Chapter 12

Labeling of Proteins for Single-Molecule Fluorescence Spectroscopy

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Abstract

Single-molecule fluorescence spectroscopy has become an important technique for studying the conformational dynamics and folding of proteins. A key step for performing such experiments is the availability of high-quality samples. This chapter describes a simple and widely applicable strategy for preparing proteins that are site-specifically labeled with a donor and an acceptor dye for single-molecule Förster resonance energy transfer (FRET) experiments. The method is based on introducing two cysteine residues that are labeled with maleimide-functionalized fluorophores, combined with high-resolution chromatography. We discuss how to optimize site-specific labeling even in the absence of orthogonal coupling chemistry and present purification strategies that are suitable for samples ranging from intrinsically disordered proteins to large folded proteins. We also discuss common problems in protein labeling, how to avoid them, and how to stringently control sample quality.

Key words Labeling, Maleimide, Cysteine, RP-HPLC, Ion-exchange chromatography, Fluorescent dye, Fluorescence spectroscopy, Single-molecule detection, Förster resonance energy transfer, FRET, Intrinsically disordered proteins, Protein folding

1 Introduction

Single-molecule Förster resonance energy transfer (FRET) has become a valuable tool for studying biomolecular structures, dynamics, and interactions [1]. As a single-molecule technique, it has the power to detect rare events, dynamics, and conformations that might otherwise be hidden in the ensemble average. FRET relies on labeling the biomolecules of interest with two fluorescent dyes with suitable spectral properties, so that radiationless energy transfer from the donor fluorophore to the acceptor fluorophore can occur. The transfer process relies on the coupling of the transition dipoles of the two fluorophores, and the resulting efficiency of energy transfer, $E(r)$, depends on the inverse sixth power of the distance, r, between the dye molecules, $E(r) = 1/(1 + (r/R_0)^6)$. This dependence makes FRET ideally suited for interrogating even

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small distance changes around the Förster radius, R_0, the distance where the transfer efficiency E equals ½ (*see* **Note 1**). Typical values of R_0 for single-molecule dyes are in the range of 4–7 nm, which makes FRET a sensitive reporter for processes on the biomolecular length scale [2]. Correspondingly, a wide range of questions have been addressed with single-molecule FRET spectroscopy [3], including the mechanisms of molecular machines [4], protein–nucleic acid interactions [5, 6], enzymatic reactions [7], protein and RNA folding [8–10], as well as the distance distributions and dynamics of unfolded and intrinsically disordered proteins (IDPs) [11–13]. Single-molecule experiments provide access to a wealth of information on biomolecular dynamics over a wide range of timescales; useful techniques are correlation spectroscopy, the analysis of broadening and exchange between subpopulations in FRET efficiency histograms, and kinetics from fluorescence trajectories of immobilized molecules or from microfluidic mixing [9]. By combining wavelength-selective photon detection with fluorescence polarization sensitivity and time-correlated single-photon counting, a multitude of complementary observables become accessible that turn single-molecule FRET into a versatile spectroscopic toolbox [14].

However, recording high-quality single-molecule data requires high-quality samples. Quality here primarily refers to labeling specificity and sample purity. Although spectroscopic approaches such as alternating excitation [15] enable a lot of sample heterogeneity to be resolved, single-molecule FRET experiments greatly benefit from the preparation of pure samples to avoid experimental artifacts, to reduce measurement time, and to be able to detect and resolve even small subpopulations unequivocally. Fortunately, sample consumption is exceedingly low, so very stringent purification procedures can be applied to accomplish this goal. To achieve site-specific labeling, many strategies have been described [16], including the labeling of cysteine residues [17, 18], the introduction of unnatural amino acids [19], or the use of enzymatic tags [20]. With the same attachment chemistry for both fluorophores, it is often not possible to resolve labeling permutants (each dye can be on either labeling site). This lack of complete site-specificity may not be essential if FRET is used as a spectroscopic ruler with a single pair of donor and acceptor, since the distance between the two dyes is the same for both permutants. However, variations in the local environment of the fluorophores can cause site-specific variations in quantum yields [21], and in advanced applications, such as 3-color-FRET [22] or FRET in combination with a fluorescence quencher [23], it is crucial to know and control the exact location of both dyes.

In spite of the wide range of sophisticated strategies for labeling with orthogonal chemistry that are now available, the most commonly used approach is still the derivatization of two reactive

cysteine residues with maleimide-functionalized dyes, primarily owing to its simplicity. In the following protocol, we describe a reliable strategy for this method that we use routinely in our laboratory and that (starting from purified unlabeled protein) usually yields samples with excellent quality in a few days. Our implementation of the method offers several advantages:

1. The strategy is simple and can be applied to a wide range of proteins, from IDPs to larger folded proteins.
2. Maleimide coupling can be carried out in a short time (<2 h) and with high yields (>90%).
3. By engineering the reactivity of the two cysteine residues and/or stringent chromatographic purification steps, even labeling permutants can often be separated.
4. Sample loss is reduced by avoiding concentration steps.
5. When RP-HPLC is used as purification method, the labeled protein is obtained in lyophilized form, simplifying storage and use in downstream applications.

We note that some of the procedures described here can also be useful for experiments closely related to single-molecule FRET, such as photoinduced electron transfer (PET) [24], or for ensemble methods requiring site-specific labeling with fluorophores, spin labels, or other chemical moieties.

2 Materials

Prepare all solutions using ultrapure water and analytical-grade reagents and test them for fluorescence impurities before use. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Instruments

1. HPLC system, equipped with a diode array detector and fluorescence module (e.g., Agilent 1200 series, Agilent Technologies). A multi-wavelength absorbance detector is the minimum requirement.
2. FPLC system, equipped with two pumps, a sample pump, a mixer, and a multi-wavelength absorbance detector (e.g., ÄKTA avant, Cytiva).
3. C18-functionalized reversed-phase chromatography columns (e.g., Reprosil Gold 200 5 μm, Dr. Maisch; Xterra RP18 5 μm, Waters; Sunfire 3.5 μm, Waters), semi-preparative scale (4.6 × 250 mm, column volume 4.15 mL). For larger proteins, C4 or C8 columns can be useful. A 2-μm inline metal frit should be used to prevent larger particles from entering and damaging the column.

4. Desalting columns (e.g., HiTrap, 5 mL, Cytiva) and ion-exchange chromatography columns (e.g., Mono Q/S, 1 mL, Cytiva).
5. UV-Vis spectrophotometer.
6. Ultrasonic bath.
7. Vacuum filtration system for buffers.
8. Lyophilizer or centrifugal evaporator with cold trap ("SpeedVac").
9. Optional: Anaerobic atmosphere (e.g., nitrogen glove box).

2.2 Reagents

1. 1 mL of a 1 M solution of Tris(2-carboxyethyl)phosphine (TCEP) in water. Adjust with sodium hydroxide to pH 7. Store in aliquots at −20 °C.
2. 1 mL of a 1 M solution of dithiothreitol (DTT) in water. Store in aliquots at −20 °C and minimize room temperature exposure to avoid oxidation.
3. RP-HPLC buffer A: 0.5 L of water with 0.1% (v/v) trifluoroacetic acid (TFA). Prepare freshly, do not use for more than 2 days. Stir to mix components properly (*see* **Note 2**).
4. RP-HPLC buffer B: 0.5 L of 100% acetonitrile (ACN).
5. Ion-exchange binding buffer (low salt): 0.5 L of 20 mM potassium phosphate pH 7.2, 20 mM potassium chloride, filtered and degassed. Optional: Add 0.001% Tween 20 after filtration (*see* **Note 3**).
6. Ion-exchange elution buffer (high salt): 0.5 L of 20 mM potassium phosphate pH 7.2, 1 M potassium chloride, filtered and degassed. Optional: Add 0.001% Tween 20 after filtration (*see* **Note 3**). Ideally, the ion-exchange buffer system should fulfill all the criteria listed under *Labeling buffer*, so the protein can be used for labeling directly as it elutes from the column.
7. Dimethyl sulfoxide, anhydrous (DMSO): Store desiccated.
8. Dimethyl formamide, anhydrous (DMF): Store desiccated.
9. Dissolve 1 mg of maleimide-functionalized dye (*see* Subheading 3.1.2) in 200 µL DMSO and sonicate in ultrasonic bath for 10 min to monomerize the dye. Split into 10–20 µL aliquots and lyophilize to obtain 50–100 µg aliquots. Store at −20 °C. Lyophilized dye can be stored for >1 year at −20 °C without significant loss in reactivity.
10. Labeling buffer (*for RP-HPLC*): e.g., 0.1 M potassium phosphate, pH 7.0. Prepare 10 mL by mixing 0.615 mL 1 M K_2HPO_4 and 0.385 mL 1 M KH_2PO_4 with 9 mL of water, followed by degassing. Store at 4 °C. Addition of salt or glycerol is possible, as well as the use of other buffer systems (e.g., HEPES). The pH should be between 7.0 and 7.4 in

order that a sufficient fraction of cysteine residues (pK_a = 8.3–8.5) are in the reactive thiolate anion form but no cross-reactivity with amines occurs [25].

The following chemicals should be avoided: (1) primary amines in the buffer (e.g., Tris), as they exhibit some reactivity towards maleimides; (2) thiols (DTT, 2-mercaptoethanol), as they compete with the cysteine for the maleimide-functionalized dye; and (3) TCEP, as it can react with maleimides [17, 26] and undergo other side reactions [27].

For ion-exchange chromatography, labeling can be performed directly in the elution buffer if the buffer system meets the abovementioned criteria.

11. Denaturing labeling buffer: 10 mL of 6 M GdmCl and 0.1 M potassium phosphate, pH 7.0. The true pH of GdmCl-containing solutions can be obtained according to Ref. 28. This buffer should only be used if efficient refolding of the protein of interest is possible and labeling under native conditions is not feasible, e.g., because of protein aggregation or surface interactions.
12. Liquid nitrogen.
13. 10 mg/mL of trypsin protease in 1 mM HCl. Store in aliquots at −80 °C.
14. Trypsin digest buffer: 50 mM HEPES, pH 7.5, and 100 mM NaCl. Store at 4 °C.

3 Methods

3.1 Selection of Labeling Sites and Fluorophores

3.1.1 Design Considerations for Introducing Cysteine Residues

1. Remove solvent-accessible cysteine residues from the protein by site-directed mutagenesis, e.g., by replacing them with serine residues. Check the stability and/or functionality of the modified protein if necessary.
2. Introduce two cysteine residues at suitable sites (e.g., replacing serine) by site-directed mutagenesis. For folded proteins, labeling sites should be solvent-exposed and separated by a distance that maximizes the change in transfer efficiency during the process of interest. A computational tool is available to identify labeling sites for best resolution [29]. In IDPs and unfolded proteins, labeling sites should be spaced ~40–100 amino acids apart, depending on the amino acid sequence and residual structure [30].
3. Avoid labeling sites where dyes can come into direct contact to prevent self-quenching [31]. Tryptophan residues in close proximity to the dyes can also quench fluorescence by photo-induced electron transfer [21, 32] and can complicate the quantitative analysis of transfer efficiencies (*see* Fig. 1c) and fluorescence correlation experiments [21]. In IDPs, such quenching effects can modulate the signal up to a sequence separation of ~20 amino acids between dye and quencher

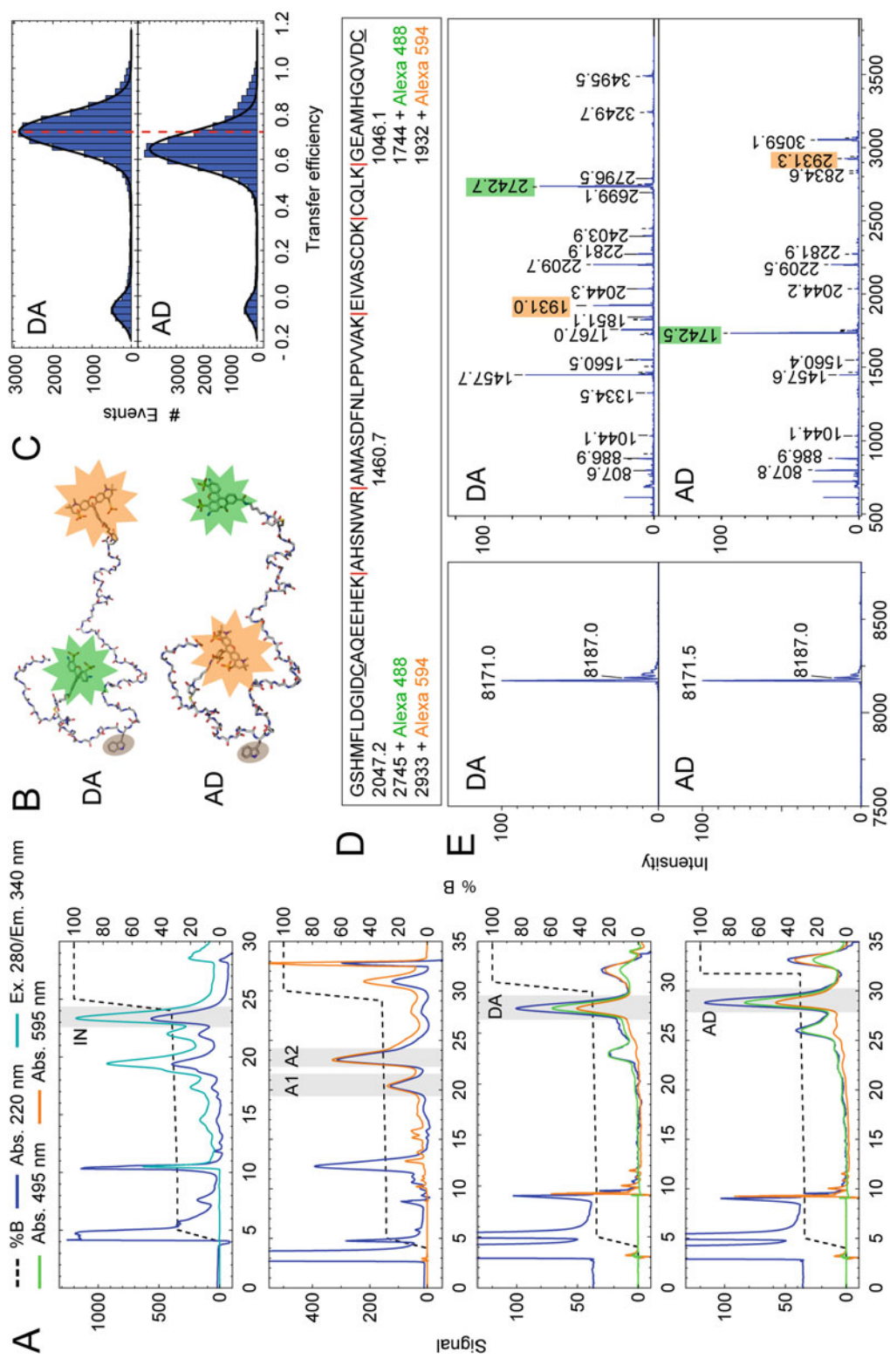

Fig. 1 Sequential labeling and RP-HPLC-based purification of the intrinsically disordered N-terminal domain of HIV-1 integrase (IN; 60 residues). (**a**) *First panel*: RP-HPLC run of unlabeled IN. Several truncation products elute before the full-length protein (shaded) and are removed before labeling. *Second panel*: IN was labeled with an 0.7:1 molar ratio of Alexa 594 dye to protein. In the RP-HPLC run, unlabeled protein (12 mL) elutes before the two single-labeled permutants

(20 and 23 mL) and double-labeled protein (31 mL). *Third panel*: Single-labeled protein from peak A1 was reacted with an equimolar amount of Alexa 488, yielding sample DA. *Fourth panel*: Single-labeled protein from peak A2 was reacted with an equimolar ratio of Alexa 488, yielding sample AD. (**b**) Backbone representation of IN DA and AD products, with dyes attached (Alexa 488: green, Alexa 594: orange). A Trp residue (brown) can quench the dye at position 11. (**c**) Single-molecule measurements of IN DA and AD. The transfer efficiency peak of AD is shifted to lower values owing to quenching of the acceptor dye in this construct [21]. The peak below zero transfer efficiency arises from molecules lacking an active acceptor dye (the shift to $E < 0$ is caused by the correction for direct excitation of the acceptor). (**d**) Sequence of IN, with tryptic fragments indicated. Additionally, the expected masses upon reaction with either Alexa 488 (+698 Da) or Alexa 594 (+886 Da) are given. The cysteine residues used for labeling are underlined. The reactivity of the other cysteine residues was suppressed by the complexation with Zn^{2+} ions. (**e**) *Left*: ESI-MS spectra of both donor–acceptor-labeled IN variants from (**a**) (expected mass: 8170.5 Da). Additional peaks with a mass of +16 suggests methionine oxidation. *Right*: Corresponding MALDI-MS spectra after trypsin digest. In the sample from peak DA, only the N-terminal fragment is detected with an Alexa 488 modification (green), and only the C-terminal fragment with an Alexa 594 modification (orange). The peaks from the other permutation are absent. In the sample from peak AD, this pattern is switched, confirming site-specific labeling in both cases

[33]. If necessary, replace tryptophan by phenylalanine by site-directed mutagenesis. Generally, rhodamine and oxazine dyes are more susceptible to quenching than cyanine dyes [34].

4. To maximize site-specific labeling, the reactivity of the individual cysteine residues can be tuned by modulating the pK_a of the thiol group [18, 35]. Positive charges in the vicinity will lower the pK_a and increase the population of the reactive sulfhydryl anion at pH 7. Conversely, negative charges near the sulfhydryl group decrease its reactivity by increasing its pK_a. Prediction tools for cysteine reactivity, pK_a, and solvent accessibility exist for folded proteins [36, 37]. For unfolded proteins, a cysteine residue at the negatively charged C-terminus will have a lower reactivity than a cysteine at the N-terminus. The difference in reactivity can be further increased by introducing charged residues as immediate neighbors. Under these conditions, adding sub-stoichiometric concentrations of dye usually results in the preferential modification of one cysteine residue and increases the overall yield of site-specifically labeled protein.

3.1.2 Choice of Dye Pair

Fluorophores suitable for single-molecule FRET spectroscopy are characterized by high photostability and quantum yield. Purchase maleimide-functionalized dyes to label the sulfhydryl groups of cysteine residues within the protein of interest. Among the best-established labels are the Alexa Fluor dye series (Thermo Fisher Scientific), particularly Alexa Fluor 488, 594, and 647; the ATTO dye series (ATTO-TEC); and the cyanine dyes (Cytiva, Thermo Fisher Scientific). The CF dye series (Biotium) includes suitable and very hydrophilic far-red dyes, in particular CF640R, CF660R, and CF680R. Some "self-healing" cyanine dyes are also commercially available (Lumidyne Technologies), offering higher photostability than their unmodified precursors [38]. Select the dyes according to the required spectral properties (e.g., excitation and detection wavelengths) and the expected inter-dye distance. The Förster radius of the dye pair should match the expected distances to yield maximum transfer efficiency resolution. When working in a high-fluorescence background environment (e.g., inside live cells), using longer excitation wavelengths has proven useful to reduce problems with background luminescence or cellular autofluorescence [39, 40]. Furthermore, take into account dye charge in the selection of the FRET pair: when charge interactions are governing the behavior of a protein, select a dye pair that perturbs the overall charge distribution as little as possible. The retention of dyes on RP-HPLC columns can be used as an indicator for the hydrophobicity of fluorophores and can thus aid the selection for hydrophilicity [41].

3.2 Preparing the Protein for Labeling

Purify the protein of interest (with two cysteine residues for labeling) according to established protocols. Include reducing agent during the purification to avoid disulfide-mediated adduct

formation and to maintain the reduced state of the cysteine residues. The requirements for efficient labeling are: (1) maintaining the reactive reduced state of the cysteine residues; (2) removing impurities and reducing agents that interfere with the labeling reaction; and (3) obtaining the protein at high concentration (>10 μM) to maximize the labeling rate (*see* **Note 4**). With the following protocols, protein samples meeting these requirements can be prepared efficiently in a single chromatography step (based on RP-HPLC or ion-exchange chromatography). The respective chromatography can also be used for further purification steps of the dye-labeled protein, using similar elution gradients throughout. The protocols are optimized for initial protein amounts of 0.1–2 mg (RP-HPLC) or 3–50 mg (ion-exchange chromatography).

3.2.1 RP-HPLC–Based Purification

RP-HPLC separates molecules by hydrophobicity. After chromatography, the protein is lyophilized and can readily be dissolved in any buffer to any concentration, which eliminates the need for buffer exchange or concentration steps and greatly enhances the overall yield (*see* **Note 5**). The low pH (≈2) of the mobile phase and the absence of oxygen during lyophilization keep the cysteine residues reduced until the protein is dissolved for labeling. As the protein is usually denatured during HPLC, this approach is useful for proteins that can be refolded or do not require refolding (peptides, IDPs) and works best for peptides and proteins with a mass <15 kDa.

The RP-HPLC gradient is designed to separate the protein of interest from impurities in a minimum amount of time. A useful starting point for the purification of most proteins is given below.

Step 1: 1 column volume (CV) at 5% B (*see* **Note 6**)—elution of all molecules that do not interact with the column (salts, reducing agents, etc.).

Step 2: 1 CV at 10% B—elution of all molecules that interact weakly with the column.

Step 3: 6 CVs with a gradient from 10% to 60% B—elution of the protein of interest (*see* **Notes 7** and **8**).

Step 4: 1 CV at a 100% B—elution of tightly bound molecules.

Estimate the percentage of buffer B where the protein elutes (%B_{el}) by calculating %B 1 CV before the detection of the protein in the absorbance channel (include the volume of the injection loop in the calculation). Adjust the gradient by increasing %B at step 2 and making the gradient in step 3 shallower. As a rule of thumb, a gradient from %B_{el}−15% to %B_{el}+5% over 5 CVs yields good separation; the protein should elute at the end of the gradient to achieve a narrow peak. If there are impurities eluting close to the protein of interest, a shallower gradient of 10% over 5 CVs is recommended.

The semi-preparative columns used here are operated with a flow rate of 1 mL/min.

A standard RP-HPLC purification protocol is as follows:

1. Use a sample that is already enriched in the protein of interest (>50%, e.g., by immobilized metal ion-affinity chromatography), ideally with protein concentrations >50 μM. The semi-preparative HPLC columns used here have a binding capacity of roughly 1 mg protein.
2. Add 5 mM TCEP or DTT to the sample. Incubate for 10 min to fully reduce the protein (*see* **Note 9**).
3. Perform a test run to identify at what percentage of buffer B the protein elutes. For that purpose, dilute 20 μL (>1 nmol) of the protein in HPLC buffer A and inject. Optimize the gradient accordingly. Detect the peptide bond absorbance at 220 nm (*see* **Note 10**), and the absorbance of aromatic amino acids at 280 nm. Excite and detect fluorescence according to the spectral properties of the aromatic residues in the protein of interest (Phe: 256/280 nm, Tyr: 275/305 nm, Trp: 280/350 nm) to distinguish it from impurities.
4. Inject 0.5–1 mg of the sample and collect the fraction(s) of interest. If desired, remove 50 μL of the elution peak for mass spectrometry. Perform multiple runs for larger amounts of protein to maintain good peak resolution (*see* **Note 11**).
5. Flash-freeze the collected fractions in liquid nitrogen immediately after collection.
6. Lyophilize samples in a lyophilizer or centrifugal evaporator and store dry at −80 °C until use. In this state, the sulfhydryl groups on the protein typically remain reactive for years.

3.2.2 Ion-Exchange–Based Purification

If a protein is too large for HPLC or its folded state must be maintained during labeling, ion-exchange chromatography is the purification method of choice. The method separates molecules by surface charge. The protein binds to the column and is eluted with a salt gradient, which concentrates dilute samples in the process.

Ion-exchange chromatography requires the choice of a suitable ion-exchange column, a corresponding working pH and an initial salt concentration. Depending on the net charge of the protein, either an anion- or cation-exchange column is selected for purification. For proteins with an isoelectric point (pI) < 7.0, we recommend using an anion-exchange column (Mono Q); for proteins with a pI > 7.5, a cation-exchange column (Mono S). The pH of the binding and elution buffers should be between 7.0 and 7.5, and at least 0.5 units higher (anion-exchange chromatography) or lower (cation-exchange chromatography) than the pI of the protein. In this way, the protein binds to the column at low salt concentrations and elutes in a buffer that is suitable for labeling. Accordingly, we recommend the use of phosphate or HEPES

buffers for ion-exchange chromatography (*see* also Subheading 2.2, **Note 12**). The optimal salt concentration in the initial binding buffer strongly depends on the net and surface charge of the protein; a typical buffer system is described in Subheading 2.2. For more information, refer to Ref. 42.

A standard ion-exchange purification protocol is as follows:

1. Buffer-exchange the sample by dialyzing against binding buffer (supplied with 1 mM TCEP or DTT) or using a desalting column (e.g., HiTrap).
2. Add 5 mM TCEP or DTT to the sample. Incubate for 10 min to completely reduce the protein (*see* **Note 9**).
3. Equilibrate the ion-exchange column with 10 CVs binding buffer, followed by 10 CVs elution buffer and 10–20 CVs binding buffer. Equilibrating with elution buffer is necessary to saturate the functional groups of the column with the chosen counter ion and to obtain a stable, linear salt gradient for elution. Record the absorbance at 280 nm (or at 220 nm if the protein does not contain aromatic residues, *see* **Note 10**).
4. Apply 3–50 mg of protein, either using a sample pump or by injection (if applying a small volume) (*see* **Note 13**).
5. Wash the column with binding buffer until the absorbance at 280 nm returns to baseline.
6. Elute the protein with a gradient of 0–40% elution buffer in 40 CVs. Optimize the gradient if required (*see* **Note 14**).
7. Use the collected fractions immediately for labeling or flash-freeze in liquid nitrogen and store at −80 °C.

3.3 Labeling Reaction

The starting material for labeling is the protein purified either by RP-HPLC or ion-exchange chromatography (*see* Subheading 3.2). Depending on the desired purity and yield of the product, different labeling strategies are possible, as outlined below. If you purify the protein with RP-HPLC, you can choose between native and denaturing labeling conditions (*see* Subheading 2.2). We recommend labeling under denaturing conditions if the protein is aggregation-prone or sticky, i.e., interacts strongly with surfaces. For purification with ion-exchange chromatography, use the dye with the highest net charge in the first labeling step to maximize resolution during purification.

There are several strategies for labeling depending on the order of addition of the two dyes to the reaction mixture. In the sequential labeling strategy, the protein is first labeled with one dye at a sub-stoichiometric ratio of dye to protein. Single-labeled protein is purified and then reacted with the second dye. The first dye will primarily couple to the more reactive cysteine residue, so choose the dye accordingly. If both cysteine residues have similar reactivities, a separation of labeling permutants can often be achieved chromatographically (*see* Figs. 1a and 2a). This approach typically

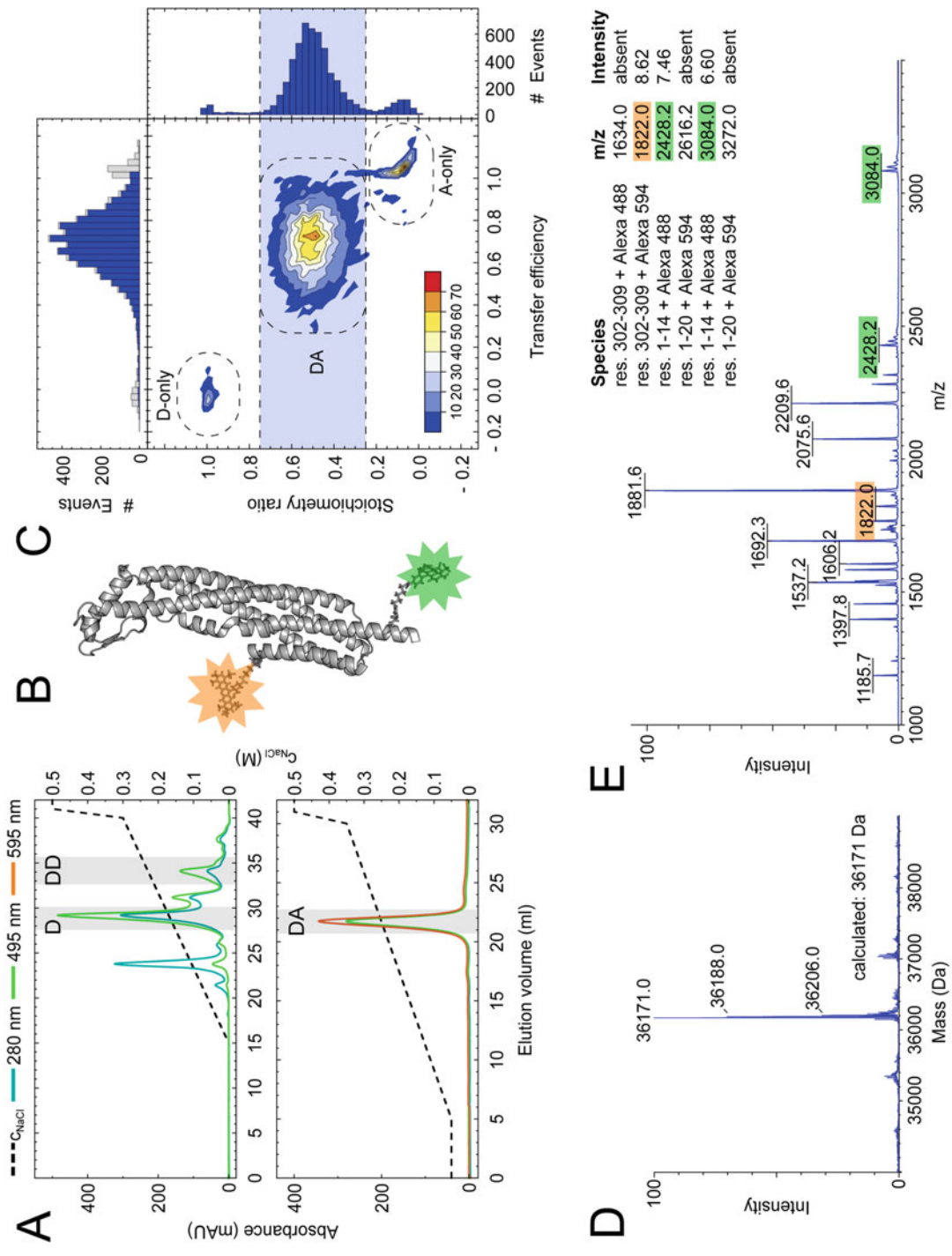

Fig. 2 Sequential labeling and anion-exchange-based purification of the pore-forming toxin cytolysin A, with reactive cysteines introduced at positions 8 and 309 (ClyA; 309 residues) [46]. (**a**) *Top panel:* ClyA was labeled with a 0.7:1 molar ratio of Alexa 488 dye to protein and purified with anion exchange

chromatography on a MonoQ column. Unlabeled protein (24 mL) elutes before the two single-labeled permutants (29 and 31 mL) and double-labeled protein (34 mL). *Bottom panel*: Anion exchange chromatography after the second labeling reaction. Single-labeled protein from peak D at 29 mL was incubated overnight with a threefold molar excess of Alexa 594. Before ion exchange chromatography, uncoupled dye was removed from the sample on a HiTrap desalting column. (**b**) Structure of ClyA (PDB 1QOY [47]) with dyes attached (Alexa 488: green, Alexa 594: orange). (**c**) Single-molecule measurement of donor–acceptor-labeled ClyA. Fluorescence was collected after pulsed-interleaved excitation, so the relative fractions of donor-only, donor–acceptor, and acceptor-labeled protein can be identified from the transfer efficiency vs. stoichiometry ratio plot. The transfer efficiency histogram (*top*) is depicted before (gray bars) and after (blue bars) selecting bursts with a stoichiometry ratio between 0.25 and 0.75 (blue-shaded area). The projected stoichiometry ratio histogram is shown to the right. (**d**) ESI-MS spectrum of donor–acceptor-labeled ClyA with peak masses indicated. The expected mass of donor–acceptor-labeled ClyA is 36,171 Da. (**e**) MALDI-MS spectrum of ClyA after trypsin digest. Only the N-terminal fragment is detected with an Alexa 488 modification (green), and only the C-terminal fragment with an Alexa 594 modification (orange). The peaks from the other permutation are absent. The remaining peaks correspond to ClyA peptides without reactive cysteine residues

yields the highest quality samples. Advantages are that site-specific labeling is possible; the product usually has the highest purity; and correctly labeled fractions are easily identified from the chromatogram. A disadvantage is that two chromatography runs are required, which might decrease the overall yield.

An alternative is to perform both labeling reactions simultaneously. In this case, both dyes are added at the same time at equimolar concentrations. This approach is particularly useful for preliminary tests of the suitability of labeling positions or if only small amounts of protein are available. An advantage is that only one chromatography run is required. The disadvantages are that no site-specific labeling is possible; the elution profile is more heterogeneous since it contains all possible labeled species, so the correctly labeled fraction might be difficult to identify from the chromatogram; and additional quality control (FRET, mass spectrometry) of several fractions is often required to identify the correctly donor–acceptor–labeled protein.

Finally, one can perform the labeling reaction in semi-sequential fashion. In this case, the first dye is added at a sub-stoichiometric ratio of dye to protein and incubated to react with the protein to completion. Afterwards, an excess of the second dye is added to the same reaction. This approach is useful if only small amounts of protein are available but site-specific labeling is important. Advantages are that only one chromatography run is required and that site-specific labeling is possible if the cysteine residues have sufficiently different reactivity. Disadvantages are that the second cysteine residue might lose reactivity due to oxidation during incubation with the first dye, especially at high protein concentrations; the elution profile is more heterogeneous, so the correctly labeled fraction might be difficult to identify; and additional quality control (FRET, mass spectrometry) of several fractions is often required to identify the donor–acceptor–labeled protein.

A standard labeling protocol is outlined here:

The following steps are carried out in a low-humidity and oxygen-free atmosphere (e.g., in a nitrogen glove box), if available.

1. Dissolve the dye in DMSO to a concentration of 10 μg/μL or in DMF to a concentration of 5 μg/μL. The solubility of dyes in DMF is generally lower than in DMSO. Use DMF for proteins prone to methionine oxidation (*see* Subheading 3.6.5). Protect the dissolved dye from light.
2. Sonicate the dissolved dye in an ultrasonic bath for 15 min to completely dissociate dye oligomers, which can increase the fraction of undesired double-donor- or double–acceptor–labeled protein.

3. Determine the protein concentration. *RP-HPLC*: Dissolve the lyophilized protein in labeling buffer (native or denaturing) to reach a protein concentration of 10–200 μM (*see* **Notes 4**, **15**, and **16**). *Ion-exchange chromatography*: Determine the protein concentration in the elution fraction. If the protein is already coupled to the first dye, use the dye absorbance to calculate the protein concentration. For an example calculation, *see* **Note 17**.

   Add the dye to the protein solution according to the respective labeling scheme and mix the labeling reaction gently. When working with aggregation-prone proteins, add the protein solution to the dissolved dye to ensure rapid mixing. Keep the percentage of organic solvent below 5% for maximum labeling efficiency. Unused dye dissolved in organic solvent can be lyophilized and reused. For an example calculation of how much dye to add to a labeling reaction, *see* **Note 17**.

   Sequential labeling with first dye: Add a 0.7:1 molar ratio of dye to the protein solution.

   Sequential labeling with second dye: Add a 1.2–3-fold molar excess of dye to the protein solution.

   Simultaneous labeling: Add both dyes at an equimolar ratio to the protein (i.e., 1:1:1).

   Semi-sequential labeling: Add a 0.7:1 molar ratio of the first dye to the protein solution. Incubate the reaction protected from light for 1–2 h at room temperature. Add the second dye in a 1.2–3-fold molar excess to the protein solution.

4. Incubate the reaction protected from light for 2–3 h at room temperature or overnight at 4 °C (which can increase the labeling yield).

5. Quench unreacted dye (to reduce its affinity to the column) and reduce oxidized cysteines by addition of 10 mM DTT. Add the labeling reaction to the DTT solution to ensure fast mixing. Incubate for 10 min at room temperature and either purify the reaction directly or flash-freeze it in liquid nitrogen and store at −80 °C until further purification.

3.4 Purification of Dye-Labeled Protein

In this step, donor–acceptor-labeled protein is purified from the labeling reaction. The most common impurities that interfere with single-molecule experiments are: (1) dye not coupled to the protein and (2) protein carrying only donor dye(s). The purification procedure is independent of the labeling strategy and needs to be carried out twice for sequential labeling.

3.4.1 RP-HPLC-Based Purification

Since dyes usually contain large aromatic moieties, dye-coupled proteins often have a larger retention time than the unmodified protein, and single-labeled protein elutes before double-labeled protein (*see* **Notes 18** and **19**; Figs. 1a and 3b). In many cases, it

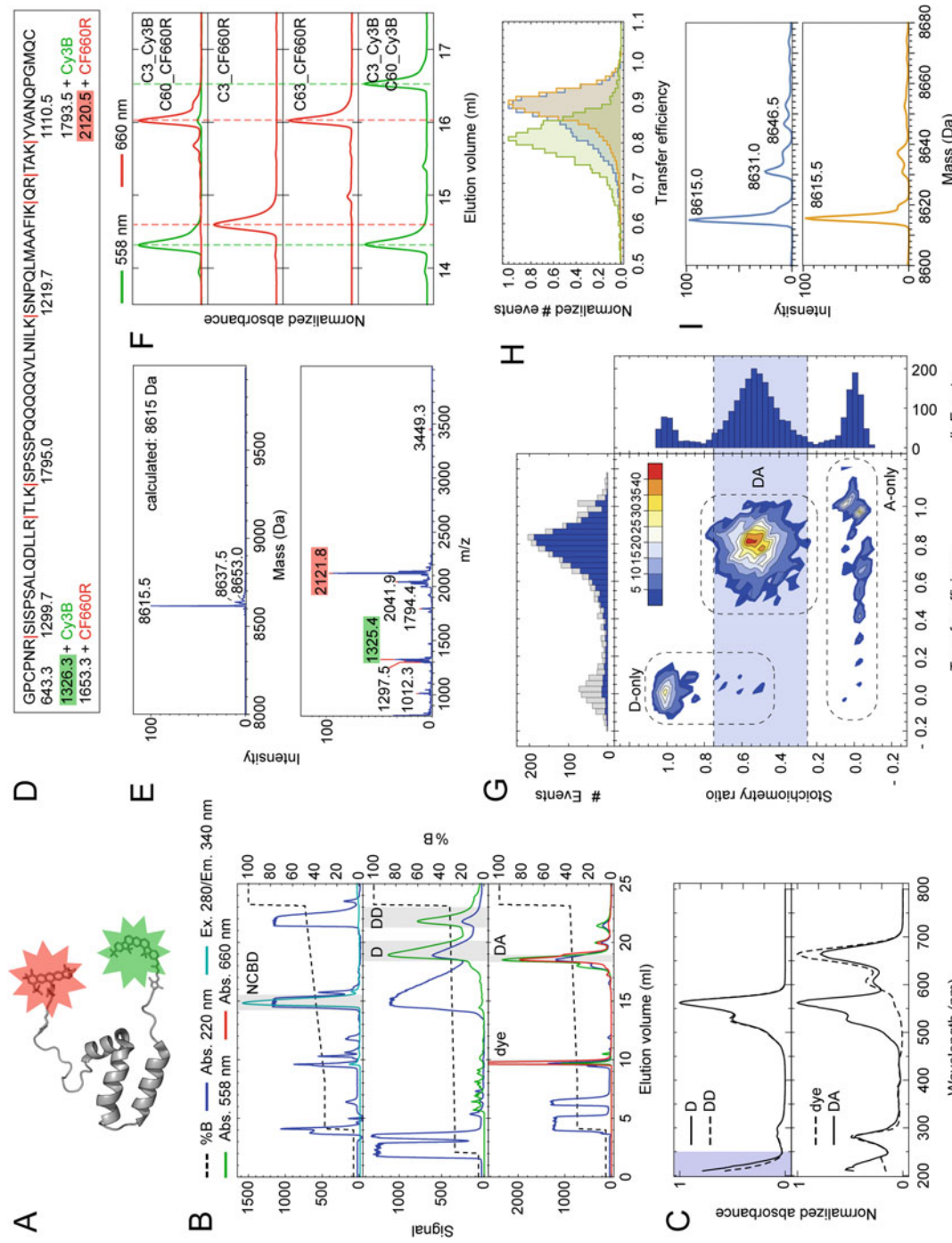

Fig. 3 Site-specific labeling, RP-HPLC-based purification, and characterization of the molten-globule-like nuclear coactivator binding domain of CBP/p300 (NCBD; 63 residues). (**a**) Structure of NCBD (PDB 2KKJ [48]), with dyes attached (Cy3B: green, CF660R: red). (**b**) RP-HPLC purification of the unlabeled, single-labeled, and

donor–acceptor-labeled protein. The fractions of interest are shaded in gray. *Top*: NCBD was co-expressed with its binding partner ACTR and can be distinguished from it and other impurities by its fluorescence peak at 340 nm (arising from a single Tyr residue). *Middle*: NCBD was incubated for 3 h with a 0.7:1 molar ratio of Cy3B to protein. Unlabeled protein elutes before single-labeled (D) and double-labeled protein (DD). *Bottom*: The single-labeled protein was incubated for 3 h with a 1.5-fold molar excess of Biotium CF660R and purified (DA). Unreacted dye elutes before the labeled protein, reflecting its hydrophilicity. (**c**) Absorbance spectra recorded during the HPLC runs in (**a**), with the corresponding elution peaks indicated. *Top*: Single-and double-Cy3B-labeled protein can be distinguished by their 220/558 nm absorbance ratios. The absorbance spectrum of the single-labeled protein (black line) has a higher relative absorbance in the peptide bond region ($<$250 nm, blue-shaded area). *Bottom*: Donor–acceptor-labeled protein (DA) can be distinguished from free dye by its absorbance spectrum. In the elution peak DA, the absorbance ratio at the emission maxima of both dyes matches the ratio of the extinction coefficients provided by the suppliers (Cy3B: 130.000 M^{-1} cm^{-1}, CF660R: 100.000 M^{-1} cm^{-1}). (**d**) NCBD sequence with trypsin cleavage sites and the masses of the individual fragments indicated. The N- and C-terminal fragments contain the cysteine residues for labeling (C3/C63). The expected molecular weights of these fragments after coupling of Cy3B (+683 Da) or CF660R (+1010 Da) are shown. (**e**) *Top*: The ESI-MS spectrum of donor–acceptor-labeled NCBD shows a dominant peak at the expected mass. *Bottom*: MALDI-MS spectrum of NCBD after trypsin digest. The N-terminal fragment is only detected with a Cy3B modification (green), the C-terminal fragment only with a CF660R modification (red). (**f**) Analytical RP-HPLC elution profiles of NCBD after trypsin digest confirm site-specific labeling close to 100%. By digesting different NCBD variants, we are able to determine the elution times of all possible labeled peptides. *First panel*: donor–acceptor-labeled NCBD; *second panel*: single-cysteine NCBD variant labeled with CF660R at C3; *third panel*: single-cysteine NCBD variant labeled with CF660R at C63; *fourth panel*: double-Cy3B-labeled NCBD. Thus, in the Cy3B/CF660R variant, we find C3 exclusively modified with Cy3B, and C63 exclusively modified with CF660R. (**g**) Single-molecule measurement of donor–acceptor-labeled NCBD. Fluorescence was collected after pulsed-interleaved excitation, so the relative fractions of donor-only, donor–acceptor, and acceptor-labeled protein can be identified from the transfer efficiency vs. stoichiometry ratio plot. The transfer efficiency histogram (*top*) is depicted before (gray bars) and after (blue bars) selecting bursts with a stoichiometry ratio between 0.25 and 0.75 (blue-shaded area). The projected stoichiometry ratio histogram is shown to the right. (**h**) Methionine oxidation in NCBD. Transfer efficiency histograms of reduced (orange), partly oxidized (blue), and fully oxidized (green) NCBD. Protein oxidation causes a pronounced shift to lower transfer efficiencies. (**i**) ESI-MS spectra of partly oxidized (blue) and reduced (orange) NCBD. In the upper MS spectrum, oxidation products with additional masses +16 and +31.5 are present, suggesting the addition of one or two oxygen atoms. This observation can be attributed to the oxidation of two methionine residues in the protein (sequence in (**d**))

is possible to separate the two labeling permutants, so site-specific labeling can be achieved (Fig. 1a). In some cases, dye isomers can lead to different elution times of the labeled protein, resulting in multiple peaks for protein carrying a single dye. When labeling with donor and acceptor dye simultaneously, these effects can lead to very complex chromatograms. We recommend a shallow gradient (a change of 5–10% B over 5 CVs) to resolve the different labeled species. Shallower gradients usually improve separation. The peak corresponding to unlabeled protein can be collected and used in another labeling reaction. If you label a large amount of protein (>0.5 mg), it is advisable to purify the labeling reaction in two runs to maintain peak separation. If the donor–acceptor-labeled protein does not have the desired quality (e.g., donor-only–labeled contamination), another RP-HPLC run on a column with different properties usually helps to isolate the desired species.

A standard RP-HPLC purification protocol is outlined here:

1. Perform a test run on the HPLC to optimize the elution gradient (the gradient from the initial purification run is a good starting point). For that purpose, dilute 5 μL of the quenched labeling reaction in RP-HPLC buffer A and inject. Detect protein absorption at 220 nm and dye absorption at the appropriate wavelength (*see* **Note 20**). If using fluorescence detection, excite at 280 nm; detect aromatic residues at 340 nm and the dye at its emission wavelength. When labeling with donor and acceptor simultaneously, excite fluorescence at the donor absorbance maximum and detect at the donor and acceptor emission maxima to identify the correctly labeled species via FRET.
2. Inject the labeling reaction and collect the appropriate peaks. If desired, take a 50 μL sample for mass spectrometry (*see* **Note 11**).
3. Flash-freeze the appropriate fractions in liquid nitrogen immediately after collection.
4. Lyophilize samples and store dry at −80 °C until use (or proceed analogously to labeling with the second dye).

3.4.2 Ion-Exchange Chromatography-Based Purification

Many commonly available dyes have a nonzero net charge to increase their hydrophilicity, so that labeling often alters the charge of the protein. Ion-exchange chromatography then allows the separation of unlabeled, single-labeled, and double-labeled reaction products. As in the case of RP-HPLC, the single-labeled permutants often elute as two separable peaks from the column, thus enabling site-specific labeling. In ion-exchange chromatography, the elution profile strongly depends on the net charge of the protein and the dyes. For example, when labeling a positively charged protein with a negatively charged dye, the double-labeled

species is expected to elute first from a cation-exchange column, followed by single- and unlabeled protein. Optimization of the gradient can lead to better separation, but for ion-exchange chromatography this is not as straightforward as for RP-HPLC. Very shallow salt gradients usually lead to peak broadening rather than improved separation. pH and counter ions can be altered systematically to optimize the separation of labeling products [42].

A standard ion-exchange purification protocol is outlined here:

1. Equilibrate the ion-exchange column with binding buffer (*see* Subheading 3.2.2). Record the absorbance at 280 nm and at the wavelength of maximum absorbance of the dyes. If using fluorescence detection, proceed analogously to RP-HPLC.
2. Dilute the labeling reaction ~3-fold in the binding buffer or exchange the buffer with a desalting column to enable binding of the labeled protein to the ion-exchange column. When labeling with an excess of dye, remove it with a desalting column prior to ion-exchange chromatography.
3. Inject the labeling reaction and wash the column with binding buffer until the absorbance at 280 nm returns to baseline.
4. Elute the protein with a gradient of 0–40% elution buffer in 40 CVs (*see* **Note 14**).
5. Use the collected fractions immediately for labeling with the second dye or flash-freeze in liquid nitrogen and store them at −80 °C until use.

3.5 Preparation of Samples for Single-Molecule Spectroscopy

1. *Lyophilized protein from RP-HPLC*: Dissolve the protein in a suitable buffer (e.g., 50 mM sodium phosphate, pH 7) at a concentration of 1–100 μM. Since the protein will be diluted >10,000-fold for single-molecule measurements, it is possible to use additives to increase protein stability or solubility in the stock solutions if necessary (e.g., glycerol, GdmCl) without interfering with the final solution conditions of the measurements. *Ion-exchange–purified protein:* Determine the concentration via the absorbance of the dyes. Add stabilizing agents if necessary. Do not include thiols in storage buffers as they can promote hydrolysis of the maleimide-sulfhydryl bond over time [43].
2. Split the protein sample into small aliquots for single use to avoid repeated freeze–thaw cycles. Flash-freeze aliquots in liquid nitrogen and store at −80 °C.
3. For more dilute aliquots (1–100 nM protein), include 0.001% Tween 20 (v/v) in the buffer to avoid loss of protein to surfaces.

3.6 Quality Control

3.6.1 Protein Identity and Impurities

The identity of donor–acceptor-labeled protein is most reliably assessed with electrospray ionization mass spectrometry (ESI-MS), as depicted in Figs. 1e, 2d, and 3e. Common contaminants are double-donor and double-acceptor–labeled species. If 2-mercaptoethanol was used as a reducing agent, peaks with an additional mass of +76 Da point towards the formation of 2-mercaptoethanol-cysteine adducts. Note that the molecular weight of the dye given by the supplier often does not match the observed shift in mass, since it includes counter ions that are usually removed during purification.

3.6.2 UV-Vis Absorption Spectrum

Record a UV-Vis absorption spectrum of the labeled protein under native and denaturing (e.g., in 6 M GdmCl) conditions. Note that the peaks might shift slightly (<10 nm) compared to free dye.

1. Check the absorption maxima of both dyes. Do their relative amplitudes match the ratio of the published extinction coefficients? If not, this might be an indication of double-donor or double-acceptor labeled species in the sample (*see* **Notes 20** and **21**).
2. Check the shape of the recorded spectra. Are they similar under native and denaturing conditions? If not, this might indicate a stacking interaction of the dyes, which will perturb their spectroscopic properties [21, 31].

3.6.3 Single-Molecule Spectroscopy

A FRET efficiency histogram from a confocal recording of freely diffusing donor–acceptor-labeled protein is a straightforward way to assess sample purity (or a camera-based recording of immobilized samples if total internal reflection fluorescence is used). Ideally, use alternating or pulsed-interleaved excitation [15, 44] to determine the amount of donor-only, donor-acceptor, and acceptor-only species in the sample (*see* Fig. 2c and 3g). Donor-only and acceptor-only peaks can even be present if the donor–acceptor-labeled protein is very pure according to chromatography or mass spectrometry, e.g., owing to background fluorescence or the inactivation of dyes in a previous passage through the confocal volume. Note that in the presence of excessive amounts of single-labeled protein, even alternating excitation may not suffice to fully isolate the signal from donor–acceptor-labeled protein.

3.6.4 Site-Specific Labeling

Site-specific labeling can be confirmed by tryptic digest of the protein, either in combination with mass spectrometry (MS) or analytical RP-HPLC (*see* Fig. 3f).

A protocol for tryptic digestion is as follows:

1. Dilute 0.1–1 nmol of donor–acceptor-labeled protein in 50 μL trypsin digest buffer. The higher the sensitivity of the fluorescence detector, the less protein is required. If unlabeled cysteine residues are present in the protein, add 1 mM TCEP to the buffer.

2. Add a sub-stoichiometric amount of trypsin protease (1:10 molar ratio of trypsin:protein) and incubate overnight in the dark at 37 °C. For IDPs, a digestion time of 1 h is usually sufficient.
3. Analyze peptide fragments with analytical RP-HPLC on a C18 column. A gradient from 5% to 50% buffer B over 15 CVs usually yields good separation. Detect absorbance at 220 nm as well as at the absorbance maxima of the dyes. Excite fluorescence at 280 nm, detect at 340 nm and at the emission maxima of the dyes of interest.
4. Analyze the chromatogram. For a site-specifically labeled protein, only one peak should appear with the absorbance and fluorescence of each dye (*see* Fig. 3f). If the protein is not site-specifically labeled, two peaks will appear for each dye. The area under these peaks (dye absorbance) is proportional to the amount of dye coupled to each site. The identity of the labeling site can often be determined using additional information about the corresponding tryptic peptide, e.g., the predicted extinction coefficient at 220 nm or the fluorescence of aromatic amino acids.

Otherwise, site-specificity can be tested by MS. Again, the protein is digested with trypsin or other proteases (sometimes a service provided at the MS facility), and the mass of the tryptic peptides is analyzed. Depending on the dye modification, the tryptic peptides will have different masses. If only the mass of one peptide with donor dye is detected, and the mass of the peptide with acceptor dye is absent from the spectrum (and vice versa), then it is a good indication that site-specific labeling was achieved (*see* Figs. 1d, e, 2e, and 3d, e). Note that the relative peak areas in the MS spectrum are not quantitative because of different mobility of the different ions. The large negative charge of many commonly used fluorophores can reduce the mobility of labeled protein in positive ion mode substantially.

3.6.5 Methionine Oxidation

Oxidation of methionine to methionine sulfoxide is a common side reaction, e.g., when incubating proteins in the presence of DMSO for an extended time [45], so DMF should be used for labeling oxidation-prone proteins. Methionine oxidation is easily recognized by a +16 shift in the mass spectrum (or multiples of it in the case of several methionine residues, *see* Fig. 3i). It can have a significant effect on protein structure and activity, as illustrated in Fig. 3h. Methionine sulfoxide can be reduced again to methionine either enzymatically (using methionine sulfoxide reductases) or with dimethyl sulfide in the presence of 7 M hydrochloric acid [45], a harsh procedure that might be unsuitable for larger proteins.

4 Notes

1. R_0 can be calculated from the spectral overlap integral, J, between the fluorophores, the orientational factor, κ^2, the quantum yield of the donor, Q, and the refractive index, n, of the medium: $R_0 = \left(\frac{9000(\ln 10)}{128\pi^5 N_A} \frac{Q\kappa^2 J}{n^4}\right)^{1/6}$, with N_A being Avogadro's number. If the fluorophores rotate freely and the relative orientation of the fluorophore averages on a timescale shorter than the donor excited state lifetime, κ^2 averages to a value of $2/3$. The validity of this approximation can be tested by fluorescence anisotropy measurements [14]. J is calculated from the donor emission spectrum, $f_D(\lambda)$ (normalized to an area of 1), and the wavelength-dependent molar extinction coefficient of the acceptor, $\varepsilon_A(\lambda)$, according to $J = \int f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$.
2. Incomplete mixing can lead to shifts in the retention time of proteins. If used infrequently, it is advisable to purchase TFA in 1-mL glass ampules. For frequent use, buy TFA in a 1-L glass bottle for maximum shelf life. Use filter tips to handle the corrosive liquid.
3. The addition of the mild detergent Tween 20 to the buffer is not strictly required but significantly reduces protein–column interactions and increases the yield of eluted protein. The stability and function of proteins is usually not affected by low concentrations of Tween 20, but suitable tests should be performed for every protein.
4. This concentration range usually gives the best results. Lower concentrations lead to a lower labeling efficiency due to hydrolysis of the dye before it reacts with the protein. Higher protein concentrations can be less efficient because of the formation of intermolecular disulfide bridges competing with the maleimide addition.
5. We do not recommend using any membrane-based concentration or filtration steps during labeling and subsequent purification, since dye-coupled proteins often have the tendency to stick to ultrafiltration membranes, leading to loss of sample.
6. Use at least 5% ACN at all times to avoid collapse of the stationary phase of the column.
7. Especially when purifying IDPs, RP-HPLC allows the separation of the full-length protein from truncations (*see* Fig. 1a). Usually, the full-length protein elutes at higher percentage of buffer B.
8. If the protein of interest does not elute in the gradient (**step 3**) or at 100% B (**step 4**), it might have excessive affinity to the column. To elute it, inject 1 mL of 6 M guanidinium chloride

while flowing a mixture of 95% A/5% B over the column, followed by an elution gradient (without injecting sample).

9. Do not use 2-mercaptoethanol as a reducing agent in this step, as it can form cysteine adducts.
10. The wavelength for detecting peptide bond absorption can be adjusted from 200 to 235 nm, depending on the amount of protein, the required sensitivity, and the absorption of the buffer.
11. To maximize column lifetime, run an elution gradient without injecting sample after the purification run to remove residual bound protein (and dye).
12. Even though phosphate buffer is not recommended for anion-exchange chromatography by some suppliers (as phosphate ions strongly interact with the anion-exchange resin), it is frequently used in this application [42]. We recommend the use of phosphate buffer because it is ideal for subsequent labeling.
13. The volume applied to the ion-exchange column only matters if the protein does not strongly bind to the resin. In this case, concentrating and applying the protein in a small volume might be necessary. Alternatively, the salt concentration in the binding buffer can be reduced to enhance binding (e.g., using only 10 mM phosphate buffer). On the other hand, proteins that bind strongly to the ion-exchange resin will be highly concentrated on the column and therefore might aggregate. Lowering the affinity by increasing the salt concentration in the binding buffer can help to prevent aggregation.
14. To increase column lifetime, take care to remove any precipitated protein before injection, e.g., by centrifugation. Ion-exchange columns should be cleaned after each use by injections of 1–3 CV 1 M sodium hydroxide and 6 M GdmCl (both filtered), each followed by a wash with 10 CVs binding buffer.
15. Minimize the amount of time between dissolving the protein and starting the labeling reaction (ideally <15 min), especially at high protein concentrations, to minimize oxidation. The shorter the time, the higher the labeling efficiency.
16. For the first labeling reaction, it is crucial to accurately determine the protein concentration. The correct dye-to-protein ratio increases labeling efficiency and avoids excessive formation of double-labeled protein (with two identical dyes). If the protein does not contain aromatic residues that absorb at 280 nm, it is advisable to determine the extinction coefficient at 225 nm, either by dissolving a known amount of protein in a certain volume or by quantifying the protein concentration via

a bicinchoninic acid (BCA) or Bradford assay. The calculated extinction coefficient can subsequently be used for the rapid quantification of protein concentration with UV spectroscopy. Once the protein is coupled to a fluorescent dye, use the dye absorbance peak and published extinction coefficient to determine the protein concentration. Note that many dyes strongly absorb at 280 nm, which affects concentration determination via ε_{280} (cf. **Note 21**).

17. Use the Lambert-Beer law to determine the protein concentration (c_{Protein}) and total amount of protein (n_{Protein}) in a sample of volume V_{Protein}:

$$c_{\text{Protein}} = \frac{A_\lambda}{\varepsilon_\lambda \cdot d} \text{ and } n_{\text{Protein}} = c_{\text{Protein}} \cdot V_{\text{Protein}}$$

here, λ is the wavelength used for concentration determination, A_λ is the measured absorbance, d is the length of the optical path, and ε_λ the extinction coefficient at λ.

Example: Measured $A_{280} = 0.082$, with an optical path of 1 mm and ε_{280}=5500 M^{-1} cm^{-1}. The total volume of the sample is 400 µL. The dye (molecular weight 721 g/mol) is dissolved in DMSO at a concentration of 10 µg/µL and is to be reacted with the protein at a 0.7-fold molar ratio.

$$c_{\text{Protein}} = \frac{A_\lambda}{\varepsilon_\lambda \cdot d} = \frac{0.082}{5500 \text{M}^{-1}\text{cm}^{-1} \cdot 0.1 \text{ cm}} = 149 \text{ µM}$$

$$n_{\text{Protein}} = c_{\text{Protein}} \cdot V_{\text{Protein}} = 149 \frac{\text{nmol}}{\text{mL}} \cdot 0.4 \text{ mL} = 60 \text{ nmol}$$

$$c_{\text{Dye}} = \frac{10 \text{ gL}^{-1}}{721 \text{ g mol}^{-1}} = 0.0139 \text{ mol L}^{-1} = 13.9 \text{ nmol µL}^{-1}$$

$$V_{\text{Dye}} = \frac{n_{\text{Protein}} \cdot (\text{dye-to-protein ratio})}{c_{\text{Dye}}} = \frac{60 \text{ nmol} \cdot 0.7}{13.9 \text{ nmol µL}^{-1}} = 3.02 \text{ µL}$$

Add the 400 µL of protein solution to 3 µL of dissolved dye and mix rapidly, e.g., by pipetting or inverting the tube.

18. Usually, single- and double-labeled species can be distinguished by the ratio of peptide bond absorption (measured at 220 nm or 235 nm) to dye absorption (Fig. 3c). Judging protein absorption at 280 nm is not advisable if protein and dye have similar extinction coefficients at this wavelength. If a diode-array detector is present, record the absorbance spectra over the whole chromatography run to facilitate post-run analysis.
19. If there is insufficient separation between unlabeled and single-labeled protein, try another column with different properties (e.g., switch from a 5-µm to a 3.5-µm pore size).

20. Protein labeled correctly with two FRET dyes can usually be identified by the ratio of the absorbance peak intensities, which should match the ratio of the extinction coefficients of the dyes. Store absorbance spectra over the entire chromatography run to facilitate analysis. In the calculation, account for acceptor dye absorbance at the absorbance maximum of the donor dye.
21. Calculate labeling efficiencies by determining the protein concentration via absorbance at 280 nm and the dye concentration at its absorbance maximum. Account for dye absorbance at 280 nm (usually provided by the supplier as "correction factor"). This approach is not reliable for proteins with a low ε_{280} ($<$20,000 M^{-1} cm^{-1}), since the dye then dominates absorbance at 280 nm.

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