no drug conditions (Kim et al., 2017). This is most likely due to the non-specific reconstitution of split TEV protease in iTANGO, where the split halves can have an inherent affinity towards each other (Kim et al., 2017). SPARK was further optimized in SPARK2 (Sanchez & Ting, 2020). In SPARK2, an optimized TEV protease called uTEV1Δ is used. This protease was evolved to have a higher catalytic efficiency, giving an 11.7-fold higher reporter signal than that of the original SPARK assay.

When choosing a genetic assay for studying PPIs, users must consider the time scale and dynamics of the PPIs. If observing fast on-off dynamics is desired, real-time assays should be chosen over integration assays. If dynamic information is not needed, but transcriptional

readout is preferred, non-temporally-gated integration assays may suffice. If users would like both transcriptional readout and the capability of detecting PPIs that occur within a specific time window, temporally-gated integration assays should be used.

CRITICAL PARAMETERS

PPI affinity

The binding affinity between the two PPI partners is a determining factor for the success of a SPARK experiment. Figure 2 shows representative images of two PPI pairs with sub-nanomolar affinity (between arrestin and activated β_2-adrenergic receptor (Gurevich et al., 1995)) and low single-digit micromolar affinity (between a nanobody and an activated μ-opioid receptor (Livingston, Mahoney, Manglik, Sunahara, & Traynor, 2018)), respectively. For the sub-nanomolar affinity PPI pair, high signal-to-noise ratio can be achieved with as short as 5-10 min stimulation. For the low single-digit micromolar affinity PPI pair, high signal-to-noise ratio can be achieved with a 20 min stimulation. Therefore, we expect PPIs with affinity in the range of tens of micromolar to give even lower SPARK signal.

Geometry of the SPARK TEVp and TF components

The SPARK TEVp and TF components each contains a PPI partner, and the geometry of the two constructs can impact the PPI and the subsequent protease cleavage. If both PPI partners are normally cytosolic, users should test both partners on the SPARK TEVp and the TF components. For the SPARK TEVp component, users should test fusing the PPI partner both before and after the TEVp, and screen different linker lengths, as described in Basic Protocol 1. For the SPARK TF component, users should also screen different linker length between the C-terminus of the PPI partner and the LOV domain, and if applicable, between the PPI partner and the membrane anchor.

Choice of the TEVp and the LOV domain

Users can choose between at least two TEVp sequences and two LOV domains, as detailed in Basic Protocol 1. The two TEVp sequences, both being truncated (1-219) and having the S219V mutation, are different in amino acid position 153, where the S153N mutation enhances the initial turnover rate and thus gives higher SPARK signal and background. The two LOV domains, eLOV and hLOV1, are different by eight mutations and hLOV1 is four amino acids shorter than eLOV at the C-terminus. hLOV1 has tighter caging on TEVcs and, therefore, gives reduced background and reduced signal. Overall, the combination of TEVp with theS153N mutation and eLOV gives the highest SPARK signal and background. Conversely, the combination of TEVp without the S153N mutation and hLOV gives the lowest SPARK signal and background. We recommend starting with these two extreme combinations and adjust based on the observed signal-to-noise ratio.

Membrane trafficking and stability of the SPARK TF component

Since the SPARK TF component contains a fully-functional transcription factor, a mistrafficked or unstable TF component could lead to high SPARK background. If using a membrane anchor, users should select one with good membrane trafficking, such as the CD4 transmembrane domain suggested in Basic Protocol 1. Users are also suggested to test using membrane trafficking signal peptides to enhance membrane trafficking/localization of the SPARK TF component. To check membrane trafficking, users can follow the immunostaining protocol (Support Protocol 2, representative data in Figure 3).

Lentivirus quality

SPARK is expression level-dependent. Any SPARK component that has a low expression level could significantly reduce SPARK performance. For first-time users, we recommend using a positive control (e.g. using β_2-adrenergic receptor in the SPARK TF component and rat β-arrestin 2 in the SPARK TEVp component, as shown in Figure 2) to ensure all procedures are properly done. Lentivirus quality can vary from batch to batch and from person to person. When users start to use a new batch of lentivirus, we again recommend using a positive control, to ensure viral quality. When preparing lentiviruses, users should start with healthy HEK293T/17 cells and use fresh aliquots of complete cell culture media for all procedures, as instructed in Support Protocol 1. Use freshly prepared plasmid DNA for SPARK components and high-quality pVSVG and Δ8.9 plasmids prepared by Maxiprep (see Support Protocol 1). Make sure to mix all plasmid DNA before adding PEI MAX. To preserve viral quality, when storing lentiviruses, aliquot and freeze in liquid nitrogen as quickly as possible; when thawing lentiviruses for transduction, thaw in a 37 °C water bath and proceed immediately after thawing.

Cell health before and after lentiviral transduction

Unhealthy HEK293T/17 cells could impact SPARK signal. Before lentiviral transduction, make sure to use overnight-passaged cells that just reaches 80-90% confluence on the day of experiment. Coat plates with HFN to enhance cell attachment. Seed cells into well plates using fresh complete cell culture media according to Basic Protocol 2 such that they are at 50% confluence before transduction. Before adding lentiviruses to the cells, ensure most cells are attached to the bottom of the wells. We have found that adding lentiviruses while cells are still in suspension can significantly reduce cell health. During incubation, leave some space between the aluminum foil and the well plates to ensure sufficient air flow. During stimulation, use fresh complete cell culture media to replace the two-day-old media in the wells, and use proper

aspiration and addition techniques as detailed in Basic Protocol 2 to avoid disturbing the attached cells.

Fluorescence imaging

Fluorescence imaging techniques are key to the proper acquisition and analysis of SPARK results. Misfocusing on the order of tens of microns could lead to drastically different SPARK images. We recommend unexperienced users to consult their microscope manufacturer or imaging core professionals. During imaging, find the optimal focal plane for each field of view using the fluorescent protein on the SPARK TEVp component, and re-focus when changing field of view. Adjust laser power and exposure time so that signal is distinct from the background.

TROUBLESHOOTING

Please see Table 1 for a list of common problems with the protocols, their causes, and potential solutions.

[*Table 1 near here]

UNDERSTANDING RESULTS

Following the protocols described above, users can expect to get SPARK results similar to those in Figure 2, and immunostaining results similar to those in Figure 3.

Figures 2A and 2B are an example of a high-affinity PPI pair (sub-nanomolar affinity between β_2-adrenergic receptor and arrestin during isoproterenol stimulation). The signal-to-noise ratio (S/N), as calculated according to Basic Protocol 2 Step 10, is over 60 between the double positive (+light+stimulant) and double negative (-light-stimulant) conditions. In comparison, a test PPI pair with micromolar affinity (Figures 2C and 2D) gives a S/N of 5.1. These two

representative PPI pairs should allow users to estimate SPARK signal for PPI pairs with affinities ranging from the nanomolar to the micromolar range.

Figure 3 shows a representative immunofluorescence image of a SPARK TF component. An intracellular V5 tag designed according to Basic Protocol 1 Step 6 was used for immunostaining. For well-trafficked SPARK TF components, users should expect to see similar images, showing clear membrane localization of the construct.

TIME CONSIDERATIONS

Basic Protocol 1: Designing constructs can take days to weeks, depending on the level of experience of the user. Obtaining plasmids from Addgene normally takes a couple of weeks, but can take months. Molecular cloning normally takes 1-2 weeks.

Basic Protocol 2: SPARK takes three days from transduction to imaging.

Support Protocol 1: Lentiviral production takes two days from transfection to harvest. Additional time may be required to prepare sufficient number of HEK293T/17 cells prior to transfection.

Support Protocol 2: Immunostaining takes 2-4 hours, excluding imaging.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Schematics of SPARK. A and B are test PPI partners. TEV protease (TEVp) only cleaves TEV cleavage site and drives gene expression when there is both light (to uncage TEV cleavage site from LOV domain) and PPI (to bring TEVp close to TEV cleavage site). Note that PPI partner B does not have to be membrane-bound itself; it can be fused to a transmembrane domain (not shown).

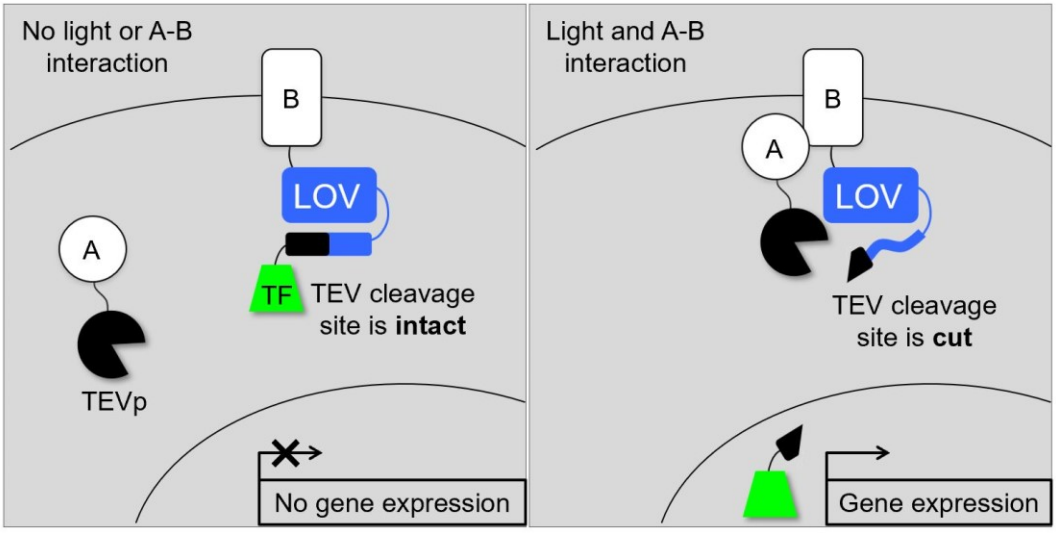


Figure 2. Representative fluorescence microscopy images and quantitative analysis of SPARK assays performed with PPI partners of different affinities. (A) A sub-nanomolar affinity PPI pair (between activated β_2-adrenergic receptor and rat β-arrestin 2). Constructs are as shown in Supplementary Text 4 in the Supplementary Information. Blue: mTurquoise2 expression marker on the SPARK TEVp component. Red: mCherry reporter gene. PPI was induced (stimulated) by 10 μM isoproterenol, a β_2-adrenergic receptor agonist. Both isoproterenol and light were administered for 10 min. (B) Quantitative analysis for (A) using the procedures described in Basic Protocol 2 Step 10. One technical replicate (one well) is shown for each SPARK condition. Each dot represents one field-of-view. The horizontal bar represents the mean of fluorescence intensity.(C) A single-digit micromolar affinity PPI pair (between a nanobody and activated μ-opioid receptor). Red: mCherry expression marker on the SPARK TEVp component. Green: citrine reporter gene. PPI was induced (stimulated) by 10 μM DAMGO, a μ-opioid receptor agonist. Both DAMGO and light were administered for 20 min. (D) Quantitative analysis for (C) using the procedures described in Basic Protocol 2 Step 10. One technical replicate (one well) is shown for each SPARK condition. Each dot represents one field-of-view. The horizontal bar represents the mean of fluorescence intensity. All images were obtained with 10× air objective. All scale bars, 100 μm.

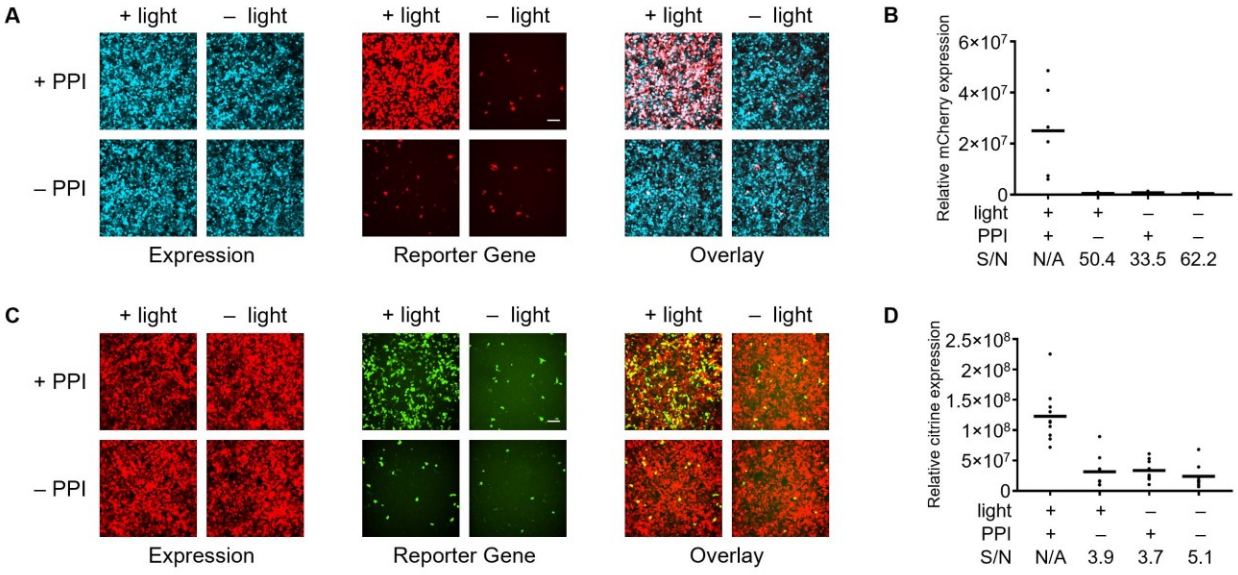
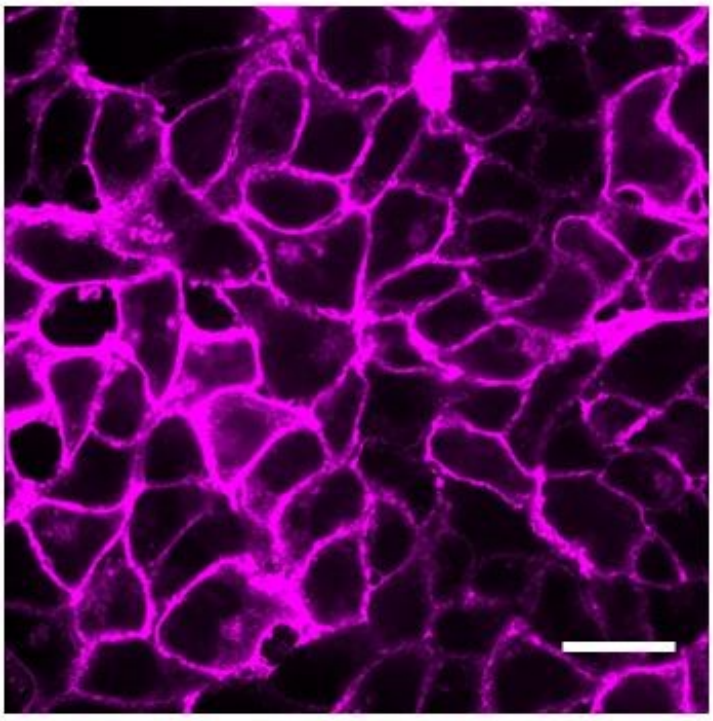


Figure 3. Representative immunofluorescence microscopy images of the SPARK TF component. Transmembrane domain: μ-opioid receptor. Primary antibody: mouse anti-V5 tag antibody. Secondary antibody: goat anti-mouse IgG antibody, Alexa Fluor 647. Image shows good expression level and membrane trafficking/localization for this construct. Imaged with 60× oil-immersion objective. Scale bar, 20 μm.



TABLES

Table 1. Troubleshooting

Issue	Possible Cause	Solution
Cells are dead after lentiviral transduction	Unhealthy cells before transduction	Always use healthy cells for lentiviral transduction. Do not use cells within 3 passages of thawing, or over 20 passages. Find the optimal culturing and passaging conditions for your batch of cells before performing SPARK experiments. In our hands, we find passaging cells one day before transduction to ~40% confluence (so that they reach ~80-90% confluence the next day) results in optimal cell health.
	Insufficient air	When wrapping with aluminum foil, leave space between the aluminum foil and the plate.

	Spraying too much ethanol on plates before wrapping in aluminum foil	Wrap plates in aluminum foil first before spraying ethanol. Or wait for plates to dry before wrapping.
	Extended time outside of incubator	When performing light stimulation, avoid taking plates out of the incubator for more than 30 min. Or perform light stimulation inside the incubator.
	Phototoxicity	Use a weaker light source. Or shine from a longer distance. Or use '5 seconds on, 20 seconds off' light intervals with a timer switch.
	Unhealthy media	Two days after transduction, aspirate lentivirus from all wells, and replace with warm fresh media.
No or weak SPARK signal	Any technical issue during lentiviral production, transduction, stimulation, or imaging	Include an inducible PPI as positive control (such as arrestin – β_2-adreneline receptor, induced by isoproterenol). We recommend all first-time users to test a positive control.
	Weak PPI	PPIs with sub-nanomolar affinity have to be maintained for a period of time in the order of minutes to produce a SPARK signal. Longer time (20 min) is required for PPIs with single-digit micromolar affinity. For PPIs with affinity higher than 10 μM, we recommend using a longer light window.
	Mismatched light window	Light has to be provided exactly when PPI occurs. Otherwise, LOV domain could revert back to the caged state within minutes. If unsure, use longer light stimulation.
High SPARK background in the absence of light	Suboptimal TEVp, TEVcs, or LOV domain	Make sure your constructs use the exact TEVp, TEVcs, and LOV domain as suggested in Basic Protocol 1.
	Strong PPI	If your PPI is strong enough to give a dark-state background as high as lit-state signal, the temporal gating of SPARK is not useful due to insufficient caging.

	Mistrafficked or miscleaved SPARK TF component	Perform a control experiment without addition of the SPARK TEVp component. If high background remains, the SPARK TF component may be mistrafficked or contains sequences cleavable by endogenous proteases. Use immunostaining to determine the localization of the SPARK TF component and screen membrane trafficking signal peptides.
Cells do not settle within 5 hours of seeding	Plate or flask surface not properly treated	Coat with 25 µg/mL of human fibronectin for 30 min or longer. Or use 50 µg/mL fibronectin. Make sure the entire plate or flask surface is covered.
	Unhealthy cells	Always use healthy cells. Do not use cells within 3 passages of thawing, or over 20 passages. Find the optimal culturing and passaging conditions for your batch of cells before experiment. In our hands, we find passaging cells one day before experiment to ~40% confluence (so that they reach ~80% confluence the next day) gives optimal cell health.
Cells are lifted after immunostaining	Harsh washing	Always aspirate liquid slowly from bottom edge of each well, and add liquid slowly to the side wall of each well. Do not drop liquid on top of cells. Do not vigorously shake the plate.
Poor lentivirus quality	DNA degradation	pVSVG and Δ8.9 lentiviral plasmids must be prepared using Maxiprep. Viruses should be made with fresh viral plasmid DNA of the SPARK components prepared within two weeks.
	Poor transfection efficiency	Thoroughly mix all plasmid DNA before adding PEI MAX. Do not pre-mix PEI MAX with any plasmid DNA. Prepare PEI-plasmid DNA mixture using DMEM without FBS. Use PEI MAX thawed and stored at 4 °C for less than a month.
	Loss of lentiviral activity	When harvesting lentivirus, aliquot and flash freeze in liquid nitrogen immediately. Do not keep the lentivirus-producing plate or flask out of incubator for prolonged periods of time. When using lentivirus, thaw at 37 °C water bath immediately after taking out of -80 °C, and use immediately after thawing.