Discovery of an F-actin–binding small molecule serving as a fluorescent probe and a scaffold for functional probes

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Actin is a ubiquitous cytoskeletal protein, forming a dynamic network that generates mechanical forces in the cell. There is a growing demand for practical and accessible tools for dissecting the role of the actin cytoskeleton in cellular function, and the discovery of a new actin-binding small molecule is an important advance in the field, offering the opportunity to design and synthesize of new class of functional molecules. Here, we found an F-actin–binding small molecule and introduced two powerful tools based on a new class of actin-binding small molecule: One enables visualization of the actin cytoskeleton, including super-resolution imaging, and the other enables highly specific green light–controlled fragmentation of actin filaments, affording unprecedented control of the actin cytoskeleton and its force network in living cells.

INTRODUCTION

Actin filaments are major components of the cytoskeleton in eukaryotic cells, functioning to maintain the shape and internal framework of cells and to provide the cells with a driving force for shape change and movement (1). Natural actin-binding small molecular inhibitors have long been recognized as valuable tools for dissecting the mechanisms of actin-related cellular functions (2).

Actin-binding molecules have also been used as platforms for functional molecules; for example, fluorescent phalloidin conjugate was originally developed in 1979 (3) and is still the gold standard for labeling endogenous actin filaments in fixed samples. Lifect is a 17-amino acid peptide aptamer derived from an actin-binding protein, and its conjugate with a fluorescent protein is widely used for transfection-mediated visualization of the actin cytoskeleton (4). Since then, a number of genetically encoded probes have been developed to visualize endogenous F-actin in living cells. On the other hand, a rationally designed and optimized analog of an actin-binding natural product, lysine-modified des-bromo-des-methyl-jasplakinolide (5), has emerged as powerful scaffold for the design of synthetic fluorescent probes. By using this scaffold as a recognition unit for F-actin, SiR-actin has been successfully developed as a first-in-class fluorescent probe that is practically applicable for live-cell super-resolution imaging (6). More recently, carbopyranine-based probes have been introduced (7–9). There is a growing demand for practical and accessible tools for dissecting the role of the actin cytoskeleton in cellular function, and thus, the discovery of a new actin-binding small molecule is an important advance in the field, offering the opportunity to design and synthesize of new class of functional molecules.

Here, we show that HMRef (10), a simple rhodol derivative bearing a hydroxymethyl group (Fig. 1A), is a new and powerful tool for actin labeling in live cells. HMRef was originally developed as a highly fluorescent and membrane-permeable fluorophore for in vivo tumor-imaging probes. Unexpectedly, we found that HMRef can clearly visualize the actin cytoskeleton, despite having no structural similarity to any of the known actin-binding natural products, such as phalloidin or jasplakinolide (2). By modifying this newly found actin-binding molecule, we have also succeeded in developing a functional molecule for optical manipulation of F-actin.

RESULTS

In vitro and in cellulo validation and elucidation of binding to F-actin

To demonstrate the binding ability of HMRef to F-actin, we first checked the in vitro interaction of HMRef with isolated F-actin/ G-actin by means of fluorescence polarization (FP) assay (Fig. 1B) (11). As expected, the FP signal increased when HMRef was incubated with isolated F-actin, i.e., HMRef has intrinsic binding affinity for F-actin and does not require assistance from actin-binding proteins. The FP signal increase was negligible in the presence of monomeric actin, suggesting specific binding of HMRef to polymeric actin. Second, we conducted costaining of fixed HeLa cells with HMRef and known F-actin binders. Fluorescence images of HMRef and Alexa Fluor 647 phalloidin merged strongly (Fig. 1C), and both phalloidin and jasplakinolide markedly and dose-dependently decreased the HMRef fluorescence response to actin in fixed cells, suggesting that their binding sites are likely to overlap, at least...
M decreased in the presence of either 1 M HMRef or binding of Alexa Fluor–labeled phalloidin (2 U/ml) was markedly for fixed cell actin staining, indicating that modification of with Alexa Fluor–labeled phalloidin (2 U/ml), it seems likely that the median inhibitory concentration values of those compounds under the conditions used are in the submicromolar range. Thus, it is plausible that the hydroxymethyl group is not required for high-affinity actin binding.

Note that HMRef and 2 provide slightly different F-actin images, albeit with similar affinity. HMRef shows bright fluorescence on F-actin with little background or off-target fluorescence, whereas 2 has a relatively higher background, leading to a loss of image contrast (Fig. 2B). We consider that the low background of HMRef is at least partially due to the environmental sensitivity of the dye. In cells, lipophilic dyes such as HMRef and 2 can accumulate in the endomembrane, but HMRef, although not 2, would exist in nonfluorescent and colorless spirocyclic form in the hydrophobic internal membrane, which would reduce off-target fluorescence (Fig. 2C).

Live-cell imaging with HMRef
While further investigations are needed to clarify how HMRef and its derivatives are recognized by F-actin, we examined the potential of HMRef as a practical actin cytoskeleton visualization tool in living cells and tissues. In vitro actin polymerization/depolymerization assay (Fig. S4) and some in cellulo experiments to examine the dose dependence of the cell response (Fig. S5) revealed that higher concentrations (a few micromolars) of HMRef affect actin polymerization (Fig. S4, A to C), depolymerization (Fig. S4D), migration rate (Fig. S5B), cell shape (Fig. S5, C and D), and cell proliferation (Fig. S5E). However, these impacts were not detected at submicromolar concentrations of HMRef, which are sufficient for visualization of the actin cytoskeleton (Fig. S5D), suggesting that HMRef in this concentration range meets the requirements for visualization of physiological actin dynamics.

We confirmed that HMRef is suitable for super-resolution imaging using the stimulated emission depletion (STED) (12, 13) and super-resolution radical fluctuation (SRRF) (14) techniques and obtained extremely detailed actin staining images (Fig. 3, A to F). Existing probes used for live-cell STED imaging are based on fluorogenic rhodamine derivatives that show yellow to red fluorescence (6–9) (emission wavelength of more than 580 nm), so HMRef is a first-in-class green fluorescent probe for STED imaging (excitation/emission maxima, 498/519 nm).

Given that actin has been one of the most highly conserved proteins during evolution, it was not unexpected that HMRef stains actin in multiple cell lines derived from various species (Fig. 3G), including Vero and Cos-7 cells, for which SiR-actin does not work well (6). In addition, as found for primary cultured cells and tissues, HMRef clearly visualized retrograde actin flow in migrating fish keratocytes (Fig. S6 and movie S1) (15, 16), as well as the contractile ring dynamics of cleavage furrow ingression during cell division in Drosophila wing disc (Fig. 3, H and I). These results indicate that HMRef is a powerful tool to visualize the actin cytoskeleton of various cells and organisms in real time.

Optical manipulation of F-actin with a synthetic small molecular probe
Next, we used HMRef as an F-actin–binding scaffold to develop a functional probe for F-actin manipulation. A tool that can ablate the actin filament network with single-cell resolution would have great potential for better defining the forces involved in adhesion and migration of multicellular assemblies (17). Although actin-targeting natural product inhibitors are already useful tools in actin-related cell biology (2), their spatial resolution is limited. One approach to circumventing this issue is the use of chromophore-assisted light inactivation (CALI), in which a suitable ligand serves to direct a

![Fig. 1. HMRef as a F-actin binding molecule.](image-url)

(A) Structure of HMRef (B) Evaluation of F-/G-actin–binding ability of HMRef by means of FP. Values are means ± SD, n = 2 (F-actin, 1 nM) or n = 3 (others). (C) Confocal imaging of fixed and permeabilized HeLa cells stained with HMRef (green) and Alexa Fluor 647 phalloidin (red). Fixed cells were incubated with Alexa Fluor 647 phalloidin (2 U/ml) in Dulbecco’s PBS for 30 min and then stained with 500 nM HMRef in Dulbecco’s PBS for 2 hours. Scale bars, 20 µm.
gradually propagated to the whole-cell area (fig. S12A and movie the photoirradiated area. Subsequently, F-actin fiber dismantling signal and subsequent F-actin fiber dismantling were observed in HeLa cells in which Lifeact (\textregistered) TagRFP was transiently expressed, 4
tion of actin filaments. When we locally illuminated GLIFin-loaded cells (\textregistered), and cell viability were unaffected (figs. S10 and S11).

As for spatial resolution, GLIFin could achieve single-cell level resolution for actin fragmentation (Fig. 4F), presumably as a result of the path length of singlet oxygen, which is as short as <100 nm in cells (23). Next, we sought to achieve precise subcellular perturbation of actin filaments. When we locally illuminated GLIFin-loaded HeLa cells in which Lifeact (\textregistered) TagRFP was transiently expressed, and localized photobleaching of the red fluorescent protein (RFP) signal and subsequent F-actin fiber dismantling were observed in the photoirradiated area. Subsequently, F-actin fiber dismantling gradually propagated to the whole-cell area (fig. S12A and movie S2). No fragmentation of actin was seen in the absence of GLIFin (fig. S12B and movie S3).

We next applied GLIFin to epithelial cell monolayers (Fig. 4G and figs. S13 and S14) where cell–cell interactions involving the actin cytoskeleton at adherens junctions and the actin–extracellular matrix mediate coordinative cell movement and morphogenesis (24). Migration measurements showed a substantial decrease in migration speed in a light- and GLIFin-dependent manner (Fig. 4H), without loss of cell viability (fig. S15). Notably, the effect of GLIFin-mediated inactivation was relatively long lasting, and the migration rate was restored only after ~12 hours. A limited forward movement of cells into open space was frequently observed in nonirradiated areas, where the cells formed a kind of “boundary layer,” although space was still available in front of the actin-inactivated cells (Fig. 4I). These observations might reflect disorder of long-range interactions involving the intracellular actin network. Last, we confirmed the applicability of GLIFin-mediated inactivation to an in vivo model, the wing disk of Drosophila larvae (fig. S16). Compared to the selective removal of cells by laser ablation, GLIFin-mediated photoinactivation offers the advantage of low invasiveness. Specifically, GLIFin allows flexible disruption of intracellular force transmission while leaving cells alive and adhesive. We believe that it will be a powerful tool in a variety of fields, including the study of supracellular organization to generate force between leader and follower cells during the cooperative movement of groups of cells (25).

**DISCUSSION**

In present study, we introduce a new F-actin–binding small molecule, HMRef, which is structurally quite simple and consequently synthetically more accessible than previously known actin-binding small molecules. On the basis of this discovery, we have established a new class of live-cell visualization and manipulation probes for F-actin.

The characteristics of HMRef for staining fixed cells are compared with those of the well-established existing methods in
table S2. As for the visualization of the actin cytoskeleton in living cells, an existing probe, SiR-actin (6), has been widely used. Nevertheless, we believe that HMRef will be a practical tool for live-cell or tissue imaging due to its nearly equal brightness ($\varepsilon \Phi_L$) to SiR-actin (table S3), as well as a number of advantages for living sample imaging. First, HMRef stains actin in multiple cell lines derived from various species (Fig. 3G), including Vero and Cos-7 cells, for which SiR-actin does not work well (6). Second, F-actin staining by HMRef can be done more quickly (~15 min) than by SiR-actin (1 hour-~) (fig. S17). This is partly due to the difference in binding rate of the dyes, suggesting that HMRef would be more suitable for real-time tracking of the dynamics of the actin remodeling process. Third, while

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**Fig. 3. HMRef as a fluorescent probe for F-actin.** (A) "STED" imaging of living COS-7 cells stained with 1 $\mu$M HMRef. A confocal image without STED ("confocal") is shown for comparison. Scale bar, 20 $\mu$m. (B) Magnified images and line profiles of HMRef-stained COS-7 cells. Line profiles show the fluorescence signal along the long axes in the insets, and the fitted Gaussian curves are also shown. Scale bar, 2 $\mu$m. (C and D) Confocal (C) and SRRF (D) images of HeLa cells stained with 1 $\mu$M HMRef. The SRRF image was reconstructed from 100 raw confocal images. HeLa cells were incubated with 1 $\mu$M HMRef in growth medium for 1 hour, and images were captured with an Andor Dragonfly confocal microscopy system. (E and F) Enlargements of the region outlined by the boxes in (C) and (D) are shown in (E) and (F), respectively. (G) Confocal images of HMRef-loaded living cells. Five hundred nanometers of HMRef for 30 min. Scale bars, 10 $\mu$m. (H) Schematic illustration of *Drosophila* wing disc. In (I), the posterior-dorsal region of the wing disc is imaged (red square). (I) Live imaging of *Drosophila* wing imaginal discs incubated with 500 nM HMRef. Selected snapshots from a movie showing HMRef (green in top and gray in bottom) and E-cad–mTagRFP (red in top and gray in middle). HMRef labels F-actin along the cell-cell junction and the contractile ring during cytokinesis (cyan arrowheads). Magenta asterisks indicate a dividing cell and its sister cells. Scale bar, 10 $\mu$m.
SiR-actin showed a biased misdistribution of staining pattern at the cellular level, typically without verapamil, HMRef-stained images tended to be uniform (fig. S18), suggesting that HMRef might enable more meaningful comparisons of F-actin amount and structure among cells. Furthermore, although both SiR-actin and HMRef stained stress fibers well, HMRef provided better actin meshwork staining images than SiR-actin (fig. S19).

As for manipulation, GLIFin induced light-dependent inactivation of the actin cytoskeleton in various cell lines (Fig. 4D and figs. S13, S16, and S20). Unlike ablation of actin using pulse laser irradiation, GLIFin-mediated actin fragmentation is a relatively slow, time-dependent process (fig. S9). When F-actin inactivation was induced in a localized subcellular area by GLIFin, rapid F-actin stress fiber dismantling was observed in the photoirradiated area, and thereafter, the stress fiber dismantling gradually propagated through the whole cell (fig. S12A). This may suggest the involvement of active processes, such as F-actin switching for oxidatively modified actin (26), and/or a mechanobiological response to the loss of tension (27). In addition, given that the effect of GLIFin-mediated inactivation was relatively long lasting and the decrease in the migration rate took at
least 12 hours to recover (Fig. 4H), it seems plausible that the damaged actin molecules cannot be reused for actin filament network assembly and that accumulation of newly expressed actin molecules is required for recovery of cell motility.

Very recently, two novel techniques for optical manipulation of F-actin have been reported using optojasps (28, 29), a conjugate between jasplakinolide and photoswitchable azobenzene, and Nvoc-CytoD (30), a photocaged cytochalasin D, which directly affect F-actin. While the inhibitory action of those drugs has been precisely defined, the mechanism underlying GLIFin-mediated actin perturbation remains to be established. Besides, GLIFin-mediated photoactivation involves generation of singlet oxygen, which would oxidize actin and perhaps its binding proteins around the actin cytoskeleton, suggesting that GLIFin would not be an appropriate tool to discuss relationship between certain aspect of actin function and biological phenomena. However, since GLIFin enables flexible designing of emasculated cell zone in multilevel assemblies, it would be a powerful tool to address how long-range and short-range interplay among cells determine individual cell behavior to maintain group integrity during multicellular process, such as collective migration and cell competition. In addition, GLIFin offers a number of advantages over those agents, as follows. First is wavelength: Manipulation with GLIFin can be achieved with a 514-nm laser, which is less toxic than a 405-nm laser (31). Second is sustained effect: Technically, once GLIFin dismantles F-actin, the GLIFin molecules can be removed, so that side effects can be minimized, whereas the photoactivatable drug approach requires of the continuing presence of active drug to maintain the effect. Third is spatial resolution: The path length of singlet oxygen is as short as <100 nm in cells (23), indicating that active species would hardly diffuse across cells, whereas activated drugs can diffuse away from the irradiated area. These advantages suggest that GLIFin might maintain a relatively physiological phenotype of nonirradiated cells in multicellular experiments, such as studies of migration in epithelial cell monolayers or of cell/tissue homeostasis, especially three-dimensional homeostasis, potentially with the use of a two-photon excitation. Thus, our approach complements existing visualization/ manipulation techniques, and we expect it to be a useful tool in our endeavor to reach a comprehensive understanding of actin function.

### MATERIALS AND METHODS

#### Cell lines and culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, or F-12 containing 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (all reagents were purchased from Life Technologies). All cell lines were maintained at 37°C in 5% CO₂. Details of the cell sources and culture media are given in text S2.

#### Preparation of HMRef derivatives

HMRef was prepared as previously described (10). Compound 1 was prepared as fig. S21. Compound 2 was prepared as fig. S22. Compound 3 was prepared as fig. S23. Compound 4 was prepared as fig. S24. Compound 5 (HMRpf) was prepared as previously described (10). Compound 6 (HMRet) was prepared as previously described (10). Compound 7 was prepared as fig. S25. Compound 8 was prepared as fig. S26. GLIFin was prepared as fig. S27.

#### Imaging of the actin cytoskeleton stained with HMRef derivatives and/or fluorescence-labeled phalloidin in fixed cells

Cells were plated on glass-bottomed eight-well chamber plates (80826, Ibidi) and incubated with growth medium for 1 day. The cells were washed with phosphate-buffered saline (PBS) three times, fixed, and permeabilized with PBS containing 4% HCHO and 0.1% Triton X-100 for 10 min. PBS was removed, and the fixed cells were washed with PBS three times and incubated in PBS containing 0.66% MeOH and Alexa Fluor 647 phalloidin (2 U/ml) (A22287, Thermo Fisher Scientific) for 30 min. For costaining with HMRef derivatives, the Alexa Fluor 647 solution was replaced with PBS containing 500 nM or 1 μM HMRef derivatives. Unless otherwise mentioned, fluorescence images were acquired with a confocal fluorescence microscope (TCS SP8, Leica) equipped with a multilayer-argon and He-Ne laser and an objective lens (HCX PL APO CS 40×/1.25 oil, Leica). The excitation and emission wavelengths were 488/510 to 550 nm for HMRef derivatives, and 633/661 to 750 nm for Alexa Fluor 647 phalloidin.

#### FP analysis

Actin (1 mg) from rabbit muscle (A2522-1MG, Sigma-Aldrich) was dissolved in 1 ml of general actin buffer (“G buffer”; catalog no. BSA01-010, Cytoskeleton Inc.). The actin solution was left on ice for 1 hour for depolymerization. Then, 100 μl of actin polymerization buffer (“P buffer”; catalog no. BSA02-001, Cytoskeleton Inc.) and 200 nmol of adenosine 5’-triphosphate (ATP) in 2.0 μl of H₂O were added and mixed. After 2 hours of incubation for polymerization, a dilution series of the F-actin solution was prepared. To each solution, HMRef (final concentration, 100 nM) was added, and aliquots of the mixtures were pipetted into sample tubes. The FP was measured using a Beacon 2000 (Panvera) (11).

#### Pyrene-labeled actin polymerization assay

Polymerization assay was carried out as previously described (6). G buffer [5 mM tris-HCl (pH 8.0), 0.2 mM CaCl₂, and 0.2 mM ATP] and P buffer [100 mM tris-HCl, 20 mM MgCl₂, 500 mM KCl, 10 mM ATP, and 50 mM guanidine carbonate (pH 7.5)] were prepared according to the manufacturer’s protocol. Briefly, a stock solution of 465 μM pyrene-labeled-G-actin (20 mg/ml; catalog no. AP05-A, Cytoskeleton Inc.) was 46.5-fold diluted with G buffer then centrifuged, providing 15 min at 15,000 rpm at 4°C, and the supernatant was collected, providing 10 μM working solution. The working solution was left on ice for 1 hour for depolymerization and pipetted into wells of a black 384-well assay plate (10 μl per well) (catalog no. 784900, Greiner Bio-One). One microliter of probe solution in dimethyl sulfoxide (DMSO) was added and mixed, and then 1 μl of P buffer and 20 nmol of ATP in 0.2 μl of H₂O were added and mixed. The time course of the fluorescence (excitation/emission, 365/407 nm) was measured using a multilabel plate reader (EnVision 2103, PerkinElmer).

#### Pyrene-labeled actin depolymerization assay

Depolymerization assay was carried out as previously described (6). Briefly, a 10-μM working solution of pyrene-labeled G-actin was prepared and pipetted into wells of a black 96-well assay plate (40 μl per well). Ten microliters of P buffer and 20 nmol of ATP in 0.2 μl of H₂O were added and mixed. The actin solution was incubated for 2 hours for polymerization. Then, 1 μl of probe solution in DMSO was added and mixed. After incubation for 5 min, the actin
solution was fivefold diluted with 160 µl of G buffer. The time course of the fluorescence (excitation/emission, 365/407 nm) was measured using a multilabel plate reader (EnVision 2103, PerkinElmer).

**STED microscopy**

COS-7 cells were cultured on glass-bottomed dishes (D11531H, Matsunami Glass) at 37°C in 5% CO₂ in DMEM (045-30285, Wako) with 10% FBS (172012, Sigma-Aldrich), 2% l-glutamine solution (073-05391, Wako), 1% sodium pyruvate solution (190-14881, Wako), and 1% penicillin-streptomycin mixed solution (26253-84, Nacalai) (12, 13). The cells were gently washed twice with Hapes-buffered saline (HBS) (pH 7.4) (25 mM Hepes, 115 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, and 25 mM glucose) and incubated at ambient temperature for 30 min in the dark in HBS containing 1 µM HMRef. Imaging was performed on a TCS SP8 STED 3X microscope (Leica) including a pulsed white light laser for excitation, a 592-nm depletion laser for STED, and a HyD detector. HMRef-stained cells were observed with a 100× oil immersion objective (HCX PL APO CS2 100x/1.40 oil) in a field of view of 8192 by 8192 pixels with a pixel size of 10 nm by 10 nm. The excitation and emission wavelengths were 488 and 500 to 570 nm, respectively.

**Live-cell imaging with HMRef derivatives, SiR-actin, and/or Lifeact-TagRFP**

Cells were plated on eight-well chamber plates (80826, Ibidi) and incubated for a day before imaging, unless otherwise mentioned. Cells were incubated in growth medium containing indicated concentrations of HMRef derivatives for 30 min, and differential interference contrast (DIC) and fluorescence images were acquired with a confoical fluorescence microscope (TCS SP8, Leica) equipped with an argon laser and an objective lens (HCX PL APO CS 40x/1.25 oil, Leica). The excitation and emission wavelengths were 488/510 to 550 nm for HMRef derivatives, 561/582 to 709 nm for Lifeact-TagRFP, or 633/653 to 780 nm for SiR-actin.

**SRRF imaging**

Dual-color SRRF imaging was performed on a spinning disc confocal microscope (14). HMRef and Alexa Fluor 647 phalloidin excitation was conducted with a 488-nm/150-mW diode laser (LM-488-150, Andor) and a 637-nm/140-mW diode laser (LM-637-140, Andor), respectively. The two lasers were fiber coupled (seven-line laser combiner, multimode ×2, single mode ×1; LC-ILE-700-M2-S1, Andor) to a spinning disk confocal unit (CR-DFLY505, Andor) equipped with a multiband dichroic mirror (DFly laser dichroic for 405/488/561/640). The fluorescence was processed with appropriate filter sets for HMRef (TR-DFLY-F525-050, Andor) and Alexa Fluor 647 (TR-DFLY-F700-075, Andor) to capture fluorescence images with a charge-coupled device (CCD) camera (iXion Life 888, Andor), driven by Fusion software (version 2.0 for Fig. 2 and version 2.2 for Fig. S19; Andor). Images were taken using a 60× objective (APON60XOTIRF, numerical aperture (NA) 1.49, Olympus), mounted on an inverted microscope (IX83, Olympus), and equipped with Z-drift compensator (IX3-ZDC2, Olympus).

**CCK8 assay**

HeLa cells were seeded in a plastic-bottomed 96-well plate (655090, Greiner Bio-One) at a density of 7.6 × 10⁴ cells per well. After 24 hours, the medium was aspirated and replaced with fresh medium containing various concentrations of probes (adjusted by diluting 10 mM DMSO stock solution). After incubation for ~20 hours, the medium was aspirated and replaced with medium containing 5% Cell Counting Kit-8 (CK04, Dojindo). After further incubation for 1 hour, the absorbance at 405 nm was measured using a plate reader (EnVision 2103 Multilabel Reader, PerkinElmer) to determine the cell viability. Values from the wells containing cells without probe and without photoradiation were taken as representing 100% living cells, and values from wells without cells were taken as representing 100% dead cells.

**GLIFin treatment**

For GLIFin treatment, the cells were stained with GLIFin for 1 hour, followed by light irradiation through a rod scope from a Xe light source, MAX301 [Band-pass (BP), 515 to 569 nm] for 1 min. The medium was replaced with 200 µl well of fresh medium. After 20 hours, CCK8 assay was performed as described above.

**HMRef imaging of fish keratocytes**

Keratocytes of Central American cichlids (Hypsophrys nicaraguensis) were cultured in culture medium (Leibovitz’s medium, L-15, L5520, Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (Nichirei, Tokyo, Japan) and antibiotic/antimycotic solution (09366-44, Nacalai Tesque, Kyoto, Japan) as previously described (16). All methods were carried out in accordance with national guidelines and the Regulation on Animal Experimentation at Yamaguchi University. All experimental protocols were approved by Yamaguchi University Animal Use Committee. Cells were treated with trypsin (0.5 g/liter) and 0.53 mM EDTA (trypsin-EDTA, 32778-34, Nacalai Tesque) for 30 to 60 s to separate any cell-cell adhesions. The single keratocytes were treated with the culture medium containing 250 nM HMRef for 10 min. Then, the medium was replaced with the culture medium containing no probe. The migrating keratocytes were observed using an inverted microscope (Ti; Nikon, Tokyo, Japan) equipped with a laser confocal scanner unit (CSU-X1; Yokogawa, Tokyo, Japan) with a 100× objective lens (CFI Apo TIRF 100×/1.49, Nikon, Tokyo, Japan). The fluorescence images were detected using an electron multiplying CCD camera (DU897, Andor, Belfast, UK).

**HMRef time-lapse imaging of Drosophila wing disc**

Drosophila melanogaster larvae expressing E-cadherin–mTagRFP (32) were dissected in Schneider’s medium (21720024, Thermo Fisher Scientific) containing 5% FBS (s1810, Biowest). The wing discs were cultured in Schneider’s medium in the presence of 500 nM HMRef for 35-mm glass-based dish (3911-035, IWAKI). After incubation for 1 hour, time-lapse imaging was performed with an inverted confocal microscope (A1R, Nikon) equipped with a 60×/NA 1.2 Plan Apochromat water-immersion objective. The excitation and emission wavelengths were 488/500 to 550 nm for HMRef and 561/570 to 620 nm for E-cadherin–mTagRFP. Images were taken with a 5-min interval for 65 min at ~25°C.

Image processing was performed using ImageJ. Briefly, the HMRef and E-cadherin signals on the adherens junction plane were extracted using a custom-made macro. The background signal was subtracted using the “subtract background” command [correlation coefficient (r) = 50] for the HMRef image.

**Ultraviolet-visible absorption and fluorescence spectroscopy**

Ultraviolet-visible absorption spectra were obtained on a Shimadzu UV-1800. Fluorescence spectra were acquired with a Hitachi F7000. The slit width was 1 nm for both excitation and emission. The
photomultiplier voltage was 400 V. Relative fluorescence quantum yields were obtained by comparing the area under the emission spectra of the test samples with standard samples and were calculated according to the following equation

\[ \Phi_X / \Phi_{st} = [A_X / A_{st}] \left[ n_X^2 / n_{st}^2 \right] [D_X / D_{st}] \]

where \( \Phi_\text{st} \) is the standard, \( \Phi_X \) is the absorbance at the excitation wavelength, \( n \) is the refractive index, and \( D \) is the area under the fluorescence spectra on an energy scale. Optical properties of probes (1 \( \mu \)M) were examined in 0.1 M sodium phosphate buffer containing 0.1% DMSO as a cosolvent. For determination of fluorescence quantum efficiency (\( \Phi_H \)), fluorescein in 0.1 M aqueous NaOH (\( \Phi_H = 0.85 \)) was used as a standard (33).

Singlet oxygen detection by near-infrared spectroscopy
Singlet oxygen was detected by measuring \( ^1\text{O}_2 \) luminescence at around 1270 nm upon laser irradiation using a near-infrared emission spectrometer (Fluorolog-3, HORIBA, Japan). Probe solution (PBS containing 0.1% DMSO as a cosolvent) was excited with monochromatic light (508 nm), and luminescence was recorded between 1220 and 1340 nm in 5-nm steps. To calculate the quantum yield of \( ^1\text{O}_2 \) generation, the luminescence signal was integrated for 7 s for each wavelength. The quantum yield was calculated using Rose Bengal in PBS as a reference (0.75) (34).

GLIFin-mediated light inactivation of F-actin
Cells were prepared as described above. They were incubated in growth medium containing GLIFin for 1 hour, followed by light irradiation using BP 515- to 569-nm light from a Xe light source, MAX301 (Asahi Spectra Co. Ltd., for global irradiation) or a TCS SP8 (514 nm, Leica). In experiments involving an incubation time of more than 1 hour after irradiation, the medium was replaced with fresh medium.

Microtubule actin costaining
After GLIFin-mediated light inactivation of F-actin as described above, cells were washed with PBS three times, fixed, and permeabilized with PBS containing 4% HCHO and 0.1% Triton X-100. After 10 min, the solution was aspirated. The fixed cells were washed with PBS three times and blocked with 1% bovine serum albumin (BSA)/PBS. After 30 min, the blocking solution was aspirated, and the fixed cells were incubated in PBS containing 1% BSA, 0.66% MeOH, Alexa Fluor 647 phalloidin (2 U/ml) (A22287, Thermo Fisher Scientific), anti-α-tubulin antibody conjugated with fluorescein isothiocyanate (FITC) (3 μg/ml; ab64503, Abcam), and 4',6-diamidino-2-phenylindole (DAPI) (3 μg/ml; D1306, Invitrogen) at ambient temperature for 1 hour. The PBS was aspirated and replaced with fresh PBS. Fluorescence imaging was done at 405/430 to 465 nm for DAPI, 488/510 to 550 nm for FITC (tubulin), and 633/661 to 750 nm for Alexa Fluor 647 (F-actin).

GLIFin manipulation of Drosophila wing cells
The wing disc was dissected and mounted as described above and incubated with Schneider’s medium containing 1 μM GLIFin and 5% FBS for 1 hour before the light irradiation. To perform the light-mediated inactivation experiment, the wing disc was irradiated for 1.5 min with 488-nm laser at 5% power. Nonirradiated wing discs were used as a control. The control and irradiated wing discs were observed at 5 min before and at 3.5 hours after the irradiation. To examine the effect of GLIFin manipulation on the F-actin intensity, the wing discs were fixed at room temperature for 30 min in PBS containing 4% paraformaldehyde. After washing with PBS containing 0.1% Triton X-100, these preparations were incubated overnight with Alexa Fluor 647 phalloidin (1:1000; A22287, Thermo Fisher Scientific). The E-cadherin and phalloidin signals on the adherens junction plane were extracted as described above.

Epithelial cell sheet migration assay
Madin-Darby canine kidney (MDCK) cells (4.0 × 10⁵ cells/ml) were plated on both sides of eight-well chamber plates (80826 or 80206-G500, Ibidi) separated with 25 Culture-Inserts two-well (80209, Ibidi) and incubated in growth medium for 1 day. After removal of the medium from the cells, GLIFin-mediated light inactivation of F-actin was carried out. DIC images were captured with a confocal fluorescence microscope (TCS SP8, Leica). Fluorescence images were acquired as described above (also see fig. S14).

Live-dead staining
Live-dead staining was carried out according to the manufacturer’s protocol. Briefly, cells were incubated in PBS containing 0.15% DMSO, 2 μM calcein-AM (L3224, Thermo Fisher Scientific), 2 μM ethidium homodimer-1 (L3224, Thermo Fisher Scientific), and DAPI (2 μg/ml; D1306, Invitrogen). Fluorescence images (488/510 to 570 nm for live and 561/650 to 750 nm for dead) were acquired with a confocal fluorescence microscope (TCS SP8, Leica) and equipped with an argon laser and an objective lens (10×/0.40 dry, Leica).

Transfection of Lifeact
A mixture of 875 μl of Opti-MEM (31985-062, Gibco), 26.25 μl of Lipofectamine LTX, 17.5 μl of PLUS Reagent (15338-100, Invitrogen), and 1 μl of pcAG-Lifeact-TagRFP (100 μg/ml; 60107, Ibidi) solution was incubated for 5 min. Then, HeLa cells were incubated in the solution for 1 to 3 days and used for the experiment (fig. S12 and movie S2).

Preparation of F-actin in vitro
Actin protein from rabbit skeletal muscle (cytoskeleton, AKL95-B) was dissolved in 2250 μl of G buffer and incubated for 1 hour on ice. Then, 250 μl of P buffer and 500 nmol ATP in 5.0 μl of H₂O were added and mixed (final concentration of F-actin, 0.4 mg/ml). After 2 hours of incubation for polymerization, the solution was used for measurement (fig. S17, A to C).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abg8585

View/request a protocol for this paper from Bio-protocol.

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.