Video Article

Spatial and Temporal Control of T Cell Activation Using a Photoactivatable Agonist

Elisa Sanchez[1,2], Morgan Huse[1]

[1]Immunology Program, Memorial Sloan-Kettering Cancer Center

[2]Weill-Cornell Graduate School of Medical Sciences

Correspondence to: Morgan Huse at husem@mskcc.org

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Abstract

T lymphocytes engage in rapid, polarized signaling, occurring within minutes following TCR activation. This induces formation of the immunological synapse, a stereotyped cell-cell junction that regulates T cell activation and directionally targets effector responses. To study these processes effectively, an imaging approach that is tailored to capturing fast, polarized responses is necessary. This protocol describes such a system, which is based on a photoactivatable peptide-major histocompatibility complex (pMHC) that is non-stimulatory until it is exposed to ultraviolet light. Targeted decaging of this reagent during videomicroscopy experiments enables precise spatiotemporal control of TCR activation and high-resolution monitoring of subsequent cellular responses by total internal reflection (TIRF) imaging. This approach is also compatible with genetic and pharmacological perturbation strategies. This allows for the assembly of well-defined molecular pathways that link TCR signaling to the formation of the polarized cytoskeletal structures that underlie the immunological synapse.

Video Link

The video component of this article can be found at https://www.jove.com/video/56655/

Introduction

T lymphocytes (T cells) play a central role in cellular immunity by recognizing antigenic peptides displayed in the context of cell surface MHC. Antigen recognition, which is mediated by the TCR, drives the differentiation of naïve T cells and promotes the delivery of cytolytic and communicative responses by effector populations. TCR engagement also induces dramatic changes in cellular architecture. Within minutes, the T cells gloms onto the side of the antigen-presenting cell (APC), forming a polarized interface known as the immunological synapse (IS)[1,2]. The IS potentiates T cell effector responses by enabling the directional release of cytokines or, in the case of cytotoxic T lymphocytes (CTLs), lytic proteins that destroy the APC.

TCR engagement by pMHC induces the rapid phosphorylation of multiple downstream adaptor molecules, including Linker for the Activation of T cells (LAT), which ultimately promotes robust remodeling of the synaptic cytoskeleton[2]. Cortical filamentous actin (F-actin) drives T cell spreading over the APC surface, and then resolves into an annular structure characterized by F-actin accumulation at the IS periphery and depletion from the center. F-actin ring formation is tightly coupled to the reorientation of the microtubule organizing center (MTOC, also called the centrosome in T cells) to a position just beneath the center of the interface. Both events occur within minutes of initial antigen recognition and establish the architectural context in which subsequent activation events and effector responses occur.

To study IS formation, various labs have developed approaches in which the APC is replaced by a glass surface that either contains immobilized TCR ligands or supports a lipid bilayer that itself contains the ligands[3,4]. T cells form IS-like contacts on these surfaces that can be imaged by total internal reflection fluorescence microscope (TIRF) or confocal microscopy, enabling high-resolution studies of early T cell activation and IS formation.

Although these approaches have allowed for excellent visualization of the fully assembled IS, much of the signaling following TCR:pMHC ligation occurs within seconds, complicating efforts to determine the sequence of events following TCR activation accurately. To circumvent this issue, a photoactivation approach has been developed, in which photoactivatable pMHC is used to achieve spatiotemporal control of TCR activation[5,6,7]. In this system, T cells are attached to glass surfaces containing photoactivatable pMHC that is non-stimulatory to the TCR until irradiated with ultraviolet (UV) light. UV irradiation of a micron sized region of the surface beneath the T cell removes the photocage creating a stimulatory zone that can be recognized by the T cell. Subsequent signaling events and cytoskeletal remodeling are then monitored using genetically encoded fluorescent reporters. Two photoactivatable versions of antigenic peptides, moth cytochrome c_{88-103} (MCC) and ovalbumin$_{257-264}$ (OVA), which are presented in the context of the class II MHC I-E^k and the class I MHC H2-K^b, respectively, have been developed (**Figure 1**). This enables

the analysis of both $CD4^+$ T cells specific for MCC- I-E^k (expressing the 5C.C7, 2B4, or AND TCRs) and $CD8^+$ T cells specific for OVA-H2-K^b (expressing the OT1 TCR).

Over the past decade, the TCR photoactivation and imaging approach has been utilized to establish the precise kinetics of early TCR signaling steps and also to identify the molecular pathways governing polarized cytoskeletal remodeling[5,6,7,8,9,10]. For example, the assay was instrumental in determining that centrosome reorientation toward the APC is mediated by a localized gradient of the lipid second messenger diacylglycerol centered at the IS. It is anticipated that this methodology will continue to be valuable for applications that demand high-resolution imaging analysis of T cell function.

Protocol

1. Preparation of Stimulatory Glass Surfaces

1. Coat eight-well chambered coverglass with biotinylated poly-L-lysine (Bio-PLL) diluted 1:500 in distilled, deionized water (ddH_2O). Incubate for 30 min at room temperature (RT).
2. Wash with H_2O.
3. Dry for 2 h at RT.
4. Block Bio-PLL coated surfaces with blocking buffer (HEPES-buffered saline [10 mM HEPES pH 7.4, 150 mM NaCl], with 2% BSA) for 30 min at RT.
    1. Dissolve 5 mg of poly-L-lysine hydrobromide in 1 mL of 10 mM $NaPO_4$, pH 8.5.
    2. Add 125 µmol (0.55 mg) of NHS-biotin (from a 100 mg/mL stock in DMSO) and check the pH of the reaction. If the pH is below 8.5, add 2 µL of 4 N NaOH to raise it to between pH 8.5 and 9.5.
    3. Vortex for 30 min.
    4. Quench the reaction with 50 µL of 100 mM glycine dissolved in 20 mM Tris pH 8.0.
    5. Spin at full power for 10 min and transfer the supernatant to a new tube.
    NOTE: Bio-PLL quality is typically compared to previous preparations by coating serial two-fold dilutions of the material onto a 96-well ELISA plate and quantifying biotin content after incubation with alkaline phosphatase coupled streptavidin.
5. Remove blocking buffer. Do not allow wells to dry. Add streptavidin (100 µg/mL in blocking buffer). Incubate for 1 h at 4 °C.
6. Wash in HBS. Fill and invert chamber slide wells 4 - 5 times, removing HBS from the wells. Do not allow wells to dry.
7. Add the biotinylated pMHC ligands and adhesion molecules to the surface.
    NOTE: MCC and OVA can be photocaged by adding ortho-nitrobenzyl based protecting groups (*e.g.,* nitrophenylethyl (NPE) or nitroveratryloxycarbonyl (NVOC)) to the ε-amino group of key lysines (K12 in MCC, K7 in OVA). Photocaged MCC and OVA can be obtained from commercial vendors. After reconstitution in aqueous buffer, they are refolded into bacterially expressed I-E^k and H2-K^b using established approaches[11,12].
8. Validation of photoactivatable MCC or OVA peptide
    1. Decage NVOC-protected peptides by UV irradiating with a handheld UV lamp (see **Table of Materials**) for 20 min at RT.
    2. Pulse 1.0×10^5 APCs with 1 µM of nonstimulatory peptide (negative control, *e.g.* Hb), unirradiated photoactivatable peptide, irradiated photoactivatable peptide, and agonist peptide (positive control, *e.g.*, MCC) for 1 h to overnight at 37 °C.
    3. To stimulate T cells expressing the 5C.C7/2B4/AND TCR, use CH12 or CH27 B cells. To stimulate OT1 T cells, use RMA-s or EL4 thymoma cells.
    4. Mix the pulsed APCs with an equal number of T cells in 96-well round bottom plates at a final volume of 200 µL. Incubate at 37 °C for 12 - 24 h.
    5. Recover the supernatants from each well and analyze IL-2 by ELISA with streptavidin-horseradish peroxidase colorimetric detection.
    NOTE: Confirmation of the caging status of all peptides by electrospray mass spectrometry prior to refolding into MHC complexes, is strongly recommended.
9. Perform the folding and purification of MHC so as to minimize exposure to light. For example, wrap folding reactions in aluminum foil and carry out gel filtration chromatography with the UV lamp off.
    NOTE: It has been found that photoactivatable MCC- I-E^k and OVA- H2-K^b induce UV independent T cell activation at high density, possibly due to incomplete functional caging. Hence, the photoactivatable pMHC is diluted into a 10 - 30x excess of nonstimulatory pMHC (*e.g.* I-E^k containing the $hemoglobin_{64-76}$ peptide (Hb)) during the immobilization step. This enhances the signal to noise ratio of the subsequent imaging experiment.
10. To photoactivate $CD4^+$ T cells, collectively use a mixture of biotinylated Hb-I-E^k (3 µg/mL), biotinylated photoactivatable MCC- I-E^k (0.1 µg/mL), and biotinylated antibody against the class I MHC H2-K^k (0.5 µg/mL). The anti-H2-K^k antibody encourages 5C.C7/2B4/AND T cells, which express H2-K^k, to spread onto the glass surface without undergoing activation.
11. For $CD8^+$ T cells, use a mixture of biotinylated H2-D^b bearing the peptide KAVYDFATL (1 µg/mL), biotinylated photoactivatable OVA-H2-K^b (0.1 µg/mL), and the extracellular domain of the adhesion molecule ICAM-1 (2 µg/mL produced by insect cell culture[13]). The ICAM-1 encourages close contact formation by engaging the integrin LFA1 on the T cell surface.
12. Apply all protein mixtures in blocking buffer, followed by incubation for 1 h at RT or at least 2 h at 4 °C.
    NOTE: The molecular density on surfaces of this kind to be ~8000 per μm^2 has been previously determined[6]. Given that photoactivatable pMHC represents ~$1/30^{th}$ of the biotinylated protein on the surface, its density will be ~267 molecules per μm^2, prior to decaging.
13. Wash as in step 1.6 and leave in HBS until ready to use.
14. Add 200,000 $CD4^+$ or $CD8^+$ T cells expressing the appropriate TCR into each well and allow cells to adhere at 37 °C for 15 min. Once cells have attached to and spread on the surface, they are ready for photoactivation and imaging.
    NOTE: Retroviral transduction of effector T cells with fluorescent imaging probes is described in detail elswhere[6,7]. Calcium signaling can also be studied using untransduced T cells loaded with the calcium sensitive dye Fluo-4[6]. The ratiometric dye Fura-2 is not recommended because it requires excitation in the UV range, which also induces decaging of the photoactivatable pMHC.

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2. Image Acquisition

1. Use an inverted TIRF microscope outfitted with a UV compatible 150X objective lens for image acquisition. UV irradiate user-defined regions using a digital diaphragm system attached to a 100 W mercury lamp (HBO). Direct UV light from this lamp onto the sample using a 400 nm long pass mirror.
2. Use image analysis software for localized photoactivation and time-lapse acquisition. In most experiments, monitor probes in both the green and red channels using 488 nm and 561 nm excitation lasers, respectively. Laser light is directed onto the sample using a dual-bandpass dichroic mirror that also transmits in the UV range (to enable decaging). Please see the **Table of Materials** and **Figure 6** for additional information on microscope configuration.
3. After mounting the chamber slide containing the T cells, adjust settings to obtain TIRF or epifluorescence illumination, as necessary. In live mode, select a field of cells that are expressing the fluorescent probe(s) of interest. Establish micron-scale regions for photoactivation beneath individual cells using software control.
4. Begin time-lapse acquisition. Typically, 80 timepoints are acquired, with an interval of 5 s between each time point. This leaves more than enough time for sequential 488 nm and 563 nm exposures, in the case of dual color experiments.
5. After 10 timepoints, photoactivate the selected regions by opening the digital diaphragm shutter for 1 - 1.5 s.
6. After the time lapse is complete, select a new field of cells and repeat the process.

3. Data Analysis

NOTE: Localized photoactivation of immobilized ligands creates a well-defined, stationary stimulatory region that can be used for quantitative analysis of signaling responses and cytoskeletal remodeling events. Analysis protocols typically involve either quantifying fluorescence intensity within the irradiated region or using the irradiated region as a positional endpoint for distance measurements (*e.g.* for assessing polarization of the centrosome to the irradiated region). Both analysis protocols are described below. Various interactive image analysis programs can be used to make intensity and distance measurements, which can then be output for additional processing and analysis.

1. **Fluorescence intensity**
    1. Determine the fluorescence intensity (FI) of a background region outside of the cell (FI_b). This will be used for background correction.
        1. Draw a micron-sized square region outside of the cell and make a mask. To determine the fluorescence intensity, click on **Analyze | Mask Statistics**. Select **Mean Fluorescence Intensity** and export the values.
        NOTE: Procedural details (*e.g.* 3.1.1.1) refer to Slidebook software (see **Table of Materials**). Implementation of this protocol using other software packages (*e.g.*, FIJI) will be slightly different.
    2. Determine the FI within the photoactivated region for each time point.
        1. Select **Mask** to highlight the region that was photoactivated. To determine the fluorescence intensity, click on **Analyze | Mask Statistics**. Select **Mean Fluorescence Intensity** and export the values.
    3. Subtract the background FI from the measured FI values within the region. Then, normalize the corrected FI measurements by dividing by the average, background corrected FI of the first 9 frames before photoactivation. $\Delta F/F = ((FI-FI_b)/mean(FI_{1\text{-}9})-FI_b))$.
    NOTE: Graph ΔF/F as a function of time.
2. **Distance**
    1. Obtain the x and y coordinates of the center of the photoactivated region.
        1. To determine the x and y coordinates of the center of the photoactivated region, select **Mask** to highlight the region that was photoactivated. Once the region of photoactivation is highlighted, select **Analyze | Mask Statistics | Center of Area** and export the values.
    2. Determine the x and y coordinates for the fluorescent probe of interest for each time point. This is typically achieved through either manual or automated particle tracking.
        1. To determine the x and y coordinates of the fluorescent probe of interest, select **Manual Particle Tracking** and click on the fluorescent probe of interest over time. Once all time points have been tracked, select **Analyze | Mask Statistics | Center of Area** and export the values.
    3. Calculate the distance between the fluorescent protein of interest and the center of the photoactivated region for each time point using the equation: $\text{Distance} = \sqrt{(x_2-x_1)^2+(y_2-y_1)^2}$, in which x_2 and y_2 are the coordinates of the fluorescent protein of interest and x_1 and y_1 are the coordinates of the center of the photoactivated region.
    4. Graph the distance as a function of time.

Representative Results

The photoactivation and imaging approach allows for observation and facile quantification of rapid, polarized signaling responses. To illustrate its capabilities, reproduced here is an experiment examining the spatiotemporal correlation between TCR-induced DAG accumulation and centrosome reorientation. 5C.C7 T cell blasts were retrovirally transduced with two fluorescent reporters: a DAG biosensor containing the tandem C1 domains from protein kinase C-θ linked to GFP (C1-GFP) and RFP-tubulin to monitor the centrosome. The T cells were then attached to chambered coverglass containing photoactivatable MCC- I-E^k. C1-GFP was imaged using TIRF microscopy and RFP-tubulin in epifluorescence mode. As shown in **Figure 2**, localized photoactivation of the surface beneath the T cell induces the accumulation of DAG in the irradiated zone. This is followed within seconds by the reorientation of the centrosome to the same region.

C1-GFP accumulation can be quantified by calculating the normalized fluorescence intensity, after background correction, at the center of the photoactivated region over time (**Figure 3**). Centrosome reorientation in response to photoactivation can be quantified by calculating the distance between the centrosome and the center of the photoactivated region as a function of time (**Figure 4**).

This method can be used to examine a wide array of rapid, polarized signaling responses, essentially anything that can be monitored using a fluorescent probe. For example, TCR photoactivation is utilized to monitor early signaling microcluster formation using fluorescently labeled Grb2 (Grb2-GFP) (**Figure 5**). Similar to the DAG accumulation and centrosome reorientation responses, Grb2-GFP polarization occurs shortly following photoactivation (**Figure 5**), and can be quantified using the normalized fluorescence intensity approach.

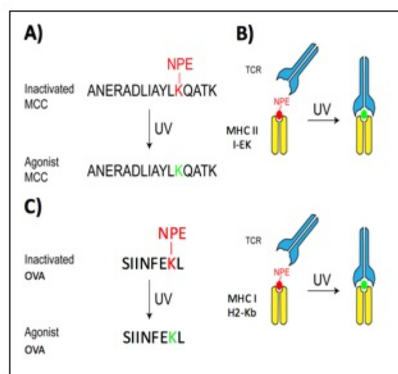

Figure 1: Photoactivation system using photocaged MCC peptide.
(A) Photocaged strategy for MCC peptide. A NPE group is attached to the lysine residue of the MCC peptide. UV irradiation results in the removal of NPE, restoring MCC to its native form. **(B)** When NPE is bound to MCC, the 5C.C7 TCR cannot bind to MCC. Removal of NPE results in recognition of the 5C.C7 TCR to the native MCC peptide. **(C)** The photocaged strategy has been adapted to work with CD8+ T cells, in which photocaged OVA peptide is used to activate the OT-1 TCR upon UV irradiation.

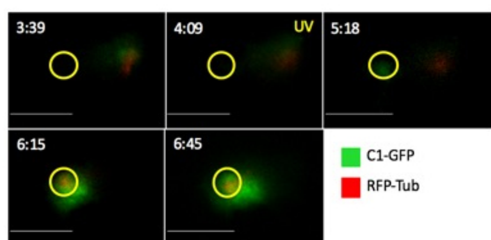

Figure 2: Time-lapse montage of DAG accumulation and centrosome reorientation following photoactivation of TCR in 5C.C7 T cell.
5C.C7 cell expressing a DAG biosensor, containing tandem C1-domains of PKC- fused to GFP (C1-GFP) and Tag-RFP Tubulin (RFP-Tub), a fluorescent reporter for the centrosome. The montages illustrate the polarized response of 5C.C7 T cells over time. The yellow oval and text indicate the time and location of photoactivation. Over time, C1-GFP accumulates at the photoactivated region, followed by centrosome reorientation towards the photoactivated region. Scale bar: 10 µm.

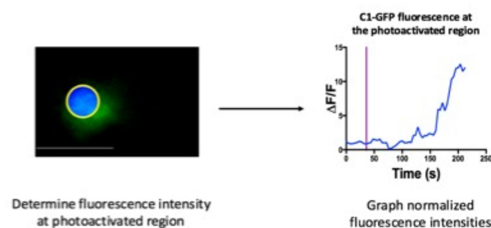

Figure 3: Quantification of DAG accumulation at the photoactivated region
DAG, C1-GFP, accumulation at the photoactivated region (left) can be quantified by calculating the normalized fluorescence intensity, after background correction, at the photoactivated region (yellow circle with blue shading) over time. Data are normalized relative to the nine frames obtained just before photoactivation. C1-GFP enrichment at the photoactivated region can be calculated by the division of the mean fluorescence intensity in the irradiated region by the mean fluorescence intensity of the entire cell, following background correction. The equation: $\Delta F/F = ((FI-FI_b)/mean(FI_{1-9})-FI_b))$, in which FI is the mean fluorescence intensity, can be used to calculate the normalized fluorescence intensity. The normalized fluorescence intensity at the photoactivated region can then be graphed as a function of time (right). Purple line indicates the time of photoactivation. Scale bar: 10 µm.

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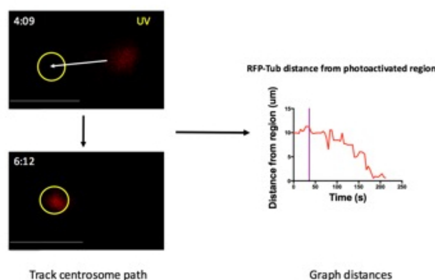

Figure 4: Quantification of centrosome reorientation.
Determine the coordinates of the centrosome (RFP-Tub) over time by manually tracking the centrosome path toward the photoactivated region (white arrow) at each time point (left). Determine the coordinates of the center of the photoactivated region (yellow circle). The distance between the centrosome and the center of the photoactivated region can be calculated at each time point using the equation for distance = $\sqrt{((x_2-x_1)^2+(y_2-y_1)^2)}$, in which x_2 and y_2 are the x and y coordinates for the center of the area of the centrosome, and x_1 and y_1 are the x and y coordinates of the center of the photoactivated region. The distance of the centrosome from the center of the photoactivated region at each time point can then be graphed as a function of time (right). Purple line indicates the time of photoactivation. Scale bar: 10 µm.

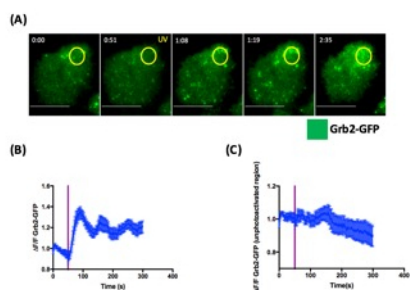

Figure 5: Grb2-GFP microcluster formation.
(A) Representative 5C.C7 cell expressing fluorescently labeled Grb2 (Grb2-GFP). The montages illustrate the polarized response of Grb2-GFP overtime. The yellow oval and text indicate the time and location of photoactivation. Over time, Grb2-GFP accumulates at the photoactivated region. (B) Quantification of Grb2-GFP fluorescence intensity at the photoactivated region over time. N = 15 cells. (C) Quantification of Grb2-GFP fluorescence intensity in a control region outside of the photoactivated zone. N = 15 cells.

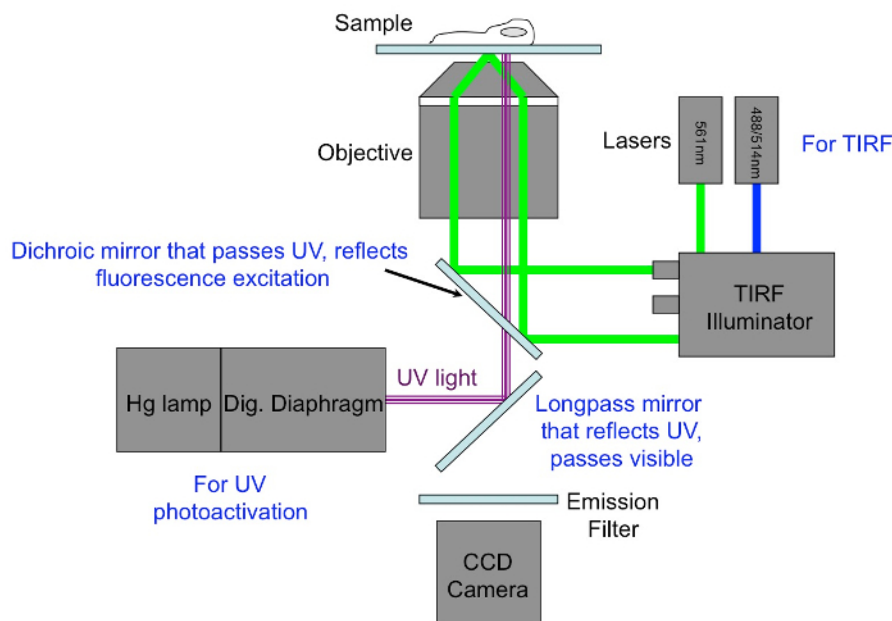

Figure 6: Microscope configuration for photoactivation and TIRF imaging.
488 nm and 561 nm lasers are used for TIRF and epifluorescence imaging of green and red probes, respectively. UV light for photoactivation is taken from a mercury (Hg) lamp attached to a digital diaphragm system capable of illuminating small, user-defined regions. Light from the Hg lamp/diaphragm is reflected onto the sample by a longpass mirror positioned beneath the dichroic mirror that reflects the imaging lasers. The dichroic mirror must be designed to pass UV light. Please click here to view a larger version of this figure.

Discussion

In recent years, light has emerged as an excellent tool for spatiotemporally controlled activation of cellular processes. Various methodologies have been developed, each with associated advantages and disadvantages. The system described here, which is based on the decaging of immobilized, extracellular ligands, is ideally suited for the analysis of rapid, subcellular, polarized signaling responses. This approach has been applied to examine IS formation in T cells as described above. Additionally, caged ligands for other receptors have been engineered, enabling us to apply the same strategy to assess inhibitory signaling in natural killing cells and nuclear translocation of the transcription factor NF-κB in response to toll like receptor signaling[14,15].

In this protocol, stimulatory ligands are immobilized to ensure that they remain as a stationary, polarized source of stimulation for the duration of the imaging experiment. This approach is well suited for studies of cell polarization. It should be noted however, that the TCR photoactivation approach is easily adapted for temporally controlled T cell activation on supported lipid bilayers. Obviously, one cannot achieve sustained, polarized stimulation in this context. This may be unnecessary, however, given the experimental objectives.

The system is also quite flexible with respect to the UV light source for decaging. Currently, a 100 W Hg lamp is used for focused illumination, however a pulsed nitrogen laser (pulse width 4 ns, pulse energy 120 mJ), was previously used[6]. At times, a handheld UV lamp (365 nm, 4 W) has been positioned over the imaging sample in order to decage larger regions of photostimulatable ligand. The specific features of the decaging system will in each case depend on the technical requirements of the experiment in question.

When implementing this system, it is important to confirm that the observed responses are specific for the photoactivation event, and not the result of spurious receptor activation or UV-induced photodamage. To rule out these artifacts, two specificity controls can be performed. First, fluorescence intensity in cells that are not directly irradiated during photoactivation experiments can be monitored. If the surface is properly caged, appreciable responses in these cells should not be observed (for example, **Figure 5C**). Second, experiments in which T cells are UV-irradiated on surfaces lacking photoactivatable ligands can be performed. This control is critical for identifying UV-induced effects that are independent of photoactivatable ligand (for example, see reference 6). During system optimization, it is also extremely helpful to have at hand a robust cellular imaging response (*e.g.,* Grb2-GFP recruitment) that is both fast and localized. Assessing correlations between signaling events and photoactivation is much more straightforward if those events are spatiotemporally proximal to receptor stimulation.

Although the photoactivation approach offers exquisite spatiotemporal control over receptor signaling, it lacks the adaptability afforded by transfectable optogenetic systems[16,17,18,19,20,21,22]. Certain optogenetic proteins are also photoreversible, providing conditional control that is not available with the photoactivation approach described here. In optogenetic systems, however, constraining the diffusion of photoactivated proteins is technically challenging. This complicates the analysis of polarized signaling, particularly in a subcellular context.

One should also note that the engaging entity of this photoactivation system is a glass surface, which cannot recapitulate all aspects of a bona fide cell-cell interaction. To circumvent this issue while maintaining spatiotemporal control of signal initiation, investigators have used an optical trap system to bring an APC into contact with a T cell, allowing for the onset of cytoskeletal rearrangements and IS maturation to be visualized in real time[23]. Although this system enables the precise initiation and visualization of cytoskeletal dynamics using an actual APC, it is relatively low throughput and is not compatible with TIRF illumination, which adversely affects image quality.

In conclusion, the method described in this protocol provides spatiotemporal control of TCR-induced signaling within the T cell, ultimately allowing for the elucidation of the fast biochemical changes following TCR activation. This system provides us with a means to better understand how the signaling events following TCR activation promote T cell polarization, IS formation, and ultimately the delivery of effector responses.

Disclosures

The authors have nothing to disclose.

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