Optogenetic Manipulation of Cell Migration with High Spatiotemporal Resolution Using Lattice Lightsheet Microscopy

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18 **Abstract**

19 Lattice lightsheet microscopy (LLSM) is modified with the aim of manipulating cellular
 20 behavior with subcellular resolution through three-dimensional (3D) optogenetic activation. In
 21 this study, we report a straightforward implementation of the activation source in LLSM in
 22 which the stimulating light can be generated by changing the spatial light modulator (SLM)
 23 patterns and the annual masks. As a result, a Bessel beam as a stimulation source is integrated
 24 into the LLSM without changing the optical configuration, achieving better spatiotemporal
 25 control of photoactivation. We show that the energy power required for optogenetic reactions
 26 is lower than 1 nW (or 24 mW/cm²), and membrane ruffling can be activated at different
 27 locations within a cell with subcellular resolution. We also demonstrate guided cell migration
 28 using optogenetic stimulation for up to 6 h, where 463 imaging volumes are collected, without
 29 noticeable damage to cells.

30

31 Introduction

32 Spatiotemporal manipulation and recording of biological processes, such as cell migration or
 33 membrane dynamics with the subcellular resolution, is crucial in understanding these
 34 fundamental cellular processes^{1,2,3}. However, it is technically challenging for cell manipulation
 35 with a precise spatiotemporal resolution, especially in three dimensions (3D)^{4,5}. A common
 36 approach for subcellular manipulation can be achieved by optogenetic tools on a conventional
 37 microscope such as wide-field or confocal microscope^{6,7,8}. Recording the three-dimensional
 38 rapid cellular response at high spatiotemporal resolution can be problematic, where a high
 39 numerical aperture (NA) objective is often used to create a tightly focused spot for better
 40 confinement of the photoactivation beam. When such a photoactivation beam propagates in the
 41 cell, it activates molecules not only in the focal spot but also along the beam path where the
 42 out-of-focus activation may not be imaged when a pinhole is used to reject the out of focus
 43 signal as in the case of confocal microscopy (the beam paths of different excitation and
 44 detection schemes can be found in Figure S1)⁹. As a result of tight focusing, an uneven photo-
 45 excitation along the beam propagation is created. Therefore, it is desirable to develop a wide-
 46 field technique with good sectioning capability to record the optogenetic response. A notable
 47 implementation in this regard is the development of lattice light-sheet microscopy (LLSM),
 48 which is constructed by an array of Bessel beams, where destructive interference of the
 49 coherent Bessel beams minimizes the contribution from sidelobes within the detection zone¹⁰.

LLSM provides several advantages for high-resolution volumetric imaging of living cells, including reduced phototoxicity in which the excitation energy is distributed over an array of the illumination beams, an optimal balance between the thickness and length of the lightsheet, enabling high spatial resolution imaging with a good optical sectioning capability, and improved signal-to-noise ratio in which the out-of-focus noise is suppressed by the coherent modulation of lattice beams^{11, 12, 13}. In addition, the lattice patterns also allow easy implementation of super-resolution microscopy using structured illumination¹⁴.

With the help of LLSM, several biological processes, including the dynamics of the immunological synapses, transcription factors, and lymphocyte migration, have been investigated in great detail^{15, 16, 17}. With an additional optical path for illumination, it has been demonstrated that photoablation/FRAP/photoactivation experiments^{18, 19, 20} can be conducted on LLSM. However, it remains challenging to perform experiments requiring optical stimulation, such as subcellular optogenetic applications, in LLSM. In this situation, the lattice beams illuminate the samples at a tilted angle, which results in poor spatial activation of the signaling molecules within a cell, especially along the lightsheet propagation direction. LLSM could be potentially useful in living cells to conduct long-term optogenetic experiments with a very high spatiotemporal resolution at very low phototoxicity if the light stimulation can be integrated into LLSM. In addition, the 3D nature of LLSM may allow local activation of biological processes in a specific subcellular compartment or organelle. In this study, we report

a novel LLSM design for optogenetic experiments. The optical configuration of our system is the same as the LLSM reported previously. In our system, the LLSM is used to monitor cellular behavior, whereas optogenetic molecules are activated by a Bessel beam, whose 3D energy distribution can be precisely controlled by the phase profiles on the SLM. Optically-induced clustering and membrane ruffling, in addition to guided cell migration, can be achieved in our system.

To demonstrate the performance of our technique, we conducted several cell-migration-related optogenetic experiments in our system. The traditional approach to inducing cell migration is to create concentration gradients of the growth factors²¹ through diffusion mimicking the native cell migration environment. However, the diffusion gradient is difficult to maintain for precise cell manipulation at the single-cell level. The newly developed optogenetic tools enable cell manipulation through light, where cells are modified genetically to respond to a specific wavelength of light^{22,23}. The most common genetic modifications for optogenetic experiments include optically induced heterodimerization (cryptochrome 2-calcium and integrin-binding N-terminal domain [CRY2-CIBN])²⁴ and optically induced clustering (CRY2olig)²⁵. In this experiment, we adapted both systems to control cellular behavior. To achieve 3D cellular manipulation in LLSM, we demonstrated that our approach offers three advantages over original LLSM, including 1) controllable activation in 3D, 2) subcellular optogenetic activation and 3) long-term cell manipulation.

88

89 **Results**

90 **Bessel beam activation in lattice lightsheet microscopy for optogenetics**

91 To conduct an optogenetic experiment in LLSM , our system consists of a large parallel array
 92 of coherently interfering Bessel beams through the SLM in which individual Bessel beams with
 93 selected wavelengths can be used as activation sources by simply changing the patterns on the
 94 SLM²⁶ located at the plane conjugated to the imaging plane (Figure 1a and 1b). With a different
 95 pattern on the SLM, the lattice beams at the imaging plane can be switched to a stimulating
 96 Bessel beam whose x, y positions can be controlled by different modulations in the SLM
 97 patterns, as shown in Figure 1b. In addition, the lattice beams can be dithered to create a
 98 uniform excitation sheet to obtain 3D images of cells with the sample scanning mode, as shown
 99 in Figure 1c. To demonstrate the advantages of using Bessel beam excitation for optogenetic
 100 applications, we developed two probes based on the CRY2 system: (1) CRY2olig and (2)
 101 CRY2-CIBN. In the optically-induced clustering probe, we fused CRY2olig with a mRuby3.
 102 As the CRY2 molecules oligomerize upon blue light illumination, the mRuby3 fluorescence
 103 proteins move closer together, forming a bright emitting spot (Figure 1d). In this experiment,
 104 we tested several activation powers ranging from 0.5 to 4 nW in which the Bessel beam was
 105 formed using maximum and minimum numerical apertures (NAs) of 0.64 and 0.56,
 106 respectively. The theoretical and experimental cross-sectional intensities of the Bessel beam

107 along the beam propagation direction are illustrated in Figure S2 of the Supplementary
108 Information. The time-lapse maximum intensity projection (MIP) images of the CRY2oligo-
109 mRuby3 transfected human bone osteosarcoma epithelial cell line(U2OS) with various
110 stimulation energies are shown in Figure 1e. At each time point, a series of images were
111 collected in which the cell was first excited by a shorter wavelength laser for activation and
112 imaged by LLSM with a 10 ms exposure time for both activation and imaging. The cells were
113 then moved to the next position, and the process was repeated until the volumetric image was
114 collected. Each imaging volume consisted of 131 planes, which took 2.6 s to collect. After
115 optical activation, a significant aggregation of CRY2oligo-mRuby3 in the middle area of the
116 cells illuminated by the Bessel beam could be found (Figure 1e and Movie 1). Since the sample
117 scan mode was used, lines of mRuby3 fluorescence signals could clearly be seen at the center
118 of the cells. The diffusion of CRY2oligo-mRuby3 into the cytoplasm caused a broadening of
119 the width of the observed CRY2oligo-mRuby3 fluorescent strips over time. The intensity
120 profiles of different Bessel beams formed by various maximum and minimum NAs in addition
121 to the corresponding time-lapse MIP images of cell responses at the excitation laser power of
122 1 nW ($\sim 24 \text{ mW/cm}^2$) are shown in Figure S3 of the Supplementary Information. Note that the
123 minimum excitation power at the focal plane required to produce a visible clustering signal
124 was 0.2 nW, which was low enough for a long-term living cell experiment. Before stimulation,
125 no clustering was observed, indicating that the 561 nm laser used for volumetric imaging in the

LLSM could not trigger the clustering of the CRY2olig-mRuby3 protein (row 1 of Figures 1e). We also tested the activation using different wavelengths (405, 445, 488, and 514 nm) at 1 nW. The activation of CRY2olig-mRuby3 was observed for all of the tested wavelengths (Figure S4 and Movie 2 in the Supplementary Information).

3D activation using shifting Bessel beams

A Bessel beam is a non-diffracted beam in which the energy concentrates within its length and diverges rapidly, as shown in figure S1. Taking advantage of this property, we demonstrated that it was possible to shift the lattice beam for fast and large volumetric imaging, during which the lattice beam could be shifted in the propagation direction by imposing a defocusing phase profile on the SLM²⁷. Therefore, it may be possible to create a Bessel beam that can locally activate molecules in the beam propagation direction. In the previous section, we demonstrated that the Bessel beam could activate the CRY2oligo-mRuby3 at a specific location in each 2D frame during volumetric imaging where the continuous activation at the same position in different frames by Bessel beams acted as a Bessel fan activation on the Y'Z' plane within one volume scan (Figure 2a, Bessel fan). The corresponding timing diagrams for switching on the activation (blue) and imaging (green) laser by two-color SLM and the movement of the sample stage (black) are illustrated at the bottom of figure 2a. To control the activation in the beam propagation direction, we generated a shifted Bessel fan in the Y direction via SLM with the phase map shown in Figure 1b. The shift distance could be adjusted by changing the amplitude

of the modulation phase profile on the SLM to locally activate the basal area of the cell (Figure 2a, shifted Bessel fan) with the defocusing Bessel pattern for activation using the same timing diagram. Projection images of the cells activated by the Bessel fan activation across or underneath the cell body at different time points are shown in Figure 2b (movies 3 and 4). The depth color image was used to illustrate the clustering of molecules and the cell edge. For the regular Bessel fan activation, the XZ' and Y'Z' projections (first row in Figure 2b) indicate that the clustering CRY2oligo-mRuby3 initially took place 3 s after stimulation in the middle of the cell. In contrast, the clustering of CRY2oligo-mRuby3 was observed 21 s after activation at the bottom of the cell when a shifted Bessel fan was used (second row in Figure 2b).

To further demonstrate the capability of our system to conduct 3D activation, we first obtained a volume image of the cell without activation to select the proper frame for activation. The single Bessel beam activation was achieved by switching activation wavelength 488 nm at a given frame within a 3D stack scan (Figure 2a single Bessel Beam) where the timing diagram indicates the activation beam is switched on once at a specific z step during volumetric imaging. Unlike the Bessel fan stimulation in which the cells were stimulated in every frame, the single Bessel beam stimulation only activated the cells once per volume scan. The MIP images of the cells activated by such single Bessel beam stimulation are depicted in Figure 2c, in which the clustering of CRY2oligo-mRuby3 in the illuminating region could clearly be seen 21 s after stimulation. Our results indicate that the clustered fluorescent proteins slowly diffused away

from the illuminated area (marked as a white cross in Figure 2c and Movie 5). To analyze the spatiotemporal behavior of the activated clusters, 3D cell volume renderings with selected CRY2oligo-mRuby3 clusters are shown in Figure 3a, where cluster 1 is located right at the activation region, clusters 2 and 3 are 2 μm and 5 μm away from the activation region, respectively. The time-dependent fluorescence intensity changes of the chosen clusters are plotted in Figure 3b, where cluster 1 shows an instantaneous increase upon the activation beam. The delayed responses are observed according to the distance from the activation spot, for example, 10 s for cluster 2 and 40 s for cluster 3. To evaluate the photobleaching effect in our system, we monitored the fluorescence signal of a pre-existing cluster (cluster 4), which was far away from the activation beam. No decrease in fluorescence intensity of cluster 4 was observed during the whole experimental period indicating that the photobleaching effect of our system is negligible. A series of raw images for the selected plane activated by a single Bessel beam are shown in Figure 3c, where the formation of the cluster over time is marked in a white box. The analysis of fluorescence intensities of different clusters activated by Bessel fan and shifted Bessel beam is shown in Figures S5 and S6. These findings clearly demonstrate that LLSM could be easily modified to conduct 3D optogenetic experiment with good spatial control using a regular and modified Bessel fan/beam as an activation source.

Subcellular optogenetic activation of membrane ruffling

The next challenge of using LLSM for optogenetic application was to induce optogenetic

183 responses that had subcellular resolution. To control cellular responses at the subcellular level,
184 we adapted the CRY2-CIBN system to trigger the regional cell membrane ruffling. In this
185 experiment, the CRY2 was fused with PI3K-catalyzed motif (p85, iSH), which could trigger
186 membrane ruffling by enhancing Rac1 and cdc42 signaling for actin filament elongation^{28, 29,}
187 ³⁰, whereas a plasma membrane-anchor peptide (CAAX motif) was attached to CIBN. To
188 observe membrane ruffling, F-tractin-mCherry was used to monitor cytoskeletal dynamics. A
189 2A-self cleaved peptide (p2a) was used to connect each component, including F-actin-mCherry,
190 CRY2-iSH, and CIBN-CAAX to ensure nearly equal expression levels in the cell. We first
191 tested CRY2-mCherry-iSH-p2a-CIBN-EYFP-CAAX transfected cells using a Bessel fan
192 activation at 488 nm to trigger the response in the selected areas. The MIP images of CRY2-
193 mCherry in the cell in XY' plane are shown in Figure S7 and Movie 6 in the Supplementary
194 Information. To demonstrate that membrane ruffling can be induced with subcellular resolution,
195 two areas within a cell that were separated by 15 μm were illuminated sequentially by the
196 activation beam (regions 1 and 2 in Figure S7), and membrane ruffling at each location could
197 clearly be seen after stimulation. To evaluate whether the function of the cells was
198 compromised by the activation process, we waited 30 min for the cells to recover to the non-
199 activated state (as shown in Movie 6). A lattice beam at 488 nm instead of a Bessel fan was
200 used to activate the whole cell. Membrane ruffling was observed over the whole-cell, unlike
201 the local membrane ruffling shown in Figure S7, indicating the function of the cell remained

the same even after activation by the Bessel fan in two subcellular locations. Since CRY2 moved from the cytosol to the membrane, the detailed motion of membrane ruffling was not clearly resolved in the volumetric images. To observe the detailed motion during membrane ruffling, we studied the distribution of filamentous actin in the cells expressing F-tractin-mCherry-p2a-CRY2-mCFP-iSH-p2a-CIBN-EYFP-CAAX. After stimulation (white cross in figure 4a region 1), the membrane ruffled around the area under stimulation and moved inward at the speed of $\sim 1 \mu\text{m}/\text{sec}$ where the moving distance of the membrane edge along the white arrow is plotted in the inset. To demonstrate the capability of controlling the location of the stimulation area, the stimulation beam was moved to the newly formed membrane (white cross in figure 4a region 2) by shifting the Bessel beam, which was achieved by changing the SLM pattern with the X-shift as shown in Figure 1b. The magnitude of membrane ruffling at region 2 was larger than at region 1 (detailed membrane ruffling can be found in Movie 7). To present the dynamics of the filament actin, we used color-coded signals at different time points, as shown in Figure 4a. To better illustrate the dynamic behavior of the filamentous actins (the enlarged part of figure 4a), the contour function of the Amira software was used to outline the shape of the cell using the fluorescent signal from F-tractin-mCherry. The membrane protrusion and retraction upon the activation, as shown in Figure S8, reveals that the membrane ruffling is presumably increased at the site of activation as compared to the rest of the cell body. However, after the second stimulation at the newly formed protrusion (region 2), the membrane

ruffled rapidly in 3D at the edge of the cell as seen by the projection images in XY' and XZ' of long-range actin movements (Figure 4a). These results demonstrate that our system is capable of monitoring and activating the optogenetic molecules at the subcellular level.

Long-term manipulation of cell migration on lattice lightsheet microscope

To validate the capability of our technique with respect to manipulating cellular behavior for a long period of time, we first established an optogenetic system to control the migration of a cell in which the epidermal growth factor receptor (EGFR) was fused with CRY2olig and three repeating mApple (EGFR-CRY2olig-mApplex3) to activate the intracellular signaling of EGFR through blue-light induced clustering^{31, 32}. By maximizing the brightness of red fluorescence protein for whole-cell imaging, the cross-talk activation could be minimized at a very low laser power. EGFR activation is known to trigger cell migration through the PI3K signaling^{33, 34}. When the cells expressing EGFR-CRY2olig-mApplex3 were activated by the Bessel fan in the trailing edge, the formation of lamellipodia in the trailing edge was observed, as shown in Figure 4b. The distribution of EGFR is color-coded at different time points. From Figure 4b and Movie 8, it can be seen that the direction of migration was altered after stimulation in which the formation of lamellipodia was observed in the original trailing edge, and the old leading edge had started to retract. These results confirmed that the direction of migration could be controlled by optically induced clustering of the EGFR-CRY2olig-mApplex3 system activated by the Bessel fan at 488 nm in LLSM.

Next, a directed migration experiment was conducted by continuous stimulation. Since the cell would migrate out of the field of view, the position of the samples every 20–30 minutes was adjusted while maintaining the same stimulated area in the cell, as shown in Movie 9. To illustrate the trajectories of the cell in the same image, we marked the boundary of the cell at each time point with different colors. The time-dependent trajectories of the cell expressing EGFR-CRY2olig-mApplex3 stimulated by a Bessel fan in LLSM are shown in Figure 5. The individual LLSM rendering images of the 3D cell and the stimulated area are depicted at the bottom of Figure 5 and Movie 10. Moreover, the volumes of the migrating cell over time are calculated and plotted in the inset, where the volume of cell reduces when migration direction changes at T=265, followed by the formation of large lamellipodia at T=292. From these results, we demonstrated that the combination of LLSM and Bessel fan stimulation was useful for continuously manipulating and monitoring EGFR-CRY2olig-mApplex3-expressing cells for more than 6 hours, where ~75,000 2D-images (or 463 volumetric images) were collected, a finding that introduces the opportunity for conducting long-term optogenetic experiments in living cells.

Discussion

In optogenetic LLSM experiments, an additional stimulating light source is used. Therefore, we would like to evaluate the energy level used in the activation process. The minimum energy required for activation of the Bessel beam at different wavelengths was measured. It was found

that the minimum energy required to induce the clustering of CRY2olig-mRuby3 molecules was 0.2 nW at 488 nm, and 1 nW was enough to activate the CRY2olig-mRuby3 system with a Bessel beam at 405, 445, 488, and 514 nm. For volumetric imaging in LLSM, the total power was 40 nW at 561 nm, which was distributed over the 145 lattice beams for the collection of fluorescent images with a signal-to-noise ratio greater than 5 for the observations of CRY2, CRY2oligo, filament-actin, and EGFR.

The lattice lightsheet is produced by constructive interference of several non-diffracting Bessel beams with a minimized energy distribution in the sidelobes. However, when a single Bessel beam is used as the activation source, the influence of the energy distribution in the sidelobes, which could deteriorate the spatial resolution for activation, should be considered. A detailed comparison between the Bessel beam and Gaussian beam for lightsheet microscopy can be found in the supplementary note. In this experiment, the spatial distribution of the optogenetic clustering of CRY2olig-mRuby3 using different Bessel beams formed by various minimum NAs with the same maximum NA was evaluated. The energy distributions of the Bessel beams at the back aperture and focal planes (XZ) can be found in the first and second row of Figure S3a of the Supplementary Information.

The LLSM images of the optogenetic clustering in U2OS cells expressing CRY2olig-mRuby3 stimulated by different Bessel fans at different time points are found in the fifth to the eighth row. Since the raw LLSM images contained all signals from CRY2olig-mRuby3, the Threshold

function in the Amira software (Thermo Fisher Scientific) was selected to calculate the spatial distribution of the optogenetic clustering (an example is shown in Figure S3b). The measured width of the Bessel fan and the width of optogenetic clustering are depicted in Figure S3c. It was observed that the spatial distributions of the optogenetic clustering were much wider than the width of the stimulating Bessel beams, with ratios ranging from 4 to 5. The measured Bessel beam profiles in dye solution using different numerical apertures (NAs), as shown in Figure S3, also confirmed the agreement between the simulations and the experimental data. To characterize the Bessel fan and single Bessel beam activation, we measured the fluorescence signals of the photoactivated CRY2oligo-mRuby3 molecules by 488 nm in figure S12 (movies 11 and 12). The weak fluoresce signals excited by 488 nm can be used to visualize the activated CRY2oligo-mRuby3 molecules within the path of the photoactivation beam, which could be used to indicate the beam profile of the photoactivation beam. In the Bessel fan activation, a strip with 2 μm width (FWHM) was observed from the contributions of main and side lobes of the illuminated Bessel beam, whereas a weak measured photoactivation was observed at the selected z slice in the single Bessel beam activation.

In the optogenetic heterodimer system (CRY2-CIBN), two independent membrane ruffling responses could be triggered in the same cell with 1 nW Bessel fan activation at 488 nm (Figure S7), which clearly demonstrates our capability for manipulating the cells with subcellular resolution. It was also found that about 30 minutes was required for the cell to recover to its

original state at 1 nW stimulation. The energy used for CRY2Olig-mRuby3 activation was similar to the CRY2-CIBN dimerization energy. To control cell migration, CRY2Olig was linked to the intracellular part of the EGFR, in which optically-induced oligomerization of CRY2Olig led to EGFR oligomerization. It is known that the existence of other EGFR molecules in close proximity with each other will promote EGFR signal activation. Therefore, the direction of migration could be reversed by activating EGFRs in the trailing end of the migrating cells, as shown in Figures 4b and 5.

To achieve cell manipulation in 3D, a focus shift was used in this experiment in which regions illuminated by the light sheet could be controlled by adding a binary phase Fresnel lens on the spatial light modulator used to generate Bessel beams filtered by a passive optical component, physical mask. As shown in figure 1b, the region illuminated by the light sheet, where its length and thickness are pre-determined by a chosen annular mask, could be moved forward or backward in the light propagation direction. Within the length of the lightsheet, the optogenetic system could be activated effectively, whereas the energy distribution of the lightsheet diverged rapidly beyond the length of the light sheet but was not enough for optogenetic system activation (energy distribution is shown in figure S2). Therefore, the optogenetic molecules located on the membranes could be activated locally at the apical or basal membranes to achieve a controllable optogenetic system in 3D. Despite the contribution from the sidelobes of a Bessel stimulation, which can be minimized by carefully engineering the beam shape

before the experiment as shown in figure S12, not only the diffusion effect of CRY2oligo-
mRuby3 but also the energy distribution in the sidelobes still contributed to the broadened
width in the activated area as shown in figure S3c. To improve the spatial confinement for
activation, two-photon activation or temporal focusing may be used^{35, 36}. However, the
integration of these techniques into LLSM may be complicated. Another challenge in
implementing 3D activation in LLSM was the line confinement activation instead of a point in
the propagation direction, which was about 20 μm at the present configuration in order to cover
the whole cell imaging. Such confinement allows us to conduct local activation of molecules
located on the apical or basal membrane by SLM manipulation but not enough to activate
molecules within a specific organelle in the cells with the fixed annular mask. An aperture-free
technique for the generation of activation beam and lattice beam at different beam lengths is
required in the future. In summary, the 3D activation capability of our approach opens the
opportunity for optogenetic activation of single cells in small animal models.

Materials and Methods

Plasmid construction

For the multiple-gene expression system, the plasmid pCR3.1-p2a-SpeI was generated from the original pCR3.1 plasmid, which was added in-frame to the porcine teschovirus-1 2A self-cleaving peptide (p2a) and SpeI cutting site to fit with the NheI cutting site of the enhanced green fluorescent protein (EGFP) series of vectors. The F-tractin-mCherry-p2a-SpeI, CRY2-mCherry-iSH-p2a-SpeI and CRY2-mCFP-iSH-p2a-SpeI were constructed based on pCR3.1-p2a-SpeI plasmid. The filamentous actin marker (a cytoplasmic actin filament reporter, the neuronal inositol 1,4,5-triphosphate 3-kinase A actin-binding domain known as F-tractin [Plasmid #58473, addgene]) was fused with monomeric red fluorescent protein to the N-terminal p2a peptide via digestion using two restriction enzymes, NheI-HF and PmlI, and ligated into pCR3.1-p2a-SpeI plasmid. Using the specific primer polymerase chain reaction (PCR) cloning, the C-terminal of Arabidopsis cryptochrome 2 (CRY2) with mCherry or mCFP (monomer cyan fluorescent protein) was linked with the phosphoinositide 3 kinase (PI3K)-catalyzed motif (iSH) to generate CRY2-mCherry-iSH-p2a-SpeI and CRY2-mCFP-iSH-p2a-SpeI plasmids. The multiple gene expression of the backbone plasmid, which contains the CRY2 interaction binding protein N-truncated version (CIBN) domain linked to the plasma membrane anchor leading sequence (CIBN-CAAX), was cut by double digestion using NdeI and NheI. The CRY2-mCherry-iSH-p2a-SpeI or CRY2-mCFP-iSH-p2a-SpeI was then cut by double digestion using NdeI and SpeI for insertion into the backbone plasmid. At the end, the

same protocol for generating CRY2-mCherry-iSH-p2a-CIBN-EYFP-CAAX and F-tractin-mCherry-p2a-CRY2-mCFP-iSH-p2a-CIBN-EYFP-CAAX plasmid was followed. On the other hand, the generation of CRY2olig-mRuby3. CRY2olig-mCherry plasmids were modified by point mutation PCR to generate an XmaI cutting site for compatible cohesiveness with the AgeI cutting site. The CRY2olig-XmaI-mCherry plasmids were then digested with XmaI and BsrGI. Using an AgeI and BsrGI double digestion, the mRuby3 was inserted into the CRY2olig-XmaI plasmid. To prepare CRY2Olig-mApplex3, the BsrGI-mApple-BsiWI-PmlI was first created by specific primer PCR to increase the repeat of mApple, and AgeI and BsiWI (compatible cohesive end with BsrGI) were used to replace the original mRuby3 site of CRY2Olig-mRuby3. The XhoI cutting site of EGFR fits in the frame with the open reading frame of CRY2Olig-mApplex3. EGFR was cut with NheI and XhoI for insertion into the N-terminus of CRY2Olig-mApplex3 for creating EGFR-CRY2Olig-mApplex3.

Imaging of optogenetic tools expressed cell line

U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, ThermoFisher), which contained 10% fetal bovine serum (FBS) from Gibco (ThermoFisher), 2 mM L-glutamine (Gibco, ThermoFisher), and 1% penicillin-streptomycin solution (Gibco, ThermoFisher). Before transfection, 5 mm glass coverslips were placed in 1X phosphate-buffered saline (PBS) buffer with 10 µg fibronectin for 2 h at room temperature. The fibronectin-coated coverslips were washed twice with 5X PBS and once with 1X PBS. U2OS

cells (1×10^5) were seeded into 12-well plates containing the fibronectin-coated glass coverslips and cultured in DMEM medium without penicillin-streptomycin to enhance transfection efficiency.

Cells were incubated for 16 h at 37 °C and 5% CO₂ before undergoing transfection with 0.8 µg plasmid DNA per well using Maestrefectin Transfection Reagent (Omics Bio). After 16 h, the transfected cells were allowed to recover for 24 h in 1% penicillin-streptomycin and 10% FBS DMEM medium.

Stimulation setting and Image acquisition

In order to standardize the stimulation conditions, we used a power meter (PD300-1W with Nova II, Ophir Photonics, USA) to detect the input power of the Bessel beam behind the pupil of the exciting objective. The required parameters for different wavelengths (405, 445, 488, and 514 nm) and power (0.5, 1, 2, and 4 nW) were adjusted according to the readings. To measure the laser power below the detection limit of the power meter, which is about 1 nW, the laser power was attenuated linearly by a combination of the acousto-optic tunable filter (AOTF) and the neutral density filters. Multi-color scanning was used in the Z-scan mode in the SPIM program (licensed by Howard Hughes Medical Institute, Janelia Research Campus) to generate multi-color 3D images, on which the signals from each channel were collected one by one from the same layer. As shown in the bottom of figure 2a, the SLM displaying two pre-loaded color patterns (488 nm and 560 nm) for either Bessel/lattice beam or shifted Bessel/lattice beam at

each step of the sample stage at the designed time sequence based on the experimental exposure time in the SPIM software. In an optogenetic experiment, the excitation source was first switched to the projected Bessel beam mode for stimulation and then followed by lattice beams for imaging. In each experiment, blank control images were collected for the first 20 time points by turning off the stimulating light source with a mechanical shutter which is placed in front of the 488 nm laser, controlled by a homemade Arduino device synchronized to the image acquisition software. For single Bessel beam stimulation, the SPIM program held the x-galvanometer without any movement, thus allowing the Bessel beam to stimulate the central area of the cells, and the activation wavelength 488 nm was illuminated on the samples at a selected z slice while performing z-stacking; the timing is clocked by Arduino device.

In the experiment requiring moving the stimulation area along the X-axial direction, the position of the Bessel pattern on the SLM could be changed to illuminate the corresponding focal plane position. Alternatively, the x-galvo could be used to move the illumination area of the Bessel beam to a specified position with an accuracy of 0.1 μm . In the single-Bessel beam stimulation experiment, a shutter was used to allow the stimulation beam to activate the cells once per volume scan. In addition, the tiling lattice lightsheet technique was used in the experiment to shift the Bessel beam along the Y-axial direction by changing the beam shift coefficient as described previously²⁷.

Image capturing and processing

408 The details for the LLSM optical scheme can be found in the supplementary note. In this
 409 experiment, a 2048 x 2048 pixel COMS camera (ORCA Flash 4.0 V2, Hamamatsu) was used
 410 in which an area of 200 x 960 pixels (20.6 μm x 98.9 μm) was used for LLSM imaging. In a
 411 typical LLSM imaging setup, the exposure time for each frame is 9 ms, the sample scanning
 412 step is 0.6 μm , and 131 planes of images are used for volumetric imaging. For time-lapse
 413 volumetric imaging, the volumetric image is recorded every 3 s. The camera settings for the
 414 directed cell migration experiment are the same (10 ms exposure time for 200 x 1024 pixels
 415 with 161 planes) for both colors 488 nm (activation) and 560 nm (imaging), where it takes ~ 3
 416 s to finish 3D optogenetic imaging. Initially, the time interval between each volumetric image
 417 was set to 6 s until we observed cellular response or membrane ruffling (in this case, 60-time
 418 points). We then changed the time interval to 1 min to observe directed cell migration for 343-
 419 time points. Note that for cell manipulation, such as induced membrane ruffling and directed
 420 cell migration experiments, the subcellular responses of cells cannot be predicted. Therefore,
 421 we cannot program the SLM pattern time profile in advance. To demonstrate the capability of
 422 local activation, the photoactivation was conducted manually. After recording the first cellular
 423 response, the program was stopped, and the activation beam was moved to the desired area
 424 either by changing the SLM pattern or the x-galvo position. The program was resumed to record
 425 the cellular response. These processes were repeated until the end of the experiment. Since the
 426 imaging plane was tilted from the normal direction of the coverslip, the images orthogonal to

the coverslip with a view angle similar to conventional microscopy were reconstructed from the raw images through the GPU program. To process the images for better presentation in Figures 4 and 5, Amira imaging process software (ThermoFisher) was used. For the directed cell migration, the timing and position were registered in the movie reconstruction, as shown in figure 5 and movie 10.

Acknowledgment

The microscope control software is licensed by the Howard Hughes Medical Institute, Janelia Research Campus. This work was supported by the Ministry of Science and Technology, Taiwan (Project No. MOST 109-2113-M-001 -034 -MY3 to P.C., 109-2628-M-001-001-MY4, 110-2321-B-002-012 to B.C.C., MOST 110-2636-B-007-011, and MOST 108-2638-B-010-001-MY2 to Y.C.L.). This work was also supported by the Academia Sinica, Taipei, Taiwan (AS-IA-110-M04 to P.C. and AS-CDA-107-M08 to B.C.C.). We also thank the DNA Sequencing Core Facility of the Institute of Biomedical Sciences, Academia Sinica, for DNA sequencing analysis. The core facility is funded by Academia Sinica Core Facility and Innovative Instrument Project (AS-CFII-108-115).

Figure Legends

Figure 1

(a) The schematic diagram of the major optical components in lattice lightsheet microscopy (LLSM). The patterns displayed by the spatial light modulator (SLM) are imaged at the pupil plane of the excitation objective through an annular mask and lens. Lattice beams, in addition to Bessel beams with different X, Y positions, can be generated at the imaging plane by imposing different patterns on the lightsheet microscopy (LSM). (b) Various patterns on the SLM for generating the corresponding excitation beams in the imaging plane. The position of the Bessel beams can be shifted in the X or Y direction by using X- or Y-shift patterns. For normal Bessel beam stimulation, the Bessel beam with a length of 20 μm illuminates the cell at a tilted angle of 32.8 degrees. With Y-shift stimulation, the Bessel beam can be moved along the propagation direction to illuminate the basal membrane. With an X-shift pattern, the Bessel beam can be moved to a different location in the x-direction with an accuracy that is better than 100 nm. (c) The illumination scheme for an optogenetic experiment. The blue beam represents the Bessel beam at 488 nm used to activate the optogenetic molecules, and the green lightsheet represents the 561 nm lattice lightsheet for imaging. The volumetric image was obtained by dithering the lattice beam and moving the sample along the Y' direction. (d) The schematic for optical induced clustering of cryptochrome 2 (CRY2)olig-mRuby3. Blue: CRY2olig, Red: mRuby3. (e) The time-lapse maximum intensity projection (MIP) images of CRY2olig-mRuby3 expressed cells with various activation energies. The interval between each stack is 3 s. Scale bar 10 μm .

Figure 2

(a) The schematic for various activations: Bessel fan, shifted Bessel fan, and single Bessel beam. The activation beam illuminates on the Y'Z' plane at an angle of 32.8°. Bottom: The corresponding timing diagrams for the SLM patterns with laser 488 nm Bessel beam (blue) and 560 nm lattice beam (green) illumination synchronized with sample piezo waveforms (black). (b) The time-lapse images of optical induced clustering of CRY2oligo-mRuby3 in XY' Y'Z' XZ' planes stimulated by Bessel fan and shifted Bessel fan activation. The depth of the oligomerization is color-coded, where the process of oligomerization occurs only at the basal membrane by the shifted Bessel beam activation. (c) The center part of the cell marked by the

white cross is illuminated by the single Bessel beam. Oligomerization occurs near the activation site. Scale bar 10 μm .

Figure 3

(a) Top view and side view of the 3D cell volume rendering image of the selected clusters of CRY2oligo-mRuby3 molecules induced by a single Bessel beam activation at the spot shown in Figure 2c. Scale bar 10 μm . (b) Time-lapse fluorescence intensity plots of the marked clusters in (a). (c) Left: Raw image of the optical induced clustering of CRY2oligo-mRuby3 in the selected plane activated by a Bessel beam marked in a white cross in Fig. 2c at $T = 120$ s. Right: Enlarged raw time-lapsed images of the green box in the left in the single Bessel beam activation experiment. Scale bar 10 μm and 1 μm , respectively.

Figure 4

(a) The cells expressing F-tractin-mCherry-p2a-CRY2mCFPiSH-p2a-CIBNEYFPcaax are illuminated by single Bessel beam activation. The cross represents the activation site. To demonstrate the dynamic behavior of filamentous actin, intensity contours were plotted before and after the stimulation. At region 1, the distance of membrane edge along the white arrow is shown in the inset. Different color represents different time points after stimulation. The top images are XY orthographic view, and the bottom images are XZ view with 32.8° rotation in X-axis. The gray images are the original images. (b). The Bessel fan was used to stimulate the endothelial growth factor receptor (EGFR)-CRY2Olig-mApplex3-expressing cells at the trailing edge. The images in the center row are the whole-cell images at different times post-stimulation. The stimulation areas are marked as cyan. The solid arrow indicates the original trailing edge, and the hollow arrow indicates the original leading edge. Images on the top and bottom rows are enlarged views of the dashed box area in the middle row. Different colors represent different time points. Scale bar 10 μm

Figure 5

The time-dependent trajectories of the cell in the guided migration experiment. The cell expressing EGFR-CRY2oligo-mApplex3 was stimulated by 1nW Bessel fan. Different colors represent different time points. The individual LLSM 3D rendering images of the cell at different times, and the stimulated areas are depicted at the bottom. The volume changes of the cell during the cell-guided migration are plotted in the inset. The interval between each stimulation and image is about 1 min. The entire experiment lasted 392 minutes. Scale bar 20 μm

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