Published in final edited form as:

Anal Chem. 2012 May 1; 84(9): 3929–3937. doi:10.1021/ac202825z.

Microfluidic flow cytometer for quantifying photobleaching of fluorescent proteins in cells

Jennifer L. Lubbeck^{1,2}, Kevin M. Dean¹, Hairong Ma^{1,2}, Amy E. Palmer^{1,*}, and Ralph Jimenez^{1,2,*}

¹Department of Chemistry & Biochemistry, University of Colorado Boulder, 215 UCB, Boulder, CO, USA. 80309

²JILA, University of Colorado Boulder and National Institute of Standards and Technology, 440 UCB Boulder, CO, USA. 80309

Abstract

Traditional flow cytometers are capable of rapid cellular assays on the basis of fluorescence intensity and light scatter. Microfluidic flow cytometers have largely followed the same path of technological development as their traditional counterparts, however the significantly smaller transport distance and resulting lower cell speeds in microchannels provides for the opportunity to detect novel spectroscopic signatures based on multiple, non-temporally-coincident excitation beams. Here, we characterize the design and operation of a cytometer with a 3-beam, probe/bleach/probe geometry, employing HeLa suspension cells expressing fluorescent proteins. The data collection rate exceeds 20 cells/s under a range of beam intensities (5 kW – 179 kW/cm²). The measured percent photobleaching (ratio of fluorescence intensities excited by the first and third beams: $S_{\text{beam3}}/S_{\text{beam1}}$) partially resolves a mixture of four red fluorescent proteins in mixed samples. Photokinetic simulations are presented and demonstrate that the percent photobleaching reflects a combination of the reversible and irreversible photobleaching kinetics. By introducing a photobleaching optical signature, which complements traditional fluorescence intensity-based detection, this method adds another dimension to multi-channel fluorescence cytometry, and provides a means for flow-cytometry-based screening of directed libraries of fluorescent protein photobleaching.

Introduction

Traditional flow cytometers employ light-scattering and fluorescence-based detection to assess spectral diversity^{1–3}, cell size⁴, fluorescence brightness^{5–7}, fluorescence lifetime^{8,9}, and analyte concentration¹⁰ on individual cells flowing through one or more tightly focused excitation beams at speeds of ~ 1–10 meter/sec. In this operating regime, the time window for optical excitation and detection is approximately a few microseconds per beam and hundreds of microseconds between beams. In contrast, the short transport dimensions and confining properties of microfluidic channels enable highly stable flows at cell speeds of 10^{–6} to 10^{–3} m/sec. We exploited these properties to develop the ability to screen with optical or photophysical properties that are manifested at longer time-scales (tens of milliseconds or slower) by implementing multi-point fluorescence excitation measurements in a microfluidic flow cell. We specifically investigate the probing of photobleaching in flow. Although it is likely to be ubiquitous in flow cytometry, few studies have investigated

Amy E. Palmer amy.palmer@colorado.edu, TEL: 303-492-1945, FAX: 303-492-1945. Ralph Jimenez rjimenez@jila.colorado.edu, TEL: 303-492-8439, FAX: 303-492-5235.

Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

photobleaching in detail, and there are no reports of fluorophore screening or sorting based on photobleaching. Previous reports by van den Engh *et al.* and Doornbos *et al.* focused on understanding photo-bleaching and photon saturation in DNA stains, fluorescein conjugates, phycoerythrin, and allophycocyanin, *via* pulse shape and power-dependence measurements, primarily with the goal of optimizing the magnitude of fluorescence signals.^{11,12} The excitation conditions in those studies accessed time windows of 2 μ sec to 2 msec, at excitation intensities of 5–3200 kW/cm². Here, we report the design of a cytometer for assessing photobleaching of genetically-encodable fluorescent proteins, at excitation intensities comparable to those used for confocal imaging and single molecule spectroscopy (10–100 kW/cm²).

Since the advent of green fluorescent protein (GFP), genetically-encodable fluorescent proteins (FPs)¹³ in a diversity of excitation and emission wavelengths have found widespread use in molecular and cell biology due to the ability to fuse them to a protein of interest, and target them to specific subcellular structures¹⁴. Despite these advantages, FPs exhibit complex excited-state dynamics which limit their photon output. In these fluorophores, irreversible photobleaching, which refers to photodestruction of the chromophore, usually occurs in the presence of reversible photobleaching, which involves transient conversion to a non-fluorescent or dimly-fluorescent state. Depending on the FP, reversible photobleaching has been attributed to triplet state conversion¹⁵, excited state proton-transfer¹⁶, or photoinduced isomerization of the chromophore and nearby side-chains^{17–19}. Subsequent ground-state recovery occurs in tens of microseconds to minutes^{20,21}, and may depend on the chromophore environment of the FP²¹. In ensemble measurements, reversible photobleaching is manifested as an initial fast decay of fluorescence that recovers when the excitation light is turned off^{14,21–23}. The magnitudes and time-constants of both reversible and irreversible photobleaching depend on the fluorophore, excitation intensity, and excitation wavelength^{14,21–23}. Although these factors make the composite photobleaching process tricky to quantify, there is a clear potential for using it in flow-cytometric screening for the development of new fluorescent proteins. This approach would be significantly faster than microplate or colony-based screening. Due to the longer timescale photophysics in FPs compared to small molecule fluorophores, a cytometry-based screening system will require that a correspondingly longer time-window be accessible to the measurement.

In recent work, we reported the use of millisecond pulse sequences to dissect the photobleaching process of FPs in individual HeLa cells²³. Here, to measure photobleaching of cells in flow, we implemented a design that quantifies photobleaching on the millisecond timescale, independent of fluorophore concentration, fluorescence quantum yield or extinction coefficient. We employ three spatially separated beams: a low-intensity probe beam (5 kW/cm²) to measure initial fluorescence, followed by a high-intensity bleach beam (5–179 kW/cm²) to initiate photodestruction of the fluorophores, followed by a second low-intensity probe beam, of equal intensity to the first, to assess the extent of photobleaching. This approach simplifies data acquisition compared to direct measurement of a time-resolved fluorescence decay because ratios of peak signal intensities are easier to fit and define than the multi-exponential decays which characterize the photobleaching process.

Here, we combine microfluidics and spectroscopy techniques in a flow cytometer for measuring the combined effect of reversible and irreversible photobleaching at a rate of > 20 cells/sec. To our knowledge, this capability has not previously been reported. We present calculations guiding the optical design of a multi-beam cytometer describing how to optimize measurement precision and alignment tolerance in a simple 2D hydrofocusing geometry. The technique is demonstrated on four different red fluorescent proteins (RFPs), under a variety of excitation conditions, which uncovered a diverse range of reversible and

irreversible photobleaching on the millisecond timescale. Lastly, we present kinetic simulations to examine the effects of reversible and irreversible photobleaching rates on the capability of our method to discriminate populations.

Experimental

The cytometer consists of four general components: (1) a microfluidic hydrofocusing chip and manifold which holds the sample and drives fluid flow, (2) an inverted microscope which serves as an optical platform for the cytometer, (3) optics to shape and align the three beams into the microscope, (4) the data acquisition and processing electronics.

Design Considerations

One of the design goals was to ensure that the distribution of measured fluorescence intensities for a population accurately reflects cellular RFP expression heterogeneity rather than instrument resolution. Even for a single-beam fluorescence measurement of each cell in flow, the excitation intensity and therefore the observed emission signal is strongly dependent on the trajectory of each cell as it traverses through the 3-D laser focus in the microchannel. Although, in principle, 3-D hydrofocusing geometries would more precisely define these cellular trajectories, we show that simpler-to-fabricate 2D hydrofocusing devices can provide sufficient precision for properly designed multi-beam excitation/detection geometries. In 2-D hydrofocusing, cells flowing past a cross-junction with two channels of sheath flow at higher pressures are laterally confined by the flow to dimensions significantly narrower than the channel width (Figure 1a)²⁴. Nevertheless, there will always be lateral and axial variation (relative to the optical axis of the microscope objective: see Figure 1) in trajectories from cell to cell. We quantify the effects of this variation on the fluorescence signal, along with the effects of slight misalignment of the two probe beams relative to the flow axis of the microfluidic. We minimized the alignment sensitivity by introducing a cylindrical lens ($f = 150$ mm) positioned ~ 190 mm before the objective lens, to shape the Gaussian beam into an elliptical profile in the microchannel, in which the focused beam size perpendicular to the flow direction (y -axis) is much larger than along the flow direction (x -axis).

To quantify the impact of cell transit variation on the fluorescence signal, we first calculated the astigmatic transformation of a Gaussian beam through a cylindrical lens and objective optical system with a number of experimental constraints. We assumed that each cell was $14\text{ }\mu\text{m}$ in diameter (the mean cell size observed for HeLa-S cells on a wide-field microscope), had a uniform RFP concentration (*i.e.*, an RFP containing sphere) and traveled between two $4\text{ }\mu\text{m}$ diameter (FWHM) laser beams (Figure 1a and b). This beam size matched our measurements of the focused probe beam sizes produced with a 20x, 0.45 N.A. air-objective (Figure 2). The observed fluorescence signal, S (eq. 1), is the convolution of the RFP density, $s(x,y,z)$, and the three-dimensional Gaussian intensity profile at the laser focus, $B(x,y,z)$:

$$S(x_o, y_o, z_o) = \iiint_{x,y,z} s(x-x_o, y-y_o, z-z_o) \times B(x, y, z) dx dy dz \quad (1)$$

Details of the analytical solution for a symmetrical Gaussian beam are provided in the supporting methods. To obtain an analytical expression for the asymmetric Gaussian beam shaped by the cylindrical lens, we used the transfer matrix system analogous to the ABCD matrix method for geometric optics²⁵, and calculated the evolution of the beam in the x and y -direction, independently.

Hence the elliptical Gaussian beam may be written as

$$B[x, y, z] = \frac{2P}{\pi w_x[z] w_y[z]} \times \exp \left[- \left(\frac{2x^2}{w_x^2[z]} + \frac{2y^2}{w_y^2[z]} \right) \right] \quad (2)$$

where P is the total power of the beam, $w_x[z]$ and $w_y[z]$ are respectively the x -axis and y -axis beam radius where the intensity drops to $1/e^2$ of its peak value. The evolution of the Gaussian beam along the propagation direction, defined as the z -axis here, is

$$w_i[z] = w_0 \left(1 + \frac{z^2}{z_{Ri}^2} \right) \quad (3)$$

where i denotes x or y -direction, and $z_{Ri} = 2\pi w_{0i}^2 / \lambda$ is known as the Rayleigh range. At $z = z_{Ri}$ the beam radius is $\sqrt{2}$ times larger than its waist value w_0 , and the beam area doubles.

The calculated beam radius at the sample, which is positioned at the x -axis waist position, is $w_x[z]|_{z=z_0} = 2 \mu\text{m}$ and $w_y[z]|_{z=z_0} = 56 \mu\text{m}$, which is consistent with the measured beam radius of $w_x = 3 \mu\text{m}$ and $w_y = 67 \mu\text{m}$. The distribution of peak signal intensities from a cell traversing a spherically and cylindrically focused beam centered on the hydrodynamic flow are shown in Figure 1b and 1d, respectively. These contour plots reveal how the observed fluorescence signal varies as a function of the cell axial and lateral position with respect to the center of the channel ($\Delta Z = \Delta Y = 0$). Figures 1c and 1e are similar, but correspond to the case in which the beam is displaced laterally ($\Delta Y = 0.5 \mu\text{m}$) with respect to the center of the channel. This displacement represents the precision of experimental alignment. Due to the relatively low NA of the optical system, the signal intensity is insensitive to cell axial positioning ($\pm 5.5 \mu\text{m}$ is the maximum range for a $14 \mu\text{m}$ diameter cell in a channel of $25 \mu\text{m}$ height) for both spherical and cylindrical focusing. However, in the lateral direction, a comparison of spherical vs. cylindrical beam shaping reveals very different sensitivity to cell position and beam alignment. In particular, if the beams are misaligned or the cell drifts by $\Delta Y = 0.5 \mu\text{m}$, the difference in the peak fluorescence intensity of the second beam relative to the first beam is significantly smaller with the cylindrical focus (Figure 1d–e).

Consequently, we consider cells transiting only along the X -axis between the two probe beams at randomly chosen axial and lateral positions in the range $\Delta Y = \pm 2 \mu\text{m}$ and $\Delta Z = \pm 5.5 \mu\text{m}$. If the two beams are perfectly aligned, then for both cases, the ratio of fluorescence intensities ($\text{beam1}/\text{beam2}$) = 1. However, for the misaligned geometry, this signal ratio depends on the cell position in the channel. Figure 1g shows that cylindrically focused beams yield a 35-fold lower dispersion in the signal intensities compared to the spherically focused beams. This result indicates that pairs of cylindrically focused beams will lead to substantially smaller variability in fluorescence measurements.

Microfluidics and Optical Layout

Microfluidic devices were built by anodically bonding a $25 \mu\text{m}$ thick $2''$ diameter silicon wafer to a 1.7 mm thick glass-slide. Silicon was etched down to the glass in the pattern of the channels using standard photolithography and plasma etching techniques²⁶. This method results in optically transparent channels of $25 \mu\text{m}$ height $\times$ $150 \mu\text{m}$ width $\times$ 1 mm length for the central interrogation channel. Sample ports of 1 mm diameter were drilled in a second, identical glass-slide before bonding to the silicon. The microfluidic was compression fit with “O” rings against a manifold constructed from polytetrafluoroethylene (PTFE; to minimize non-specific adsorption of cells) with $200 \mu\text{L}$ sample reservoirs (Supporting Figure S-1). The microfluidic device and combined manifold assembly were mounted onto the stage of a

commercial inverted microscope. Flow was driven using three closed-loop air-pressure controllers connected by PTFE tubing to the sample ports. By independently varying the pressures on all three inlets, the hydrodynamic focal width was kept constant at 15 μm as measured by imaging the fluorescence from a dye flowing in only the center cell channel²⁴. The cell speed in the interrogation region was varied from 1–15 mm/s to control exposure time to the bleach beam. The speed was calculated from measurements of cell transit times between probe beams 1 and 2 using fluorescence signals, and measurements of spatial separation of the beams (Figure 2; typically $240 \pm 3 \mu\text{m}$). The cell speed distribution typically had a standard deviation of 1%. The flow was visualized with a CMOS camera and wide-field trans-illumination was provided by a home-built condenser.

The three-beam geometry consisted of two equal intensity probe beams measuring the peak fluorescence from a cell before and after a higher-intensity photobleaching beam. To implement this experimental geometry, a 2W 532 nm continuous wave laser was split into three beams by a series of beam splitters (30:70 and 50:50) and waveplate-polarizer pairs, thereby allowing independent control of each beam's excitation intensity. After splitting, all beams were shaped by a cylindrical lens (150 mm focal length), directed into the microscope, reflected from a 532 nm dichroic mirror, and focused inside of the microfluidic channels by a 20x, 0.45 NA air-objective (Figure 2a). Shaping the beams with the cylindrical lens results in elliptical beams (75 μm length $\times$ 3.5 μm width, FWHM, as measured by imaging light scattered from the sample focal plane onto a CMOS camera) that stretch the entire width of the hydrofocus. The beams were distributed over a 240 μm distance with the bleach beam located mid-way between the two probe beams (Figure 2b). The probe beam intensity was 5 kW/cm² and the bleach beam was 170 kW/cm² (calculated from the FWHM of the beam dimensions and laser power measured at the sample plane ($\pm 5\%$)). The probe beams were matched in intensity before each experiment. Fluorescence was collected through the same objective and separated from excitation light by the 532 nm dichroic mirror and a 545 nm long-pass filter. The emission was detected by a red-sensitive photomultiplier tube (PMT, Hamamatsu*, R9880U-20) on the primary imaging port of the microscope. At this port, the fluorescence signals from the three beams are spatially resolved, which allowed for placement of a mask at the focal plane which blocks the photobleaching beam. A lens is used to refocus fluorescence from the two probe beams onto the PMT.

Data Acquisition and Processing

The PMT photocurrent was processed by a custom-built AC-coupled transimpedance operational amplifier, which improves the signal-to-noise ratio by removing high and low frequency noise components outside the band pass of 160 mHz - 106 kHz. The resulting voltage levels were digitized at 250 kHz with a PC-based data acquisition board (16 bit ADC) and custom software (LabView, National Instruments). After fitting each peak to a Gaussian, the peak fluorescence signals for the first and second probe beams (S_{beam1} and S_{beam3}) were recorded. Typical fluorophore transit times through each beam varied from 0.2–3.5 ms depending on the cell velocity (1–15 mm/s) and neglecting fluorophore diffusion within the cell.

Sample Preparation

HeLa suspension (HeLa-S, mean diameter = 14.4 μm , CV = 21.8%, as measured from images taken on a widefield microscope) cells were maintained in spinner flasks at 37 °C in

*Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

a 5 % CO₂ atmosphere using spinner-modified Dulbecco's Modified Eagles Medium, 10% fetal bovine serum, and 1% penicillin-streptomycin. HeLa-S cells were virally transduced according to manufacturer's protocols with an FP either under a constitutive cytomegalovirus promoter (TagRFP, and TagRFP-T in pCLNCX) or an inducible tet-responsive promoter (mCherry, mOrange2, pCL-TRE). Briefly, virus was generated by transfecting the appropriate combination of DNA (pCLNCX-FP, pCLTREFP, pCL-Ampho, pCL-TetOn, pVSV-G) into HEK293FT cells. After two days, the viral containing supernatant was collected, passed through a 0.45 μm cellulose acetate filter, and added to HeLa suspension cells with 12 μg/ml polybrene, and if appropriate, expression was induced with 1 μg/ml doxycycline. To establish the cell lines, the fluorescent population was enriched once by fluorescence-activated cell sorting (FACS) with a Dako Cytomation Mo-Flo cell sorter, to eliminate cells that were not infected with virus (further details provided in Supporting Methods). To avoid biasing the fluorescent populations evaluated in our experiments, no further enrichments by FACS were performed.

Aliquots of cells were concentrated *via* swinging-bucket centrifugation at 1000 rpm for 5 min. To prevent clumping and settling within the microfluidic reservoirs, cell pellets were resuspended in a density-matched medium using a commercially available density matching solution and HEPES-buffered Hanks' balanced salt solution (HHBSS), pH 7.4 solution supplemented with 1% bovine serum albumin. Experiments involving beads utilized 6 μm diameter fluorescently labeled beads from a Invitrogen LinearFlow Deep Red Flow Cytometry Intensity Calibration Kit, suspended in a density matched 20% (v/v) glycerol in water solution. The microfluidic channels were passivated with a 1% solution of bovine serum albumin prior to each run. Cell suspensions were loaded into the center reservoir in 150 μL aliquots at a concentration of ~5×10⁵ cells/mL. The side channels were filled with 150 μL aliquots of HHBSS, pH = 7.4 for the sheath flow.

Results and Discussion

The measured quantity in all experiments described below is the %bleach, which is defined in terms of the measured peak fluorescence signal for the first and third beams (S_{beam1} and S_{beam3} , respectively), as

$$\%bleach = \left[1 - \left(\frac{S_{\text{beam3}}}{S_{\text{beam1}}} \right) \times \left(\frac{S_{\text{beam3}}}{S_{\text{beam1}}} \right) \right] \times 100\% \quad (4)$$

With

Bleach

Beam

No

Bleach

Beam

To correct for small differences in beam intensity, and lateral misalignments of probe beams, the %bleach is defined as function of reference measurements taken in the absence of the bleach beam. Note that %bleach may be comprised of a combination of reversible and irreversible photobleaching, as dictated by the excited-state dynamics of the fluorophores. To shed light on the molecular origins of the measured %bleach in terms of the rate constants for reversible and irreversible photobleaching, we present and discuss numerical simulations of the signals in terms of a four-state model of RFP photophysics

Single-RFP Population Photobleaching

We first performed multi-beam fluorescence measurements on fluorescently labeled beads to verify the precision of the measurements matched predictions from the design-considerations discussed above. Data from one probe beam yields a fluorescence intensity coefficient of variation (CV) ranging from 6 to 16 %, depending on the fluorescence intensity of the bead type (higher intensity beads yielded lower CV, Supporting Figure S-2b). The CV value averaged over all three populations of beads, was within 10% of the average value obtained on a Dako Cytomation Mo-Flo FACS (Supporting Information, page S-4). This variability is lower than many other 2D-focusing microfluidic cytometers (CV of 25–30%)^{27,28} and comparable to 3D-focusing microfluidic cytometers (CV of 1–9%)^{29,30} but remains larger than state-of-the-art flow cytometers (*e.g.* CV < 3%, BD FACS Aria). For a two probe beam measurement with a mixture of beads, a plot of $S_{\text{beam}3}$ vs. $S_{\text{beam}1}$ was linear, with a coefficient of determination, $R^2=0.99$ with a 7% CV in the ratio of $S_{\text{beam}3}$ vs. $S_{\text{beam}1}$ for greater than 3,000 events (Supporting Figure S-2a).

Two-beam measurements (without a bleach beam) on HeLa-S cells expressing TagRFP-T, were fit to a line with a coefficient of determination of 0.99 and a 11% CV in the ratio of $S_{\text{beam}3}$ vs. $S_{\text{beam}1}$ (Figure 3).

In principle, for two probe beams of identical intensity, and in the absence of photobleaching, we expect a slope of 1 for a plot of $S_{\text{beam}3}$ vs. $S_{\text{beam}1}$. In general, we observed slopes of slightly less than 1 for both the beads (0.93, CV = 7%, Supporting Figure S-2a) and TagRFP-T expressing cells (0.94, CV = 11%) which were statistically the same by an unpaired t-test ($T = 1.42$). Under these probe beam conditions, we expect photobleaching of the beads and even the less photostable RFPs, to be negligible. For example, using photobleaching kinetics parameters measured for TagRFP-T at 532 nm in immobilized HeLa cells at similar intensities,²³ we estimate 0.4% photobleaching occurs. It seems likely that the non-unity slope occurs primarily due to a slight mismatch in the probe beam power transmitted through the objective, which we observe to be highly sensitive to alignment into the microscope. In our definition of %bleach, we account for this mismatch in probe beam intensities, to provide for a corrected measure of the bleaching magnitude. For TagRFP-T cells, with a bleach beam intensity of 170 kW/cm², and flow speed of 27.9 mm/sec (exposure time of 125 μ s), the slope decreases to 0.52 (Figure 3) which indicates a significant amount of bleaching (%bleach = 51%, from equation 4). The same performance for all measurements were reproduced with the beams intersecting the hydrofocused stream anywhere along the ~1 mm length from immediately after the hydrofocus to the end of the interrogation channel.

Photobleaching of Fluorescent Protein Populations

To evaluate the ability to resolve subpopulations on the basis of photobleaching, 3-beam measurements were performed on a mixture of HeLa-S cells expressing TagRFP³¹, TagRFP-T²², mOrange-2²², and mCherry³². Photophysical properties of these FPs are summarized in Supporting Table S-1. With a bleach beam intensity of 170 kW/cm², and flow speed of 12.9 mm/sec (exposure time of 270 μ s), the beam spacing resulted in an 8 ms average cell transit time between beams. This timescale permits complete recovery from higher-ordered excited states and dark states²³ (Supporting Table S-2). Under these conditions, four populations of cells were apparent in the plots of $S_{\text{beam}3}$ vs. $S_{\text{beam}1}$ (Figure 4a). Each RFP-expressing cell population was identified by measurements on the individual cell types under identical flow and intensity conditions. A histogram of %bleach for the ~1891 cells in this sample also reveals four subpopulations, corresponding to the four RFPs (Figure 4b). The rank order of average fluorescence intensities for the cell lines measured in the microfluidic cytometer agreed with those measured by FACS (TagRFP-T = TagRFP >

mOrange2 > mCherry). The differences in fluorescence brightness for different RFP expressing cell lines may result from differences in the relative absorption cross-section at 532 nm (Supporting Table S-1), or from differences in cellular RFP concentrations, which in turn may result from incomplete chromophore maturation and differences in the transcription promoter strength. As stated earlier, the cells assayed in the cytometer were not pre-screened or enriched for brightness, therefore a large range (CV > 130%) of intensities were screened. Tuning the PMT gain to optimize detection of weakly fluorescent cells would permit improved resolution of the photobleaching response in these cells³³. In Figure 4c, we plot the measured %bleach vs. pre-bleach fluorescence intensity for the cell mixture. These data show a resolution of the mixture into four populations, and demonstrate that the measured %bleach depends on the RFP, but is largely independent of the fluorophore concentration (as given by the pre-bleach emission level). The ability of %bleach to resolve the mixture of 4 RFPs may be quantified by fitting the histogram (Figure 4b) to a sum of four Gaussians. The fit parameters (Figure 4 caption) reveal that the mean %bleach values for mOrange2, mCherry and TagRFP were separated by at least 1σ , whereas the TagRFP-T population was separated from the others by at least 2σ . The percentages of cells that could be uniquely assigned to one population with at least 99.9% confidence were obtained by determining the confidence interval of the Gaussian fit for a given cell population which has less than 0.1% overlap from the Gaussian fits for the other cell populations. This confidence interval defines the percentage of cells in a population that can be assigned to a given Gaussian fit with 99.9% confidence. The percentages resolved by this criterion are 1% of the mOrange 2 cells were resolved from mCherry cells, 43% of the mCherry cells from mOrange 2 and TagRFP cells, 10% of TagRFP cells from mCherry and TagRFP-T cells, and 100% of the TagRFP-T cells were resolved from the others. More details on this resolution can be found in the Supplementary Information.

Photokinetic Simulations

To understand the connection between microfluidic photobleaching measurements and fluorophore photophysics, we performed numerical simulations based on a four state system consisting of the ground state (S_0), the first excited state or bright state (S_1), and two dark or weakly fluorescent states (D_0 and D_1) (Figure 5a). Table 1 contains a summary of the reactions and kinetic parameters. Photobleaching was permitted out of S_1 and D_1 . Recently we used this 4-state model to describe the excited-state dynamics of RFPs in immobilized single cells exposed to a series of millisecond timescale excitation pulses, and demonstrated that this model faithfully captured trends for reversible and irreversible photobleaching for a panel of 13 FPs²³. A similar model has been used to examine reversible photobleaching (*i.e.* blinking) of GFP³⁴. We modified our previous simulations by approximating the excitation profile as a sum of Gaussian pulses that replicate the durations and excitation rates experienced by the fluorophores as they flow through the three beams in the cytometer.

Previous investigations of photodynamics in flow cytometry primarily focused on photobleaching and photon saturation,^{11,12} which are the dominant processes operative at the $\sim 10^3$ kW/cm² intensities and microsecond timescales considered in those investigations. Photon saturation occurs when the average time between excitation-photon absorption approaches the time the fluorescent molecule spends in the excited state. We estimate that the average arrival time between excitation photon for our highest intensity beam (170 kW/cm²) beam was 1.7 photons/ μ sec. Since the excited-state lifetime of the RFPs are in the range of 2–3 ns²³, photon saturation is negligible under the conditions employed here. We therefore focused on photobleaching and dark-state conversion processes.

The excitation rate was calculated using the measured, average-excitation intensity to find the peak-excitation intensity, which was then used to calculate the maximum rate of excitation for a representative RFP (TagRFP-T, which was chosen because its photophysical

properties represent the median of the four proteins assayed) using its extinction coefficient at 532 nm ($52000 \text{ M}^{-1} \text{ cm}^{-1}$) and the Beer-Lambert law:

$$k_{ex} = \frac{\sigma I \lambda}{hc} = 2.303 \times 1000 \left(\frac{\epsilon I \lambda}{N hc} \right) \quad (5)$$

where σ is the absorption cross-section, I is the light intensity, λ is the wavelength, h is Planck's constant, c is the speed of light, ϵ is the decadic molar extinction coefficient, and N is the number of molecules.

The fluorophores first experience an excitation rate corresponding to the first probe beam. The rate of excitation increases and then decreases in a Gaussian profile in time from zero up to the peak rate of excitation ($8 \times 10^4 \text{ s}^{-1}$) and then back to zero over the course of 0.54 ms. The excitation rate remains at zero for 20 ms (as mentioned previously, ground state recovery is complete after 8 ms) before experiencing the excitation of the bleach beam (maximum rate of $1.6 \times 10^6 \text{ s}^{-1}$) and, lastly, the third probe beam. In accord with the calculations and measurements on the cylindrical beam shaping, the excitation profile was assumed to be constant in the direction perpendicular to the cell's travel. The FWHM of the laser spatial profile was transformed into time coordinates assuming an average cell velocity of 6 mm/s which is approximately the mid-point of the range of speeds used in these experiments. The peak of the time-dependent fluorescence profiles from the first and third excitation beams was then used to calculate the %bleach.

The values of the rate constants for each step in the 4-state model were taken from our previous work (Table 1)²³. In particular, three parameters were varied individually and the magnitude of photobleaching was calculated for each simulation. First, the rate of bleaching out of the higher-energy dark-state (k_{DIBleach}) was varied while k_{Rev} and k_{S1Bleach} were held at $5 \times 10^5 \text{ s}^{-1}$. A negligible increase in %bleach was observed for all but extremely large rate constants ($1 \times 10^{10} \text{ s}^{-1}$) indicating that, in this model, the dark state acts photoprotectively, *i.e.* the fluorophore does not bleach out of the dark state. Next, the rate of bleaching out of the first excited state (k_{S1Bleach}) was increased from 0 – $5 \times 10^6 \text{ s}^{-1}$ while k_{Rev} was held at $5 \times 10^5 \text{ s}^{-1}$ and k_{DIBleach} was held at 0. This perturbation resulted in an expected increase in %bleach since the increased rate of bleaching allowed the bleaching process to compete more successfully with the other S_1 depopulation pathways. Lastly, the rate of reversible photobleaching (k_{Rev}) was increased over the same range while k_{S1Bleach} was held at $5 \times 10^5 \text{ s}^{-1}$ and k_{DIBleach} was held at 0, leading to a decrease in %bleach. This trend shows that reversible and irreversible photobleaching are competing processes since increases in the rate of either process leads to opposite impacts on the observed %bleach. Although the results of our model indicate that the k_{S1Bleach} has a greater effect than k_{Rev} on the observed values of %bleach, note that in general, the rates of both processes are known to change with excitation intensity, pulsed *vs.* continuous wave illumination, and excitation wavelength^{21–23}.

For the 4 RFPs investigated here, the rates of reversible and irreversible photobleaching vary over 1–2 orders of magnitude across the range of excitation intensities characteristic of widefield and confocal microscopies (10 W/cm^2 – 1 kW/cm^2) (Supporting Table S-1)²². Therefore, for completeness, these calculations were performed using a range of rate values (Table 1), covering both experimental and modeling estimates^{12,21,23,34–36}. Our kinetic simulations indicate it is likely that the rates of both reversible and irreversible photobleaching are in the range from 1×10^5 to $1 \times 10^6 \text{ s}^{-1}$ since experimental and modeling results for the observed %bleach are in agreement in this range. Furthermore, these simulations predict that, for the current set of measurements, the magnitudes of reversible and irreversible photobleaching are anti-correlated (Figure 5b). The RFPs examined have

rate constants that vary by an order of magnitude or less and our modeling results indicate that, over this small range, the effects of reversible photobleaching significantly influence the observed extent of irreversible photobleaching.

The four proteins examined in this study represent closely related fluorophores. In particular, TagRFP and TagRFP-T differ by only one point mutation and have similar fluorescence spectra and quantum yields (Supporting Table S-1). However, as emphasized by the resolution of the populations in Figure 4, the FPs differ significantly in their photostabilities and propensities for reversible photobleaching. Our photokinetic simulations show that although the four cell populations are differentiated with our 3-beam geometry, they are not resolved solely on the basis of reversible or irreversible photobleaching but rather, by a combination of both processes. For this reason, additional resolving power will be necessary if the two processes are to be separated. For example, according to pulsed photobleaching experiments performed on stationary, individual cells (Supporting Figure S-3 and Supporting Tables S-1 and S-2), mCherry is less prone to both irreversible and reversible photobleaching than mOrange2. However, the significant contribution of reversible photobleaching for mOrange2 causes it to appear very similar to mCherry, if only %bleach is considered. Building on the multi-beam geometry described here, other excitation schemes may be devised to separately measure the rates of both processes.

Conclusions

To our knowledge, this is the first cytometer designed to quantify photobleaching in mixed populations. This multi-beam flow cytometer capitalizes on the spatiotemporal properties of the cells in microfluidic flow to measure photobleaching with a ratiometric approach, which inherently differs from previous experiments with one or two beams. We demonstrated the capability to characterize the individual cells in within a mixed population, and note that our experiment resolves two RFPs which (Tag-RFP³¹ and Tag-RFP-T²²) that cannot be resolved by previously available spectral signatures (*e.g.*, fluorescence lifetime, excitation/emission spectra, absorption).

As revealed in these experiments and simulations, this 3-beam pulse sequence probes both irreversible and reversible photobleaching. These two processes are highly interdependent and must be considered in tandem. To discriminate reversible from irreversible photobleaching, the excitation pulse sequence would need to operate on timescales that create a steady-state dark-state population. Generally, by controlling the cell velocity, excitation intensity, dimension of the beam and fluidic channel, and employing time-resolved fluorescence detection, it will be possible to implement specific probes of other photophysical dynamics, at high throughput. Furthermore, building on a recent suggestion in the literature³⁷, the method reported here can be integrated with measurements of fluorescence lifetime^{8,9} and microfluidic cell-sorting techniques^{38,39} to enable the screening of genetic libraries of FPs on the basis of photostability and fluorescence quantum yield. This work, which is currently underway in our laboratory, will enable the development of a next generation of more photostable FPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors received generous funding from the University of Colorado Molecular Biophysics Training Grant (T32 GM-065103), National Science Foundation Computational Optical Sensing and Imaging Integrative Graduate Education and Research Traineeship (0801680), as well as the National Institutes of Health (GM083849 to A.E.P.

and R.J.). R.J. is a staff member in the Quantum Physics Division of NIST. The authors thank Dr. Roger Tsien for providing TagRFP-T, mCherry, and mOrange2.

References

1. Shapiro HM. Cytometry. 1983; 3:227. [PubMed: 6185284]
2. Wade CG, Rhyne RH, Woodruff WH, Bloch DP, Bartholomew JC. J Histochem Cytochem. 1979; 27:1049. [PubMed: 110874]
3. Goddard G, Martin JC, Naivar M, Goodwin PM, Graves SW, Habbersett R, Nolan JP, Jett JH. Cytom Part A. 2006; 69A:842.
4. Mullaney PF, Vandilla MA, Coulter JR, Dean PN. Rev Sci Instrum. 1969; 40:1029. [PubMed: 5797881]
5. Hulett HR, Bonner WA, Barrett J, Herzenbe La. Science. 1969; 166:747. [PubMed: 4898615]
6. Bonner WA, Sweet RG, Hulett HR, Herzenbe La. Rev Sci Instrum. 1972; 43:404. [PubMed: 5013444]
7. Hulett HR, Bonner WA, Sweet RG, Herzenbe La. Clin Chem. 1973; 19:813. [PubMed: 4200173]
8. Steinkamp JA, Yoshida TM, Martin JC. Rev Sci Instrum. 1993; 64:3440.
9. Houston JP, Naivar MA, Freyer JP. Cytom Part A. 2010; 77A:861.
10. Engelhard HH, Krupka JL, Bauer KD. Cytometry. 1991; 12:68. [PubMed: 1900228]
11. Vandenengh G, Farmer C. Cytometry. 1992; 13:669. [PubMed: 1280553]
12. Doornbos RMP, deGroot BG, Greve J. Cytometry. 1997; 29:204. [PubMed: 9389437]
13. Tsien RY. Annu Rev Biochem. 1998; 67:509. [PubMed: 9759496]
14. Shaner NC, Patterson GH, Davidson MW. J Cell Sci. 2007; 120:4247. [PubMed: 18057027]
15. Donnert G, Eggeling C, Hell SW. Nat Methods. 2007; 4:81. [PubMed: 17179937]
16. Abbyad P, Childs W, Shi XH, Boxer SG. P Natl Acad Sci USA. 2007; 104:20189.
17. Henderson JN, Ai HW, Campbell RE, Remington SJ. P Natl Acad Sci USA. 2007; 104:6672.
18. Mizuno H, Mal TK, Walchli M, Kikuchi A, Fukano T, Ando R, Jeyakanthan J, Taka J, Shiro Y, Ikura M, Miyawaki A. P Natl Acad Sci USA. 2008; 105:9227.
19. Andresen M, Stiel AC, Trowitzsch S, Weber G, Eggeling C, Wahl MC, Hell SW, Jakobs S. P Natl Acad Sci USA. 2007; 104:13005.
20. Heikal AA, Hess ST, Baird GS, Tsien RY, Webb WW. Proc Natl Acad Sci U S A. 2000; 97:11996. [PubMed: 11050231]
21. Sinnecker D, Voigt P, Hellwig N, Schaefer M. Biochemistry-US. 2005; 44:7085.
22. Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, Davidson MW, Tsien RY. Nat Methods. 2008; 5:545. [PubMed: 18454154]
23. Dean KM, Lubbeck JL, Binder JK, Schwall LR, Jimenez R, Palmer AE. Biophys J. 2011; 101:961. [PubMed: 21843488]
24. Knight JB, Vishwanath A, Brody JP, Austin RH. Phys Rev Lett. 1998; 80:3863.
25. Verdeyen, JT. Laser electronics. 3. Prentice Hall; Englewood Cliffs, N.J.: 1994.
26. Li, D. Encyclopedia of Microfluidics and Nanofluidics. Springer-Verlag; Boston, MA: 2008.
27. Wang Z, El-Ali J, Engelund M, Gotsaed T, Perch-Nielsen IR, Mogensen KB, Snakenborg D, Kutter JP, Wolff A. Lab Chip. 2004; 4:372. [PubMed: 15269807]
28. Schrum DP, Culbertson CT, Jacobson SC, Ramsey JM. Anal Chem. 1999; 71:4173. [PubMed: 21662848]
29. Morimoto Y, Tan WH, Takeuchi S. Biomed Microdevices. 2009; 11:369. [PubMed: 19009352]
30. Mao XL, Lin SCS, Dong C, Huang TJ. Lab Chip. 2009; 9:1583. [PubMed: 19458866]
31. Merzlyak EM, Goedhart J, Shcherbo D, Bulina ME, Shcheglov AS, Fradkov AF, Gaintzeva A, Lukyanov KA, Lukyanov S, Gadella TWJ, Chudakov DM. Nat Methods. 2007; 4:555. [PubMed: 17572680]
32. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Nature Biotechnology. 2004; 22:1567.
33. Lakowicz, JR. Principles of fluorescence spectroscopy. 3. Springer; New York: 2006.

34. Dickson RM, Cubitt AB, Tsien RY, Moerner WE. *Nature*. 1997; 388:355. [PubMed: 9237752]
35. Hendrix J, Flors C, Dedeker P, Hofkens J, Engelborghs Y. *Biophys J*. 2008; 94:4103. [PubMed: 18234806]
36. Schenk A, Ivanchenko S, Rocker C, Wiedenmann J, Nienhaus GU. *Biophys J*. 2004; 86:384. [PubMed: 14695280]
37. Subach FV, Piatkevich KD, Verkhusha VV. *Nat Methods*. 2011; 8:1019. [PubMed: 22127219]
38. Wang MM, Tu E, Raymond DE, Yang JM, Zhang HC, Hagen N, Dees B, Mercer EM, Forster AH, Kariv I, Marchand PJ, Butler WF. *Nature Biotechnology*. 2005; 23:83.
39. Chen CH, Cho SH, Tsai F, Erten A, Lo YH. *Biomed Microdevices*. 2009; 11:1223. [PubMed: 19649710]

Figure 1.

(a) Schematic of hydrodynamically-focused cells traveling through two circularly shaped Gaussian laser beams where ΔZ and ΔY refer to the cell axial and lateral displacement from the center of the channel. (b–e) Contour plots showing peak fluorescence signal as a function of the cell displacement (ΔZ and ΔY) as it flows through circularly (b–c) and elliptically (d–e) shaped Gaussian beams. (b and d) Represent the fluorescence signal if the two beams are perfectly centered in the channel, whereas (c and e) represent the fluorescence signal if the second beam is displaced $0.5 \text{ }\mu\text{m}$ along the y-axis relative to the center of the channel. (f) For the misaligned beams, a scatter plot of the ratio of peak

fluorescence signals (I_1/I_2) from 500 cells randomly displaced from the center of the channel (within $\Delta Y = \pm 2 \mu\text{m}$ and $\Delta Z = \pm 5.5 \mu\text{m}$) shows the signal variance from elliptically shaped beams (red) is 35-fold smaller than that from circularly shaped beams (black).

Figure 2.

3-Beam microfluidic cytometer experimental design. **(a)** Schematic of the optical setup. Relevant components include: 20X 0.45 N.A. air immersion objective (Obj.); 532 nm dichroic mirror (DM); 545 nm long-pass filter (LPF); red-enhanced photomultiplier tube (PMT); 150 mm focal length, cylindrical lens (CL, placed 19 cm from back aperture of objective); half-wave plate ($\lambda/2$); Glan-Thompson polarizer (P); 70:30 beam splitter (BS1); 50:50 beam splitter (BS2). **(b)** Schematic of the microfluidic channel geometry at the interrogation region. Cells were hydrodynamically focused to a width of 15 μm before encountering the elliptical bleach and probe beams (FWHM $3 \times 75 \mu\text{m}$).

Figure 3.

Single cell photobleaching of HeLa-S cells transduced with TagRFP-T interrogated with the two probe beams indicate that the cytometer's response is linear with respect to fluorescence intensity and the signals from each probe beam are highly correlated ($\text{CV} = 11\%$, $R^2 = 0.997$, Non-Bleached Slope = 0.94). Upon addition of the bleach beam, the slope decreases (Bleached Slope = 0.52), indicating that photobleaching occurs. Here, each point represents a measurement performed on a single cell.

Figure 4.

Resolving RFPs based on photobleaching in a microfluidic cytometer. **(a)** A mixture of cells expressing one of four RFPs was resolved on the basis of photobleaching. Each point represents an individually assayed cell and the slope of the $S_{\text{beam}1}$ versus $S_{\text{beam}3}$ plot yields the %bleach for each RFP. **(b)** The mean %bleach for each RFP-expressing cell line (upon measurement of 200–300 cells) was 4.4 (CV = 145%) %bleach for mOrange2, 26.8 (CV = 49%) %bleach for mCherry, 52.0 (CV = 9%) %bleach for TagRFP, and 77.3 (CV = 4%) %bleach for TagRFP-T as determined using a fitting program which fit a sum of four Gaussian functions to the histogrammed data. **(c)** Plot of %bleach vs. pre-bleach intensity, showing the resolving power provided by bleaching measurements. The signal level corresponding to cellular autofluorescence is below the baseline of 27.8 mV.

Figure 5. Kinetic modeling results **(a)** Jablonski diagram depicting four-state model used for photokinetic modeling results. **(b)** Simulated percent photobleaching as a function of $k_{S1Bleach}$ and k_{Rev} . Increasing rates of irreversible photobleaching out of the first excited singlet state and reversible photobleaching have opposite effects on observed photobleaching.

Table 1

Summary of reactions and equations used in photokinetic model.

Process	Reaction	Reaction Rate	Rate Constant Value (s^{-1})
Emission and Non-Radiative Decay	$S_1 \rightarrow S_0$	$[S_1] K_{Em}$	$K_{Em} = 1 \times 10^9$
Reversible Photobleaching	$S_1 \rightarrow D_1$	$[S_1] K_{Rev}$	$K_{Rev} = \text{Varied} = 0 - 1 \times 10^6$
Dark-State Decay	$D_1 \rightarrow D_0$	$[D_1] K_{DSD}$	$K_{DSD} = 1 \times 10^9$
Ground-State Recovery	$D_0 \rightarrow S_0$	$[D_0] K_{GSR}$	$K_{GSR} = 1 \times 10^4$
S_1 Bleach	$S_1 \rightarrow \text{Null}$	$[S_1] K_{S1Bleach}$	$K_{S1Bleach} = \text{Varied} = 0 - 5 \times 10^6$
D_1 Bleach	$D_1 \rightarrow \text{Null}$	$[D_1] K_{D1Bleach}$	$K_{D1Bleach} = \text{Varied} = 0 - 5 \times 10^{10}$
Excitation	$S_0 \rightarrow S_1$	$[S_0] K_{Ex}$	$K_{Ex} = \text{Gaussian}$