Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy

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ABSTRACT Biological structures span many orders of magnitude in size, but far-field visible light microscopy suffers from limited resolution. A new method for fluorescence imaging has been developed that can obtain spatial distributions of large numbers of fluorescent molecules on length scales shorter than the classical diffraction limit. Fluorescence photoactivation localization microscopy (FPALM) analyzes thousands of single fluorophores per acquisition, localizing small numbers of them at a time, at low excitation intensity. To control the number of visible fluorophores in the field of view and ensure that optically active molecules are separated by much more than the width of the point spread function, photoactivatable fluorescent molecules are used, in this case the photoactivatable green fluorescent protein (PA-GFP). For these photoactivatable molecules, the activation rate is controlled by the activation illumination intensity; nonfluorescent inactive molecules are activated by a high-frequency (405-nm) laser and are then fluorescent when excited at a lower frequency. The fluorescence is imaged by a CCD camera, and then the molecules are either reversibly inactivated or irreversibly photobleached to remove them from the field of view. The rate of photobleaching is controlled by the intensity of the laser used to excite the fluorescence, in this case an Ar+ ion laser. Because only a small number of molecules are visible at a given time, their positions can be determined precisely; with only ≈100 detected photons per molecule, the localization precision can be as much as 10-fold better than the resolution, depending on background levels. Heterogeneities on length scales of the order of tens of nanometers are observed by FPALM images of PA-GFP on glass. FPALM images are compared with images of the same molecules by widefield fluorescence. FPALM images of PA-GFP on a terraced sapphire crystal surface were compared with atomic force microscopy and show that the full width at half-maximum of features ≈86 ± 4 nm is significantly better than the expected diffraction-limited optical resolution. The number of fluorescent molecules and their brightness distribution have also been determined using FPALM. This new method suggests a means to address a significant number of biological questions that had previously been limited by microscope resolution.

INTRODUCTION

Although biological structures span many orders of magnitude in length scale (1), resolution in many types of biological light microscopy is limited. The limit on resolution of point sources imposed by the finite size of the diffraction-limited illumination volume in a far-field optical system (2) is quantified by the Rayleigh criterion (3):

\[ r_R = 0.61 \times \lambda / NA, \]

where \( \lambda \) is the wavelength of the detected photons and \( NA \) is the numerical aperture of the lens system. To adequately visualize structure, most fluorescence imaging methods rely on observation of a large number of molecules simultaneously, which is inherently limited because the distance between observed molecules is much less than \( r_R \). New imaging methods such as 4Pi microscopy (4–9), patterned illumination microscopy (10,11), stimulated emission depletion (STED) microscopy (12–14), and other types of reversible saturable optical fluorescence transition (RESOLFT) microscopies (15) can increase resolution by reducing the size of the observation volume or, correspondingly, by increasing the accessible Fourier space and therefore increasing the number of accessible spatial frequencies. 4Pi microscopy (6,9) provides a significant increase in axial resolution (≈100 nm), but its advantages are more difficult to apply toward examination of thick or scattering samples, or lateral molecular distributions such as in biological membranes. Although STED does not rely on image deconvolution as 4Pi and patterned illumination microscopy do, both STED and 4Pi depend on nonlinear excitation under high-intensity pulsed illumination. Furthermore, they typically derive their information from large numbers of molecules observed simultaneously, and therefore may obscure crucial behavior that is only discernable at the single-molecule level. Nonlinear structured illumination microscopy (10) has so far achieved resolution (full width at half-maximum (FWHM) of the point spread function (PSF)) of 50 nm and can in principle be improved further, but is limited by the photobleaching properties of the fluorophore, and uses excitation intensities near saturation, where photobleaching may be more pronounced than at low intensity (10). Near-field scanning optical microscopy is also capable of spatial resolution better than the classical diffraction limit, but is typically used to image surfaces and is difficult to use to image in aqueous
solution (16). Fluorescence resonance energy transfer can access the molecular length scale, but has a limited range of distances over which it is sensitive and depends on a large number of variables, including molecular orientation and distance, dielectric constant, spectral overlap, and excited-state lifetime (17–19). Other single-molecule methods can resolve multiple fluorescent objects at distances longer than those accessible to fluorescence resonance energy transfer and closer than \( r_{R} \), but only in limited numbers, and only if a very large number of photons is collected per object (e.g., \( 10^{3} \) photons to resolve two objects separated by 10 nm) (20). Thus, even the most advanced fluorescence microscopy and spectroscopy methods continue to be limited in their range of accessible length scales.

Fluorescent speckle microscopy (FSM) (21–24) provides an additional means to image subdiffraction objects such as actin and microtubules by localizing single molecules as they intermittently emit fluorescence. FSM also exploits (typically stochastic) intermittency of those single molecules, which allows large numbers of molecules (\( >10^{5} \)) to be visualized, but does not allow direct control over the number of fluorescent molecules within the field of view. In FSM, the number of visible particles must be controlled by changing the concentration or density per unit area of the fluorophore-labeled molecules themselves, which in the case of green fluorescent protein (GFP)-transfected cells may not be a trivial proposition, especially if the desired density is low. Instead, in fluorescence photoactivation localization microscopy (FPALM), the number of active fluorophores, as will be explained, can be increased or decreased photophysically by changing the rates of photoactivation and photobleaching, even if the density of fluorophore-labeled molecules is much higher than one fluorophore per \( \mu \text{m}^{2} \).

We present a novel method by which fluorescence microscopy may be performed to obtain an image with greatly enhanced ability to resolve large numbers of fluorescent molecules. Unlike previous approaches, this method does not suffer from the standard resolution limits because it does not exclusively rely on resolution for visualization of multiple molecules. Rather, single molecules are localized in modest numbers, allowing position localization, which is far more precise than the resolution limit. We demonstrate this principle using photoactivatable green fluorescent protein (PA-GFP) molecules that are initially found in a dark (weakly fluorescent) state, can be either reversibly or irreversibly activated by one excitation wavelength, and then can be visualized by excitation with a second wavelength. A small number of stochastically photoactivated molecules, which are spatially separated from each other (on average) by several times the resolution, are then localized using single-molecule detection methods, in this case a high-quantum efficiency charge-coupled device (see Fig. 1). After a (stochastically determined) number of photons has been collected from the molecule, the readout cycle ends by photobleaching of that particular molecule, and the process is repeated until the total number of localized molecules is very large. The positions of readout (observed and localized) molecules are then tabulated and plotted to construct a two-dimensional map (image). The acquisition of this single-molecule information also permits determination of the fluorescence brightness and mean molecular velocity during the acquisition time.

In principle, this method can be used for any photoactivatable molecular species whose photophysical properties are sufficient under the following five photoactivation excitation and detection conditions.

1. The spontaneous interconversion rates into and out of the activated (fluorescent) state must be low compared to the light-controlled activation rate.
2. For irreversible photoactivation, the photobleaching quantum yield must be finite and, when multiplied by

![FIGURE 1 Fluorescence photoactivation localization microscopy (FPALM).](image-url)
the excitation rate at saturation, must give a photo-
bleaching rate equal to or greater than the activation rate.
3. For reversible photoactivation, the light-controlled deac-
tivation plus photobleaching rate must be equal to or
greater than the activation rate.
4. The average number of detected photons per molecule
before photobleaching must be large enough to provide
the desired resolution enhancement.
5. The fluorescence of the inactive state must be low com-
pared to that of the active state under the given detection
conditions; that is, the contrast ratio (25) must be large.

**THEORY**

The spatial precision of localization of single fluorescent
objects has been studied extensively (16,20,26–29). Thompson
et al. calculate the localization precision $\sigma_s$ for point-like
objects imaged in two dimensions by fluorescence micros-
copy to be

$$
\sigma_s^2 = \frac{r_0^2 + q^2/12}{N} + \frac{8\pi r_0^2 b^2}{q^2 N^2},
$$

(1)

where $r_0$ is the standard deviation of the point spread function,
$N$ is the total number of photons collected, $q$ is the size of an
image pixel, and $b$ is the background noise per pixel. From
Eq. 1, it is clear that molecular localization may be sig-
nificantly more precise than the optical resolution by a factor
of as much as $\sqrt{N}$. Thus, the number of detected photons will
be crucial to the enhancement of localization precision com-
pared to resolution, as stated by condition 4. For a single
molecule capable of emitting $-10^5$ visible photons (e.g.,
emission wavelength $\sim 500$ nm) before photobleaching,
the position localization could be as precise as $\sim 500$ nm/$10^5$
or a few nanometers. Considering the finite detection efficiency
(typically $\sim 5\%$) of modern single-molecule fluorescence
microscopes, an estimate of the number of collected photons is
$N_{\text{coll}} = \phi_{\text{det}} / \Phi_B$, where $\Phi_B$ is the photobleaching quantum
yield for the molecule and $\phi_{\text{det}}$ is the detection efficiency, and
the two-dimensional localization precision becomes

$$
\sigma_s^2 = \frac{r_0^2 + q^2/12}{N_{\text{coll}}} + \frac{8\pi r_0^2 b^2}{q^2 N_{\text{coll}}^2} + \frac{\Phi_B(r_0^2 + q^2/12)}{\phi_{\text{det}}} + \frac{8\pi r_0^2 b^2 \Phi_B^2}{q^2 \phi_{\text{det}}^2}.
$$

(2)

Since an increased $b$ will increase $\sigma_s^2$, it is crucial that
background be minimized, including background from in-
active protein molecules (condition 5). Consider, now, $N$ photo-
activatable molecules subdivided into inactive (I), active (A),
and bleached (B) states, with interconversion rates as follows:

$$
k_A \Phi_A + k_0 \xrightarrow{k_{BC} A \Phi_B \xrightarrow{k_{BC} B} B,}
$$

where $k_A$ is the activation excitation rate, $\Phi_A$ is the activation
quantum yield, $k_0$ is the spontaneous activation rate, $k_{BC}$ is the
spontaneous and light-dependent inactivation rate, $k_\lambda$ is the
fluorescence excitation rate, and $\Phi_B$ is the photobleaching
quantum yield. These rate equations are similar to those shown
to describe reversible photoconversion kinetics in related GFP
mutants in the absence of photobleaching (30). Here, consid-
ering photobleaching and photoactivation, three linear coupled
first-order differential equations then describe A, B, and I, the
numbers of molecules in each state, respectively:

$$
\frac{dI}{dt} = -I(k_A \Phi_A + k_0) + k_{BC} A
$$

(4A)

$$
\frac{dA}{dt} = -A k_\lambda \Phi_B + I(k_A \Phi_A + k_0) - k_{BC} A
$$

(4B)

$$
\frac{dB}{dt} = A k_\lambda \Phi_B.
$$

(4C)

Alternatively, one of the three equations (4A–4C) could
be replaced by the constraint that $A + B + I = \text{Const.}$
Considering Eqs. (4A–4C), $k_A$ and $k_\lambda$ can be chosen such
that $A$, the number of active molecules within the illuminated
region, is sufficiently sparse to allow simultaneous resolution
and localization of those active molecules, and $\frac{dI}{dt} = 0$. The
steady-state condition results in

$$
A = I \left[ \frac{k_A \Phi_A + k_0}{k_\lambda \Phi_B + k_{BC}} \right] = \rho I,
$$

(5)

where $\rho = \frac{k_A \Phi_A + k_0}{k_\lambda \Phi_B + k_{BC}}$ describes a characteristic ratio of photo-
activation to (reversible and irreversible) photobleaching that
should span as wide a range of values as possible for maximum
control over molecular readout. It should be pointed out that performing FPALM does not strictly require $dA/dt = 0$, since it will be possible to localize molecules as
long as they are sparse enough to not have overlap in their
point spread functions, but estimation of the conditions that
yield an appropriate density of molecules (i.e., a certain
favorable value for $A$) will be easier if $dA/dt \approx 0$ during
the measurement. Typically, to control (limit) the number of
active molecules at a given time (under irreversible photo-
activation), the rate of photobleaching and spontaneous
inactivation should be equal to or larger than the rate of
activation, or $\rho \ll 1$. At saturation, where $k_\lambda$ is at or near
maximum, the denominator of $\rho$ will be maximum, and so $\rho$
will be at its minimum (at a given activation rate), and that
minimum $\rho$ must be $< 1$ (condition 2). Under this condition, if
the number of inactive molecules $I$ is approximately constant
because $\frac{dI}{dt} \approx I$ (which should be satisfied anyway if $dA/dt$
$= 0$), if $k_0 \ll k_A \Phi_A$, and $k_{BC} \ll k_\lambda \Phi_B$ (condition 1), the
ratio $A/I$ can be adjusted over a wide range of values. The
value of $k_{BC}$ is expected to be very small for PA-GFP, since its
activation process is irreversible (25,31).

Considering reversible photoactivation, it will be neces-
sary under some condition for $k_{BC} \gg k_\lambda \Phi_B$, so that the
dominant path for limiting the number of activated mole-
cules is by reversible deactivation rather than photobleach-
ing. Also, to limit the number of active molecules, the
deactivation plus photobleaching rate $k_{BC} + k_\lambda \Phi_B$ needs to

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be equal to or larger than the activation rate, which is again equivalent to \( \rho \ll 1 \), or condition 3.

We may estimate the desired value of \( A \) (the number of active molecules) by considering the area/molecule in comparison to the expected area of the molecule given by the \( 1/e^2 \) radius of the point spread function \( (r_0) \) and the area covered by molecular (assuming nondirected) diffusion during the molecular readout time \( t_m \):

\[
S / A \gg r_0^2 + 4Dt_m, \tag{6}
\]

where \( D \) is the diffusion coefficient, the FWHM of the PSF is \( f_0 \sim 0.58h / NA \) (32), and \( r_0 = f_0 \sqrt{2ln2} \). \( \lambda_m \) is the emission wavelength, \( NA \) is objective numerical aperture, and \( S \) is the area of the illuminated region.

To ensure that Eq. 6 is satisfied by a constant dimensionless factor \( \gamma \gg 1 \), where \( \gamma \) is approximately the ratio of the area/molecule to the area of the PSF (e.g., \( \gamma \sim 16 \) for molecules to be separated by approximately an average distance of four times the size of their image on the relevant timescale), let the limit for straightforward simultaneous resolution and localization of particles require that

\[
S / A = \gamma(r_0^2 + 4Dt_m) \tag{7}
\]

or,

\[
A = \frac{S}{\gamma(r_0^2 + 4Dt_m)}. \tag{8}
\]

From Eqs. 5 and 8, it follows that

\[
\rho l = \frac{S}{\gamma(r_0^2 + 4Dt_m)} \tag{9A}
\]

and

\[
\rho = \frac{S}{l \gamma(r_0^2 + 4Dt_m)}. \tag{9B}
\]

Similarly, an estimate of \( \chi \), the maximum range of \( \rho \), yields

\[
\chi = \frac{\rho_{\text{max}}}{\rho_{\text{min}}} = \frac{k_A^{\text{max}} \Phi_A + k_0}{k_B^{\text{max}}} : \frac{k_A^{\text{max}} \Phi_B + k_0}{k_B^{\text{max}}} \tag{10}
\]

which reflects the degree of control over the photoactivation readout and should be maximized experimentally. Thus, minimizing both \( k_B^{\text{max}} \) and \( k_0 \) is essential for optimization of photoactivation readout. It also follows that in cases where \( k_A^{\text{max}} \Phi_A \gg k_0 \) and \( k_A^{\text{max}} \Phi_B \gg k_B^{\text{max}} \) (i.e., the spontaneous rates are small compared to the light-induced rates), maximization of the quantum yield for activation is helpful for control of the number of active molecules. Furthermore, a nonzero photobleaching quantum yield is also necessary, since otherwise the number of active molecules will become too large for resolution and localization. However, a large photobleaching yield will also reduce the total number of photons that can be detected per molecule and therefore reduce localization precision.

**Estimation of imaging requirements for 80-nm and 20-nm localization precision**

Using the theoretical model described above, the desired properties for a candidate PA-GFP and the parameters of the imaging system can be summarized. Consider now two cases: 1) imaging immobilized molecules at the demonstrated localization precision of 80 nm, and 2) imaging molecules mobile in two dimensions with \( D \sim 0.08 \mu m^2/s \) such as for influenza hemagglutinin (33) with a goal of \( \sim 20 \) nm precision.

Case 1. To have a representative sampling of molecules within a structure of size \( \sim 80 \) nm, one would roughly require \( \sim 100 \) molecules per \( 80 \) nm \( \times \) \( 80 \) nm region within the image. For molecules uniformly distributed over a \( 10 \mu m \times 10 \mu m \) area, this would require localization of \( 1.6 \times 10^6 \) molecules. However, typical confocal images of cells contain highly heterogeneous distributions of molecules, and in many cases large regions of the image do not contain significant concentrations of fluorophore. Thus, estimating that a fraction of the image (e.g., \( 10\% \)) contains labeled features of interest, the number of molecules needed \( N_{\text{tot}} \) is effectively lower (\( N_{\text{tot}} \sim 1.6 \times 10^5 \)) molecules. Now, to estimate the acquisition timescale for such a number of immobile molecules, we employ the theory section derived previously in the text. Given that the image is \( \sim 10 \mu m \times 10 \mu m \), but \( \sim 10\% \) of the image contains the majority of molecules, the area in which the molecules are effectively distributed is \( \sim 0.1 \times 100 \mu m^2 = 10 \mu m^2 \). To ensure that molecules are separated by \( 4 \times r_0 \) on the average, we choose \( \gamma = 16 \) (Eqs. 7 and 8), which yields (from simulations) an \( \sim 9.7\% \) chance of having two molecules within \( r_0 \) of each other. Using Eq. 8, \( \gamma = 16 \), and \( r_0 = 0.281 \mu m \), one estimates that \( A \sim 8 \) molecules/frame within the \( 10 \mu m^2 \) area should be activated. If one is willing to discard any molecules that are within \( r_0 \) of each other, the number of frames then is equal to \( N_{\text{tot}}/A \sim 2 \times 10^4 \) frames.

The next crucial factor determining time resolution is the imaging frame rate. The upper limit on frame rate is dictated by detected photon rate per molecule (\( \eta \)) and the photobleaching rate of molecules. The photobleaching rate should be optimized by using an excited laser intensity where the photobleaching time is close to the image acquisition time (photobleaching times of \( \ll 1 \) s are achieved routinely using high-intensity laser beams). The most obvious limitation in this case would be laser power at the readout wavelength. Second, the value of \( \eta \) at saturation for the fluorophore is crucial. Given a background noise estimated from measurements on coverslips to be \( b \sim 1.02 \) photons/frame/pixel (including readout noise), and pixel size \( q = 0.121 \mu m \), one can solve Eq. 2 to find that one needs \( N_{\text{coll}} \sim 49 \) detected photons/molecule to achieve...
80 nm localization precision. For EGFP, $\eta_{\text{EGFP}} \sim 4 \times 10^3$ photons/s/molecule detected is typical at saturation in fluorescence correlation spectroscopy measurements (S. Hess, unpublished). Using the detected $\eta$ for EGFP, it should be possible to detect 49 photons within a time of $\sim$1.2 ms. Allowing for PA-GFP to have even a 10-fold lower saturating emission rate ($\eta_{\text{PA-GFP}} \sim 4 \times 10^3$ photons/s/molecule including average brightness reduction due to flicker), 12 ms/frame should be enough to collect sufficient photons for the desired localization precision. Thus, the total acquisition time for 2 $\times$ 10$^4$ frames will be $\sim$240 s. In the case of the sapphire sample, estimating a twofold higher background yields a $N_{\text{coll}} \sim 90$ photons, an acquisition frame time of 22.5 ms, and a total acquisition time of 450 s, which is the same order of magnitude as the actual acquisition time of 500 s. Note that this calculation assumes that the optimum (saturating) detected photon emission rate is achieved by the excitation (readout) laser. Thus, for a goal of 80 nm localization precision, the following parameters should be sufficient: $q = 0.121 \mu m$, $\eta > 4 \times 10^3$ photons/s/molecule, $b < 2$ photons/s/pixel, frame acquisition time of $>20$ ms, and a total number of detected photons before photobleaching of $N_{\text{coll}} = \phi_{\text{det}}/\Phi_B > 100$ (i.e., $\Phi_B < 2 \times 10^{-4}$ using $\phi_{\text{det}} \approx 2\%$). In practice, a number of parameters of the photoactivatable protein fluorophore, such as fluorescence quantum yield, extinction coefficient, fluorophore emission spectrum, flicker, and photobleaching properties, will affect the number of photons that are emitted. Detector quantum efficiency, objective numerical aperture, dichroic mirror and emission filters, and excitation laser intensity will also determine the number of emitted and detected photons.

Case 2. Now, to resolve features on $\sim20$ nm length scales (i.e., $\sigma_x < 20$ nm) in samples with molecules randomly diffusing in two dimensions with $D = 0.08 \mu m^2/s$, $N_{\text{det}}$ increases to $2.5 \times 10^6$ for 10% coverage of a 100 $\mu m^2$ area at a density of 100 activated molecules within each area of 20 nm $\times$ 20 nm. Reading out the same number of molecules per frame ($\sim$8), the number of frames increases to $\sim3 \times 10^5$ frames. Given a background level of 2.0 photons/s/pixel, the required number of detected photons per molecule increases to $N_{\text{coll}} \sim 440$, which will then require 110 ms/frame if the molecules are immobile, and an acquisition time of $3.3 \times 10^4$ ms, or $>9$ h. If the molecules are mobile, an acquisition time of 0.2 s will be required because the molecular image will be spread by diffusion at $D = 0.08 \mu m^2/s$ such that $4D t_m \sim 0.064 \mu m^2$ (from Eq. 6), which effectively blurs the image from a 1/e$^2$ radius of $r_0 \sim 0.281 \mu m$ to a larger radius $r_1 = \sqrt{r_0^2 + 4Dt_m} \approx 0.38 \mu m$, and requires an increase of $N_{\text{coll}} \sim 800$ photons, yielding a total acquisition time of $\sim$17 h. Thus, such measurements will require that the number of emitted photons before photobleaching (with detection efficiency $\phi_{\text{det}} \approx 2\%$) be $4 \times 10^4$, which will require $\Phi_B < 2.5 \times 10^{-5}$, not a trivial constraint.

**METHODS**

**Sample preparation**

Samples of PA-GFP were prepared from stocks kindly provided by Dr. George Patterson (National Institute of Child Health and Human Development), by dilution 1:100, 1:400, or 1:1000 in high-purity water (HPLC grade, Sigma-Aldrich, St. Louis, MO) for a final concentration of $\sim$400 nanomolar. A single one-microliter droplet of diluted PA-GFP solution was deposited on a No. 1.5 glass coverslip (Corning Life Sciences, Corning, NY) and allowed to slowly evaporate. The sample was then placed on the microscope stage and illuminated from below by the objective lens.

**Illumination, photoactivation, and detection**

Fig. 1 illustrates the principle of the method. Photoactivatable molecules (initially in an inactive, nonfluorescent state) are illuminated continuously by the “readout light”, in this case an Ar$^+$ ion laser. Next, the “activation light”, a 405-nm diode laser, is turned on, which causes some of the inactive molecules (presumably a stochastically determined subset) to become active, but only a small enough number that they can all be resolved from one another. After those active molecules have been imaged for some time, they will spontaneously photobleach and become (permanently) nonfluorescent (or, in the case of reversibly photoactivatable molecules, they will be inactivated by the readout light). Images taken of the molecules as a function of time are then used to individually localize each fluorescent molecule and, after sufficient numbers have been analyzed, to construct an image at higher resolution than diffraction would ordinarily allow.

**Spectroscopy**

Fluorescence was focused by the tube lens and reflected out the left side port into an optical fiber with 1-mm diameter, then detected by a PC-controlled spectrometer (USB2000FL, Ocean Optics, Dunedin, FL). Raw spectra were recorded every 100 ms unless otherwise noted, without boxcar or averaging, background-subtracted using a blank spectrum determined under the same conditions, then smoothed by boxcar with full width 4 nm.

**Photoactivation microscopy**

Fluorescence was focused by the tube lens and reflected out the right side port onto a CCD camera (Quantifire, Indigo Scientific, Baldock, Herts, UK)
interfaced by firewire to a PC. Images were collected by the CCD at 1,003 frames/s, gain of 11.4, and no binning (1 × 1) unless otherwise noted, then saved in raw (BMP or uncompressed TIF) format and analyzed using MATLAB scripts designed for particle recognition and localization. In brief, the algorithm searches for all pixels within the image above a threshold, assigning a single square region of interest (which in width is ~3 times the FWHM of the measured PSF) for each such pixel above the threshold, excluding all pixels within any other region of interest (ROI) closer than ~1.7 times the box half-width from any other ROI. The total number of pixels above a second threshold within each ROI is then determined. Those ROIs with at least 20 pixels above the second threshold are analyzed to determine the background-subtracted centroid. Only those pixels within the ROI that were above the second threshold are used to calculate the centroid. Because comparison of various algorithms for single-particle localization reveals that the centroid algorithm is susceptible to offset bias and degraded localization accuracy even at modest signal/noise ratios (S/N ~ 5) (28), the center of mass coordinates are then used as the initial guess for a least-squares Gaussian fit of the image of the fluorescent object (26):

\[ I(x, y) = B + I_0 e^{-2[(x-x_0)^2 + (y-y_0)^2]/\sigma^2}, \]

(11)

where \( B \) is the background pixel value, \( I_0 \) is the peak pixel value, \( x_0 \) and \( y_0 \) are estimated coordinates of the center of the fluorescent object, and \( e^{-2(\sigma^2)} \) is the 1/e\( ^2 \) radius of the point spread function. Although the PSF of a high numerical aperture lens is not strictly Gaussian (34,35), for distances less than the 1/e\( ^2 \) radius, a Gaussian is a reasonable approximation of the PSF, and is frequently used to fit the image of a point-like fluorescent object (16,26,27).

The overall magnification of the optical system results in a pixel size of 0.121 μm, which is within a factor of 2 of the standard deviation of the PSF (~0.20 μm) recommended for optimal localization precision in the presence of background (27). Analysis of mean pixel value as a function of the variance of the pixel value under the binning (1 × 1) and gain (11.4) settings used to obtain images yielded a slope of 11.7 ± 0.4 pixel values per photon and an intercept of ~1.8 ± 3.3 (i.e., zero within uncertainty).

In practice, when the sample is first visualized, a number of molecules may already be activated by exposure to room light or spontaneous activation processes. The density of molecules may be higher than is optimal for localization of individual molecules, but as photobleaching progressively decreases the number of active molecules (in the absence of the activation laser), the density approaches a more manageable level and acquired frames can be analyzed to determine molecular positions. As the density drops below the desired value (e.g., 0.8 molecules/μm\(^2\)), the activation laser is pulsed at high intensity or turned on continuously at low intensity (such that the total dose per frame is between 4 and 40 J/cm\(^2\)). From that point on, frames are acquired until the majority of activatable molecules have been read out and photobleached. The density of molecules that are already activated at the beginning of the experiment (i.e., accidentally activated) depends largely on the age of the PA-GFP sample and whether it has been exposed to room light or left in buffer at room temperature for a significant time (i.e., days). Frozen protein stocks appear to last for approximately months without significant spontaneous activation (data not shown).

Simulations

For simulations, molecules were positioned randomly (100 at a time) within a 512 × 512-pixel region by generating a floating-point random number for both \( x \) and \( y \) coordinates of the molecule (not rounded off to the nearest integer). The image of the molecules was generated to include shot noise by stochastically distributing 1000 photons/molecule using a random number generator to determine whether a given pixel would receive a photon. The probability of detecting a photon at a given position was given by the point spread function (in this case a Gaussian centered at the \( x \) and \( y \) coordinates of the molecule, with 1/e\( ^2 \) radius of 1.6 pixels). Each pixel in the image then had a pixel value equal to the number of photons from the given distribution of molecules. Background of 10 ± 3 photons at each pixel was also added by a random number generator to the photons that form the image of the molecules.

Calibration sample

A 1 cm × 1 cm × 0.5-mm wafer of single-crystal sapphire (Princeton Scientific, Princeton, NJ) within 0.05° of R-cut was cleaned with acetone, isopropanol, then water, then annealed in air at 1700°C for 10 h, cooled to room temperature, then cleaned again by the same procedure. A 1-μL drop of PA-GFP (original stock diluted 1:100 in high-purity water) was then deposited on the surface and allowed to dry in air at room temperature in a darkened room. After drying, the sample was inverted and placed on top of a No. 1.5 coverslip (crystal surface with PA-GFP facing down) and was imaged from below by the objective. The sample was then imaged by FPALM as described above, using a Z806/10× laser cleanup filter (Chroma) to limit the excitation from the Ar+ ion laser to just the 496.5-nm line, yielding a power of 1.60 mW at the sample (496.5 nm line only), or an intensity of ~105 W/cm\(^2\). Activation was achieved by an initial 2-s pulse of illumination at 405 nm with intensity ~40 W/cm\(^2\) and by continuous illumination at 405 nm with ~4 W/cm\(^2\) after the molecular density had reduced due to photobleaching (~500 s later); ~1000 frames were acquired at ~0.999 frames/s with an integration time of 0.983 s, gain of 11, and 2 × 2-pixel binning. The latter 500 frames were analyzed to determine the presented distribution of PA-GFP molecules.

Atomic force microscopy was performed using a JPSA (Manchester, NH) XE-100 microscope in contact mode on the same sapphire sample in various 1.5 μm × 1.5 μm areas of 256 × 256 pixels (not necessarily the same areas imaged by FPALM) with a stage with 50 μm travel, 1 line/s (256 s/image) rate, a new tip (radius <10 nm) with aluminum reflective coating (Budget Sensors, Sofia, Bulgaria), in air at room temperature.

RESULTS

Fluorescence photoactivation and emission under 405 nm and Ar+ ion laser illumination

Fig. 2 shows emission spectra from PA-GFP molecules dried on a glass coverslip and illuminated continuously by ~4.5 μW of power from the Ar+ ion laser and for brief (10-s) pulses of ~197 μW of 405 nm illumination. Negligible fluorescence was observed on coverslips without PA-GFP illuminated under the same conditions. During and after 405 nm illumination, the fluorescence emission within the detected wavelength range 510–565 nm significantly increased (Fig. 2, C and D). The observed emission maximum at 518 ± 2 nm (not corrected for detection or filter transmission efficiency), matches the expected emission of PA-GFP of 517 nm (25). The increase in fluorescence after 405 nm illumination indicates that a significant fraction of previously inactive PA-GFP molecules were activated. Under continuous Ar+ illumination in the absence of 405 nm activation, the PA-GFP fluorescence photobleached at a rate of 0.046 ± 0.002 s\(^{-1}\). Successive activation attempts became less effective as a larger fraction of the original molecules had already been activated or photobleached. The fluorescence visible before application of the first 405-nm pulse (Fig. 2 B) indicates that a small fraction of the PA-GFP had already been activated.

Fluorescence microscopy of immobilized PA-GFP

Fig. 3 shows a typical time series of fluorescence images of a glass coverslip coated with a 1-μL drop of 1 nM PA-GFP,
activated previously by 10 s of illumination at 405 nm at an intensity of 7.3 W/cm² (~197 µW at the sample). The sample was excited continuously by 64 W/cm² of Argon ion laser illumination (3.5 mW at the sample, including both the 476.5-nm and 497.5-nm lines) and imaged by CCD using 1-s exposures (Fig. 3A). The discrete on-off behavior of various spots as a function of time (Fig. 3, B–F), and their FWHM of typically 0.30 ± 0.06 µm, which is consistent with the expected FWHM of 0.55 λm/NA ~ 0.237 µm (32) at the maximum emission wavelength λm = 517 nm, indicates that the observed spots are single fluorescent molecules.

Localization of single molecules

Simulation results

Fig. 4A shows results from the algorithm for identification and localization of bright spots in fluorescence images of PA-GFP molecules. Briefly, pixels within the image with intensity above a threshold (pixel value 30) are identified and marked with an ROI of 7 × 7 pixels (0.85 × 0.85 µm²). Any ROI which was <0.73 µm (6/7 times the full width of the box) from any other ROI was excluded (distance measured center to center). ROIs are further selected for those with at least 20 pixels above a second threshold (pixel value 3) after background subtraction. Selected boxes are analyzed for centroid (Fig. 4A, blue lines) and the centroid coordinates are then used as an initial guess for an unweighted Gaussian least-squares fit of the intensity within the box using Eq. 11 and a fixed r₀ = 1.6 pixels = 193 nm.

Fig. 4B compares the precision of the localization routine using simulated images and either a 1), centroid-after-threshold-and-background-subtraction algorithm, or 2), Gaussian-fit-after-threshold-and-background-subtraction algorithm with initial position guess given by the centroid. The histograms of position deviation are shown for 19,706 total simulated molecules arranged randomly in a 512 × 512 image with 1000 photons each and PSF 1/e² width of 1.6 pixels. The distribution of deviations for the centroid show significantly (>20-fold) larger systematic offset 0.0273 ± 0.004 pixels compared to 0.00116 ± 0.00012 pixels for the same objects localized by Gaussian fit. The 1/e² width of the distribution of deviations (0.088 pixels for centroid and 0.105 pixels for Gaussian fit) was similar, consistent with the high signal/noise ratio of the simulated particle images. The values are slightly higher than the expected 1/e² width of 2/√N = 0.063 pixels in the ideal case without background and indicate that the algorithms can certainly be improved, but also reflect additional sources of noise such as pixelization.
Fig. 5 shows the results of a simulated distribution of molecules placed along periodic vertical strips 60.5 nm wide separated by regions 60.5 nm wide without molecules. Although such a structure would be difficult to resolve using standard light microscopy, the periodicity is visible in the plot of localized molecular positions.

Experimental results

An FPALM image of a drop of PA-GFP dried on a coverslip, obtained after brief 405-nm illumination and acquired under continuous Argon ion laser illumination is shown in Fig. 6. After an initial period of intense fluorescence emission from a relatively large number of activated molecules, photobleaching reduced the number of fluorescent molecules to yield a low enough density for resolution and localization of between 50 and 200 molecules per acquired (1-s) frame. Fig. 6 A shows the tabulated and plotted positions of 48,746 PA-GFP molecules localized during a continuous illumination and image acquisition of 399 s (400 frames). Presentation of these positions at successively higher zoom is shown in Fig. 6, B–D. Fig. 7, A–C, presents the molecular positions as dark circles on a light background and zooms in on a different region. Fig. 7 D overlays the position of each localized molecule with a circle equal in radius to $r_0$, the calculated $1/e^2$ radius of the point spread function for the 1.2-NA objective and a 520-nm emission wavelength. Note that many of the spots due to single molecules are closer together than $r_0$ and would be difficult to resolve if they were emitting simultaneously. Note also the nonuniform distribution of molecules on the coverslip surface, which presumably results from the evaporation pattern of the water droplet containing the hydrogel.
the PA-GFP. Features and structures much smaller than the diffraction-limited resolution ($r_0 \approx 0.61 \lambda/\text{NA} = 264 \text{ nm}$) are clearly visible, especially in the higher zoom panels. Molecular positions in Fig. 6 are plotted on top of the sum of all widefield images obtained during the acquisition. The summed image is essentially equivalent to the widefield image of the same measured distribution of molecules if they had all been fluorescent simultaneously, and were imaged using the same laser illumination profile. As expected, much of the structure observed by fluorescence photoactivation localization microscopy is lost when the images are summed.

Fig. 8 shows a plot of measured molecular positions of PA-GFP dried on an annealed sapphire crystal surface that contained terraces spaced by 200–630 nm, as imaged by atomic force microscopy (AFM). The terraces in most cases consisted of a vertical step of $0.33 \text{ nm}$, which is close to the expected step thickness for single atomic monolayers in sapphire in this crystal orientation, followed by a gradual linear decline in height, which when repeated over many micrometers resembles a sawtooth pattern. The PA-GFP was observed to often localize along periodic arrays of parallel lines, which can be seen in Fig. 8 A. Note also the asymmetry of the distribution on either side of the ridges, which demonstrates a higher molecular density approaching the ridge from one side and a lower density on the other side of the ridge.

The PA-GFP molecules were observed to activate slowly (with an ~5- to 10-s timescale) under continuous unattenuated Ar+ laser illumination, in the absence of 405 nm illumination. The wavelength intensity spectrum of the Ar+ laser measured at the sample was found to contain a small fraction (~3%) of its intensity at $\lambda < 470 \text{ nm}$, which presumably causes activation in the absence of 405 nm illumination. Activation was not observed in the absence of 405 nm light when a cleanup filter (Z496/10×3) was included in the Ar+ laser path that passed only the 496.5 nm line. A portion of the PA-GFP was found to be activated before we illuminated it with the 405-nm laser (data not shown), which contributed to the initially large number of molecules in the field of view.
In addition to high-resolution imaging of the molecular distribution, FPALM can be used to quantify other single-molecule properties of the fluorophores, which are inaccessible to standard widefield fluorescence microscopy. Fig. 9 shows the brightness distribution of localized molecules and the time dependence of the number of localized molecules. Under continuous Ar\textsuperscript{+} illumination, the number of fluorescent particles decayed as a single exponential with a 1/\(e\) time of 125 \(\pm\) 3 s.

**DISCUSSION**

FPALM is capable of imaging entire cells or multiple cells, with localization precision of the order of tens of nanometers, and potential for even better precision. The experimental setup consists of a fluorescence microscope, a CCD camera, and two commercially available CW lasers. Images can be acquired with modest alignment and setup time. Single molecules are localized individually, allowing their position and brightness to be measured and analyzed to extract spatial and kinetic information that is inaccessible when large numbers of molecules are observed simultaneously. Unlike other single-molecule imaging methods, the maximum labeling density suitable for localization can be orders of magnitude larger than the \(\sim 1\) fluor/\(\mu\)m\(^2\) (for each nonoverlapping spectroscopic channel) dictated by the optical Rayleigh criterion.

This allows for a much more complete picture of the spatial distribution of the species of interest, while still providing single-molecule sensitivity. In principle, position analysis of a given molecule as a function of time can be achieved to estimate the velocity. FPALM does not require scanning either the beam or the sample, as is necessary in RESOLFT (including STED), 4Pi, and standard confocal microscopy (6,13,15). Furthermore, the sample is illuminated by a relatively low intensity (<100 W/cm\(^2\)) compared to that used in confocal microscopy (i.e., \(\sim 10^4\) to \(10^5\) W/cm\(^2\)), reducing the deleterious effects of unwanted photobleaching and cell damage.

FPALM has been demonstrated as a means of imaging structures on subdiffraction length scales, which has been confirmed by atomic force microscopy. The distribution of
molecular positions at the edge of a terrace shows a $1/e^2$ width of $\sim 73 \pm 3$ nm, or a FWHM of $86 \pm 4$ nm, which is significantly less than the diffraction limit in normal wide-field microscopy. AFM imaging revealed that the sharp side of the vertical steps from terrace to terrace occurred over a lateral distance of $79 \pm 6$ nm, in good agreement with the value determined by FPALM. Although the above FWHM shows that subdiffraction features can be resolved, the FWHM is also degraded by background from the sapphire, which was higher than in the samples of PA-GFP on coverslips, and therefore the ultimate resolution of FPALM may be much better than has been shown here. Nonetheless, the profile of the features and their spacing as determined by FPALM are consistent with AFM results, confirming that the method can be used to visualize structures on length scales shorter than the diffraction limit.

The time dependence of the fluorescence emission of single fluorophores can complicate localization analysis (26). Due to the relatively long ($\sim 1$-s) integration times in the FPALM results presented here, the flicker and blinking that occur on the microsecond and millisecond timescales is averaged over, and therefore not directly observed. However, some blinking on longer timescales (Fig. 3) is still observed. In addition, long integration times will allow for significant movement to occur if the molecules are mobile. When random molecular diffusion occurs on timescales shorter than the integration time per frame (here $\sim 1$ s), the image of the molecule will be blurred, with fewer detected photons per pixel distributed over a larger number of pixels. Molecules moving in three dimensions during the acquisition time will be extremely difficult to localize by the current implementation of FPALM. On the other hand, consider molecules constrained to move in two dimensions (i.e., within the focal plane of the microscope). In this case, under random diffusion, the mean position of each molecule is expected to remain unchanged, whereas the variance is expected to increase according to $r^2 = 4Dt_m$, where $r_0$ is the $1/e^2$ radius of the diffraction-limited PSF (see also Eqs. 6–8). Because of the larger variance and reduced signal/noise and signal/background ratios per pixel, the ability to determine the mean position will be significantly diminished. In the special case of random two-dimensional motion with a very small diffusion coefficient ($D < 10^{-10}$ cm$^2$/s), localization may in principle still be possible.

If significant nonrandom motion occurs during the acquisition time, the image of a given molecule will no longer appear circular, also making analysis much more difficult; new methods of analysis will likely need to be developed. Although this complicates the determination of the mean position within a single image, successive images can be analyzed to determine molecular velocities (as is done in FSM), which is certainly of biological relevance (21–24). FPALM is particularly well-suited to the study of proteins and other structures in fixed cells, and is possibly applicable to a subset of membrane proteins in living cells, if their lateral mobilities are at least two orders of magnitude smaller (33) than those of soluble (i.e., cytosolic) GFP-tagged proteins (36–38). Successful examples of single-molecule total internal reflection fluorescence (TIRF) detection with high signal/noise ratio in agarose (39) and membranes (40) offer a promising complement to single-particle tracking.

![Image](https://example.com/image.png)

**FIGURE 9** Additional information, not available to ensemble fluorescence microscopy methods that image large numbers of molecules ($\gg 100$) simultaneously, is obtained by FPALM. (A) The number of molecules fluctuates and decays with time as FPALM frames are acquired. The number of fluorescent molecules within the observation volume in a widefield or confocal fluorescence microscope is difficult to determine without a calibration standard, but is obtained for every image in FPALM. A single exponential time constant ($r = 125 \pm 3$ s) described the decay of the number of molecules observed, consistent with depletion of a single reservoir of inactivated PA-GFP molecules. (B) The distribution of single-molecule fluorescence intensities is determined by FPALM and converted into the detected photon count rate at the center of the diffraction-limited image of the molecule. All amplitudes had a constant subtracted to account for background. Such information is useful in determining the degree of heterogeneity within a population of molecules and is typically difficult to obtain from measurements that sample large numbers of molecules simultaneously.
measurements that have revealed time-dependent lateral heterogeneities in biological membranes (41,42).

The power offered by genetic manipulation of fluorescent proteins (43–46) is a significant advantage for FPALM, since any protein of interest can be labeled by splicing the gene for that protein into a PA-GFP or other photoactivatable fluorescent protein (PAFP) construct. Multiple labeling is possible using combinations of photoactivatable proteins with different activation and emission spectra. Labeling of other cellular species, such as lipids, will rely on development of conjugates with photoactivatable organic dyes, PAFP-tagged antibodies, or lipid binding proteins such as the pleckstrin homology domain that binds PIP2 (47). As the variety of available PAFPs increases (31), multi-color labeling experiments using FPALM will become even more flexible. Mutagenesis of existing PAFPs can also be used to reduce blinking and undesirable types of spectral switching that complicate the use of many types of fluorophores, such as quantum dots, in single-molecule imaging. The quantum yield of PA-GFP (0.79) is already quite high in comparison to most other PAFPs (25,31). Comparison of fluorescence intermittency of PA-GFP mutants by fluorescence correlation spectroscopy has shown significant variation in the degree to which these proteins flicker, which can limit the number of photons emitted per second per molecule (S. Hess and G. Patterson, unpublished results). Improvement of the photophysical properties of PAFPs to reduce intermittency and increase photon emission rate will allow even greater localization precision to be achieved.

The irreversible photobleaching that fluorophores inevitably undergo is advantageous in FPALM since it helps control the number of fluorescent molecules in the field of view during each image frame (time point). Although it is expected that photobleaching will lead to the production of chemically reactive species that could be toxic to living cells, the number of bleached molecules produced is of the same order of magnitude as those produced in confocal microscopy experiments (it is not uncommon for a significant portion of the fluorophore to be bleached during the acquisition of a 2-stack with frame averaging, for example).

The time required by a fluorophore to be photoactivated (the "settling time" mentioned by Hoffman et al.) can also limit imaging rate in scanning photoactivation microscopy (15) but is not as significant an impediment in FPALM because the entire field is imaged at once, and each frame is acquired over a timescale of seconds or fractions of a second, rather than the typical microsecond voxel dwell time in confocal and other scanning microscopies. Also, under some conditions in other types of photoactivation microscopy, it becomes difficult to increase the activation rate past a certain point (15). In comparison, activation rates for PA-GFP much larger than the frame rate (>10 s⁻¹) have been achieved with modest activation laser intensities (~100 W/cm², data not shown) and since it is not anticipated that the frame rate would need to exceed ~10² s⁻¹, the same kind of limitation for FPALM using PA-GFP may not exist. Further study of the activation rate as a function of activation laser intensity is needed, since the activation rate and photobleaching rate can limit the acquisition frame rate.

Although many fluorescent proteins are known to spontaneously aggregate, point mutations such as L221K can be introduced into PA-GFP (which is of the family of green fluorescent proteins and is less susceptible to aggregation, with Kd ~ 0.11 mM) to raise (improve) the Kd to ~9 mM (48).

Recent work in single-molecule fluorescence microscopy has focused on improving the ability to resolve multiple fluorophores by spectral separation (16); nanoparticles such as quantum dots offer somewhat narrower emission spectra and therefore can be ‘‘packed’’ in larger numbers into the same range of visible wavelengths (49). However, in comparison to fluorescent proteins, quantum dots have so far proven to be more difficult to target to some intracellular compartments, especially the nucleus (50). FPALM obviates the need for multiple detection channels by controlling the number of particles that are fluorescent at a given time, such that even at high inactive probe concentrations, the distance between active (fluorescent) molecules is on the average much larger than the resolution. It should be noted that extending this technique to multiple spectral channels is possible, and would result in a modest increase in the possible labeling density, proportional to the number of spectral channels, but a much more significant advantage of labeling with multiple, spectrally distinct photoactivatable species is simply to be able to visualize multiple species at high resolution.

**Estimated signal/noise ratio**

We use the definition of signal/noise ratio S/N = (I₀ − Iₐ)/δ, where δ is a representative noise level and I₀ − Iₐ is the difference in mean intensity between the object and the background (Cheezum et al.), to obtain for the molecule in Fig. 3 E: I₀ − Iₐ ~ 161 photons and δ ~ 25.6 photons, for S/N ~ 6.3, which is high enough to allow localization with distance precision >100 nm using a Gaussian algorithm, but not necessarily with other algorithms (Cheezum et al.). The expected excitation rate kₑ = σI, where σ is the one-photon excitation cross section and I is the excitation intensity in photons/unit area/s, can be estimated theoretically considering the excitation laser (Ar⁺) power (6.7 mW) in the back aperture, the objective transmission coefficient (~0.5) for the appropriate wavelengths (476.5 nm and 496.5 nm), the 1/e² radius of the illuminated area (41.6 ± 0.5 μm), the resulting intensity ~46 W/cm² at 496.7 nm and ~16 W/cm² at 476.5 nm, the energy per photon, and the estimated extinction coefficient at 476.5 nm and 496.7 nm (using ε = 17,400 M⁻¹ cm⁻¹ at 504 nm and wavelength dependence from Patterson et al. (25)), to obtain excitation rates kₑ(497) ~ 6.2 × 10³ s⁻¹ and kₑ(477) ~ 1.3 × 10⁴ s⁻¹. The expected detected photon count rate per molecule (η) is estimated by
\[ \eta = \phi_B \left( \frac{k_s^{(497)}}{k_s^{(477)}} \right) \phi_{\text{det}}, \]

where \( \phi_B = 0.79 \) is the fluorescence quantum yield, and \( \phi_{\text{det}} \) is the overall detection efficiency, estimated as \( 2\% \), to yield the \( \sim 120 \) photons/s expected, which compares fairly well with the average (measured) collected photon rate of \( \sim 40-180 \) s\(^{-1}\) estimated from the histogram of detected molecular counts rate (Fig. 9 B) and the FWHM of the PSF. The average time before photobleaching, \( \tau_B \sim (k_s \phi_B)^{-1} \), is calculated using estimated photobleaching quantum yield, \( \Phi_B \sim 5 \times 10^{-5} \), slightly higher than for EGFP (25) to obtain \( \tau_B \sim 2.7 \) s, which is consistent with our observation of PA-GFP molecules for a few frames before photobleaching. For simplicity we use the same photobleaching yield at both 477 nm and 497 nm, although experimental results do show some dependence of \( \Phi_B \) on excitation wavelength in EGFP (51).

**Constraints on activation and deactivation rate constants**

Given that we need to activate molecules at a rate of \( \sim 0.8/\mu\text{m}^2 \) per frame (for sufficient spatial separation), using Eq. 4B, the number of activated molecules/\( \mu\text{m}^2 \)/s in the imaging area \( S \) will be \( \frac{1}{S} \frac{\text{d}t}{\text{dt}} \), which should roughly equal \( 0.8/\mu\text{m}^2 \) times the frame acquisition rate. For example, for a 50-Hz frame rate, one would need \( \frac{1}{S} \frac{\text{d}t}{\text{dt}} \sim 40/\mu\text{m}^2 \) s. Since observed activation times of less than a few milliseconds in PA-GFP have been reported in a confocal geometry (25), it will usually be possible to meet the desired rate by adjusting the excitation laser power. In contrast, ensuring that the rate of activation is low enough is more difficult due to the possibility of a spontaneous activation rate \( (k_0) \) and readout-beam-induced activation, which could become particularly significant when a large reservoir of inactive molecules is present. The product of \( k_0 \times I/S \ll 0.8/\mu\text{m}^2 \) will ensure that most activated molecules are separated by more than the diffraction-limited radius of the PSF. As the inactive reservoir is depleted, the activation laser intensity could be increased to keep the number of activated molecules per frame constant (8 in this case), which will minimize delay in readout of a sufficient number of molecules. Although the spontaneous deactivation rate is expected to be very low in PA-GFP due to irreversible decarboxylation of the chromophore during photoactivation (25), further study of deactivation rates in photoactivatable proteins using single-molecule methods and fluorescence correlation spectroscopy would likely be useful.

**Comparison to other high-resolution fluorescence methods**

Methods such as nanometer localized multiple single-molecule fluorescence microscopy can resolve and localize multiple (>5) molecules within close proximity, but the precision of localization will degrade as larger numbers of molecules fall within the same diffraction-limited spot. FPALM is able to localize in principle a very large number of molecules within a diffraction-limited region as long as they are activated and localized in small numbers at a time. For example, PA-GFP molecules were localized (within one region) at a density of 83 spots within a 0.1 \( \mu\text{m} \times 0.12 \mu\text{m} \) area (Fig. 6), or \( \sim 6000/\mu\text{m}^2 \). The acquisition rate so far (\( \sim 50,000 \) molecules localized in \( \sim 400 \) s) is slow compared to confocal microscopy, but comparable to other "super-resolution" techniques such as 4Pi and nanometer localized multiple single-molecule (6,16).

In principle, the localization precision can be significantly improved. Demonstrated precisions in the range of 2 nm have been realized (52,53). One of the current limitations with this work (using PA-GFP) is the number of detected photons before photobleaching, and the background generated by fluorescence of the inactive form of the protein (among many sources).

Because of the low excitation intensities used in FPALM compared to STED, there is no need to engineer complex optics to align a stimulated emission beam intensity null with the center of the focus of another beam. Power levels similar to those described in "low-power" RESOLFT-type microscopies can be used.

A short photoactivation time (less than the pixel dwell time) is important in scanning RESOLFT-type microscopies (15) whenever photoactivation or other relevant photophysics must occur during the pixel dwell time. Although the photoactivation time can be long (>1 s) in FPALM, since it is not a scanning method, rapid molecular readout followed by rapid inactivation or photobleaching is important because it limits the temporal resolution of the method, which can influence spatial resolution. For example, if molecules are moving nonrandomly, then the localization algorithm will have trouble determining the center of the image of the molecule and therefore the precision of position determination will be reduced. However, if the molecules are diffusing randomly in two dimensions, they can still be localized with the same algorithm and their positions determined with precision higher than the diffraction-limited resolution. A further constraint to such a procedure is that the mean-squared distance between fluorescent (active) molecules still must exceed (by a large factor) the resolution-squared plus the mean-squared displacement due to diffusion during the acquisition time per molecule (i.e., the time per frame).

Activation and readout by a single (Ar+) laser beam is advantageous because it obviates the need for alignment of multiple excitation sources (simplifying alignment), but it also reduces the degree of control over relative intensities of the activation and readout illumination, and therefore reduces the degree of control over the number of active molecules visible at a given time. The inactive form of PA-GFP absorbs from <350 nm to ~450 nm (54), and activation at 435 nm by one photon has been observed (54), suggesting that activation is possible using wavelengths other than 405 or 413 nm.

These results and the rapidly developing field of nonlinear subdiffraction microscopies point to a need for fluorescent probes with nonlinear properties (i.e., strong STED or strong photoconversion). The conditions for effective FPALM also
reveal a need for minimal spontaneous photoconversion rates in PA-FPs, as well as for RESOLFT microscopies that use PA-FPs (15).

FPALM is much more straightforward for two-dimensional imaging of molecular distributions, where the molecules of interest are all located between two planes separated by \( d \ll z_0 \) and \( z_0 \) is the depth of field (axial extent of the point spread function). FPALM does not come close to the time resolution offered by three-dimensional single-particle tracking (29) but could potentially be extended to three-dimensional distributions. STED offers improved axial resolution compared to this form of FPALM, but it is interesting to consider a modified FPALM that uses TIRF or 4Pi (two-photon) excitation to improve axial resolution and confine activation. Such a method would be ideal for studies of membrane protein distributions where lateral resolution is crucial and reduced background (e.g., due to out-of-focus contributions) is highly desirable. Additionally, STED and TIRF could be used in combination to spatially control the excitation profile (13,55). The procedures for velocity and position analysis of large numbers of single molecules (~10^5) already developed for use with FSM and TIR-FSM (21,22) could be used to analyze FPALM images as well.

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At the time of publication, a related work by E. Betzig et al. had recently appeared online in the journal Science (56).

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