Two-color Photoactivatable Probe for Selective Tracking of Proteins and Cells

Arkadiusz Welman, Alan Serrels, Valerie G. Brunton, Mark Ditzel, and Margaret C. Frame
From the Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, Scotland, United Kingdom

We report the development and application of photoactivatable Green Cherry (GPAC), the first genetically encoded “continuously red-photoactivatable green” two-color probe for live cell imaging. GPAC is unique in that it enables real-time tracking of selected subpopulations of proteins and organelles in the cell or of cells within tissues and whole organisms, with constant reference to the entire population of the probe. Using GPAC-zyxin as proof of utility, we obtained new insights into the dynamic movement of the cytoskeletal protein zyxin. We show that zyxin is continuously and rapidly recruited from the cytosol into established focal adhesions. It can also move rapidly within a given focal adhesion and “hop” between adjacent focal adhesions, emphasizing the dynamic nature of proteins within these structures. The in vivo utility of GPAC is exemplified by tracking hemocyte movements using a versatile transgenic Drosophila model engineered to express GPAC in tissues and cells of interest under the control of the GAL4-inducible promoter.

Since the discovery of green fluorescent protein, fluorescent proteins have revolutionized many aspects of biomedical research and have become the detection probes of choice in multiple cell imaging applications (1, 2). Recently, a second generation of fluorescent proteins, the so-called fluorescent highlighter proteins (FHPs), have become available (3–5). FHPs are unique in that they enable real-time tracking of selected subpopulations of proteins and organelles in the cell or of cells within tissues and whole organisms, with constant reference to the entire population of the probe. Using GPPA-C-zyxin as proof of utility, we obtained new insights into the dynamic movement of the cytoskeletal protein zyxin. We show that zyxin is continuously and rapidly recruited from the cytosol into established focal adhesions. It can also move rapidly within a given focal adhesion and “hop” between adjacent focal adhesions, emphasizing the dynamic nature of proteins within these structures. The in vivo utility of GPAC is exemplified by tracking hemocyte movements using a versatile transgenic Drosophila model engineered to express GPAC in tissues and cells of interest under the control of the GAL4-inducible promoter.

We report the development and application of photoactivatable Green Cherry (GPAC), the first genetically encoded “continuously red-photoactivatable green” two-color probe for live cell imaging. GPAC is unique in that it enables real-time tracking of selected subpopulations of proteins and organelles in the cell or of cells within tissues and whole organisms, with constant reference to the entire population of the probe. Using GPPA-C-zyxin as proof of utility, we obtained new insights into the dynamic movement of the cytoskeletal protein zyxin. We show that zyxin is continuously and rapidly recruited from the cytosol into established focal adhesions. It can also move rapidly within a given focal adhesion and “hop” between adjacent focal adhesions, emphasizing the dynamic nature of proteins within these structures. The in vivo utility of GPAC is exemplified by tracking hemocyte movements using a versatile transgenic Drosophila model engineered to express GPAC in tissues and cells of interest under the control of the GAL4-inducible promoter.

Since the discovery of green fluorescent protein, fluorescent proteins have revolutionized many aspects of biomedical research and have become the detection probes of choice in multiple cell imaging applications (1, 2). Recently, a second generation of fluorescent proteins, the so-called fluorescent highlighter proteins (FHPs), have become available (3–5). FHPs are unique in that, in response to appropriate stimuli, e.g. irradiation with violet light, they undergo structural changes that result either in acquisition of a “switched on” bright fluorescence state (photoactivatable fluorescent proteins) or in a shift of fluorescence emission wavelength (photoconvertible fluorescent proteins). The main advantage of FHPs over the first generation fluorescent proteins is their ability to pulse label subpopulations of molecules or cells. This enables sophisticated spatiotemporal analysis of their dynamics (6).

Studying protein dynamics using FHPs involves three essential steps: (i) generation of a functional FHP-linked probe and its genetic introduction into cells or tissues under investigation, (ii) precise determination of the pool of interest and its photoactivation/photoconversion, and (iii) subsequent fluorescence-based imaging and data analysis. The currently available photoactivatable fluorescent proteins include photoactivatable green fluorescent protein (PAGFP) (7), photoactivatable monomeric RFP1 (8), KFP1 (9), Dronpa (10), and photoactivatable mCherry (11). Although their lack of visibility in the non-activated state and bright fluorescence after activation provide a good contrast welcomed in fluorescence microscopy, a limitation that remains is to select specific intracellular subpopulations to be activated for dynamic analysis. To do this, photoactivatable proteins are often used in combination with other fluorescent markers of cellular structures. The identification of transfected cells and specified intracellular pools of the probe is much easier in the case of photoconvertible proteins such as Dendra (12), Kaede (13), photoswitchable CFP (14), monomeric EosFP (15), and KikGR (16) because they are fluorescent both before and after photoconversion (4). Unfortunately, some of these proteins suffer from oligomeric state, inefficient photoconversion rates, relatively low brightness, and fast photobleaching (4). In addition, photoconversion is associated with the loss of initial color, so computer-based methods must be applied to visualize the entire population of the probe (to combine the highlighted and not highlighted pools). This is not optimal because fluorescence intensities and photobleaching rates are often different for two different fluorescence channels. Undoubtedly, the development of additional FHPs with new and improved properties is eagerly awaited and would contribute to further advances in the field.

For these reasons, we have devised a novel photoactivatable fluorescent probe, photoactivatable Green Cherry (GPAC), which enables efficient highlighting and tracking of selected pools of proteins, organelles, and cells with parallel continuous visualization of the entire population of the probe. GPAC is well suited for fluorescence studies both in vitro and in vivo. We provide proof of principle that this is a probe with great potential, utility, and versatility by demonstrating proof of concept tracking and dynamic movement of the focal adhesion protein zyxin and by tracking hemocyte movements between distinct regions of intact larvae of Drosophila melanogaster.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Vector Plasmids—**The pArek1 mammalian expression plasmid was constructed from the pEGFP-N1 plasmid (Clontech) by replacing the BglIII-NotI
Photoactivatable Green Cherry

fragment including enhanced green fluorescent protein (EGFP) coding sequence with a short DNA containing BglII, EcoRV, EcoRI, BamHI, SacII, Swal, Xhol, and NotI sites. This short DNA was made by synthesis and annealing of two complementary oligonucleotide strands, oligonucleotides 1 and 2. (The sequences of all oligonucleotides used in this study are provided under supplemental “Methods”.)

To generate G\textsuperscript{PAC} variants 1 (\textit{G\textsuperscript{PAC}v1}) and 2 (\textit{G\textsuperscript{PAC}v2}), we first amplified PAGFP and mCherry using the following combinations of primers: (a) primers 3 and 6 (PAGFP-m1), (b) primers 5 and 7 (PAGFP-m2), (c) primers 4 and 6 (mCherry-m1), and (d) primers 5 and 8 (mCherry-m2). The P\textit{PAGFP-N1} and pmCherry-N1 plasmids (kind gifts from Jennifer Lippincott-Schwartz (Cell Biology and Metabolism Branch, National Institutes of Health) and Roger Y. Tsien (University of California San Diego), respectively) were used as templates for the PCRs. Each of the amplified modules was subcloned into the pGEM-T Easy shuttle vector (Promega catalog no. A1360).

The following oligonucleotides were used: 9 and 10 (L20aaK-Ras4B), and 11 and 12 (NLS), 13 and 14 (PTS). After PCR, the amplified DNA was cloned into the pGEM-T Easy plasmid using EcoRV-SacII restriction enzymes. This resulted in creation of pGEM-T Easy-\textit{G\textsuperscript{PAC}v2} and pGEM-T Easy-\textit{G\textsuperscript{PAC}v1}, respectively. The \textit{G\textsuperscript{PAC}v2} and \textit{G\textsuperscript{PAC}v1} cassettes were recloned into the pArek1 plasmid using EcoRV-SacII restriction enzymes to generate pArek1-\textit{G\textsuperscript{PAC}v1} and pArek1-\textit{G\textsuperscript{PAC}v2}, respectively. The cDNA sequences of \textit{G\textsuperscript{PAC}v1} and \textit{G\textsuperscript{PAC}v2} are provided under supplemental “Methods.” The pArek1-\textit{EGFP} vector used as a positive control was identical to the pArek1-\textit{G\textsuperscript{PAC}v1} plasmids but contained \textit{EGFP} instead of \textit{G\textsuperscript{PAC}v1}. \textit{EGFP} was amplified from p\textit{EGFP-N1} using oligonucleotides 3 and 7 to introduce 5' and 3' termini identical to \textit{G\textsuperscript{PAC}v1} and \textit{G\textsuperscript{PAC}v2}.

The \textit{G\textsuperscript{PAC}v1}/\textit{G\textsuperscript{PAC}v2}/\textit{EGFP}/L20aaK-Ras4B, -nuclear localization signal (NLS), and -peroxisomal targeting sequence (PTS) constructs were made by hybridization of the indicated oligonucleotides and their ligation into BamHI-Xhol-digested pArek1-\textit{G\textsuperscript{PAC}v1}, pArek1-\textit{G\textsuperscript{PAC}v2}, and pArek1-\textit{EGFP} vectors. The following oligonucleotides were used: 9 and 10 (L20aaK-Ras4B), and 11 and 12 (NLS), 13 and 14 (PTS). The F15aaYes-\textit{G\textsuperscript{PAC}v1}/\textit{G\textsuperscript{PAC}v2}/\textit{EGFP} constructs were made using an identical strategy, but BglII-EcoRV restriction sites and oligonucleotides 15 and 16 were utilized. The \textit{G\textsuperscript{PAC}v1}/\textit{G\textsuperscript{PAC}v2}/\textit{EGFP}-\beta-actin and -zyxin fusion proteins were generated by amplification of \beta-actin and zyxin open reading frames using primers 17 and 18 (\beta-actin) and 19 and 20 (zyxin). After PCR, the amplified DNA was cloned into the pGEM-T Easy plasmid and then recloned into pArek1-\textit{G\textsuperscript{PAC}v1}/\textit{G\textsuperscript{PAC}v2}/\textit{EGFP} using BamHI-Xhol sites.

The p\textsuperscript{[UAST]} plasmid is designed for \textit{GAL4}-mediated temporal and spatial control of transgene expression in \textit{D. melanogaster} (17). The p\textsuperscript{[UAST]}-\textit{G\textsuperscript{PAC}v1} and p\textsuperscript{[UAST]}-\textit{G\textsuperscript{PAC}v1-NLS} plasmids were constructed by recloning the \textit{G\textsuperscript{PAC}v1} and \textit{G\textsuperscript{PAC}v1-NLS} open reading frames from pArek1-\textit{G\textsuperscript{PAC}v1} and pArek1-\textit{G\textsuperscript{PAC}v1-NLS} into the multiple cloning site of p\textsuperscript{[UAST]}. The BglII and Xhol sites were used.

Cell Culture and Transfection.—HEK293T and Ref52 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 \mu g/ml streptomycin. They were maintained at 37 °C in a humidified incubator containing 5% CO\textsubscript{2}.

Transfections were performed using Amaxa Nucleofector technology (Lonza, Cologne, Germany) according to the manufacturer’s protocols. The cells were plated onto glass-bottomed 35-mm tissue culture dishes (catalog no. FD35-100, World Precision Instruments Inc., Sarasota, FL) and analyzed 24–48 h later.

Transgenic Drosophila Models.—The initial p\textsuperscript{[UAST]}-\textit{G\textsuperscript{PAC}v1} and p\textsuperscript{[UAST]}-\textit{G\textsuperscript{PAC}v1-NLS} transgenic lines were generated by injection of purified DNA into embryos of strain w\textsuperscript{1118} using standard procedures. They were screened for w\textsuperscript{+} expression and balanced over TM3, Sb, or CyO chromosomes (Bestgene Inc.). Transgenic fly stocks were maintained at 18 °C, and crosses were raised at 25 °C in humidified incubators. The flies were fed a cornmeal/yeast diet.

The \textit{Hemene-GALA4} line (18) was a gift from Michael J. Williams (Umeå Centre for Molecular Pathogenesis, Umeå University, Umeå, Sweden); the \textit{da-GALA4} line (19) was obtained from the Bloomington Drosophila Stock Center at Indiana University.

Antibodies, Gel Electrophoresis, and Western Blot Analysis—SDS-PAGE was performed using 10% polyacrylamide gels. Proteins were blotted onto nitrocellulose transfer membrane (Whatman Protran BA85), incubated with the appropriate primary antibody, a horseradish peroxidase-coupled anti-rabbit secondary IgG (catalog no. 7074, Cell Signaling), and visualized using the ECL Plus Western blotting detection system (catalog no. RPN2132, Amersham Biosciences). The following primary antibodies were used: polyclonal rabbit anti-green fluorescent protein (catalog no. 3999-100, BioVision Research Products, Mountain View, CA) and polyclonal rabbit anti-DsRed (catalog no. 632496, Clontech).

Microscopy.—Microscopy experiments were performed using an Olympus FV1000 confocal microscope with an SIM scanner. \textit{In vitro} life cell imaging experiments were performed using a 60× objective with a numerical aperture of 1.35. The cell culture medium was replaced with fresh phenol red-free medium, and the cells were maintained at 37 °C in a temperature-controlled chamber. The following settings were used: pixel dwell time, 4 \mu s/pixel; pixel resolution, 512 × 512; 5% 488 nm laser power; and 5% 559 nm laser power. Photoactivation was achieved using 7% 405 nm laser power, a 40 \mu s/pixel dwell time, and a 200-ms activation pulse.

For \textit{in vivo} imaging, L3 larvae were selotaped to the bottom of a glass-bottomed tissue culture dish to restrict their movements. Imaging was performed using a 20× (numerical aperture of 0.50) or 40× (numerical aperture of 0.80) water objective (as indicated in the figure legends) and the following settings: pixel dwell time, 4 \mu sec/pixel; pixel resolution, 512 × 512; 20% 488 nm laser power; and 20% 559 nm laser power. Photoactivation was achieved using 90% 405 nm laser power and a 3-s activation pulse.

Steremicroscopic photographs of larvae were made using a Nikon AZ100 microscope equipped with a 130-watt mercury light source (Nikon UK Ltd., Kingston-on-Thames, United Kingdom).
RESULTS

Construction and Characterization of G^PA^C—To develop new two-color FHPs with improved applicability for both in vitro and in vivo tracking of subpopulations of proteins or cells, we combined the advantages of the previously described mCherry and PAGFP fluorescent proteins (7, 20). mCherry is a monomeric fluorescent protein with excitation and emission peaks of 587 and 610 nm, respectively. Taking into account brightness, photostability, and folding efficiency at 37 °C, it is one of the best red fluorescent proteins available for long-term imaging (1). PAGFP represents a photoactivatable protein with very good activation efficiency, high brightness, and excellent contrast enhancement following activation (7). We aimed to generate a fluorescent probe that would be clearly visible in the red fluorescence channel, enabling simple determination of the region of interest and, after photoactivation, well suited for independent observation of an activated pool in the green fluorescence channel. Optimally, the red fluorescence should remain unaffected by photoactivation allowing continuous visualization of the entire probe population with unchanged microscope settings both before and after photoactivation. Such a probe would be advantageous in vivo (good penetration of red light) and very easy to use in vitro. To achieve this goal, we generated several mCherry-PAGFP fusion constructs employing different linker sequences (data not shown). This strategy was associated with some risk because green fluorescent protein and mCherry have been previously described to constitute a good fluorescence resonance energy transfer (FRET) donor acceptor pair, especially in tandem construct experiments (21). It was possible that PAGFP-derived fluorescence would be short-lived and of low intensity. Indeed, some of the fusions tested displayed this kind of behavior (data not shown). However, following the initial screen, we found two constructs that showed good photoactivation and undetectable FRET (Fig. 1 and supplemental Fig. 1). We refer to them as GPACv1 and GPACv2. Importantly, cells expressing GPACv1 maintained their activated status for at least a few hours after violet light irradiation. Thus, we generated the first continuously red and photoactivatable green two-color probe suitable for live cell imaging with the unique ability to monitor protein and cell tracking with continuous reference to total probe pools.

Utility of Green Cherry as a Fluorescent Highlighter in Vitro—Although G^PA^C probes were correctly expressed and displayed favorable fluorescent properties (Fig. 1 (B–E) and supplemental Fig. 1), their utility required thorough testing. To check whether both variants are able to accept additional peptide sequences at their N and C termini without compromising their functionality, we made G^PA^C fusion constructs with either the N-terminal 15 amino acids of the non-receptor tyrosine kinase c-Yes (F15aaYes) or the C-terminal 20 amino acids of the oncoprotein K-Ras4B (L20aaK-Ras4B). These sequences undergo a series of complex post-translational modifications to function correctly. The N-terminal region of c-Yes undergoes removal of the initiator methionine, covalent linkage of saturated fatty acid myristate, and palmitoylation of a cysteine residue (22). The C-terminal domain of K-Ras4B is farnesylated in the cytosol and binds to the endoplasmic reticulum membrane. After enzymatic cleavage of the terminal three amino acids of the so-called CAA_X box, the farnesylated cysteine is methylated (23). These modifications are essential for targeting of c-Yes and K-Ras4B into diverse cellular membranes, including the plasma membrane. Importantly, analogous EGFP fusion constructs are known to display correct functionality, so we were able to use them as positive controls (24).

As illustrated in Fig. 1 (F and G), the F15aaYes-G^PA^C and G^PA^C-L20aaK-Ras4B fusion proteins showed localization identical to F15aaYes-EGFP and EGFP-L20aaK-Ras4B, respectively (including both plasma membrane distribution and intracellular membranes), indicating that G^PA^C did not compromise the post-translational processing or localization function of these short peptides. The G^PA^C probes also maintained their two-color photoactivatable properties, indicating that they may represent robust fluorescent tags with little interference with the function of the peptides they are fused to (Fig. 1 and supplemental Fig. 1).

To further characterize the utility of G^PA^C as a two-color photoactivatable fluorescent marker, we created G^PA^C fusions with the NLS derived from the SV40 large T antigen (25) and with the evolutionary conserved PTS (26). As shown in Fig. 1 (H and I), G^PA^C-NLS and G^PA^C-PTS displayed the expected nuclear and peroxisomal localizations, demonstrating that G^PA^C molecules can be correctly imported into these organelles without loss of function. Moreover, selected peroxisomes and nuclei were able to be tracked following photoactivation (Fig. 1 (H and I), supplemental Video 1, and data not shown).

We also made fusion proteins between G^PA^C and two cytoskeletal proteins, actin and zyxin. Actin is an essential component of the microfilament system, whereas zyxin is a major focal adhesion protein often used as a marker of these structures (27, 28). As shown in Fig. 1 (J and K) and supplemental Fig. 1, all fusion proteins displayed correct expression and localization. Importantly, the distribution of G^PA^C-actin and G^PA^C-zyxin resembled that of EGFP-actin and EGFP-zyxin, which are believed to reflect the physiological behavior of intracellular actin and zyxin (27). Taken together, these data validate G^PA^C as a robust photosensitive fluorescent probe that is well suited to study the dynamic movement of specific subcellular pools of proteins and organelles in tissue culture in vitro.

Application of G^PA^C to Analyze Zyxin Dynamics—Although focal adhesions are important cytoskeleton-extracellular matrix interfaces, many aspects of their behavior remain elusive (29). This is partially due to technical challenges associated with studying dynamic movements of their components in relation to total cellular pools or movement between distinct cellular locales (27). We decided to employ G^PA^C-zyxin to address a few questions related to zyxin dynamics. We used Ref52 cells because they have well defined focal adhesions and represent an established model to study these structures (30). First, we investigated whether zyxin can be recruited from the cytosol into pre-existing focal adhesions. As shown in Fig. 2A, the cytosolic and focal adhesion-localized pools of G^PA^C-zyxin (as well as membrane ruffles and other areas containing G^PA^C-zyxin) could be easily identified using red fluorescence of the probe.
Photoactivatable Green Cherry

A
GC (Variant 1)

B
Empty
EGFP
PA-GFP
GC V1
GC V2
mCherry

C
Empty
EGFP
PA-GFP
GC V1
GC V2
mCherry

D
Pre activation
0 min
3 min

E

F
GC-L20aaK-Ras4B
EGFP-L20aaK-Ras4B

G
F15aaYes-GC
EGFP-F15aaYes-EGFP

H
GC-NLS
EGFP-NLS

I
GC-PTS
EGFP-PTS

J
GC-β-actin
EGFP-β-actin

K
GC-Zyxin
EGFP-Zyxin
This allowed us to precisely define a region of interest to be activated within a cytoplasmic region devoid of focal adhesions. A single 200-ms pulse of violet light was sufficient to efficiently activate \( \text{G}^{\text{PaC}} \)-zymxin in this region to emit green fluorescence. The contrast between the activated area and its surroundings was clear (Fig. 2B). The activated \( \text{G}^{\text{PaC}} \)-zymxin pool rapidly redistributed from the activated area into other regions of the cell. Importantly, accumulation of the activated probe in previously established focal adhesions was obvious within seconds (Fig. 2B and supplemental Video 2). After \( \sim 2 \) min, activated \( \text{G}^{\text{PaC}} \)-zymxin was visible in all focal adhesions in the cell. During the same time, there was little observable change in the overall organization of cellular focal adhesions when cells were inspected using red fluorescence (Fig. 2 (A and B) and supplemental Video 2). Similar data were obtained with multiple (>50) cells in several independent experiments, indicating that \( \text{G}^{\text{PaC}} \)-zymxin molecules were continuously and rapidly recruited from the cytosol into established focal adhesions. Because the observed recruitment of \( \text{G}^{\text{PaC}} \)-zymxin to focal adhesions was not associated with any consistent increases in the size of these structures (data not shown), we analyzed the dynamics of focal adhesion-associated pools of \( \text{G}^{\text{PaC}} \)-zymxin. Again, the red fluorescence of the probe was substantial for precise focusing of the activating 405 nm laser beam within the selected regions of focal adhesions. In agreement with previous studies (31), we observed some retrograde movement of zyxin from focal adhesions (data not shown). We also frequently observed rapid redistribution of \( \text{G}^{\text{PaC}} \)-zymxin within a given focal adhesion and accompanying exchange of the construct between focal adhesions (Fig. 2 (C and D) and supplemental Video 3). To our knowledge, this rapid exchange of a cytoskeletal protein between adjacent focal adhesions has not been reported before. Interestingly, in addition to a fast moving pool of \( \text{G}^{\text{PaC}} \)-zymxin, a subpopulation of activated \( \text{G}^{\text{PaC}} \)-zymxin remained in the initial region of activation within the focal adhesions (at least on the time scale of imaging, \(< 5 \) min) (Fig. 2, C and D).

Utility of \( \text{G}^{\text{PaC}} \) as a Fluorescent Highlighter in Vivo—The experiments described so far demonstrated that the \( \text{G}^{\text{PaC}} \) probe represents a valuable tool to track the fate of selected subpools of proteins in the context of total cellular pools in tissue culture in vitro. To validate \( \text{G}^{\text{PaC}} \) applicability for in vivo studies, we generated two transgenic \( \text{D}. \text{melanogaster} \) models. In the first model (\( \text{Drosophila} \ \text{G}^{\text{PaC}} \)), the \( \text{G}^{\text{PaC}} \) probe (variant 1) was placed downstream of five optimized binding sites for the yeast transcriptional activator \( \text{G}^{\text{A}} \text{AL4} \), followed by the HSP70 TATA box and transcriptional start site, and upstream of the SV40 transcriptional terminator (17). The second model (\( \text{Drosophila} \ \text{G}^{\text{PaC-NLS}} \)) was identically designed but contained \( \text{G}^{\text{PaC-NLS}} \) instead of \( \text{G}^{\text{PaC}} \) alone. Several independent transgenic lines with different chromosome integration sites were developed within each model. Although the \( \text{G}^{\text{PaC}} \) and \( \text{G}^{\text{PaC-NLS}} \) genes are present in all cells of \( \text{Drosophila} \ \text{G}^{\text{PaC}} \) and \( \text{Drosophila} \ \text{G}^{\text{PaC-NLS}} \), respectively, they remain silent unless the \( \text{G}^{\text{AL4}} \) transcriptional activator is introduced. Multiple \( \text{Drosophila} \) lines expressing \( \text{G}^{\text{AL4}} \) in diverse tissues of interest and under the control of different promoters have been described in the literature, and they are readily available (32).

To determine whether \( \text{G}^{\text{PaC}} \) and \( \text{G}^{\text{PaC-NLS}} \) can be expressed in \( \text{Drosophila} \) without any adverse effects on development or viability, we crossed \( \text{Drosophila} \ \text{G}^{\text{PaC}} \) and \( \text{Drosophila} \ \text{G}^{\text{PaC-NLS}} \) with flies expressing \( \text{G}^{\text{AL4}} \) under the control of the ubiquitous \( \text{daughterless} \) (\( \text{da} \)) promoter (19, 33). The progeny from these crosses (\( \text{Drosophila} \ \text{G}^{\text{PaC/da-GAL4}} \) and \( \text{Drosophila} \ \text{G}^{\text{PaC-NLS/da-GAL4}} \)) was viable and fertile and, most importantly, expressed \( \text{G}^{\text{PaC}} / \text{G}^{\text{PaC-NLS}} \) in multiple organs and tissues as evidenced by strong red fluorescence (Fig. 3). To analyze the functionality of the \( \text{G}^{\text{PaC}} \) and \( \text{G}^{\text{PaC-NLS}} \) probes in an in vivo situation, we subjected the selected areas of live \( \text{G}^{\text{PaC/da-GAL4}} \) and \( \text{G}^{\text{PaC-NLS/da-GAL4}} \) larvae to activation with a 405 nm laser beam. As shown in Fig. 3 (C and D), no green fluorescence following 488 nm excitation was detected before a 405 nm laser pulse. After 405 nm laser irradiation, the selected region of interest emitted strong green light in the presence of 488 nm excitation light. The red fluorescence following 559 nm excitation was visible clearly with unchanged intensity both before and after a 405 nm laser pulse and was very helpful in the selection and anatomical identification of the regions to be activated. Even small groups of cells or single cells in precisely defined locations could be activated and observed at relatively high magnification (Fig. 3 (E, E’, and F) and data not shown).

To demonstrate that the \( \text{G}^{\text{PaC}} \) probe can be utilized to study dynamic processes in an intact organism, we crossed \( \text{Drosophila} \ \text{G}^{\text{PaC-NLS/Hemese-GAL4}} \) flies expressing \( \text{G}^{\text{AL4}} \) in hemocytes (18). Hemocytes are \( \text{Drosophila} \) blood cells that are involved in the immune response, but their behavior in vivo is still poorly understood (34, 35). \( \text{Hemese-GAL4} \) drives strong expression in circulating hemocytes that can be observed through the cuticle of the larva and in sessile hemocytes that are found settled under the epidermis and in large clusters at the posterior end of the larva (18). The sessile hemocytes and the circulating hemocytes are morphologically indistinguishable (18). As shown in Fig. 4, the nuclei of the hemocytes in \( \text{G}^{\text{PaC-NLS/Hemese-GAL4}} \) crosses were readily visible in the red fluorescence channel. We activated a small area in the post-

---

**FIGURE 1. Characteristics of photoactivatable green cherry probes.** A, schematic illustrating the structure and fluorescent properties of \( \text{G}^{\text{PaCv1}} \). GC, Green Cherry. B and C, Western blots showing expression of \( \text{G}^{\text{PaCv1}} \) and \( \text{G}^{\text{PaCv2}} \) in transiently transfected HEK293T cells. Polyclonal anti-red fluorescent protein (B) and anti-GFP (C) antibodies were used. EGFP-, PAGFP-, and mCherry-transfected cells served as controls for antibody specificity. D, photographs showing red and green fluorescence of \( \text{G}^{\text{PaCv1}} \) in transiently transfected HEK293T cells before and after photoactivation with 405 nm light. A representative cell was selected. E, graph showing the intensity of red (*) and green (**) fluorescence of \( \text{G}^{\text{PaCv1}} \) in HEK293T cells before and after activation. The graph provides quantitative fluorescence data for the cell shown in D. F–K, localization and fluorescent properties of \( \text{L20aaK-Ras4B} \) (F), \( \text{F13aalyes} \) (G), NLS (H), PTS (I), actin (J), and zyxin (K) fused to \( \text{G}^{\text{PaCv1}} \) and \( \text{EGFP} \). For \( \text{G}^{\text{PaCv1}} \)-linked probes, the images before activation are shown in the upper panels, and the images after activation are in the lower panels. For both \( \text{G}^{\text{PaCv1}} \)- and \( \text{EGFP} \)-linked probes, red fluorescence images are shown in the left panels, and green fluorescence images are shown in the right panels. HEK293T (J–I) or Ref52 (J and K) cells were used. Note that only one nucleus was activated in H. A movie for I is provided in Supplemental Video 1, and supplemental Fig. 1 provides results obtained using the \( \text{G}^{\text{PaCv2}} \) probe. The coding sequences of \( \text{G}^{\text{PaCv1}} \) and \( \text{G}^{\text{PaCv2}} \) are provided under supplemental "Methods." Scale bars = 10 \( \mu \text{m} \).
Photoactivatable Green Cherry

A

Pre-activation (green)  Pre-activation (red)  Activation (overlay)  92 sec (overlay)

B

Activation  2 sec  10 sec  92 sec

C

Pre-activation  Pre-activation  Activation (overlay)

D

Activation  2 sec  18 sec  56 sec

Activation  2 sec  18 sec  56 sec
The migration of some of the “activated” cells from that region into the bloodstream was obvious on a time scale of tens of seconds after activation (Fig. 4 (A and B) and supplemental Video 4). We subsequently activated a larger population of cells in the same posterior area to track long distance cell redistribution. The release of cells from the activated region was obvious, and it seemed to be influenced by muscle contractions (Fig. 4C and supplemental Video 5). About 15 min after the initial activation of the cell subpopulation in the posterior end of the larva, we searched other remote areas of the same larva for the presence of activated cells (coincident green and red signals). Cells activated in the posterior end could be...

FIGURE 2. Selected aspects of zyxin dynamics in Ref52 cells analyzed using the G\textsuperscript{488C}-zyxin probe. A, green and red channel images of a representative cell before photoactivation (left two panels). The region of interest to be activated is indicated by the white circle. A also contains overlays of green and red fluorescence at the point of activation and 92 s later (right two panels). B, redistribution of G\textsuperscript{488C}-zyxin from the activated region visualized using green fluorescence channel (upper panels). Note the movements within the activated focal adhesion and to the neighboring focal adhesions. The corresponding red channel images are shown in the lower panels. Movies for C and D are provided in supplemental Video 3. Scale bars = 5 μm.

FIGURE 3. Fluorescent properties of Drosophila G\textsuperscript{488C/da-GAL4} and G\textsuperscript{488C-NLS/da-GAL4} larva. A and B, bright-field (left panels) and red fluorescence (right panels) stereoscopic images of G\textsuperscript{488C/da-GAL4} (A) and G\textsuperscript{488C-NLS/da-GAL4} (B) larvae and negative control larvae. C, red (left panels) and green (middle panels) fluorescence of a region within the anterior part of the G\textsuperscript{488C/da-GAL4} larva before (upper panels) and after (lower panels) photoactivation. Overlays of red and green fluorescence are shown in the right panels. D, red (left panels) and green (middle panels) fluorescence of a region within the middle body part of the G\textsuperscript{488C-NLS/da-GAL4} larva before (upper panels) and after (lower panels) photoactivation. Overlays of red and green fluorescence are shown in the right panels. E and E', lower (×1 optical zoom) and higher (×3 optical zoom) magnification images, respectively, of a group of activated cells located in the direct proximity of the tracheole in a G\textsuperscript{488C/da-GAL4} larva. F, image showing a small group of activated nuclei in the muscles of a G\textsuperscript{488C-NLS/da-GAL4} larva. Confocal microscope images shown in C–E, E', and F were taken using a ×40 water objective; all pictures were superimposed over the corresponding reflected light images (collected using a 405-nm filter) to visualize some additional structural details. Note that only the left half of the image was photoactivated in C and D. Scale bars = 1 mm (A and B), 100 μm (C–F), and 20 μm (E').
Photoactivatable Green Cherry
easily detected in other areas of the body, e.g. in the proximity of the anterior spiracles (trachea openings). Some of them were rapidly moving in the bloodstream, whereas others displayed more settled sessile hemocyte-like behavior (Fig. 4D and supplemental Video 6). These observations indicate that hemocytes can rapidly (within minutes) relocate from the posterior end of the larva and infiltrate remote tissues.

Taken together, the data obtained using fruit flies indicate that the GPA-C probe is well suited for in vivo applications. The developed Drosophila GPA-C and GPA-C-NLS models provide a versatile and powerful platform for future cell fate-oriented studies in this organism.

DISCUSSION

In their activated state, the vast majority of currently available FHPs are visible in one fluorescence channel only (e.g. they emit fluorescence of only one color) (4). This can be restrictive in certain applications, particularly in vivo, where focusing and distinction between the real signal from the probe and unspecified background fluorescence are sometimes difficult. Also, the visualization of the entire probe population (activated and not activated) is impossible without employing computer-based methods, eliminating the possibility of any direct analysis of the activated pool in the context of the total pool of the probe. The only known photoconvertible protein, which, following photostimulation, is still visible at both the pre-activation and post-activation wavelengths, is the recently described Phamret (albeit the pre-activation fluorescence wavelength intensity is significantly reduced). Phamret is a rationally designed fusion construct of mscCFP and PAGFP, and its functionality is based on the FRET between mscCFP and the activated form of PAGFP (36). Phamret enables imaging of very fast cellular processes in vitro but is less suited for in vivo applications because it relies on the use of FRET and cyan fluorescence, which has weak tissue penetration compared with light of longer wavelengths (36).

The new probe we describe here represents rationally designed fusion between two well characterized fluorescent proteins: mCherry (20) and PAGFP (7). We overcame several technical difficulties to generate tandem constructs that preserve the desired fluorescent properties of both of their components without any unwanted FRET interference. Importantly, although the molecular mass of the probes (~52 kDa) is relatively high (but still comparable with some other available FHPs), the probes are efficiently expressed and serve well as fluorescent tags with multiple peptides and proteins tested (Fig. 1 and data not shown). A major advantage of GPA-C is that, using the red fluorescence channel, an investigator can continuously follow the dynamics of the entire probe population in the course of the experiment (both before and after activation) without any need to change microscope settings. Additionally, the intensity of red color directly indicates the intensity of the signal expected after photoactivation because of the 1:1 ratio between mCherry and PAGFP. These features facilitate initial focusing, selection of the region of interest, and potential microscope adjustments during the experiment. They could also enable relative quantification of the ratio between the activated pool of the probe and the total pool of the probe in selected regions of interest at different time points after photoactivation. Taking into account unique two-color photoactivatable characteristics, high expression levels, and no detectable toxicity in vivo, GPA-C constructs compare favorably with other FHPs described to date and may represent the probes of choice in many experiments aiming to analyze different aspects of cellular dynamics. We demonstrated the utility of GPA-C by analyzing zyxin dynamics in focal adhesions of mammalian cells and by studying hemocyte migration in D. melanogaster. Considering the fact that GPA-C probes functioned equally well in both systems, one can expect that they will also be applicable to other experimental models. Of course, the utility of GPA-C should be thoroughly validated in each individual case for every single protein. The relatively high molecular mass of the probe may represent a limitation in some applications (for example, due to sterical hindrance of the function of the protein it is fused to). Also, one cannot exclude that, in the case of some GPA-C fusion constructs, quenching with natural ligands of specific proteins could affect the applicability of this new tool.

The unique features of GPA-C-zyxin enabled us to show that zyxin molecules are continuously and rapidly (within seconds) recruited from the cytosol into existing focal adhesions. These dynamics are much faster than the reported times of focal adhesion turnover, which are in the range of tens of minutes (37). The focal adhesion-localized zyxin not only rapidly redistributes within a given focal adhesion but also displays a fast “hopping” to neighboring focal adhesions, a kind of behavior that has never been described before. In Drosophila, we used GPA-C-NLS to show that hemocytes activated at the posterior end of the larva can rapidly relocate with the bloodstream to other distant places. Some of them are able to settle in remote locations within minutes after activation. Because we used intact larvae, it is unlikely that the observed “settlement” was associated with the immune response. It is more reasonable to assume that circulating hemocytes may occasionally infiltrate other tissues to become sessile hemocytes and vice versa.

In summary, we constructed novel photoactivatable proteins with unique “continuously red-photoactivatable green” two-color characteristics. These proteins are well suited for protein, organelle, and cell tracking in vitro and in vivo. We utilized them to provide new insights into the dynamics of the cytoskel-

---

**FIGURE 4. Hemocyte dynamics in Drosophila GPA-C-NLS/Hemese-GAL4.** A. A group of photoactivated hemocyte nuclei in the posterior region of a GPA-C-NLS/Hemese-GAL4 larva (white circles). B, images of the same region taken 18 s later. Note the two hemocytes leaving the initial area of activation (white arrows). C, redistribution of hemocytes (white arrow) following activation of a much bigger area (white ellipsoids) in the posterior end of the same larva as in A and B. D, colonization of the anterior spiracle area of the same larva by hemocytes photoactivated in the posterior end. Note the migrating hemocytes (white arrows) and more settled ones (yellow arrows). See also supplemental Videos 6. In A–D, the red fluorescence image is in the left panels, the green fluorescence image is in the middle panels, and overlay is provided in the right panels. All displayed confocal microscope images were taken using a ×20 water objective, and pictures were superimposed over the corresponding reflected light images (collected using a 405-nm filter) to visualize some additional structural details. Movies for each panel are provided in supplemental Videos 4–6. Scale bar = 100 µm.
et al. protein zyxin and to analyze hemocyte migration patterns in intact larvae of *D. melanogaster*. We believe that the GFPAC probes described here have great promise as robust novel tools for precisely controlled studies on protein and cellular dynamic movements, with constant reference to total pools. This will provide greater versatility than other available probes for such studies.

Acknowledgments—We thank Jennifer Lippincott-Schwartz and Roger Y. Tsien for pPAGFP-N1 and pmCherry-N1 plasmids. We are grateful to Michael J. Williams for the Drosophila Hemese-GAL4 line and to Paul Perry for help with stereomicroscopic imaging.

REFERENCES

1. Shaner, N. C., Steinbach, P. A., and Tsien, R. Y. (2005) *Nat. Methods* 2, 905–909
2. Chudakov, D. M., Lukyanov, S., and Lukyanov, K. A. (2005) *Trends Biotechnol.* 23, 605–613
3. Shaner, N. C., Patterson, G. H., and Davidson, M. W. (2007) *J. Cell Sci.* 120, 4247–4260
4. Lukyanov, K. A., Chudakov, D. M., Lukyanov, S., and Verkhusha, V. V. (2005) *Nat. Rev. Mol. Cell Biol.* 6, 885–891
5. Lippincott-Schwartz, J., and Patterson, G. H. (2009) *Trends Cell Biol.* 19, 555–565
6. Giepmans, B. N., Adams, S. R., Ellisman, M. H., and Tsien, R. Y. (2006) *Science* 312, 217–224
7. Patterson, G. H., and Lippincott-Schwartz, J. (2002) *Science* 297, 1873–1877
8. Verkhusha, V. V., and Sorkin, A. (2005) *Chem. Biol.* 12, 279–285
9. Chudakov, D. M., Belousov, V. V., Zaraisky, A. G., Novoselov, V. V., Staroverov, D. B., Zorov, D. B., Lukyanov, S., and Lukyanov, K. A. (2003) *Nat. Biotechnol.* 21, 191–194
10. Ando, R., Mizuno, H., and Miyawaki, A. (2004) *Science* 306, 1370–1373
11. Subach, F. V., Patterson, G. H., Manley, S., Gillette, J. M., Lippincott-Schwartz, J., and Verkhusha, V. V. (2009) *Nat. Methods* 6, 153–159
12. Gurskaya, N. G., Verkhusha, V. V., Scheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S., and Lukyanov, K. A. (2006) *Nat. Biotechnol.* 24, 461–465
13. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 12651–12656
14. Chudakov, D. M., Verkhusha, V. V., Staroverov, D. B., Souslova, E. A., Lukyanov, S., and Lukyanov, K. A. (2004) *Nat. Biotechnol.* 22, 1435–1439
15. Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Röcker, C., Salih, A., Spindler, K. D., and Nienhaus, G. U. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 15905–15910
16. Tsutsumi, H., Karasawa, S., Shimizu, H., Nukina, N., and Miyawaki, A. (2005) *EMBO Rep.* 6, 233–238
17. Brand, A. H., and Perrimon, N. (1993) *Development* 118, 401–415
18. Zettervall, C. J., Anderl, I., Williams, M. J., Palmer, R., Kurucz, E., Ando, I., and Hultmark, D. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 14192–14197
19. Giebel, B., Stüttem, I., Hinz, U., and Campos-Ortega, J. A. (1997) *Mech. Dev.* 63, 75–87
20. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) *Nat. Biotechnol.* 22, 1567–1572
21. Albertazzi, L., Arosio, D., Marchetti, L., Ricci, F., and Beltram, F. (2009) *Photochem. Photobiol.* 85, 287–297
22. Resh, M. D. (1994) *Cell* 76, 411–413
23. Silvius, J. R. (2002) *J. Membr. Biol.* 190, 83–92
24. Welman, A., Burger, M. M., and Hagmann, J. (2000) *Oncogene* 19, 4582–4591
25. Jans, D. A., and Hübner, S. (1996) *Physiol. Rev.* 76, 651–685
26. Subramani, S. (1998) *Physiol. Rev.* 78, 171–188
27. Worth, D. C., and Parsons, M. (2008) *Int. J. Biochem. Cell Biol.* 40, 2397–2409
28. Lindberg, U., Karlsson, R., Lassing, I., Schutt, C. E., and Höglund, A. S. (2008) *Semin. Cancer Biol.* 18, 2–11
29. Lock, J. G., Wehrle-Haller, B., and Strömblad, S. (2008) *Semin. Cancer Biol.* 18, 65–76
30. Thomas, S. M., Hagel, M., and Turner, C. E. (1999) *J. Cell Sci.* 112, 181–190
31. Gao, W. H., and Wang, Y. L. (2007) *Mol. Biol. Cell* 18, 4519–4527
32. Elliott, D. A., and Brand, A. H. (2008) *Methods Mol. Biol.* 420, 79–95
33. Cronmiller, C., Schedl, P., and Cline, T. W. (1988) *Genes Dev.* 2, 1666–1676
34. Märkus, R., Laurinyecz, B., Kurucz, E., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., and Andó, I. (2009) *Proc. Natl. Acad. Sci. U.S.A.* 106, 4805–4809
35. Williams, M. J. (2007) *J. Immunol.* 178, 4711–4716
36. Matsuda, T., Miyawaki, A., and Nagai, T. (2008) *Nat. Methods* 5, 339–345
37. Block, M. R., Badowski, C., Millon-Fremillon, A., Bouvard, D., Bouin, A. P., Faurobert, E., Gerber-Scokaert, D., Planus, E., and Albigez-Rizo, C. (2008) *Eur. J. Cell Biol.* 87, 491–506

Photoactivatable Green Cherry