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Chromophore maturation and fluorescence fluctuation spectroscopy of fluorescent proteins in a cell-free expression system

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Abstract

Cell-free synthesis, a method for the rapid expression of proteins, is increasingly used to study interactions of complex biological systems. GFP and its variants have become indispensable for fluorescence studies in live cells and are equally attractive as reporters for cell-free systems. This work investigates the use of fluorescence fluctuation spectroscopy (FFS) as a tool for quantitative analysis of protein interactions in cell-free expression systems. We also explore chromophore maturation of fluorescent proteins, which is of crucial importance for fluorescence studies. A droplet sample protocol was developed that ensured sufficient oxygenation for chromophore maturation and ease of manipulation for titration studies. The kinetics of chromophore maturation of EGFP, EYFP, and mCherry were analyzed as a function of temperature. A strong increase in the rate from room temperature to 37 °C was observed. We further demonstrate that all EGFP proteins fully mature in the cell-free solution and that brightness is a robust parameter specifying stoichiometry. Finally, FFS is applied to study the stoichiometry of the nuclear transport factor 2 in a cell-free system over a broad concentration range. We conclude that combining cell-free expression and FFS provides a powerful technique for quick, quantitative study of chromophore maturation and protein-protein interaction.

Keywords

Brightness analysis; NTF2; EGFP; mCherry; Temperature; Chromophore formation

Cell-free protein expression is a simple and flexible method for the rapid synthesis of folded proteins. These systems represent an attractive alternative for producing difficult-to-express proteins, such as membrane proteins or proteins that seriously interfere with cell physiology [1]. Recently, cell-free systems have been used to engineer complex biological processes [2]. The inherent open nature of the cell-free system allows direct access and control of the biological system, which cannot be attained by cell experiments. Thus, the cell-free system is emerging as a versatile platform for systems biology experiments [3]. In addition, cell-free expression has been used lately to study the maturation kinetics of fluorescent proteins [3,4].

Because knowledge of protein–protein interactions is of critical importance for developing a molecular description of biological processes, we investigate the potential of fluorescence fluctuation spectroscopy (FFS)¹ to directly quantify these interactions in the cell-free

system. FFS utilizes the intensity fluctuations of fluorescent molecules passing through a very small optical observation volume to determine concentration, diffusion coefficient, and brightness of the molecules [5]. Brightness reflects the average fluorescence signal of a single particle [6]. Suppose two monomeric proteins that are labeled with enhanced green fluorescent protein (EGFP) associate to form a dimer. Because the dimer carries two fluorescent labels, it will appear twice as bright as the monomeric protein. This simple relationship between brightness and stoichiometry has been successfully used to quantify homo- and hetero-protein complexes inside cells [7,8].

The principle of brightness analysis should be directly applicable to FFS measurements of cell-free systems. Just as in cells, expression of EGFP-tagged proteins provides a convenient method for introducing fluorescent markers. However, there are a number of potential challenges for FFS studies in cell-free systems. The fidelity of cell-free expression and of chromophore maturation needs to be checked, because the presence of nonfluorescent or incompletely expressed protein leads to biased interpretation of FFS experiments [9]. We develop a droplet-based assay for FFS experiments in a cell-free extract. The droplet format not only ensures a small sample volume, but also provides sufficient oxygenation of the sample for efficient chromophore maturation.

The widespread use of fluorescent proteins as markers in biological and medical research rests on the unique ability of the expressed protein to form a chromophore on its own. The formation of the chromophore is referred to as maturation and only requires molecular oxygen as an external reagent [10]. Although the kinetics of chromophore formation has been the subject of numerous studies, there is significant inconsistency in the reported maturation times of individual fluorescent proteins. Here we investigate the temperature dependence of the maturation process, which has received little attention so far. Specifically, we conduct FFS experiments in batch mode on EGFP, EYFP, and mCherry using an *Escherichia coli*-based cell-free system. We observe a pronounced temperature dependence of the maturation rates of the fluorescent proteins, model the kinetics by transition state theory, and compare our results with other maturation studies based on de novo protein synthesis. Because EGFP exhibits a very fast maturation at 37 °C, we consider EGFP to be a suitable reporter for many kinetic studies in the cell-free system.

We further use EGFP and a tandem-dimer EGFP (EGFP₂) to check the fidelity of expression and maturation of the *E. coli*-based cell-free system and to demonstrate the feasibility of protein titration experiments with the droplet-based assay. Nuclear transport factor 2 (NTF2) is used as a model protein to explore brightness characterization of protein-protein interactions in the cell-free system. NTF2 plays a key role in nucleocytoplasmic transport and maintains the cellular Ran gradient which drives the transport process [11]. Depletion of NTF2 affects the Ran gradient and can lead to cell death [12]. The mechanism by which NTF2 performs its function is not well understood. Because there are conflicting reports regarding the oligomeric state of NTF2 [13–15], we examine the stoichiometry and concentration dependence of NTF2 in cell-free solution and compare the result with the literature.

Materials and methods

Experimental setup

A mode-locked Ti:sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA) serves as a source for two-photon excitation through a modified Zeiss Axiovert 200 microscope

¹Abbreviations used: EGFP, enhanced green fluorescent protein; EGFP₂, tandem-dimer EGFP; FFS, fluorescence fluctuation spectroscopy; NTF2, nuclear transport factor 2.

(Thornwood, NY) as previously described [7]. FFS measurements are taken from 30 to 120 s and use two-photon excitation of the sample at 1000 nm. Excitation light is focused through a Zeiss 63 × C-Apochromat water immersion objective (N.A. = 1.2) or a 63× Plan Apochromat oil immersion objective (N.A. = 1.4). An excitation power of 0.3 mW is used to avoid saturation and photobleaching effects. Dual-color experiments are recorded onto separate detectors using a 580 nm dichroic mirror (T580LXPR, Chroma, Rockingham, VT). Photon counts are detected with an avalanche photodiode (Perkin-Elmer, SPCM-AQ-14) and recorded by a data acquisition card (ISS, Champaign, IL), which stores the complete sequence of photon counts using sampling frequencies ranging from 50 to 200 kHz. The photon counts are analyzed with programs written in IDL 6.0 (Research Systems, Boulder, CO).

Cell-free sample preparation

We use the S30 T7 High-Yield Expression System (Promega, Madison, WI) as our cell-free solution system. We mix DNA into 5- to 15-μL samples of cell-free solution according to the Promega protocol, and let the synthesis reaction run at room temperature for 2–4 h. Reactions were subsequently stopped by the addition of 0.1% RNase A (Sigma-Aldrich, St. Louis, MO) and then spun down at 18,000g for 20 min to remove any large particles. Samples were transferred into a ring of vacuum grease (Dow Corning, Midland, MI) to prevent spreading inside an eight-well coverglass chamber slide (Nagle Nunc International, Rochester, NY). The eight-well chamber is sealed with a rubber stopper or an additional coverslip and vacuum grease seal. Glass surfaces are treated with SigmaCote (Sigma-Aldrich) and measurements are taken 10 μm above the coverglass to avoid surface adhesion effects.

Expression vectors, purified protein, and buffers

Cell free—EGFP was amplified from the pEGFP-C1 plasmid (Clontech, Mountain View, CA) with a 5′ primer that encodes a BamHI restriction site and a 3′ primer that encodes an XhoI site. The result was cloned into the pRSET B vector (Invitrogen, Carlsbad, CA). We refer to this plasmid as pB-G. A tandem dimeric EGFP was constructed by cloning a second EGFP between the EcoRI and HindIII restriction sites of the pB-G plasmid. NTF2 was amplified from human NTF2 (GenBank Accession Number: BC002348) and cloned into the EcoRI/HindIII site of pB-G. A monomeric NTF2 was generated by mutating the methionine residue 118 to glutamate [15].

Solution—EGFP was expressed in *E. coli* and purified according to protocol using QiaExpress Ni-NTA Fast Start kit (Qiagen, Valencia, CA). Cell-free and aqueous dilution experiments were performed using HBS-EP buffer (Biacore, Uppsala, Sweden) or Dulbecco's PBS with calcium and magnesium (Biowhittaker, Walkersville, MD).

Brightness analysis—The time-dependent fluorescence intensity $F(t)$ of the FFS measurement is used to determine its autocorrelation function,

$$G(\tau) = \frac{\langle \delta F(t+\tau) \delta F(t) \rangle}{\langle F(t)^2 \rangle}, \quad (1)$$

where $\delta F(t) = F(t) - \langle F \rangle$ is the fluctuation in fluorescence intensity about the average. A fit of the autocorrelation function to a simple diffusion model determines the autocorrelation amplitude $G(0)$, which is related to the average number of molecules N_{PSF} present in the excitation volume,

$$G(0) = \frac{\gamma}{N_{PSF}}, \quad (2)$$

with γ representing the shape factor of the FFS observation volume [16]. We convert N_{PSF} into a concentration by $c = N_{PSF}/V_{PSF}$. The value of V_{PSF} is determined from an FFS measurement of a dye sample with known concentration [7]. The brightness is calculated from the fluctuation amplitude and the average fluorescence intensity,

$$\lambda = \frac{G(0) \cdot \langle F \rangle}{\gamma}, \quad (3)$$

and reflects the average fluorescence intensity received per particle. The brightness of a monomeric fluorescent protein, such as λ_{EGFP} , serves as reference value and is determined by a calibration measurement of a sample containing only the fluorescent protein. Association of two EGFP-labeled proteins to form a dimer results in a doubling of the brightness, because the protein complex contains two EGFP proteins. This example illustrates that brightness encodes the stoichiometry of a protein complex. It is useful to define a normalized brightness b by dividing the brightness λ of a protein sample by the brightness of EGFP,

$$b = \frac{\lambda}{\lambda_{EGFP}}, \quad (4)$$

The normalized brightness b provides an experimental measure of the stoichiometry of the protein complex. For example, $b = 2$ indicates a dimeric protein.

Kinetics of chromophore maturation

Protein expression is stopped with 0.1% RNase and quickly transferred to the sample chamber. The observed increase in fluorescence intensity reflects chromophore maturation of previously synthesized proteins and is fit by

$$F(t) = F_0 + \Delta F (1 - e^{-t/\tau}), \quad (5)$$

where F_0 is the average fluorescence intensity at the time the reaction is stopped, ΔF is the change in average fluorescence intensity from the subsequently maturing chromophores, and τ is the characteristic maturation time. The maturation rate coefficient k is the inverse of the maturation time, $k = (1/\tau)$.

The temperature dependence of the maturation rate coefficient is modeled using transition state theory. The Eyring equation relates the reaction rate coefficient k to temperature T ,

$$k = \frac{k_B T}{h} \exp\left(-\frac{\Delta H^\ddagger - T\Delta S^\ddagger}{RT}\right), \quad (6)$$

where $\Delta H^\ddagger$ is enthalpy of activation, $\Delta S^\ddagger$ is entropy of activation, k_B is the Boltzmann constant, h is Planck's constant, and R is the gas constant. The reaction rate data are plotted as $\ln(k/T)$ vs $1/T$ and fit to a straight line. The slope and intercept of the fitted line determine the activation enthalpy and entropy. Sample temperatures are adjusted using an ASI 400 air

stream incubator (Nevtek, Williamsville, VA) or a VWR Polyscience chiller recirculator (Niles, IL) connected to home-built coils around the microscope objective and sample holder. The sample temperatures reported in this work were recorded using a thermocouple placed in a droplet or water reservoir adjacent to the measured sample.

Results

Cell-free synthesis of EGFP

Because cell-free samples are typically 5–50 μL , the measurement protocols must be adapted for small volumes. Our first approach was to place the cell-free solution between two coverslips. On expressing EGFP we observed that the sample was only fluorescing around the edges of the sample, which is in contact with the surrounding air. Because oxygen is required for maturation of the chromophore, our result indicated that good contact of the sample with the surrounding air is crucial. To address this challenge we adopted a configuration where a drop of cell-free solution is placed on a chambered coverglass slide. Before placing the droplet, a small circle of vacuum grease is applied on the glass surface to prevent spreading of the solution across the slide. This protocol results in a well-formed droplet with a large surface area in contact with air for efficient uptake of oxygen. Expression of EGFP in the droplet solution resulted in the appearance of fluorescence throughout the sample as confirmed by an axial scan [17] through the sample with a long-working distance objective.

Because the measurement of cell-free expression reactions may take several hours, we evaluated the long-term stability of the droplet sample. A droplet sample containing an aqueous solution of the fluorescent dye Alexa 488 was measured as a control by FFS for several hours (data not shown). We observed a continuous increase in the fluorescence intensity. FFS analysis showed that the brightness of the dye remained constant, but the dye concentration increased. The increase in concentration is consistent with the loss of sample volume due to evaporation. To circumvent this problem we placed the droplet into a sealed chamber together with an additional water droplet (Fig. 2D). The water serves to establish vapor pressure equilibrium after the top opening has been sealed with a rubber stopper, thereby preventing the evaporation of the sample. The sealed chamber and water reservoir are prepared in advance and the cell-free reaction mixture is added later via syringe through the rubber stopper. We tested the sealed chamber using an aqueous droplet sample containing the dye Alexa 488, which was measured for more than 6 h. The fluorescence intensity remained constant over the whole measurement period, demonstrating that evaporation effects are negligible. Thus, all experiments described here have been performed using the sealed chamber configuration.

We study cell-free expression of EGFP using FFS to monitor the course of protein synthesis in real time. Initial experiments established that the cell-free extract contains aggregates that interfere with later FFS analysis of the data. Fortunately, such complexes are relatively easily removed through centrifugation. Centrifuging the sample at 18,000g for 5–10 min pellets the detritus, and the clean cell-free supernatant is easily removed. The centrifugation step can be conducted before or after the protein expression reaction. Immediately after adding the DNA for EGFP to the centrifuged reaction mixture, the sample is placed on the microscope. Sixty-second measurements are taken at regular intervals over the next 5 h (Fig. 1A). The intensity increases after an initial lag phase. The reaction eventually begins to run down as the synthesis process exhausts the available energy [18]. Brightness analysis of these data allow us to extract time courses for the average number of molecules in the observation volume and the brightness of EGFP (Fig. 1B and C). The number of molecules increases overtime demonstrating that proteins are being produced as expected. More importantly, the brightness (Fig. 1C) is constant throughout the reaction which shows that

there are no transitional or alternate brightness states. Unfolded and unmaturing EGFP proteins are nonfluorescent and so invisible to FFS measurements. Once the protein is completed, it “turns on” at the characteristic brightness of EGFP and remains stable and independent of protein concentration. This is further confirmed by comparing FFS measurements on cell-free expressed EGFP, recombinant EGFP purified from transformed *E. coli*, and EGFP in living mammalian cells (Table 1). Measured under the same excitation power, all three EGFP samples have the same brightness. This robustness of EGFP brightness makes it a useful and reliable tool for quantitatively examining protein interactions. Table 1 also displays the diffusion time or average residence time within the optical volume. This information is acquired through autocorrelation analysis of the data and reflects the differing solution environments of the three methods. Purified EGFP in aqueous buffer solution has the fastest diffusion time. Both EGFP in cells and in cell-free solution have a slower diffusion time. Fluorescent dye added both to the buffer solution and to basic cell-free solution shows a similar change in diffusion time (data not shown). This indicates that the difference in diffusion time reflects the changes in viscosity of the solutions.

Kinetics of chromophore maturation

We investigate the kinetics of chromophore maturation using cell-free expression in a free-standing droplet (~10 μ L). After spinning down the cell-free solution, DNA is added and left to synthesize for 10–30 min. The reaction is stopped with 0.1% RNase and quickly transferred to the sample chamber. By adding RNase to the droplet the synthesis reaction is rapidly stopped [3]. Any subsequent increases in the fluorescence intensity can be attributed to chromophore maturation of previously synthesized proteins. Since each chromophore's maturation is independent, fluorescence intensity increase can be described by a single-exponential model. Fig. 2A shows the EGFP maturation intensity as a function of time fit with Eq. (5). At room temperature (20 °C), the characteristic maturation time, $\tau_{EGFP} = 15 \pm 3.5$ min is established by repeating the experiment several times ($n = 6$). In addition to EGFP, we also investigated the maturation of EYFP ($n = 3$) and mCherry ($n = 4$). We found EYFP at 19 °C to have a significantly longer maturation time, $\tau_{EYFP} = 78 \pm 12$ min and mCherry at 20 °C to be rather slow at $\tau_{mch} = 155 \pm 10$ min (Fig. 2B and C).

Oxygen diffuses within ~100 s from the edge to the center of a 10 μ L droplet. As such, we anticipate that oxygen availability is not a limiting factor for maturation reactions with reaction times longer than 100 s. As a control experiment we measure the maturation of EGFP in a smaller droplet (1 μ L). With a diffusion time to the center of only ~20 s the rate to replenish oxygen is much faster than for the large droplet. The maturation time of EGFP measured in the 1 μ L droplet at 19 °C ($\tau_{EGFP} = 15$ min) is identical to the time observed in the 10 μ L droplet. This result demonstrates that oxygen availability is not a limiting factor for our assay.

Literature values for chromophore maturation times of EGFP cover a broad range of values and involve a variety of experimental approaches [19–23]. To further complicate matters individual studies have been performed at different temperatures. However, a quantitative study that evaluates the influence of temperature on maturation is not readily available. We decided to expand our study in order to address the temperature dependence of chromophore maturation. Measurements of the fluorescence intensity of the maturation process were taken at selected temperatures up to 37 °C and fit with Eq. (5) to recover the characteristic maturation time τ and rate coefficient $k = 1/\tau$. The results of the temperature study are plotted as $\ln(k/T)$ vs $1/T$ (Fig. 3). The Eyring plot shows that maturation rates increase with temperature. Maturation of EGFP at 37 °C proceeds sufficiently fast (Fig. 2D) that we approach the practical limit of the experimental setup. Thus, the temperature dependence of EGFP maturation was performed by cooling the solution below room temperature. The straight lines through the data points of Fig. 3 represent a fit of the rate coefficients to

transition state theory. The slope and y-axis intercept determine the activation enthalpy $\Delta H^\ddagger$ and activation entropy $\Delta S^\ddagger$ of the maturation process of each protein. The activation enthalpies for EGFP, EYFP, and mCherry are 65 ± 1.6 , 46 ± 4.3 , and 43 ± 1.7 kJ/mol, respectively. These values fall in the range expected for solution oxidation reactions. The differences in activation enthalpy between the proteins are relatively small, particularly between EYFP and mCherry. The differences in the activation entropies (-79 ± 5 , -156 ± 15 , -175 ± 6 J/(mol K) for EGFP, EYFP, and mCherry) follow a similar pattern. Overall, the ratios of the maturation kinetics for different fluorescent proteins are approximately independent of temperature, because of the similarity of the activation enthalpies. Calculating from the fits, the characteristic maturation times at 20 °C are $\tau_{EGFP} = 15$ min, $\tau_{EYFP} = 82$ min, $\tau_{mCh} = 157$ min, and at 37 °C these values become $\tau_{EGFP} = 3$ min, $\tau_{EYFP} = 27$ min, $\tau_{mCh} = 57$ min. Note that the fast maturation rate measured for EGFP at 37 °C (Fig. 2D) agrees with the prediction from transition state theory (upper leftmost cross in Fig. 3).

Brightness and titration studies

After a protein is expressed and matured, its properties can be studied by FFS. Here we focus on demonstrating the feasibility of brightness measurements as a function of protein concentration with the droplet setup. For the simple addition of a ligand, enzyme, or other chemical, the rubber stopper and syringe method is very effective. However, this approach is inconvenient for performing repeated dilutions of the droplet. Thus, instead of the rubber stopper a coverslip was placed as a lid on top of the chambered coverslide. A thin layer of vacuum grease applied to the contact area of the coverslip seals the sample chamber. A 10 μ L droplet of purified EGFP is placed onto the coverslip. Dilutions are performed by unsealing the coverslip, adding additional buffer with a pipette, pipetting up and down, and finally removing an equivalent volume of the mixture and replacing the coverslip. At each dilution step an FFS measurement is taken to determine brightness and concentration (Fig. 4, inset). The brightness is concentration independent and reflects that EGFP is a monomeric protein. The same result is obtained for EGFP expressed in cell-free solution (Fig. 4). An important control for brightness experiments is the demonstration of brightness doubling with the tandem-dimer EGFP₂, formed by encoding two EGFPs connected with a short linker sequence [7]. After expressing EGFP₂ in cell-free solution, a droplet was placed on the coverslip followed by FFS measurements in between dilution steps. The brightness of EGFP₂ is concentration independent and twice the brightness of monomeric EGFP, because of the presence of two EGFP molecules. These results demonstrate that the droplet sample provides a quick and reliable method for titration experiments. It further establishes that the quality and fidelity of the cell-free expression system are suitable for brightness studies.

With the cell-free system characterized and brightness analysis validated, we apply this method to a biological system. The protein NTF2 establishes the cellular Ran gradient, which is crucial for nucleocytoplasmic transport [24]. Ran exists in the form of RanGTP at high concentrations in the nucleus and at low concentrations in the cytoplasm, where RanGTP is converted to RanGDP. This gradient provides directionality for nucleocytoplasmic transport. The transport factor NTF2 maintains the gradient by returning RanGDP to the nucleus, where it is converted back into RanGTP. NTF2 has been identified as a dimer in previous studies [13,14] with one study reporting a monomer/dimer equilibrium with a dissociation constant of ~ 1 μ M [15]. A recent FFS study of NTF2 from our lab finds NTF2 to be a dimer in cells [17]. However, only cells with high protein concentration were used for the cell study. We now take advantage of the cell-free system to measure the oligomeric state of NTF2 over a wide concentration range using the same FFS approach as the cell study. We perform brightness titration studies on EGFP and EGFP₂ expressed in cell-free solution (Fig. 5) as control experiments. Next, we repeat the dilution experiment on EGFP-NTF2 expressed with the cell-free system. The brightness of EGFP-

NTF2 aligns with EGFP₂ (Fig. 5), indicating that NTF2 is dimeric over the entire measured concentration range (~30–1400 nM). We also performed a brightness titration of the mutant EGFP-NTF2.M118E expressed in cell-free solution, because it has been previously reported that the point mutation M118E prevents dimerization of NTF2 [15]. Our brightness data confirm that the point mutation abolishes dimerization and show that EGFP-NTF2.M118E is a monomer at all measured concentrations (Fig. 5). Thus, the brightness analysis of NTF2 in the cell-free system agrees with the earlier cell data [17] and indicates that NTF2 continues to be a dimer well below 1 μ M.

Discussion

There are several factors that are crucial for FFS measurements on cell-free systems. The presence of aggregates in cell-free expression systems is an obstacle to FFS measurements. Some of the aggregates are fluorescent, which leads to spikes in the fluorescence intensity and prevents meaningful brightness analysis. In the case of two-photon excitation we occasionally trapped large fluorescent aggregates in the laser beam. Furthermore, some of the nonfluorescent aggregates are large enough to exclude sufficient volume when passing through the observation volume, so that the fluorescence signal is reduced. While this effect is less noticeable in the raw data, because no spikes are generated, it biases brightness analysis. The presence of large particulates ((x02A7E) μ m) was directly confirmed by viewing the sample solution through a 63 $\times$ objective under bright-field conditions. Aggregates seem to be present in most cell-free systems. It is speculated that some of these aggregates build up because cell-free solutions do not have the active processes which clean up unused or discarded reaction material. The bulk of the aggregates can be removed by an additional centrifugation step after reaction solution has been reconstituted and/or after the reaction has finished. Without this step, it is difficult to perform FFS experiments in the cell-free environment.

A unique feature of fluorescent proteins is their ability to form a chromophore by posttranslational modifications. This maturation process only requires molecular oxygen as an external reagent. The amount of oxygen dissolved in air-saturated water under normal atmospheric conditions is ~200 μ M at room temperature, which exceeds the concentration of expressed fluorescent protein by more than two orders of magnitude. Despite this apparent excess of oxygen over protein, we observe that a cell-free expression solution sandwiched between two coverslips failed to develop fluorescence except at the edges of the solution that were in contact with air. This observation indicates that other processes exhaust the oxygen reservoir of the solution. In fact, it has been previously reported that oxygen is efficiently depleted within minutes in a cell-free reaction [25]. We choose a droplet as our sample geometry to ensure efficient oxygen transfer at the solution/air interface. The volume of droplets (typically 10–15 μ L) permits oxygen to diffuse from the interface to the center of the droplet in ~100 s. Oxygen diffuses from the periphery to the center of a 1- μ L droplet 5 times faster than through a 10- μ L droplet. Measurement of EGFP maturation under both conditions returns the same results and demonstrates that oxygen is not a limiting factor in our experiments. In fact, we succeeded in measuring maturation rates as fast as 3 min with the droplet setup (Fig. 2D). A hydrophobic ring prevents spreading of the sample droplet across the hydrophilic coverglass. This setup is important for the titration experiments, because by preserving the droplet shape it is straightforward to add, mix, and remove solution. Measurements on droplets can be performed for many hours (Figs. 1 and 2) without loss of sample. Evaporation is prevented by sealing the measurement chamber as described earlier.

The process of chromophore formation, particularly of GFP, has been carefully studied. The green chromophore pathway (GFP, EGFP, EYFP) has three primary stages: cyclization,

oxidation, and dehydration. The red chromophore pathway (DsRed, mCherry) includes an additional oxidative step for the formation of an acylimine linkage in the polypeptide backbone. Studies suggest that the primary rate-limiting step is oxidation for the green pathway. While the steps for the red pathway are less distinctly time separated, a recent report states that the final dehydration is partially rate limiting [26–28]. We model EGFP and EYFP maturation with a single exponential rate process, consistent with prior studies. The maturation kinetics of mCherry is also well-modeled by an exponential process. However, the intensity traces of chromophore maturation are affected by experimental imperfections, such as drift in the axial focus and variations in the laser power. These imperfections, although minor, currently limit our ability to investigate whether any small deviation from the single-exponential rate model exists. Nevertheless, the analyses of the maturation kinetics and its temperature dependence demonstrate that a single-exponential kinetic model provides a satisfactory description of the data.

While a coherent mechanism of chromophore formation has emerged, a consistent picture of the overall kinetics is not yet available. The maturation kinetics of fluorescent proteins has been studied by various methods, such as (i) de novo protein expression, (ii) triggered protein folding of solubilized inclusion bodies, and (iii) reoxidation after chemical reduction of the chromophore. The results obtained by each method are inconsistent with each other. For example, the maturation time of EGFP is reported as ~60 min (by folding of solubilized inclusion bodies), as ~130 min (by reoxidation), and as ~8 min (by de novo protein expression). Although it is not clear why the reported rates differ that widely, each method requires unique sample conditions, which may contribute to the diversity of reported values. It seems prudent to compare studies that utilize the same experimental method. Because our study is based on cell-free expression, we focus in this paper on de novo protein expression methods.

However, even studies based on the same method report different maturation rates for the same fluorescent protein. Thus, additional experimental factors influence the maturation process. Although it has been noted that the kinetics of maturation of GFP is different at 25 and 37 °C [29], the influence of temperature on chromophore maturation has not received further attention. We performed, to the best of our knowledge, the first quantitative characterization of maturation kinetics as a function of temperature. Published maturation studies have been conducted at specific temperatures, ranging from room temperature to 37 °C. Because the rate of maturation changes ~5-fold over this temperature range, one needs to account for this change when comparing maturation experiments.

Chromophore maturation by de novo protein expression is usually performed in one of two ways. Most studies employ anaerobic expression conditions followed by the addition of oxygenated solvent to complete the maturation process. Some studies utilize aerobic expression conditions and terminate protein expression while monitoring the subsequent increase in fluorescence due to maturing protein, as was done in this case. Adding RNaseA to the solution stops gene expression almost instantly and allows the determination of maturation times of less than 1 min as has been previously demonstrated [3].

A large number of experiments probing maturation kinetics exist in the literature. To ensure a meaningful comparison we only consider studies based on de novo protein expression of the proteins EGFP, EYFP, or mCherry. We make no distinction between de novo protein expression by bacterial, cell-free, or eukaryotic cell expression systems. For EYFP a maturation time τ of 56 min measured at 25 °C has been reported, while a second study determined a time constant of 23 min measured at 37 °C [4,30]. These published data are in excellent agreement with the values determined from Fig. 3 with τ = 58 min at 25 °C and τ = 27 min at 37 °C. The maturation of mCherry has been measured independently by two

groups at 37 °C, who reported values of 22 and 57 min [31,32]. Our maturation time for mCherry at 37 °C is $\tau = 57$ min, which matches the value reported by Merzlyak et al. [32]. Finally, the maturation lifetime of EGFP by cell-free expression has been reported as $\tau = 14$ min at 37 °C and as $\tau = 8$ min at 29 °C [3,4], while our own data yield lifetimes of $\tau = 3$ min at 37 °C and as $\tau = 7$ min at 29 °C. Thus, our result is in close agreement with the value reported by Shin and Noireaux [3]. Surprisingly, EGFP is the fastest maturing protein among the ones tested. This observation is in contrast to its reputation of being a relatively slow maturing protein as established by previous denaturation and refolding studies. Because EGFP exhibits very fast maturation kinetics with de novo protein synthesis, it is important to ensure that a sufficiently fast rate of solvent oxygenation is guaranteed. Otherwise, the measured kinetics could be slowed down by the limited availability of oxygen.

EGFP's rapid maturation makes it an excellent reporter for studies of protein reactions and interactions conducted in a cell-free expression system, ensuring that the delay between expression and onset of fluorescence is minimal. In addition to traditional fluorescence methods, such as fluorescence lifetime and polarization, it is feasible to conduct fluorescence correlation experiments with cell-free systems. Expanding the repertoire of techniques in cell-free systems to FFS measurements of brightness requires a few precautions. The quality of protein expression of the cell-free system must be fairly good. Misfolding of the fluorescent protein or failure to mature creates a nonfluorescent component that interferes with the interpretation of brightness experiments. Similarly, the presence of long-lived dark states would bias the interpretation of brightness studies. Observing the brightness of EGFP₂ provides a sensitive test of the completeness of protein maturation. For example, if 30% of EGFP molecules did not mature or existed in a long-lived dark state, a subpopulation of EGFP₂ would exist with only one of the two EGFPs fluorescing. This, in turn, lowers the brightness from 2 to 1.7. A detailed discussion of the influence of dark states and flickering of fluorophores on brightness experiments is available [9]. The brightness of EGFP and EGFP₂ in mammalian cells is routinely measured as a control to establish that maturation and protein expression are not compromised. The brightness doubling observed with EGFP₂ (Fig. 4) ensures that virtually all EGFP molecules of the cell-free system mature and acquire fluorescence [9]. The result further confirms the absence of long-lived dark states for the EGFP-fluorophore in two-photon excitation. In addition, our brightness experiments on NTF2-EGFP establish that protein interactions can be quantitatively assessed in a cell-free system over a wide concentration range.

In summary, cell-free expression offers a quick and convenient method for performing fluorescence studies of protein–protein interactions with no need for further purification of the product. The commercial *E. coli*-based cell-free system has proven to be suitable for brightness studies. Combining cell-free expression with the droplet setup ensures sufficient oxygenation of the sample and provides an excellent platform for chromophore maturation studies of fluorescent proteins as a function of temperature. Maturation kinetics exhibits a pronounced temperature dependence, which is conveniently characterized by determining the activation enthalpy and entropy. This information should prove useful for future comparison of maturation measurements and hopefully provides a first step toward establishing definitive rate coefficients for individual fluorescent proteins.

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Fig.1.

Cell-free protein synthesis. (A) EGFP DNA plasmid is added to the cell-free reaction mixture and FFS measurements are taken at intervals. The average fluorescence intensity is plotted as a function of time. (B) Further analysis of the data shows that the number of EGFP molecules tracks with intensity and increases with time as new proteins are synthesized. (C) The normalized brightness, however, remains stable throughout the reaction demonstrating a single, robust EGFP brightness state.

Fig.2.

Chromophore maturation time. Cell-free EGFP synthesis is initiated at room temperature and then stopped with RNase. Any subsequent intensity increase is the result of a previously synthesized protein completing the maturation process. The curve is fit with a single exponential rate process. (A) Single maturation curve of EGFP with fit. (B) Single maturation curve of EYFP with fit. (C) Single maturation curve of mCherry with fit. The mean maturation time of each protein and its standard deviation are determined from repeated measurements at the same temperature and are quoted in the text. (D) The final panel shows an EGFP sample maturing at 37 °C with $\tau = 3 \text{ min}$. Overlaid is a cartoon of the sample setup. A small sample droplet (~10 μL) is injected into a sealed chamber slide containing a water reservoir to establish vapor pressure equilibrium.

Fig.3. Temperature dependence of maturation rate coefficient k . The fitted maturation rate coefficient k and sample temperature T are graphed in an Eyring plot for EGFP (crosses), EYFP (triangles), and mCherry (asterisks). The fitted curve determines the activation enthalpy $\Delta H^\ddagger$ and activation entropy of the reaction.

Fig.4. Droplet dilutions. Cell-free expressed and *E. coli* purified EGFP are measured in 10- μ L droplets. The dilutions are performed using HBS buffer. The inset shows *E. coli* purified EGFP (crosses) with a normalized brightness of ~ 1 plotted over 4 orders of magnitude of concentration. The FFS measured concentrations are in good agreement with the 1.5 $\times$ and 2 $\times$ dilutions that were executed. The main plot is the dilution of cell-free expressed proteins. EGFP (diamonds) and EGFP₂ (triangles) are plotted with normalized brightness versus concentration. EGFP₂ has $b = 2$ independent of concentration, demonstrating that cell-free brightness is a robust parameter that reports protein stoichiometry.

Fig.5.

Cell-free NTF2, EGFP (asterisks) and EGFP₂ (crosses) are expressed in cell-free solution and act as brightness controls plotted as normalized brightness versus concentration. We show that EGFP-NTF2.M118E (squares) is also monomeric in our cell-free system. EGFP-NTF2 data (diamonds) lie on top of EGFP₂ revealing that NTF2 is dimeric over the observed concentration range (~30–1400 nM).

Table 1

EGFP brightness in different environments.

Sample	Brightness (photon counts/s)	Diffusion time (ms)
Cell-free EGFP	3300 $\pm$ 110	0.75 $\pm$ 0.09
Purified EGFP	3200 $\pm$ 200	0.27 $\pm$ 0.05
Cellular EGFP	3200 $\pm$ 330	1.26 $\pm$ 0.38

EGFP is expressed in cell-free solution, is His-tag purified from *E. coli* into PBS buffer, or is expressed in U2OS cells. FFS measurements on all three samples report the same brightness within error. The changes in the diffusion time reflect the changes in viscosity of each sample.