SPARK: A Transcriptional Assay for Recording Protein-Protein Interactions in a Defined Time Window

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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 analysis, 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. © 2021 Wiley Periodicals LLC.

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: optogenetics • protein-protein interaction • sensor • transcriptional assay • temporal gating

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INTRODUCTION

Protein-protein interactions (PPIs) play fundamental roles in regulating cellular processes and biological functions, including signal transduction, enzyme activities, metabolism, the 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 (Specific Protein Association tool giving transcriptional Readout with rapid Kinetics; Kim et al., 2017) that converts transient, binary PPIs into gene expression has recently been reported. In

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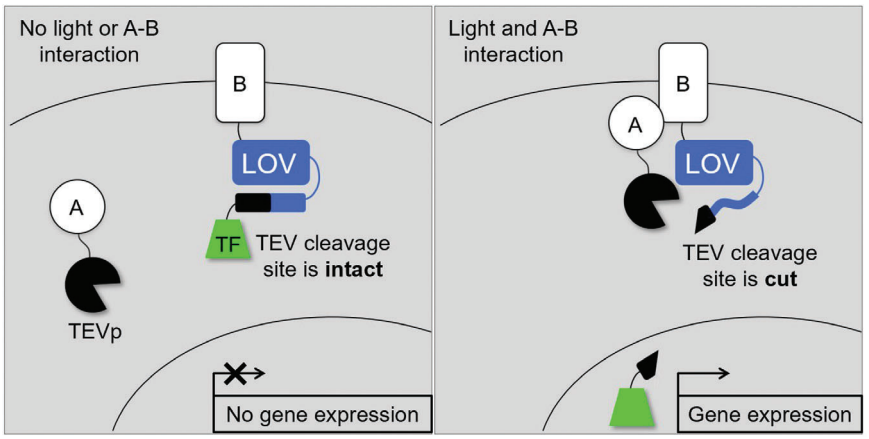

Figure 1 Schematics of SPARK. A and B are test PPI partners. TEV protease (TEVp) only cleaves the TEV cleavage site and drives gene expression when there is both light (to uncage the TEV cleavage site from the LOV domain) and PPI (to bring TEVp close to the TEV cleavage site). Note that PPI partner B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

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) (Fig. 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 a 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 a 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, one with sub-nanomolar affinity and one with low single-digit micromolar affinity. Of note, the interaction within both PPI pairs described here is 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.

BASIC PROTOCOL 1

DESIGNING DNA CONSTRUCTS FOR SPARK

This protocol details the design of the three SPARK components (Fig. 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, and not a step-by-step protocol on how to perform the

actual cloning. For cloning protocols, readers are directed to other Current Protocols articles (Kramer & Coen, 2006; Lohman, Tabor, & Nichols, 2011). Software such as ApE and SnapGene can be used to design the DNA.

The Supporting Information (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 prior work (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 (pLX208 vector backbone for the SPARK TF and TEVp components, Addgene, plasmid #104846, RRID:Addgene_104846; pFPGW vector backbone for the SPARK reporter gene component, Addgene, plasmid #125232, RRID:Addgene_125232; both are gifts from Alice Ting). To replace the open reading frame (ORF) in the pLX208 vector, users can digest the plasmid with *Bam*HI (*Bam*HI-HF, New England BioLabs, cat. no. R3136S) and *Mlu*I (*Mlu*I-HF, New England BioLabs, cat. no. R3198S) restriction enzymes. To replace the ORF in the pFPGW vector, users can digest the plasmid with *Age*I (*Age*I-HF, New England BioLabs, cat. no. R3552S) and *Asc*I (New England BioLabs, cat. no. R0558S) restriction enzymes. Standard cloning techniques such as polymerase chain reaction (see Current Protocols article; Kramer & Coen, 2006), ligation reaction (see Current Protocols article; Lohman et al., 2011), and Gibson assembly can be used to clone in new ORFs. 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 in each construct to allow further modifications.

Materials

None

Designing the DNA construct for the SPARK TEVp component

1. To begin designing the DNA construct, 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 the 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, respectively, in this component. Note that mTurquoise2 is not compatible with the nuclear stain DAPI, which may be used together with immunostaining (see 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 Fig. 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 fusing the PPI partner both 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 Text 1 in the Supporting Information. For an example, see Text 4 in the Supporting 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 an 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 SPARK systems, with one or the other of the PPI partners 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 of 10 and 20 amino acids in length to ensure that 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 (Text 1 in the Supporting 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 Text 2 in the Supporting Information. For an example, see Text 4 in the Supporting 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 one or the other 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 (Text 2 in the Supporting Information). For G protein–coupled receptors, 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, more recently 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, or 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 (LOV) sensing domain (LOV) with a 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 Text 2 in the Supporting Information. For an example, see Text 4 in the Supporting 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 TFs not naturally found in HEK293T/17 cells can also be used as the TF. However, we have found that the use of stronger TFs 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 (see Text 2 in the Supporting Information).

6. Select an epitope tag. Include this 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 a 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 of 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 Text 3 in the Supporting Information. For an example, see Text 4 in the Supporting 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 component.

   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 ORF of three SPARK components (with rat β-arrestin 2 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 Text 4 in the Supporting 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 cell seeding, 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 an 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 (see Current Protocols articles; Kramer & Coen, 2006; Lohman, Tabor, & Nichols, 2011), and have produced lentiviruses of the three components according to Support Protocol 1.

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, the volumes of all reagents and the number of cells should be adjusted accordingly, unless otherwise specified.

Materials

HEK293T/17 cells (ATCC, cat. no. CRL-11268)
Complete cell culture medium (see recipe), 37°C
25 µg/ml human fibronectin (HFN; see recipe)
0.4% (w/v) trypan blue solution (Gibco, cat. no. 15250061)
Lentiviruses for three SPARK components (prepared according to Support Protocol 1)
Complete cell culture medium (see recipe) containing stimulant, 37°C

T25 cell culture flasks (Alkali Scientific, cat. no. TVN0025)
48-well plastic plates (Greiner, cat. no. 677102)
Hemocytometer (Hausser Scientific, cat. no. 3100)
37°C water bath (Thermo Scientific, cat. no. TSGP10)
Aluminum foil
Red light source
White or blue LED light source or ambient room light source with ≥0.5 mW/cm^2 light power
Fluorescence microscope with appropriate capacities [e.g., 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; ORCA-Flash 4.9 LT+sCMOS camera; and compact four-line laser source with 405 nm (100 mW), 488 nM (100 mW), 561 nM (100 mW), and 640 nm (75 mW)]
Image analysis program

Additional reagents and equipment for immunostaining (see Support Protocol 2; optional)

NOTE: All steps should be performed in a biosafety level 2 (BSL-2) laboratory following standard regulatory procedures.

NOTE: All solutions and equipment in contact with cells must be sterile.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator (e.g., Eppendorf, cat. no. Galaxy 170 S) unless otherwise specified.

1. One day before lentiviral transduction, passage HEK293T/17 cells to 40% confluence in 5 ml pre-warmed complete cell culture medium in a T25 cell culture flask.

   One T25 (25 cm^2) is sufficient for all wells in a 48-well plate (1 cm^2/well). HEK293T/17 cells at 40% confluence will grow to 80% to 90% confluence overnight. We have found 80% to 90% confluent cells to be most healthy for lentiviral transduction.

2. Coat 48-well plastic plates with 200 µl of 25 µg/ml HFN per well. For each set of SPARK experiments, prepare two plates, for light and dark conditions. In each plate, coat at least two wells, for with-stimulant and no-stimulant conditions. Incubate plates at 37°C for ≥10 min and then aspirate HFN.

   We routinely use plastic 48-well plates for imaging purposes, but glass-bottom 24-well plates (Cellvis, cat. no. P24-1.5H-N) can be used for obtaining higher-quality images. HFN helps with cell attachment and ensures good cell health after lentiviral transduction.

3. Resuspend the 80% to 90% confluent cells from step 1 in pre-warmed complete cell culture medium, count cells with 0.4% trypan blue solution and a hemocytometer, dilute to 500,000 cells/ml in medium, and seed 200 µl cells per well. Shake plates gently back and forth and then left and right a couple of times to ensure that the cells distribute evenly. Avoid circular motion. Let plates sit in the incubator for 1 to 5 hr until most cells are settled.

   Users can proceed to the next step when most cells are attached to the wells, which usually happens within 2 hr. The initial cell confluence is ∼50%. For optimal cell health, we recommend using fresh aliquots of complete cell culture medium (that has not been previously subjected to warm-cool cycles) to perform this step.

4. Thaw lentiviruses for the three SPARK components (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 100 µl lentivirus for each SPARK component together for each well in a 48-well plate (i.e., for each well, prepare ∼300 µl viral master mix, containing 100 µl of each virus).

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

5. Add 280 µl viral master mix to each well. Shake plates gently back and forth and then left and right a couple of times to ensure that the 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 plates and the aluminum foil to ensure sufficient air flow. Incubate for 40 to 56 hr (2 days).

   Light should be avoided for both plates during incubation by wrapping the 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 of the plates (dim light from computer monitors will not stimulate SPARK). For no-stimulant wells, aspirate and replace medium with 200 µl pre-warmed complete cell culture medium. For with-stimulant wells, aspirate medium

and replace with 200 µl pre-warmed complete cell culture medium containing stimulant. Wrap the dark plate in aluminum foil and shine light on the light plate using a white or blue LED light source or an ambient room light source with $\geq$0.5 mW/cm^2 light power. After stimulation for the desired length of time, wash with-stimulant wells three times with 200 µl pre-warmed complete cell culture medium each in the dark room, wrap both plates with aluminum foil, and incubate for 18 to 30 hr (overnight).

Aspirating lentivirus from all wells and replacing with fresh cell culture medium can enhance cell health. To avoid disturbing the cells, use a manual pipet to slowly draw the medium from the bottom edge of each well and add medium by slowly dispensing to the side wall of each well. Do not draw or dispense too quickly or drop medium directly on top of cells.

The LOV domain requires only <0.5 mW/cm^2 light power to be uncaged (Pudasaini & Zoltowski, 2013), and it has been previously found that light stimulation with an ordinary room light source has the same effect as a blue LED array (Kim et al., 2017). In our hands, cells can be stimulated continuously for $\geq$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 s on, 20 s off" light intervals using a timer switch (programmable digital timer outlet from Nearpow, Togoal, or a similar brand).

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 with a fluorescence microscope with appropriate capacities. For each well, acquire 6 to 12 fields of view using a 10× or 20× objective with a region of interest (ROI) of 11 × 11 mm. For each field of view, acquire images using two different channels, acquiring 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 that in 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 the laser power and exposure time so that signal is distinct from the background.

   We use a Nikon inverted confocal microscope equipped as detailed in the Materials list above.

9. If applicable, perform immunostaining according to Support Protocol 2 and re-image 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 using an image analysis program as follows. Apply a mask or threshold to images with the SPARK reporter gene component such that it is just above the background. Calculate total fluorescence intensity above the threshold from each field of view by multiplying mean fluorescence intensity by total object area. Then, subtract total background intensity (calculated by multiplying the mean intensity value of a no-reporter-expression area by the total object area) from total fluorescence intensity. For each of the four SPARK conditions (+light+stimulant, +light-stimulant, -light+stimulant, -light-stimulant), calculate background-subtracted total fluorescence intensity from all fields of view collected and display as a dot plot.

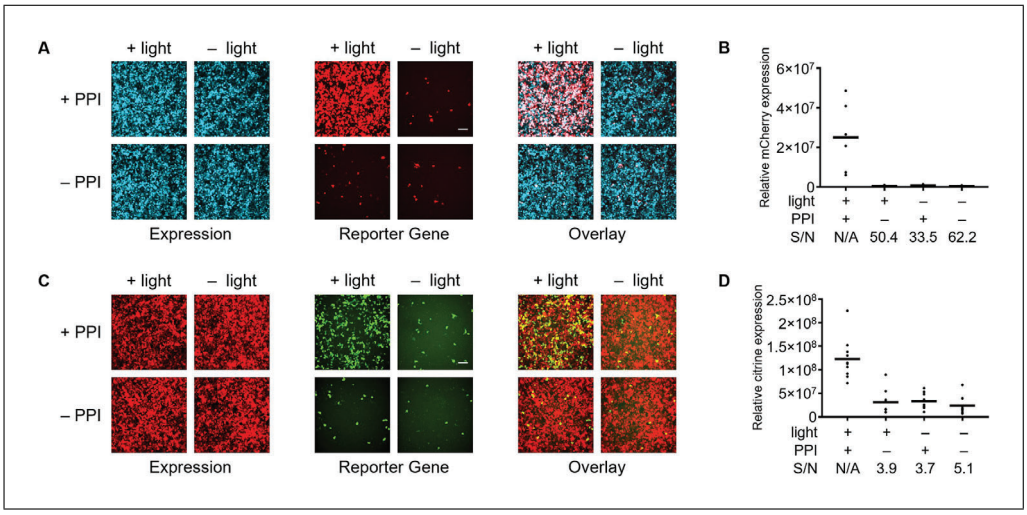

Figure 2 Representative fluorescence microscopy images and quantitative analysis of SPARK assays performed with PPI partners of different affinities. (**A**) A sub-nanomolar-affinity PPI pair (between activated β_2-adrenergic receptor and rat β-arrestin 2). Constructs are as shown in Text 4 in the Supporting 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 bars represent the mean of fluorescence intensity. S/N, signal-to-noise ratio. (**C**) A PPI pair with single-digit micromolar affinity (between a nanobody and an 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 bars represent the mean of fluorescence intensity. All images were obtained with a 10× air objective. All scale bars, 100 μm.

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.

LENTIVIRUS PREPARATION

SUPPORT PROTOCOL 1

This protocol describes the procedures for preparing second-generation lentiviruses for the three SPARK components (Basic Protocol 1) for HEK293T/17 cell transduction (Basic Protocol 2). We find that this protocol robustly produces high-quality lentiviruses for SPARK assays. If users prefer to use third-generation lentiviruses, they are directed to a prior Current Protocols article (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, the volumes of all reagents and the number of cells should be adjusted accordingly, unless otherwise specified.

Additional Materials (also see Basic Protocol 2)

Dulbecco's modified Eagle's 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 for three SPARK components (designed according to Basic Protocol 1)
1 mg/ml PEI MAX (see recipe)
Liquid nitrogen

1.5-ml Eppendorf tubes

NOTE: All steps should be performed in a biosafety level 2 (BSL-2) laboratory following standard regulatory procedures.

NOTE: All solutions and equipment in contact with cells must be sterile.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator (e.g., Eppendorf, cat. no. Galaxy 170 S) unless otherwise specified.

1. On the day before lentivirus preparation, passage HEK293T/17 cells to 40% confluence in 5 ml pre-warmed complete cell culture medium in a T25 cell culture flask.

   One T25 cell culture flask will produce 6 ml lentivirus, enough for lentiviral transduction for 60 wells of cells growing in 48-well plates (1 cm^2/well). HEK293T/17 cells at 40% confluence will grow to 80% to 90% confluence overnight. We have found 80% to 90% confluent cells to be most healthy for lentivirus preparation.

2. Coat a T25 cell culture flask with 1 ml of 25 µg/ml HFN. Incubate plates at 37°C for ≥10 min and then aspirate HFN.

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

3. Resuspend the 80% to 90% confluent cells from step 1 in pre-warmed complete cell culture medium, count cells with 0.4% trypan blue solution and a hemocytometer, dilute to 700,000 cells/ml in medium, and seed 1 ml cells per T25 cell culture flask. Shake flask gently back and forth and then left and right a couple of times to ensure that the cells distribute evenly. Avoid circular motion. Let flask sit in the incubator for 1 to 5 hr until most cells are settled.

   Users can proceed to the next step when most cells are attached to the flask, which usually happens within 2 hr. The initial cell confluence will be ∼80%. For optimal cell health, we recommend using fresh aliquots of complete cell culture medium (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 DMEM [no fetal bovine serum (FBS)] with 250 ng pVSVG lentiviral envelope plasmid and 2250 ng of Δ8.9 packaging (helper) plasmid.

   pVSVG and Δ8.9 are a lentiviral envelope plasmid and a packaging (helper) plasmid, respectively. To ensure high plasmid DNA quality, pVSVG and Δ8.9 must be prepared using a maxiprep. We routinely use a Qiagen Plasmid Maxi Kit (HiSpeed Plasmid Maxi Kit, Qiagen, cat. no. 12662 or 12663) and store the plasmid DNA in ultrapure water. Plasmid DNA amplified by maxiprep and stored in ultrapure water can be stored ≤1 year at 4°C.

5. For every T25 flask, mix 250 µl lentiviral plasmid master mix with 2500 ng of the appropriate plasmid DNA, i.e., the plasmid for a SPARK component. 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 ≤3 years at room temperature or 4°C.

6. Add 25 µl of 1 mg/ml PEI MAX to each mix. Thoroughly mix by pipetting. Let mixture sit at room temperature for ≥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 pre-warmed complete cell culture medium to each mix and transfer entire mixture to each T25 flask. Gently shake flasks back and forth and then left and right a couple of times to ensure that the PEI/plasmid DNA mixture distributes evenly.
8. Incubate for 48 to 56 hr (2 days).
9. Aliquot lentivirus-containing supernatant into 1.5-ml Eppendorf tubes and discard 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 lifting by naked eye, users should centrifuge the supernatant for 2 min at 200 × g in a spinning-bucket centrifuge (e.g., Eppendorf, cat. no. 5810R) to remove solid particles. Alternatively, 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 ≤1 year at −80°C. We do not titer lentiviruses. If desired, users may measure transduction efficiency using immunostaining and nuclear staining (see Support Protocol 2).

IMMUNOSTAINING OF SPARK COMPONENTS

SUPPORT PROTOCOL 2

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, step 9). Alternatively, users can transduce individual components into HEK293T/17 cells and perform immunostaining 2 days after transduction. Always include an untransduced control. 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, the volumes of all reagents should be adjusted accordingly, unless otherwise specified.

Materials

HEK293T/17 cells in 48-well plate from Basic Protocol 2, after step 6 or step 9
Immunostaining fixative with 4% formaldehyde (see recipe)
1× phosphate-buffered saline (PBS; see recipe)
Methanol, −20°C
Primary antibody (not all antibodies listed may be needed):
  Mouse anti–FLAG tag M2 antibody (MilliporeSigma, cat. no. F3165)
  Rabbit anti–HA tag antibody (Cell Signaling Technology, cat. no. 3724S)
  Mouse anti–V5 tag antibody (Invitrogen, cat. no. R960-25)
0.01 g/ml bovine serum albumin (Dot Scientific, cat. no. DSA30075-100) in 1× PBS
100 μg/ml DAPI (see recipe)

Secondary antibody (not all antibodies listed may be needed):
Goat anti-mouse IgG antibody, Alexa Fluor 488 (Invitrogen, cat. no. A-11001)
Goat anti-mouse IgG antibody, Alexa Fluor 568 (Invitrogen, cat. no. A-11004)
Goat anti-mouse IgG antibody, Alexa Fluor 647 (Invitrogen, cat. no. A-21235)
Goat anti-rabbit IgG antibody, Alexa Fluor 488 (Invitrogen, cat. no. A-11008)
Goat anti-rabbit IgG antibody, Alexa Fluor 568 (Invitrogen, cat. no. A-11011)
Goat anti-rabbit IgG antibody, Alexa Fluor 647 (Invitrogen, cat. no. A-21244)

Tilting or orbital plate shaker (Thermo Fisher, cat. no. 88861025, or Benchmark Scientific, cat. no. BT3001)

CAUTION: The 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.

NOTE: All steps should be performed in a biosafety level 2 (BSL-2) laboratory following standard regulatory procedures.

NOTE: All primary and secondary antibodies in this list can be diluted with PBS to 1 mg/ml and stored ≤1 year at 4°C. For long-term storage, follow the manufacturers' instructions.

1. Aspirate medium from wells with HEK293T/17 cells in a 48-well plate from Basic Protocol 2, after step 6 or step 9, by using a manual pipet to slowly draw medium from the bottom edge of each well.
2. Add 200 µl immunostaining fixative with 4% formaldehyde gently to side wall of each well in the 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 aspiration and addition procedures (see steps 1 and 2, respectively).

   After washing with PBS, samples may be stored ≤16 hr (overnight) at 4°C 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 side wall of each well. Immediately incubate plate at −20°C for 5 min. Avoid any shaking, tilting, or disturbance to sample.
5. Aspirate methanol immediately after incubation. Wash three times with 200 µl of 1× PBS each time.
6. Dilute primary antibody to a final concentration of 1 µg/ml each in 0.01 g/ml bovine serum albumin in 1× PBS. If also performing nuclear staining, add 100 µg/ml DAPI to 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.

   The 0.01 g/ml bovine serum albumin in 1× PBS can be stored ≤1 year at −20°C and is good for at least three freeze-thaw cycles.
7. Aspirate PBS. Add 200 µl primary antibody/DAPI mixture to each well. Incubate at room temperature under room light for 30 min with gentle shaking on a tilting or orbital plate shaker.
8. Aspirate primary antibody/DAPI mixture. Wash each well three times with 200 µl of 1× PBS each time.

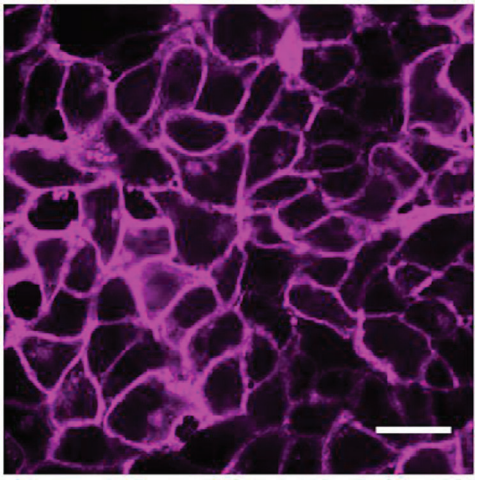

Figure 3 Representative immunofluorescence microscopy image of the SPARK TF component. The TF is tethered to a transmembrane domain: μ-opioid receptor. Primary antibody: mouse anti–V5 tag antibody. Secondary antibody: goat anti-mouse IgG antibody, Alexa Fluor 647. The image shows a good expression level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

9. Dilute secondary antibody to a final concentration of 1 μg/ml each in 0.01 g/ml bovine serum albumin in 1× PBS.

   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 under room light for 20 min with gentle shaking on a tilting or orbital plate shaker.

    Prolonged incubation with secondary antibody can lead to nonspecific 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 1× PBS in each well.

    The samples are now ready for fluorescence imaging (see Basic Protocol 2, step 9).

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

REAGENTS AND SOLUTIONS

Complete cell culture medium

500 ml DMEM (Gibco, cat. no. 11885084)
500 ml minimum essential medium (MEM; Gibco, cat. no. 11095080)
100 ml FBS (Biowest, cat. no. S1620)
20 ml 1 M HEPES (Gibco, cat. no. 15630080)
10 ml pen-strep (with 10,000 U/ml penicillin and 10,000 μg/ml streptomycin; Gibco, cat. no. 15140122)
In cell culture hood, mix all reagents and filter into sterile 1-L bottle with 0.45-μm PES filter (Alkali Scientific, cat. no. VH50045)
Store ≤6 months at 4°C

We recommend aliquoting into sterile 50-ml tubes to reduce warm-cool cycles.

DAPI, 100 µg/ml

Dissolve DAPI nuclear staining dye (Bio-Rad, cat. no. 1351303) to a final concentration of 100 µg/ml in ultrapure water. Store ≤3 years at 4°C.

Human fibronectin (HFN), 25 µg/ml

Dissolve HFN (MilliporeSigma, cat. no. FC010) in ultrapure water (Invitrogen, cat. no. 10977) to 0.5 mg/ml and aliquot. Store ≤3 years at −20°C. Before use, dilute 0.5 mg/ml HFN to 25 µg/ml in ultrapure water. Store ≤6 months at 4°C.

Immunostaining fixative with 4% formaldehyde

20 ml 10% (w/v) formaldehyde (Macron Fine Chemicals, cat. no. H121-05)
5 ml 10× PBS (see 1× PBS recipe)
25 ml autoclaved distilled water
Store ≤2 weeks at room temperature

CAUTION: Formaldehyde is a health hazard. Store fixative in a fume hood.

PEI MAX, 1 mg/ml

Dissolve PEI MAX (polyethylenimine hydrochloride; Polysciences, cat. no. 24765-100) to 1 mg/ml in ultrapure water and adjust pH to 7.0 with a base. Store ≤3 years at −20°C. Thawed PEI MAX can be stored ≤1 month at 4°C.

Phosphate-buffered saline (PBS), 1×

400 g sodium chloride
10 g potassium chloride
72 g disodium phosphate
12 g monopotassium phosphate
Dissolve in 5 L deionized water to make 10× PBS
Store 10× PBS ≤1 year at room temperature
To get 1× PBS, dilute 10× PBS 10-fold with deionized water
Store 1× PBS ≤1 year at room temperature

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 assays (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 readout. Additionally, multiple PPI detection methods are often required to study a particular PPI because each method often has 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 assays (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 control dark and no-drug conditions (Kim et al., 2017). This is most likely due to the nonspecific reconstitution of split TEV protease in iTANGO, where the split halves can have an inherent affinity toward 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, one with sub-nanomolar affinity (between arrestin and activated β_2-adrenergic receptor; Gurevich et al., 1995) and one with low single-digit micromolar affinity (between a nanobody and an activated μ-opioid receptor; Livingston, Mahoney, Manglik, Sunahara, & Traynor, 2018). For the sub-nanomolar-affinity PPI pair, a high signal-to-noise ratio can be achieved with as short as 5- to 10-min stimulation. For the PPI pair with low single-digit micromolar affinity, a 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 contain 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 lengths 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 the S153N 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 adjusting based on the observed signal-to-noise ratio.

Membrane trafficking and stability of the SPARK TF component

Because the SPARK TF component contains a fully functional TF, 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 advised 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 Fig. 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 Fig. 2) to ensure that 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 medium 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 in Basic Protocol 2, make sure to use overnight-passaged cells that have just reached 80% to 90% confluence on the day of the experiment. Coat plates with HFN to enhance cell attachment. Seed cells into well plates using fresh complete cell culture medium according to Basic Protocol 2 such that they are at 50% confluence before transduction. Before adding lentiviruses to the cells, ensure that 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 medium to replace the 2-day-old medium 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 in Basic Protocol 2. Misfocusing on the order of tens of microns could lead to drastically different SPARK images. We recommend that unexperienced users 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 the 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.

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 from 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, 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 (Figs. 2C and 2D) gives a signal-to-noise ratio 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

Table 1 Troubleshooting Guide for SPARK

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 three 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 that passaging cells 1 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 before spraying ethanol or wait for ethanol-sprayed plates to dry before wrapping.
	Extended time outside of incubator	When performing light stimulation, avoid taking plates out of the incubator for >30 min. Alternatively, perform light stimulation inside the incubator.
	Phototoxicity	Use a weaker light source, shine light from a longer distance, or use "5 s on, 20 s off" light intervals with a timer switch.
	Unhealthy medium	Two days after transduction, aspirate lentivirus from all wells and replace with warm fresh medium.
No or weak SPARK signal	Any technical issue during lentiviral production, transduction, stimulation, or imaging	Include an inducible PPI as a positive control (such as arrestin/β_2-adrenergic receptor, induced by isoproterenol). We recommend that all first-time users test a positive control.
	Weak PPI	PPIs with sub-nanomolar affinity have to be maintained for a period of time on 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 >10 μM, we recommend using a longer light window.
	Mismatched light window	Light has to be provided exactly when PPI occurs. Otherwise, the 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 that 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 hr of seeding	Plate or flask surface not properly treated	Coat with 25 μg/ml HFN for ≥30 min or use 50 μg/ml fibronectin. Make sure that the entire plate or flask surface is covered.

(*Continued*)

Table 1 Troubleshooting Guide for SPARK, *continued*

Issue	Possible cause	Solution
	Unhealthy cells	Always use healthy cells. Do not use cells within three passages of thawing or over 20 passages. Find the optimal culturing and passaging conditions for your batch of cells before the experiment. In our hands, we find that passaging cells 1 day before the experiment to ∼40% confluence (so that they reach ∼80% confluence the next day) gives optimal cell health.
Cells lifted after immunostaining	Harsh washing	Always aspirate liquid slowly from the 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 a maxiprep. Viruses should be made with fresh viral plasmid DNA for the SPARK components prepared within 2 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 ≤1 month at 4°C.
	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 the incubator for prolonged periods of time. When using lentivirus, thaw in a 37°C water bath immediately after taking from −80°C and use immediately after thawing.

of experience of the user. Obtaining plasmids from Addgene normally takes a couple of weeks but can take months. Molecular cloning normally takes 1 to 2 weeks.

Basic Protocol 2: SPARK takes 3 days from transduction to imaging.

Support Protocol 1: Lentiviral production takes 2 days from transfection to harvest. Additional time may be required to prepare a sufficient number of HEK293T/17 cells prior to transfection.

Support Protocol 2: Immunostaining takes 2 to 4 hr, excluding imaging.

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Author Contributions

Lequn Geng: Data curation, Methodology, Writing-original draft, Writing-review and editing. **Kayla E. Kroning**: Data curation, Methodology, Writing-review and editing. **Wenjing Wang**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing-review and editing.

Conflict of Interest

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.

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