Single-molecule studies of DNA replication
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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ABSTRACT: Single-molecule fluorescence microscopy is a powerful approach to observe biomolecular interactions with high spatial and temporal resolution. Detecting fluorescent signals from individual, labeled proteins above high levels of background fluorescence remains challenging, however. For this reason, the concentrations of labeled proteins in \textit{in vitro} assays are often kept low compared to their \textit{in vivo} concentrations. Here, we present a new fluorescence imaging technique by which single fluorescent molecules can be observed in real time at high, physiologically relevant concentrations. The technique requires a protein and its macromolecular substrate to be labeled each with a different fluorophore. Then, making use of short-distance energy-transfer mechanisms, the fluorescence from only those proteins bound to their substrate are selectively activated. This approach is demonstrated by labeling a DNA substrate with an intercalating stain, exciting the stain, and using energy transfer to have the fluorescence from the stain activate the fluorescence of only those labeled DNA-binding proteins bound to the DNA. Such an experimental design allowed us to observe the sequence-independent interaction of Cy5-labeled interferon-inducible protein 16 (IFI16) with DNA and the sliding via one-dimensional diffusion of Cy5-labeled adenovirus protease (pVic-AVP) on DNA in the presence of a background of hundreds of nM of Cy5 fluorophore.


\textit{Manuscript submitted}
6.1 Introduction

Recent developments in single-molecule fluorescence microscopy have allowed remarkable insights into the dynamic properties of biomolecular processes. The high spatial and temporal resolution of fluorescence microscopy has enabled the visualization of intermediates and time-dependent pathways in biochemical reactions that were difficult or impossible to extract from experiments at the ensemble-averaged level. However, one of the important technical challenges in single-molecule fluorescence imaging is the visualization of individual fluorescently labeled molecules at high concentrations. Using conventional, diffraction-limited optics, the fluorescence of individual molecules can only be resolved if they are farther apart than the diffraction limit — ~250 nm in the lateral and ~500 nm in the axial direction. As a consequence, the highest concentration at which single fluorescently labeled molecules can still be resolved at a sufficiently high signal-to-background ratio is in the order of 10-100 nM. This concentration limit reduces the applicability of single-molecule fluorescence imaging to study the dynamics of biomolecular interactions to systems with dissociation constants in the nanomolar range or lower (60). A common and straightforward strategy to circumvent this limitation is to use a partially labeled population of the molecules of interest and supplement with a high concentration of unlabeled molecules. However, when complicated pathways involving many binding partners are studied, or those involving rare molecular transitions, there is a need for approaches that allow the visualization of all events instead of merely a small fraction.

Several recent experimental approaches in single-molecule fluorescence imaging have overcome this concentration limit (150), either by confinement of the molecules (151), reduction of the fluorescence excitation volume (152), or by temporal separation of fluorescent signals (153). Confinement of labeled molecules in a closed volume, considerably smaller than the diffraction limit, enables the detection of single molecules at concentrations much higher than the fluorescence concentration limit. As an example, trapping of proteins inside nanovesicles, with a volume of about $5 \times 10^{-19}$ liter, allows single molecules to be visualized at an effective protein concentration of approximately 3 µM (154). Alternatively, the excitation volume itself can be reduced to below the diffraction limit. Zero-mode waveguides, for example, are widely employed nanophotonic devices that enable an effective excitation of a volume much smaller than the diffraction limit and support single-molecule detection at fluorophore concentrations of up to 10 µM (152,155).

Another emerging approach to visualize biomolecular interactions at high concentrations leverages the stability of biomolecular complex formation. In the technique of PhADE (PhotoActivation, Diffusion and Excitation), photoactivatable fluorescent proteins in the detection volume are activated and subsequently imaged after first allowing the activated proteins that did not bind the surface-immobilized
substrate to diffuse away. This approach allowed the visualization of micrometer-scale movement of single molecules on replicating DNA templates at concentrations up to 2 µM (153). Although all of the above-mentioned techniques have dramatically pushed the limits of single-molecule imaging at high concentrations, experimental difficulties and temporal limitations have hindered the development of a generally applicable method to observe single-molecule dynamics at high background fluorophore concentrations. The trapping of molecules in nanovesicles or nanophotonic devices allows the fluorescence of single molecules amidst a high background concentration to be detected at high temporal resolution, but the spatial confinement precludes the visualization of large-scale movements of the fluorescently labeled species. Whereas PhADE removes these spatial constraints, it introduces limits on time resolution as a result of the time needed for activated but non-interacting proteins to diffuse away from the observation volume.

Here, we present a fluorescence imaging technique that is based on the incorporation of an activator molecule into the binding target of a molecule and the selective photoactivation by energy transfer of only those molecules that bind the target. The free molecules remain in their dark state, and only those involved in complexes are switched on and their fluorescence is observed. We demonstrate our method by visualizing large-scale motions of individual DNA-binding proteins at protein concentrations exceeding the previous concentration limit by an order of magnitude.

6.2 Results

Our approach to visualize bimolecular interactions at high fluorophore concentrations is based on generating fluorescence from only those molecules that have formed a bimolecular complex; all other fluorophores will not fluoresce. As a photoswitchable fluorophore we use Cy5, a red carbocyanine dye that has been shown to act as an efficient reversible single-molecule photoswitch supporting hundreds of cycles of switching between a dark and a bright state (156,157). Our method is based on the selective activation of the fluorescence of only those few molecules that successfully associate to a target substrate, with the remainder of the fluorophores left in their dark state. As such, at the start of an imaging experiment all fluorophores need to be placed in a dark, fluorescently inactive state. Darkening of Cy5 can be achieved chemically upon interaction with NaBH₄ (158), a phosphine like tris(2-carboxyethyl)phosphine (TCEP) (159), or sulphydryls combined with red-light illumination (160). These chemicals are presumed to target the polymethine bridge of Cy5, resulting in disruption of the conjugated π-system and a drastic blue shift of the Cy5 fluorescence (159). The bright, red-emitting state of Cy5 is recovered upon the dissociation of the chemical darkening moieties, an event that can be triggered by irradiation at shorter wavelengths (156) or by excitation of a nearby secondary fluorophore whose emission spectrally overlaps with the Cy5
absorption (157,161). Excitation of such a secondary fluorophore offers the opportunity to specifically recover the bright state of only those Cy5 molecules in close proximity to that secondary fluorophore. We present here the use of a DNA-intercalating stain to locally activate only those Cy5 fluorophores that are close to a DNA template, while leaving the background population of Cy5-labeled proteins in their dark state (see Figure 22a). Such a Local Activation of Dye (LADye) allows the selective activation of fluorescence of only those labeled proteins that are bound to a DNA substrate molecule. The short activation distance of around 1 nm (158) results in very small effective excitation volumes. This small excitation volume enables single-molecule experiments on DNA-interacting proteins in the presence of high background concentrations of labeled proteins while still being able to follow the large-scale motions of the protein on DNA.

As a proof of principle, we labeled a 60-bp dsDNA oligonucleotide with the DNA-intercalating stain Sytox Orange and used its fluorescence emission to photo-activate a dark Cy5 fluorophore that is covalently coupled to the same DNA (Figure 22b). The ability of the Cy5 molecule to switch on and off was studied both by direct activation upon irradiation with 404-nm light and by indirect, proximal activation upon excitation of the Sytox Orange with 532-nm light. Throughout the entire experiment, the sample was continuously excited with 643-nm laser light, resulting in the emission of red fluorescence whenever the Cy5 was present in the photo-activated state. Irradiation at this same wavelength, in the presence of β-mercaptoethanol (BME), eventually resulted in the darkening of Cy5. Repeats of this sequence enabled reactivation of the Cy5 and its prolonged imaging. Both 404-nm and 532-nm laser pulses resulted in similar recoveries of the red Cy5 fluorescence, indicating that photo-activation of Cy5 is equally efficient for direct activation by 404-nm irradiation and for indirect activation by Sytox Orange (Figure 22b). Analysis of single-molecule fluorescence trajectories indicates efficient activation of Cy5 with 58% and 64% fluorescence recovery for a single 404-nm and 532-nm pulse, respectively (see Figure 22c, black trace). As a control, Figure 22c (red trace) shows that in the absence of Sytox Orange, no Cy5 switching was observed for 532-nm pulses, though 404-nm pulses remained effective in Cy5 activation (45% per single 404-nm pulse). Assays in the absence of thiols (BME) completely eliminated Cy5 switching (Figure 23c, blue trace).
Figure 22: Fluorescence switching of Cy5 bound to stained DNA. a) Schematic representation of Local Activation of Dye (LADye): Prior to the experiment the entire population of Cy5 molecules is darkened. Green excitation (532 nm) allows the DNA-bound intercalating stain to fluoresce, which in turn results in photoactivation of the DNA-proximal Cy5 molecules. Subsequently, Cy5 is visualized using red laser light (643 nm), which eventually brings the Cy5 molecules back to the dark state when BME is present. b) Single-molecule Cy5 fluorescence recovery upon a 0.4 s pulse with either 404-nm light, causing a direct activation of the Cy5 fluorescence, or 532-nm laser light, leading to selective activation of only those Cy5 molecules close to the DNA. Left represents the Cy5 fluorescence before the laser pulse, and right shows the Cy5 fluorescence recovery right after the laser pulse (Cy5 fluorescence is excited at 643-nm). Scale bar is 1 µm. c) Reversible activation of Cy5 upon alternating 404-nm and 532-nm laser pulses. Shown in black is a representative Cy5 fluorescence trajectory in the regular reaction buffer (see Methods), whereas the red and blue traces represent the Cy5 fluorescence in a reaction buffer lacking Sytox Orange and BME, respectively. The lack of photo-activation by 532-nm excitation in the absence of Sytox Orange or BME confirms the role of Sytox Orange as a local activator.
Next, we investigated whether excitation of an intercalating stain bound to DNA could result in the activation of the fluorescence of a Cy5-labeled, DNA-bound protein. As a model system, we studied the DNA-dependent fluorescence activation of Cy5-labeled interferon-inducible protein 16 (IFI16), an 82-kDa human protein that acts as a cytosolic viral DNA sensor. IFI16’s physiological role includes non-specific binding to cytosolic foreign dsDNA, thereby triggering an innate immune response that activates cell death (162). We labeled IFI16 with Cy5, pre-darkened the Cy5 prior to the experiments, and allowed the labeled IFI16 to bind nonspecifically to a circular dsDNA template (based on phage M13 DNA, with a circumference of 7.3 kb) that had been coupled to a glass surface and stained with Sytox Orange (Figure 23a). A pulsed excitation of the DNA stain with 532-nm light while continuously irradiating the sample with 643-nm light resulted in fluorescence activation and visualization of the Cy5-labeled proteins, as can be seen by the appearance of fluorescent spots. Repeated photo-activation of one or more Cy5-IFI16 proteins per single DNA template is shown in Figure 23b. Investigation of the fluorescence intensity of the black trace in Figure 23b uncovered a number of discrete intensity levels with each level an integer multiple of a constant intensity (4200 ± 500 counts), suggesting the binding of an integer number of individual molecules to the DNA (Figure 23c).

Next, we imaged individual Cy5-IFI16 proteins on flow-stretched λ-phage DNA to visualize the spatial distribution and movement of the proteins on longer DNA substrates (163). Continuous illumination of Cy5-IFI16 by 643-nm light and pulsed excitation of the DNA stain by 532-nm laser light allowed the activation and visualization of individual IFI16 proteins bound to the DNA (Figure 23d). With continuous 532-nm excitation, the total number of fluorescently labeled proteins observed on individual DNA molecules was increased five-fold over the number activated with pulsed 532-nm excitation (Figure 23e). This increase in the detected number of fluorescent proteins demonstrates that the illumination conditions (intensity and pulse duration) can be optimized to maximize the probability of photoactivation while keeping fluorescence background to a minimum.
Figure 23: Photoactivation of Cy5-labeled IFI16 proteins bound to DNA. a) Cy5-coupled IFI16 proteins (1 nM) in the dark state bind the circular double-stranded M13 DNA template (left panel), and are switched to a bright state upon a 0.4-s 532-nm pulse (right panel). Scale bar is 1 µm. b) Fluorescence intensity over time of Cy5-labeled IFI16 on individual M13 dsDNA templates. Every 4 seconds (green bars), a 0.2-second green pulse is applied to recover the bright state of the Cy5 fluorophores. c) Histogram of the Cy5 fluorescence intensity of the lower trace in b). Multiple Gaussian fits (red) revealed 6 different, equidistant fluorescence intensity levels corresponding to 0 to 5 IFI16 proteins binding to the M13 DNA. d) Schematic of local activation of Cy5-labeled IFI16 proteins on a λ-phage DNA template upon application of a 532-nm laser pulse. e) Kymographs depicting the positions of Cy5-labeled IFI16 as a function of time on individual flow-stretched λ-phage DNA templates. Photo-activation was achieved by pulsed excitation of the Sytox Orange (upper kymograph) or by continuous excitation followed by pulsed 532-nm illumination (lower kymograph).
Next, we determined whether LADye can be used to visualize single, labeled proteins in the presence of a high concentration of fluorophore. As a proof of principle, we imaged individual Cy5-labeled DNA-binding proteins interacting with DNA in the presence of several hundreds of nM of free Cy5 in solution. A key requirement to visualize single fluorophores amidst high backgrounds is the efficient initial darkening of the entire population of fluorophores in solution. Thus, only those molecules that are switched to the bright state by photoactivation through energy transfer from the DNA intercalating stain will contribute to the observed fluorescence. Darkening of a solution of Cy5 (158) by NaBH$_4$-mediated caging resulted in a fluorescence reduction of 97% in comparison to conventional imaging of uncaged Cy5 at the same concentration. This efficient darkening allowed for the visualization of the fluorescence of single, surface-bound Cy5 molecules in the presence of 270 nM Cy5 in solution (Figure 24a, right panel); these conditions are not compatible with conventional single-molecule fluorescence imaging (Figure 24a, left panel).

As a model system to visualize proteins moving along DNA amidst a high background of Cy5, we chose the adenovirus protease AVP, which is a protein able to diffuse one-dimensionally along dsDNA after association with the 11-residue peptide pVIc (164). The pVIc-AVP complex binds to DNA without any sequence specificity and performs a one-dimensional random walk along the duplex to locate and process DNA-bound protein substrates (164). Using darkened Cy5-labeled pVIc-AVP and pulsed 532-nm photo-activation, we specifically visualized the Cy5-pVIc-AVP bound to DNA, even in the presence of 270 nM Cy5 in solution (Figure 24b). The kymograph shows the spatio-temporal behavior of the protein complex as it moves along the DNA. The time-dependent position of the Cy5-pVIc-AVP along the DNA was tracked by determining the center of a two-dimensional Gaussian function, fit to the fluorescent spot at every frame. Subsequently, the diffusion constant of the Cy5-pVIc-AVP was calculated by fitting the mean-square displacement of the Cy5-pVIc-AVP over time and was found to be $(2.8 \pm 0.4) \times 10^7$ bp$^2$/s (Figure 24c). This diffusion constant agrees well with previously obtained pVIc-AVP diffusion constant of $(2.1 \pm 0.2) \times 10^7$ bp$^2$/s (164), demonstrating the feasibility of LADye to investigate binding kinetics and activity of individual molecules in the presence of high background concentrations of fluorophores.
Figure 24: Visualization of single Cy5-labeled adenovirus protease (Cy5-pVlc-AVP) molecules sliding along DNA. a) Caging of Cy5-pVlc-AVP and Cy5 in solution drastically reduces the fluorescence intensity. On the left side, conventional imaging of 270 nM Cy5 resulted in saturation of the camera whereas caging of the Cy5 in solution allows single non-specifically surface-bound molecules to be resolved, under the same illumination and acquisition conditions (right). b) Kymograph of the sliding motion of individual Cy5-pVlc-AVP molecules on λ-phage DNA over time. DNA-bound Cy5-pVlc-AVP was visualized by frequent photoactivation whereas the 270-nM caged background Cy5 remained dark. c) Mean-square displacement of a single sliding Cy5-pVlc-AVP, as presented in b), over time. The diffusion constant of this labeled pVlc-AVP was determined by linear fitting of the data and found to be \((2.8 \pm 0.4) \times 10^7 \text{ bp}^2/\text{s}\). 
6.3 Discussion

Single-molecule fluorescence microscopy has provided remarkable insights on how biological macromolecules interact. However, the application of this technique to systems with labeled molecules at their concentrations inside the cell (60) has been made difficult due to high background fluorescence. Here we describe a new approach to circumvent this concentration limit by selectively visualizing only the subpopulation of labeled proteins that have productively bound to a substrate and leaving the unbound population in a non-fluorescent, dark state. Chemically darkened Cy5 fluorophores coupled to proteins of interest were triggered to recover their bright state by excitation of the spectrally overlapping DNA intercalating stain Sytox Orange, resulting in the activation of only those Cy5-labeled proteins proximal to a DNA substrate. This local activation of Cy5 dyes allowed us to observe, in real time, the interaction of single proteins with DNA at concentrations of several hundreds of nM of fluorophores in solution.

Total internal reflection fluorescence (TIRF) microscopy is commonly used in single-molecule fluorescence imaging to limit the penetration depth of the excitation light to ~ 100 nm above the coverslip surface to reduce fluorescence contributions from the rest of the solution. However, long DNA molecules that are surface anchored on one end and stretched by flow, are tilted away from the surface sufficiently so that for most of their length the DNA is farther than 100 nm away from the coverslip. To allow the visualization of the full length of these molecules, we were not able to use a TIRF-based excitation scheme but instead had to apply epifluorescence illumination. Under these conditions, the detection volume has diffraction-limited dimensions of 250 x 250 x 500 nm³, 5 times larger than the detection volume achieved in TIRF imaging. Despite such a significantly larger detection volume, we demonstrate here the visualization of single molecules bound to DNA in the presence of 270 nM fluorophores in solution. This concentration implies the ability of our LADye technique to background concentrations of as high as 1 µM in a TIRF scheme with a 6.25·10⁶-nm³ detection volume.

Here, we also demonstrate efficient fluorescence activation of a darkened Cy5 molecule coupled to a stained double-stranded DNA molecule. However, switching was found to be less efficient for Cy5-labeled proteins interacting with stained DNA. This observation is consistent with previous reports that the switching efficiency of Cy5 has a distance dependence much shorter and steeper than that reported for Förster resonance energy transfer (157). The physical size of the protein places the Cy5 at a slightly larger distance away from the DNA, thereby reducing the efficiency of fluorescence activation by the DNA-bound intercalating stain. However, the previously demonstrated linear dependence of switching efficiency on intensity and duration of the activation beam (157) provides a readily accessible experimental
parameter to optimize the extent of switching in a population of labeled molecules (Figure 23e).

An experimental concern in the application of fluorescence microscopy is the photo-induced degradation of biomolecules. In particular, excitation of an intercalating stain has been shown to induce DNA cleavage through free radical formation (165). However, at the low intensity of 532-nm illumination required for excitation of the intercalating stain and photo-activation of the Cy5 (0.49 W cm$^{-2}$ at 532 nm), no DNA degradation was detected over tens of minutes.

At this point, the NaBH$_4$ caging efficiency determines the upper limit of experimentally usable fluorophore concentration. The 97% darkening efficiency implies an upper dye concentration at which individual fluorophores can still be resolved of ~1 µM. However, the steep distance dependence of the Cy5 switching efficiency upon Sytox Orange excitation drastically reduces the volume in which Cy5 fluorescence can be activated to a cylinder around the DNA with a radius of approximately 1 nm. Theoretically, this activation volume allows for the observation of single molecules in the presence of up to millimolar concentrations of fluorophores in solution.

In principle, LADye offers a generally applicable method to study biomolecular processes including, but not limited to, DNA-based systems. The method requires only a substrate labeled with a switchable fluorophore and an immobilized binding partner coupled to a fluorophore that spectrally overlaps with the substrate’s fluorophore. LADye could, for example, be employed to visualize the movement of actin- or microtubule-based motor proteins by staining the filaments with the activator dye. Alternatively, binding of ligands to transmembrane proteins could be visualized by placing activator dyes in the membrane. LADye could also be used to complement switchable FRET (166) studies by allowing not only the observation of subunit interactions within the molecular complex, but also enabling the visualization of recruitment of subunits from solution. The ability to specifically visualize only those fluorescently labeled molecules that interact with their binding partners while the fluorescent molecules in solution remain dark enables real-time, single-molecule observations of low-affinity biomolecular interactions under equilibrium conditions that approach the in vivo concentration of the reactants.
6.4 Methods

6.4.1 Dyes and proteins

Recombinant IFI16 was synthesized in *E. coli*, purified, and subsequently labeled with the Cy5 dye by maleimide chemistry, as described (167). Cy5-IFI16 was used at a concentration of 1 nM in combination with the M13 DNA template and at 30 nM in the experiments with λ DNA.

The gene for AVP was expressed in *E. coli* and the protein purified as described previously (168,169). AVP concentrations were determined using a calculated extinction coefficient (170) of 26,510 M⁻¹cm⁻¹ at 280 nm. The peptide pVlc (GVQSLKRRRCF) was purchased from Invitrogen (Carlsbad, CA), and its concentration was determined by titration of the cysteine residue with Ellman’s reagent (171) using an extinction coefficient of 14,150 M⁻¹cm⁻¹ at 412 nm for released thionitrobenzoate. Octylglucoside was obtained from Fischer Scientific (Faden, NJ) and endoproteinase Glu-C from Sigma (St. Louis, MO). Disulfide-linked AVP-pVlc complexes were prepared by overnight incubation at 4°C of 75 µM AVP and 75 µM pVlc in 20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.1 mM EDTA and 20 mM β-mercaptoethanol. Initially, during the overnight incubation, the β-mercaptoethanol reduces most oxidized cysteines in AVP and in pVlc. Then, the remaining β-mercaptoethanol evaporates or is oxidized by atmospheric oxygen, thereby allowing Cys104 of AVP and Cys10’ of pVlc to form a disulfide (172,173).

Disulfide-linked AVP-pVlc complexes, 75 µM, were labeled in 25 mM HEPES (pH 7.0), 50 mM NaCl, and 20 mM ethanol by the addition of Cy5 maleimide (GE Healthcare, Piscataway, NJ) to 225 µM. Labeling reactions proceeded at room temperature in the dark for 2.5 h. Excess reagents were removed from the labeled sample by passage through Bio-Spin 6 chromatography columns (Bio-Rad; Hercules, CA) equilibrated in the labeling buffer. The degree of labeling was determined using a molar extinction coefficient for AVP at 280 nm of 26,510 M⁻¹cm⁻¹, for Cy5 at 649 nm of 250,000 M⁻¹cm⁻¹, and a correction factor at 280 nm of 0.05. The ratio of labeled AVP-pVlc to total AVP-pVlc was determined to be about 0.8. The labeled materials were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF).

Specific enzymatic digestions followed by MALDI-TOF mass spectrometry were used to locate cysteinyl-Cy5 conjugates in AVP-pVlc complexes. Labeled AVP-pVlc complexes, 1.2 µg, were digested by incubation with 0.01 µg each of endoproteinase Glu-C or trypsin at 21 °C in 25 mM Tris-HCl (pH 7.5). At 1, 2, 4, and 22 h, 0.5 µL of each reaction was removed and added to 4.5 µL of a saturated matrix solution (α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile and 0.1% TFA. The matrix-analyte solution was then immediately spotted onto a 100-well stainless-steel sample plate. The sample plate was calibrated using Applied Biosystems peptide calibration
mixtures 1 and 2. Mass spectrometric characterization was carried out on a Voyager-DE Biospectrometry Workstation (Applied Biosystems, Foster City, CA). The $m/z$ peak list generated for each chromatogram was analyzed by the FindPept Tool (174). The Cy5 modification was entered as a post-translational modification. AVP-pVIc complexes were found to be labeled exclusively at Cys199 (data not shown).

Cy5 mono-reactive NHS ester (GE Healthcare) was dissolved in DMSO and stored at -20 °C. For the high Cy5 background experiments, 270 nM of this Cy5 was caged with 1 mg/mL NaBH₄ and added to the single-molecule imaging buffer.

### 6.4.2 DNA construct

A biotinylated 60-bp duplex oligo DNA with a 3’ amine-end modification was purchased from Integrated DNA Technologies. The amine group of the oligo was covalently linked to Cy5 mono-reactive NHS ester (GE Healthcare) by adding a 125-fold excess of Cy5 to the oligo in reaction buffer PBS (pH 7.5) at room temperature. The labelling reaction was performed for 1 h. Unreacted fluorophores were removed by immediately running the reaction products through a NAP5 column (GE Healthcare). The degree of labeling, determined by absorbance spectroscopy, was 0.8 fluorophores/oligo.

Single-stranded M13mp18 (New England Biolabs) was biotinylated by annealing a complementary biotinylated oligo to the M13 template. Subsequently, the primed M13 was filled in by adding T7 DNA polymerase (New England Biolabs), dNTPs and a replication buffer containing MgCl₂. Replication proteins were removed from the filled-in DNA products by phenol/chloroform extraction, and it was stored in 10 mM Tris-HCl, 1 mM EDTA (TE) buffer (105).

λ DNA substrates were constructed by standard annealing reactions. The linearized DNA has 12-nt single-stranded overhangs at each end. A biotinylated oligo was annealed to one end of the λ DNA to allow surface attachment to the functionalized glass coverslip (105).

### 6.4.3 Experimental setup

Single-molecule measurements used an Olympus IX-71 microscope equipped with a 100x TIRF objective (Olympus, UApoN, NA = 1.49 (oil)). The sample was continuously excited with a 154 W·cm⁻² 643 nm laser (Coherent) and frequently pulse-photodeactivated with a 0.49 W·cm⁻² 532 nm laser (Coherent) and a 62 W·cm⁻² 404-nm laser (Cube) controlled by a home-build shutter program. Images were captured with an EMCCD camera (Hamamatsu) using Meta Vue imaging software (Molecular Devices) with a typical frame rate of 5 frames per second for the Cy5-oligo DNA and Cy5-IFI16 experiments and 32 frames per second for the Cy5-pVIc-AVP measurements.
All experiments were carried out in home-built flow cells. PEG-biotin functionalized coverslips were treated with a streptavidin solution to enable DNA template binding (175). A PDMS flow cell with 0.5-mm wide channels was adhered on the top of the glass coverslip and an inlet and outlet tube were inserted into the PDMS. After thoroughly washing the flow cell, the DNA was flushed through and subsequently the reaction buffer was flowed through at 10 µL/min. As soon as the reaction buffers entered the flow cells, fluorescence imaging was started.

6.4.4 Buffers for single-molecule measurements

LADye requires the pre-darkening of the Cy5 fluorophore, which was achieved by caging with 1 mg/mL NaBH\textsubscript{4} in 20 mM Tris at pH 7.5, 2 mM EDTA, and 50 mM NaCl prior to the fluorescence imaging experiments. The buffers used in the single-molecule imaging experiments were designed to suppress blinking and reduce photobleaching rates in caged Cy5 and Cy5-labeled proteins. The experiments on Cy5-oligo DNA and Cy5-IFI16 switching were done in a 20 mM Tris at pH 7.5, 2 mM EDTA, and 50 mM NaCl buffer, and the Cy5-pVlc-AVP experiments were done in a 10 mM Hepes at pH 7.0, 2 mM NaCl, 5% glycerol, 20 mM EtOH, and 50 µM EDTA buffer. To increase Cy5 photostability and facilitate switching, all buffers contained 0.45 mg/mL glucose oxidase, 21 µg/mL catalase, 10% (w/v) glucose, 1 mM Trolox (118) and 143 mM β-mercaptoethanol (BME). In addition, 50 nM Sytox Orange (Molecular Probes) was added to the reaction buffers to stain the DNA to enable Cy5 switching.