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Novel 1:1 Labeling and Purification Process for C-Terminal Thioester and Single Cysteine Recombinant Proteins Using Generic Peptidic Toolbox Reagents

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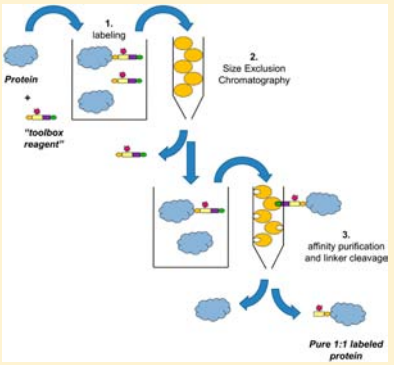
Supporting Information

ABSTRACT: We developed a versatile set of chemical labeling reagents which allow dye ligation to the C-terminus of a protein or a single internal cysteine and target purification in a simple two-step process. This simple process results in a fully 1:1 labeled conjugate suitable for all quantitative fluorescence spectroscopy and imaging experiments. We refer to a "generic labeling toolbox" because of the flexibility to choose one of many available dyes, spacers of different lengths and compositions which increase the target solubility, a variety of affinity purification tags, and different cleavage chemistries to release the 1:1 labeled proteins. Studying protein function in vitro or in the context of live cells and organisms is of vital importance in biological research. Although label free detection technologies gain increasing interest in molecular recognition science, fluorescence spectroscopy is still the most often used detection technique for assays and screens both in academic as well as in industrial groups. For generations, fluorescence spectroscopists have labeled their proteins of interest with small fluorescent dyes by random chemical linking on the proteins' exposed lysines and cysteines. Chemical reactions with a certain excess of activated esters or maleimides of longer wavelength dyes hardly ever result in quantitative labeling of the target protein. Most of the time, more than one exposed amino acid side chain reacts. This results in a mixture of dye−protein complexes of different labeling stoichiometries and labeling sites. Only mass spectrometry allows resolving the precise chemical composition of the conjugates. In "classical" ensemble averaging fluorescent experiments, these labeled proteins are still useful, and quantification of, e.g., ligand binding experiments, is achieved via knowledge of the overall protein concentration and a fluorescent signal change which is proportional to the amount of complex formed. With the development of fluorescence fluctuation analysis techniques working at single molecule resolution, like fluorescence correlation spectroscopy (FCS), fluorescence cross correlation spectroscopy (FCCS), fluorescence intensity diffusion analysis (FIDA), etc., it became important to work with homogeneously labeled target proteins. Each molecule participating in a binding equilibrium should be detectable when it freely fluctuates through the confocal focus of a microscope. The measured photon burst for each transition contains information about the size and the stoichiometry of a protein complex. Therefore, it is important to work with reagents that contain an exact number of tracers per protein at identical positions. The ideal fluorescent tracer−protein complex stoichiometry is 1:1. While genetic tags such as fluorescent proteins (FPs) are widely used to detect proteins, FPs have several limitations compared to chemical tags. For example, FPs cannot easily compete with organic dyes in the flexibility of modification and spectral range; moreover, FPs have disadvantages in brightness and photostability and are therefore not ideal for most biochemical single molecule studies. We present the synthesis of a series of exemplaric toolbox reagents and labeling results on three target proteins which were needed for high throughput screening experiments using fluorescence fluctuation analysis at single molecule resolution. On one target, Hu-antigen R (HuR), we demonstrated the activity of the 1:1 labeled protein in ribonucleic acid (RNA) binding, and the ease of resolving the stoichiometry of an RNA-HuR complex using the same dye on protein and RNA by Fluorescence Intensity Multiple Distribution Analysis (FIMDA) detection.



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INTRODUCTION

Over the past decade, fluorescence based techniques have been developed extensively, sparking tremendous interdisciplinary developments in biology, chemistry, and physics. Most importantly, since the integration of fluorescence fluctuation analysis at single molecule resolution and true single molecule spectroscopy, as well as imaging into biomedical sciences, molecular interactions can be characterized based on a combination of molecular parameters in high resolution. Binding events between small molecules and labeled proteins are followed by measuring changes in translational and rotational diffusion parameters, intensities in one or multiple colors, molecular distances, fluorescence lifetime, and several combinations thereof.[1] An ideal labeling reagent for a single molecule spectroscopy experiment, dye or fluorescent protein, should be as bright and photostable as possible. Protein labeling should be site specific and should result in a known dye−protein stoichiometry, ideally 1:1. The resulting labeled protein to be used in single molecule experiments must be free of any remaining unlabeled target to allow the deduction of valid thermodynamic and kinetic parameters from spectroscopy. Although genetic encoding allows labeling with absolute specificity,[2−4] fluorescent proteins are large moieties (>25 kDa) which are not as bright or photostable as small molecule fluorophores and the spectral range that can be covered is much narrower. While they represent the first choice for cellular imaging, fusion proteins are not ideal for spectroscopy at single molecule resolution. In particular (but not only) for in vitro spectroscopy, protein labeling with small fluorescent dyes has thus continued to attract great attention. The widespread protein labeling methods with small molecular lysine- or cysteine-reactive succinimidyl ester or maleimide dye tracers, respectively, largely produce randomly labeled targets.[5,6] Unlabeled protein fractions are difficult to quantitatively remove by chromatographic methods. This leads to the generation of a mixed population with combinations of stoichiometries and incorporation sites. In order to get better control of the labeling, enzyme fusion proteins like Halo-Tags,[7] SNAP tags,[8−10] acyl carrier protein (ACP),[11] dihydrofolate reductase (DHFR) based and β-lactamase (BL) based tags were recently developed.[12,13] Here, the protein of interest is expressed as fusion conjugate with an enzyme designed to form a covalent bond with synthetic ligands. Despite the elegance of these approaches which made small molecular dyes a generic tool for site specific 1:1 protein labeling, the tag size and separation issues between labeled and unlabeled proteins remained. Probe incorporation mediated by enzymes techniques (PRIME) use the fusion of the target protein with a short sequence such as a ligase acceptor peptide to reduce the size of the labeling site. This method has been used to selectively label proteins with fluorophores or a biotin,[14−18] although the removal of the unlabeled fraction of the protein is again an often encountered issue. To avoid enzyme reactions, genetically encoded and site specifically introduced short peptides which present a structural motif for fluorophore complexation were developed as regio-selective labeling tools.[19,20] Well-known examples of complexes derived from this approach include: Oligo-Asp Tag/Zn(II),[21,22] tetracysteine/organoarsenide,[23−26] tetraserine/bis-boronic acids,[27] and His-tag/nitrilotriacetic acid (NTA).[28,29] Specific labeling can also be performed by the use of biocompatible organic chemistry approaches,[30] such as Staudinger ligation or terminal cysteine labeling with α,β-unsaturated aldehydes,[31−33] sometimes requiring the genetic introduction of noncanonical amino acids such as propargylglycine or azidohomoalanine for Huisgen "click" dipolar cycloaddition,[32,33] formylglycine for oxime fluorophore labeling,[34] or *m*-acetyl-L-phenylalanine for hydrazone formation.[35] Methods making use of dyes with specific reactivity to the *N*-terminus of proteins have also been reported.[36] The variable efficiencies of these chemical reactions, however, almost always result in certain percentages of unlabeled proteins, triggering difficult purification steps.

In an attempt to address these issues, we have established a methodology allowing a generic preparation of pure, C-terminally, or internally 1:1 labeled recombinant proteins that is applicable to virtually any desired small molecular label and target protein sequence. The approach is based on the specific ligation of the target protein to an appropriately designed multifunctional synthetic peptidic reagent. The chemical tag carries features enabling not only its specific, regioselective, high-efficiency ligation to the protein, but also the separation of the labeled protein from the unreacted protein, as described in Figure 1. A "generic labeling toolbox" (Figure 2) was created,

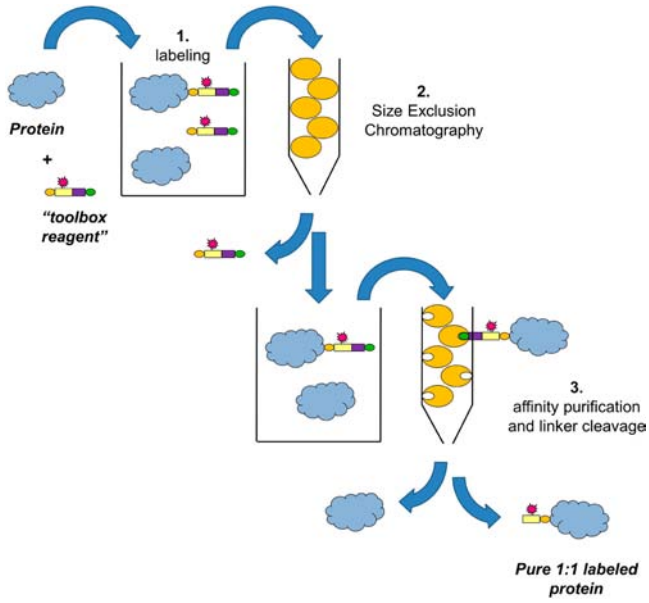

Figure 1. Principle of the catch and release strategy using the toolbox reagents.

making use of the thioester transesterification reaction for C-terminal labeling and a maleimide−thiol reaction at a cysteine residue for internal labeling of target proteins. The synthesis and application of our 1:1 labeling and 2-step purification "toolbox reagents" using different spacers, different dyes, and affinity tags for purification purposes will be described on three target proteins for high throughput nanoscreening.[37] On one target protein, Hu-antigen R (HuR),[38,39] we describe the production of a soluble 1:1 labeled full length protein as well as the internal cysteine labeling of a shortened version of HuR only containing the first two RNA binding domains, RNA Recognition Motifs (RRM) 1 and 2 (HuR12). The activity of the labeled protein was then demonstrated by interaction of labeled HuR12 with an RNA oligonucleotide labeled with the same dye as demonstrated by fluorescence intensity multiple distribution analysis (FIMDA).[40]

RESULTS

Twenty-two protein labeling toolbox reagents were prepared, all based on a common design described in Figure 2. The tags all incorporated a functional terminus used as a handle to mediate

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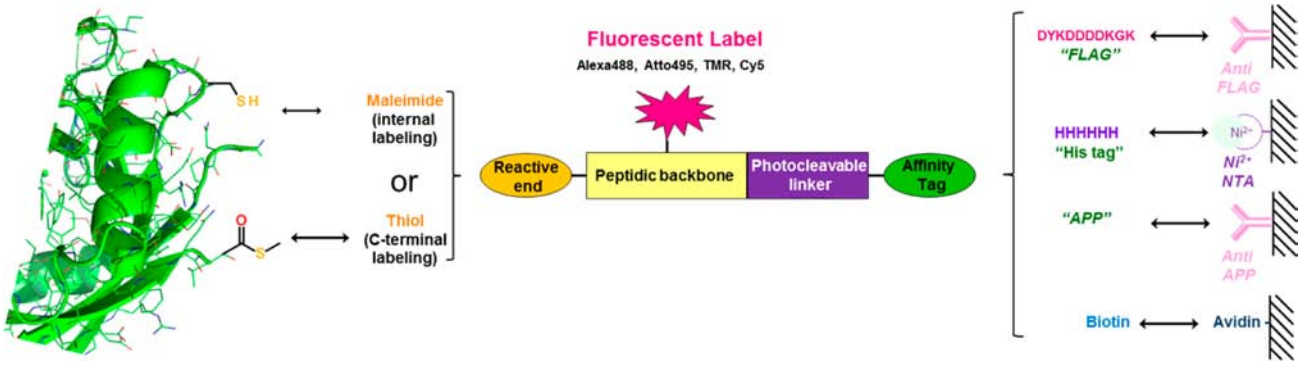

Figure 2. General structure of the toolbox reagents.

ligation to the protein, a dye moiety, several spacing units, as well as an affinity tag for purification purposes via attachment to solid supports. In some examples, a cleavable linker was used to link the affinity tag to the functional terminus and the dye moiety. By coupling of purification and labeling agents into a single molecule, the toolbox reagents enable the specific isolation of only the labeled proteins, thereby producing a 1:1 stoichiometry of label and target proteins containing single cysteines (see Figure 1). In a first step, the protein was reacted with the toolbox reagent, allowing its column or bead immobilization via the affinity tag to separate it from the unreacted starting materials. In a second step, photocleavage or disruption of the interaction between the affinity tag and the solid support released the labeled protein from the solid support. Practically, photostable dyes with high quantum yields were favored, such as sulfobenzindocyanine, rhodamine, or acridine dyes like Cy5, TMR, Alexa488, and Atto495, with excitation wavelengths in the range from 490 to 650 nm and emission wavelengths from 511 to 670 nm. Four affinity tags were used: biotin and His_6 were chosen for their very efficient binding, respectively, to avidin and Ni^{2+}-NTA supports.[41] A Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Gly-Lys decapeptide, termed FLAG tag, was also used for affinity purification using immobilized anti-FLAG antibody,[42] as well as the APP hexapeptide (binding to anti-APP antibody).[53] The introduction of *N*-Fmoc-8-amino-3,6-dioxaoctanoic acid (ADO) as a versatile hydrophilic 8-atom unit building block was chosen for generating variable spacer lengths between the affinity tags and the photocleavable linker or the dye and the functional terminus, respectively. Modulation of spacer length was used not only to introduce the required distance between the protein, the dye, and the affinity tag, but also to modulate the solubility of the conjugate when needed. This allowed an optimal toolbox reagent design for the two critical steps, efficient chemical coupling to the proteins and efficient photocleavage of the fluorescently labeled protein after affinity purification. Based on the general toolbox reagent outline, 20 tags for C-terminal labeling were synthesized on solid support. These tags were based on a Gly-Lys-Gly tripeptide central core for Lysine side chain fluorescent labeling with TMR, Cy5.0, Alexa488, and Atto495. These also included an *N*-terminal cysteine to be reacted with the C-terminal thioester target protein in a native chemical ligation reaction.[43,44] This approach enables the traceless labeling of the protein with minimal perturbation. In constructs **1a**−**f** and **2a**−**j**, a 4-[4-{1-(9-Fmoc-amino)ethyl}-2-methoxy-5-nitrophenoxy]butanoic acid was incorporated as a photocleavable linker building block for its faster cleavage kinetics compared to previous 4-alkyl-5-nitro-benzoic acid derivatives (see Figure 3).[45,46] Finally, three different affinity tags were used: biotin was used in tags **1a**−**f** for

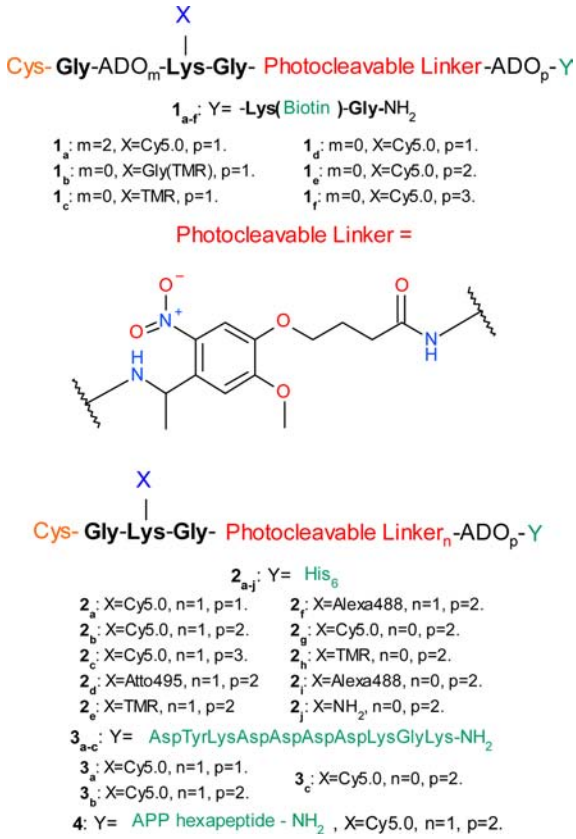

Figure 3. Structures of affinity tags synthesized for C-terminal labeling.

avidin surface immobilization, His_6 was used in **2a**−**j** for NTA surface immobilization, as well as FLAG (compounds **3a**−**c**) and APP (compound **4**) tags for respective anti-FLAG and anti-APP based surface immobilization.[42] The C-terminal labeling toolbox reagents were used on four proteins: a truncated variant of the human SH_2-domain protein signaling lymphocyte activation molecule (SLAM) associated protein (SAP)[47] encompassing amino acids 1−104 of the 128 amino acid full length sequence, the BIR-3 domain encompassing amino acids 249−358 from human XIAP,[48] as well as the human RNA-binding protein HuR (326 amino acids) and a shortened variant encompassing the *N*-terminal region and the first two RRMs (amino acids 1−189, HuR12).[38] The well-described intein mediated protein ligation was used for preparing all four C-terminal thioester recombinant proteins.[49,50] In brief, it is based on the formation of an intermediate internal thioester in the protein splicing process, which is liberated by trans-thioesterification with added thiols like DTT

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or 2-MESNA. The chemical stability of the C-terminal thioesters generated with 2-MESNA was high in contrast to the ones prepared with DTT: more than 98% of the SAP, HuR, and HuR12 proteins as well as 75% of the BIR-3 protein were recovered in their thioester forms. In contrast, in all cases, the use of DTT induced hydrolysis of more than 60% of the thioester conjugates. The labeling of SAP, BIR-3, HuR (full length), and HuR12 was then carried out using tags **1d**, **2f**, **2g**, and **1d**, respectively, by native chemical ligation between the thioester protein and sulfhydryl functionality on the tags.[43,44] The selection of the specific toolbox reagents was based on their chemical availability at the time of use and on combinations of dyes suitable for fluorescence fluctuation analysis. The test proteins were selected based on the need to prepare 1:1 labeled targets for early hit discovery projects. The ligations were performed by simply mixing the construct in DMF with the target protein in an aqueous buffer under inert atmosphere for 48 h at 4 °C. Variation from pH = 6 to pH = 8 did not appear to introduce any significant difference in the labeling efficiency. Although a starting average molar ratio of 20:1 to 25:1 of the tag reagent to the protein of choice was chosen, it was found that this tag excess could be reduced to 5-fold while retaining the labeling efficiency. Even at a tag to protein molar ratio of 2:1, a coupling efficiency of >55% was achieved after 48 h of reaction time for all tested proteins. A detailed comparison of the coupling efficiency for all test proteins was performed by RP-HPLC and at a tag to protein molar ratio of 10:1. It was found to be 30%, 55%, 31%, and 98%, for SAP, BIR-3, HuR (full length), and HuR12, respectively. Two tags for internal labeling at a cysteine residue were also synthesized on solid support, both incorporating a maleimide functionality on their *N*-terminus as well as a photocleavable linker for future release of the labeled protein. Compound **5** was labeled with TMR, and biotin was used as an affinity tag, whereas **6** made use of a Cy5.0 dye, using the APP peptide as the affinity tag. All tags were easily synthesized by solid phase synthesis techniques and obtained in >99% purity after RP-HPLC purification. Toolbox reagent **5** was used for labeling of the internal cysteine of HuR12. The sulfhydryl side chain of the single Cys residue at position 13 in the native sequence of HuR12 was coupled to the maleimido functionality on construct **5** to form a stable thioether covalent bond with at least 35% efficiency. In both C-terminal and internal labeling approaches, the unreacted peptide reagent was then easily removed by size exclusion chromatography or dialysis after the labeling step prior to protein purification. In the representative examples, streptavidin sepharose gel was added to the solution of labeled SAP and the C-terminally labeled HuR12 (tag **1d**) protein samples after gel filtration. Both proteins were shown to be >99% captured by RP-HPLC analysis. Subsequently, the beads were washed to remove any unspecifically bound proteins. The final on-bead photocleavage yield for the SAP-Cy5 conjugate was 22% based on UV/vis analysis, bringing the overall recovery yield to 7%, with >99% RP-HPLC fluorescence detected purity. Due to the low amount of protein, HPLC/ESI-MS and SDS-PAGE could not be carried out on this example. The labeling experiment was carried out in duplicate with HuR12, followed by on bead photocleavage and gave yields of 40% and 74%, respectively, based on UV/vis analysis, therefore making the overall recovery 38% and 72%, respectively. RP-HPLC quality control of C-terminally labeled HuR12 revealed excellent >99% purity (see Figure 5B). The HuR12-Cy5 conjugate showed a single fluorescent band on denaturing, reducing SDS-PAGE. HPLC/ESI-MS results revealed the expected mass, confirming the 1:1 stoichiometry of the labeling (see Figure 5C). According to the

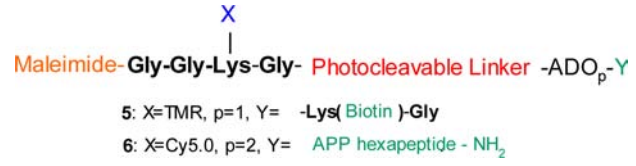

Figure 4. Structures of affinity tags synthesized for internal labeling at Cys.

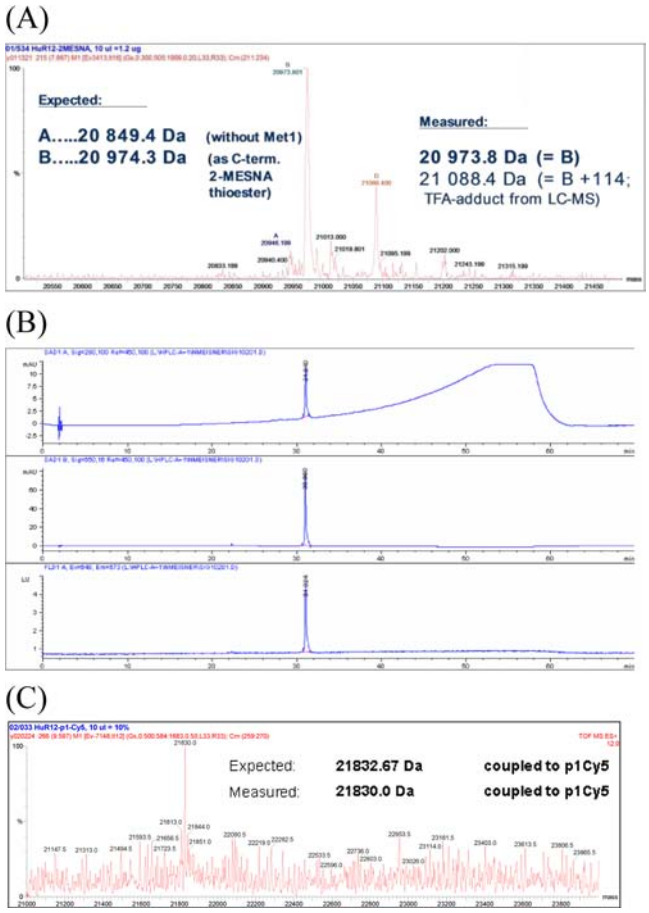

Figure 5. C-terminal labeling of HuR12 with 1 d. (A) ESI-MS of 2-MESNA thioester activated HuR12 which was used for labeling. (B) RP-HPLC analysis of the labeled protein (final product). (C) ESI-MS analysis of the labeled protein (final product).

results from CD-spectroscopy, the secondary structure of the protein was not affected by the labeling procedure (see Supporting Information for full analytical data).

With regard to His_6-tagged representative examples, such as HuR and BIR-3, both proteins were shown to be >99% captured on the Ni^{2+}-NTA affinity matrix. Photocleavage yields were 89% for HuR, and >99% for BIR-3, therefore significantly higher than for biotin constructs, based on UV/vis analysis. The overall recovery of the protein was therefore 27% for HuR and 54% for BIR-3. Both HuR-Cy5.0 and BIR-3-Alexa488 conjugates were obtained in >99% RP-HPLC purity and showed a single fluorescent band on denaturing, reducing SDS-PAGE. HPLC/ESI-MS analysis showed the expected mass for labeled BIR-3, confirming the 1:1 stoichiometry of the labeling (see Supporting Information for full analytical data). Thanks to the presence of two ADO units in the toolbox reagent **2g**, the labeling allowed the full length HuR to be retained in solution at moderate concentrations of up to 5 μM. A subsequent experiment was

carried out to perform the labeling of HuR with the non-fluorescent toolbox reagent **2j**. This conjugate showed a similar increase in solubility.

Finally, internally labeled HuR12 was captured onto a streptavidin affinity matrix with 97% efficiency, based on UV/vis analysis. The photocleavage experiment was carried out in duplicate, with respective yields of 57% and 50%, generating internally labeled HuR12 in excellent RP-HPLC purity of >99%. The HuR12-TMR conjugate showed a single fluorescent band on denaturing, reducing SDS-PAGE (see Figure 6). The overall protein recovery was 20% in both experiments.

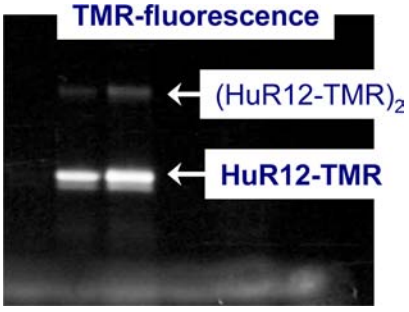

Figure 6. Internal labeling of HuR12 with 5 at Cys13. SDS-PAGE of labeled HuR12. As usually also observed for unlabeled HuR12, a minor amount of labeled HuR12-dimer was detected on reducing, denaturing SDS-PAGE. The "shadow" band corresponds to remaining C-terminal thioester of the labeled protein.

To demonstrate the usefulness of the toolbox reagents in confocal fluctuation experiments, we show a simple example of resolving a 1:1 binding stoichiometry between our HuR12-TMR protein and a synthesized 5′-terminally TMR labeled AU-rich element oligonucleotide of TNF-α. Proteins containing exactly one dye tracer are particularly important for resolving the affinity, stoichiometry, and the cooperativity of complexes with ligands. In this analysis, fluorescence intensity multiple diffusion analysis (FIMDA) was used as the detection method. FIMDA is a fluorescence fluctuation method that combines the features of one-dimensional fluorescence intensity distribution analysis (FIDA) with elements of fluorescence correlation spectroscopy (FCS). FIMDA thus allows the 2-dimensional evaluation of the sample molecules, based on a simultaneous determination of the translational diffusion time as well as the molecular brightness. 5′-Terminally TMR labeled AU-rich element of TNF-α was incubated with TMR-labeled HuR12 (HuR12-TMR) in a ratio of 1:90. Three populations were detected as shown in Figure 7. Based on a previous analysis of the individual components, two of the detected populations can be attributed to TMR-labeled RNA and singly TMR-labeled HuR12. The more globular HuR12-TMR showed a considerably shorter translational diffusion time (0.64 ms) compared to the extended 34mer TMR-labeled RNA (0.82 ms). Noteworthy, the TMR label was slightly more quenched on the protein (22 kHz molecular brightness) compared to the more solvent exposed TMR dye on the RNA (28 kHz). A third population of minor abundance was characterized by a significantly increased translational diffusion time and almost precise additive molecular brightness of 47 kHz, most probably corresponding to the RNA-HuR12 complex. As no population with increased translational diffusion time and increased molecular brightness was detected, a 1:1 stoichiometry for the TMR labeled AU-rich element of TNF-α and HuR12-TMR was supported.

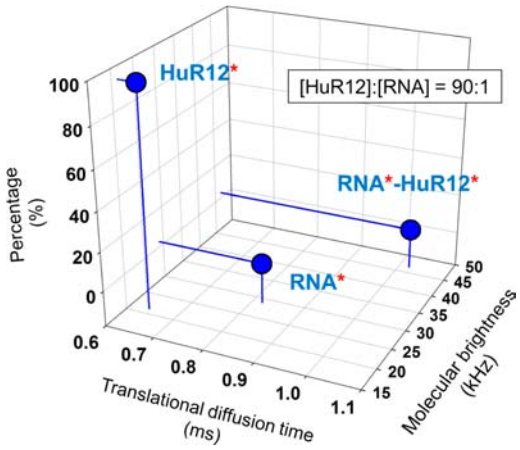

Figure 7. Binding of TMR-labeled HuR12 to TMR-labeled RNA analyzed by FIMDA. The three-dimensional graph reveals the results from one single experiment. The more globular HuR12-TMR mixed in a 90:1 molecular ratio with RNA shows a considerably shorter translational diffusion time (0.64 ms) compared to the extended 34mer RNA-Oligonucletide-TMR (0.82 ms). The TMR label is slightly more quenched on the protein (22 kHz molecular brightness) compared to the more solvent exposed TMR dye on the RNA (28 kHz). The complex between HuR12-TMR and TNF-α RNA-TMR is most likely 1:1 as shown by the almost precise additive molecular brightness of 47 kHz.

DISCUSSION

A total of 22 protein labeling toolbox reagents were synthesized in excellent purity, 20 for C-terminal native chemical ligation labeling of proteins and 2 for internal cysteine labeling. Most tags contained a photocleavable linker and all tags incorporated either a biotin or an affinity tag, therefore enabling the labeled proteins to be captured on an affinity matrix to remove the unlabeled protein and their subsequent release by either photocleavage or disruption of affinity. There are two issues which may arise with using the photocleavable linker: (a) Photocleavage can be harmful to proteins. With the cleavage conditions in this study no issues were detected by follow-up analysis of cleaved proteins by PAGE or HPLC. (b) If a dye is located closely to a photocleavable linker, the cleavage efficiency might be reduced. This is indeed an issue which we saw particularly when Rhodamine dyes were used. Further studies will be needed to systematically investigate the problem. Most likely, it will be beneficial to have the photocleavable linker separated from the dye further in the chemical design of the toolbox reagent. The C-terminal labeling toolbox reagents were then implemented on four example proteins, SAP, HuR, HuR12, and BIR-3. The proteins were first prepared as C-terminal thioesters by intein mediated protein ligation. They were then easily labeled using a 10-fold excess of toolbox reagent, for comparison, followed by a straightforward catch and release purification strategy using biotin or His_6/Ni^{2+}NTA affinity capture. The release of the protein from the affinity matrix via photocleavage (constructs **1d** and **5**) turned out to show lower efficiency and greater variability compared to His_6/Ni^{2+}NTA affinity disruption followed by solution photocleavage of the affinity tag (constructs **2f** and **2g**). The possibility that the difference in efficiency between photocleavage and affinity disruption is linked to the number of ADO units used in the tag was ruled out by additional experiments. The attachment of a hydrophilic toolbox reagent to a protein of choice, in labeled and unlabeled form, significantly increased its aqueous solubility. This added an extremely useful feature to the toolbox method for

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the generation of soluble targets with simple extensions. HuR, for example, consists of 3 RRMs, and like many other RNA regulatory proteins has limited solubility and is highly prone to aggregation as full length protein at physiologic pH in vitro. The usual solution to this problem is to use glutathione S-transferase (GST) fusion constructs or work with truncated variants encompassing only the two first RRMs responsible for RNA binding.[38] The introduction of a solubility increasing tag via our toolbox reagents, **2f** and **2g**, produced a full length HuR which was soluble enough for the concentrations needed for confocal fluorescence spectroscopy, thereby enabling RNA binding experiments to be carried with the reagents closer to the natural state. In addition, applying FIMDA as a quick technique for the measurement of complexation with the same dye used on ligand and receptor, we demonstrated the usefulness of controlled and quantitative stoichiometric labeling of binding partners for HuR12-TMR, as shown in Figure 7. We are aware of evidence in the literature for 2:2 binding,[39] as well as oligomerization of HuR on the mRNA,[51] and we appreciate that the data from our FIMDA experiment show a different stoichiometry. The difference between observed 1:1 and 2:2 stoichiometry originates, most likely, from the different HuR variants used, full length versus truncated HuR protein. However, the primary purpose of the experiment in the scope of the presented study was not to resolve a biological question but to demonstrate that the proteins used in the labeling examples are still functional and homogeneously 1:1 labeled at the single molecule level, applicable for quantitative single molecule studies such as FIMDA.[39,51]

CONCLUSION

A powerful method for the C-terminal and the internal labeling of proteins based on peptidic toolbox reagents successfully afforded the 1:1 labeling of four example proteins in good yield. In addition to the excellent purity of all conjugates, an increase of the solubility of one of the example proteins was shown, also demonstrating the efficacy of the approach as a protein solubilizing method. As the variable efficiency of the photocleavage step, particularly on the solid matrix, turned out to be crucial in governing the overall yield of the process, a generically more reliable approach would be to further expand on the use of reversible affinity interaction options, such as $His_6/Ni^{2+}NTA$, FLAG/anti-FLAG, or APP/anti-APP where the purified labeled protein is recovered by elution from the affinity matrix. The photocleavage step would then be performed in solution and the tag removed by size exclusion chromatography. With a photocleavable linker placed after a solubility spacer, 1:1 labeling and increased hydrophilicity of the protein conjugate could be achieved.

EXPERIMENTAL PROCEDURES

Toolbox Tag Synthesis. All compounds were synthesized on Rapp TentaGel-S-RAM 90 μm beads with acid labile Rink amide linker, with a loading of 0.23 mmol/g, in 50−2000 mg batches. Commonly used activation methods were employed to synthesize the backbone structures for the tags (see Supporting Information for detailed experimental procedures). 1-(4,4-Dimethyl-2,6-dioxocyclo-hexylidene)-3-methylbutyl (ivDde) group deprotection was carried out with hydrazine (3−5% solution in *N*,*N*-dimethylformamide (DMF), 3 × 10 min). Monomethoxytrityl (Mmt) deprotection was performed by repeated treatments with 5% trifluoroacetic acid (TFA)/CH_2Cl_2 for a total of 30 min. 5-Carboxy-tetramethylrhodamine (TMR) *N*-hydroxysuccinimidyl ester was purchased from Molecular Probes, Atto495 succinimidyl ester was purchased from Atto-Tec (Gießen, Germany), and Cy5.0-carboxylic acid was prepared in house following a known procedure (see Supporting Information for detailed experimental procedures).[52] Cy5.0-COOH or Atto495-COOH was coupled with (benzotriazol-1-yloxy)-tripyrrolidinophosphonium-hexafluorophosphate (PyBOP) and diisopropylethylamine (DIPEA). All tags were characterized by ESI+/MS and MALDI analyses and quantification was carried out by UV/vis spectroscopic analysis (see Supporting Information for full analytical data). In practice, six different constructs **1a**−**f** with biotin affinity tags were prepared (Figure 3). In all cases, an 8-amino-3,6-dioxaoctanoic acid-Lys(Biotin)-Gly tripeptide was built up on the resin by successive peptide couplings with *N*-Fmoc-8-amino-3,6-dioxaoctanoic acid, Fmoc-Lys(Biotin)-OH and Fmoc-Gly-OH. Constructs **1e** and **1f** then respectively included one and two additional 8-amino-3,6-dioxaoctanoic acid (ADO) moieties. The photocleavable linker was then added, followed by the Lys(ivDde)-Gly motif, all by successive peptide couplings. Two ADO units were then included into **1a**. Fmoc-Gly-OH and *N*-Boc-Cys(Trt) were successively coupled to the tags. Finally, substitution of the side chain of the Lysine residue was accomplished after ivDde deprotection and reaction of the free amine with either Cy5.0-COOH (constructs **1a**, **1d**−**f**) or isomer free 5-carboxy-TMR (construct **1c**). For tag **1b**, the free amine was first coupled to Gly, followed by reaction with TMR. The six compounds were cleaved off the resin with TFA/CH_2Cl_2/triisopropylsilane (TIS)/H_2O/1,2-ethanedithiol (EDT) (50:45:1:2.5:2.5 (v/v)) and purified by reverse phase high performance liquid chromatography (RP-HPLC) and obtained in moderate to good yield (8−39%) and very high purity (>95% as determined by RP-HPLC/Ultra Violet (UV) absorption at 215 nm—see Supporting Information for analytical data). Ten constructs **2a**−**j** were synthesized using procedures similar to those above but with hexahistidine affinity tags (Figure 3). The His_6-tags were prepared by linear solid phase peptide synthesis using Fmoc-His(Trt)-OH. The constructs were then assembled in a way analogous to the procedure above (see Supporting Information for detailed experimental procedures). Four different dyes were used: Cy5.0-COOH (constructs **2a**−**c**, **2g**), Atto495-COOH (construct **2d**), isomer free 5-carboxy-TMR (construct **2e**, **2h**), and Alexa488 (construct **2f**, **2i**). The 11 compounds were cleaved off the resin with TFA/DCM/TIS (95:5:2 v/v/v) for 1 h, then a mixture of 10 mL TFA/phenol/thioanisole/H_2O/1,2-EDT (10:750:0.5:0.5:0.25 v/m/v/v) for 1 h and purified by RP-HPLC and obtained in moderate to good yield (4−18%) and very high purity (>94% as determined by RP-HPLC/UV absorption at 215 nm—see Supporting Information for analytical data). A FLAG affinity tag was used in three other constructs **3a**−**c**,[42] as well as the so-called antibody affinity sequence hexapeptide from amyloid precursor protein (APP) tag in construct **4** (Figure 3).[53] The FLAG peptide, a Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Gly-Lys decapeptide, was prepared using Fmoc-Asp(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Gly-OH. The APP hexapeptide (proprietary) was prepared using Fmoc protected amino acids. The constructs were then assembled in a way analogous to the method above (see Supporting Information for detailed experimental procedures). The four compounds made use of a Cy5 dye and were cleaved off the resin with TFA/CH_2Cl_2/TIS/H_2O/1,2-EDT (50:45:1:2.5:2.5 (v/v)) and purified by RP-HPLC, resulting in low yield (1−5%) and very high purity (>95% for all compounds determined by RP-HPLC/UV absorption at 215 nm—see Supporting Information for analytical data).

Compound **5** was prepared using the same synthetic route as compound **1c** up to the coupling of the last Gly residue, which was kept protected (Figure 4). Substitution of the side chain of the lysine residue was then performed by Mmt deprotection and subsequent reaction of the free amine with isomer free 5-carboxy-TMR. Fmoc deprotection exposed the free amine, which was then coupled to *N*-maleoyl-γ-aminobutyric acid. Finally, compound **6** was prepared similarly to compound **4**, making use of the APP tag (Figure 4). All compounds were cleaved off the resin with TFA/CH_2Cl_2/triisopropylsilane (TIS)/H_2O/1,2-ethanedithiol (EDT) (50:45:1:2.5:2.5 (v/v)) and purified by RP-HPLC and resulted in low to moderate yield (1−25%) and very high purity (>95% for all compounds determined by RP-HPLC/UV absorption at 215 nm—see Supporting Information for analytical data).

Labeling of Proteins. *Protein Production.* C-terminal thioester proteins were generated in *E. coli* by using the IMPACT system from New England Biolabs (see Supporting Information for detailed experimental procedures).[50,54] The target protein was cloned with a fusion tag composed of a chitin-binding domain (CBD) and an intein domain. With the help of the CBD, the fusion protein was affinity-purified and captured on chitin beads. Subsequently, the target protein was released from the fusion tag by inducing the self-splicing activity of the intein with thiol-reagents such as dithiothreitol (DTT) or 2-mercaptoethanesulfonic acid (2-MESNA). The recombinant protein with an activated C-terminal thioester was finally eluted from the affinity column, whereas the intein-tag together with the chitin-binding domain remained bound to the chitin-agarose. Using this method, a truncated variant of the human Src-homology 2 (SH_2) domain protein signaling lymphocyte activation molecule (SLAM) associated protein, (SAP), encompassing amino acids 1−104 of the 128 amino acid full length sequence was amplified from cDNA (human T-lymphocytes), cloned into the (NdeI)/SAPI restriction sites of the vector pTXB1 (IMPACT-system, New England Biolabs) and purified. Intein-mediated on-column cleavage was induced by addition of 2-MESNA (Na-salt; 50 mM final concentration).[47] According to HPLC/ESI-MS analysis, the recovered protein was to >98% present as C-terminal MESNA-thioester (see Supporting Information for detailed experimental data). The protein used for subsequent labeling experiments was stored at −80 °C in a buffer of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 6.0, 500 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and at a concentration of 40−45 μM. Similarly, the Baculovirus Inhibitor of Apoptosis Protein repeat domain (BIR-3) encompassing amino acids 249−358 from human X-linked inhibitor of apoptosis protein (XIAP) was cloned into the NdeI/SAPI restriction sites of the vector pTYB1 (IMPACT-system, New England Biolabs) and purified.[48] Intein-mediated on-column cleavage was induced similarly to SAP. According to HPLC/ESI-MS analysis, the recovered protein was to >75% present as C-terminal MESNA-thioester (see Supporting Information for detailed experimental data). The protein used for subsequent labeling experiments was stored at −80 °C in a buffer of 20 mM HEPES pH 8.0, 500 mM NaCl, 1 mM EDTA, 50 mM 2-MESNA and at a concentration of 14.2 μM. The human RNA-binding protein HuR was PCR-amplified from cDNA (human T-lymphocytes), cloned into the NdeI/SAPI restriction sites of the vector pTXB1 (IMPACT-system, New England Biolabs) and purified.[55] In the final purification step, cleavage from the Intein-CBD tag was induced by addition of 2-MESNA (Na salt) in a buffer of 20 mM Tris/HCl pH 8.0, 800 mM NaCl, 1 mM EDTA, and 0.2% (w/v) Pluronic F-127. According to HPLC/ESI-MS analysis, the recovered protein was to >98% present as C-terminal MESNA-thioester (see Supporting Information for detailed experimental data). The protein used for subsequent labeling experiments was stored at −80 °C in and the cleavage buffer at a concentration of 400−450 nM. A shortened variant of the protein HuR encompassing the *N*-terminal region and the first two RRMs as well as the *N*-terminal peptide (amino acids 1−189) was subcloned into the NdeI/SAPI restriction sites of the vector pTWIN1 (IMPACT-system, New England Biolabs) and purified essentially as described for full length HuR. According to HPLC/ESI-MS analysis, the recovered protein was to >98% present as C-terminal MESNA-thioester (see Figure 5). The protein used for subsequent labeling experiments was stored at −80 °C in a buffer of 20 mM Tris/HCl, 800 mM NaCl, 1 mM EDTA, pH 8.0 and at a concentration of 20−40 μM.

Protein Labeling (Representative Procedures). SAP labeling: 215 nmol of reagent **1d** were dissolved in 25 μL anhydrous DMF under argon atmosphere and added to 500 μL of 42.5 μM SAP in a buffer of 20 mM HEPES pH 6.0, 500 mM NaCl, 0.1 mM EDTA, corresponding to a molar reagent/protein ratio of 10.1:1. The reaction was allowed to proceed overnight at 4 °C, protected from light and under gentle mixing. In order to remove any unreacted peptide, the sample was passed over a DG-10 gel filtration column (BIO-RAD) previously equilibrated with the coupling buffer (without 2-MESNA). The coupling efficiency as measured by RP-HPLC was found to be 30%.

BIR-3 labeling: 508 nmol of reagent **2f** was dissolved in 20 μL anhydrous DMF under argon atmosphere and added to 3.4 mL of 14.9 μM BIR-3 in a buffer of 20 mM HEPES pH 8.0, 500 mM NaCl, 1 mM EDTA, 50 mM 2-MESNA, corresponding to a molar reagent/protein ratio of 10:1. The reaction was allowed to proceed for 48 h at 4 °C, protected from light. In order to remove any unreacted peptide, the sample was passed over a DG-10 gelfiltration column (BIO-RAD) previously equilibrated with a buffer of 50 mM NaH_2PO_4 pH 8,0, 300 mM NaCl. The coupling efficiency as measured by RP-HPLC was found to be 55%.

HuR labeling: 51 nmol of the peptide reagent **2g** was dissolved in 100 μL anhydrous DMF under argon atmosphere (to prevent oxidation of the *N*-terminal Cysteine) and added to 12 mL of 420 nM thioester-activated HuR in a buffer of 20 mM Tris/HCl pH 8.0, 800 mM NaCl, 1 mM EDTA, 50 mM 2-MESNA, 0.2% Pluronic F-127 (Molecular Probes), corresponding to a molar reagent/protein ratio of 10.2:1. The reaction was allowed to proceed for 48 h at 4 °C on the horizontal shaker, protected from light. Finally, the sample was transferred into a Slide-A-Lyzer dialysis cassette (MWCO = 10 000 Da; 3−15 mL sample volume; PIERCE) and dialyzed against phosphate buffered saline (PBS) at 4 °C (protected from light), in order to remove any unreacted peptide and to adapt the buffer conditions to the subsequent His_6/Ni-NTA agarose affinity purification. The coupling efficiency as measured by RP-HPLC was found to be 31%.

HuR12 C-terminal labeling: 350 nmol of the peptide reagent **1d** was dissolved in 50 μL anhydrous DMF under argon atmosphere and added to 1 mL of 35 μM HuR12 in a buffer of 20 mM Tris/HCl pH 8.0, 800 mM NaCl, 1 mM EDTA, 50 mM 2-MESNA, corresponding to a molar reagent/protein ratio of 10:1. The reaction was allowed to proceed overnight at 4 °C, protected from light and under gentle mixing. In order to remove any unreacted peptide, the sample was passed over a DG-10 gel filtration column (BIO-RAD) previously equilibrated with the coupling buffer (without 2-MESNA).

HuR12 internal labeling: 251 nmol of the peptide reagent **5** was dissolved in 10 μL anhydrous DMF under argon atmosphere

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and added to 200 μL of 126 μM HuR12 in PBS, corresponding to a molar reagent/protein ratio of 10:1. The reaction was allowed to proceed overnight at 4 °C, protected from light and under gentle mixing. In order to remove any unreacted peptide, the sample was passed over a DG-10 gel filtration column (BIO-RAD) previously equilibrated with PBS. The coupling efficiency as measured by RP-HPLC was found to be 98%.

Protein Affinity Purification. Biotin-tagged products (C-terminally and internally labeled HuR12, SAP) were purified as follows: after removal of unreacted peptide reagent, streptavidin sepharose (Amersham Pharmacia; capacity: 8.6 nmol Biotin-BSA/100 μL gel) was equilibrated with the protein buffer and added to the sample, providing a capacity of at least twice the amount of labeled protein. The suspension was incubated for 30 min at 4 °C on the horizontal shaker, or until the supernatant had turned colorless. Subsequently, the beads were washed with at least 10 bed volumes of coupling buffer to remove any unspecifically bound proteins. The beads were then transferred into sealable glass vials and on-bead photocleavage was performed in a Stratalinker 1800 UV Cross-linker (Stratagene) at 365 nm and 3 mW cm^{-2}, for 60 min (under stirring and cooling for beads in suspension). Tinfoil was put beneath the vial to reflect the light and increase the cleavage efficiency. The supernatant was transferred into a fresh tube and the beads were rinsed once with the corresponding buffer. The recovered solution contained the pure, C-terminally labeled protein.

His_6-tagged products, HuR and BIR-3, were purified as follows: after removal of the unreacted peptide reagent and any buffer components which affect the His_6/Ni interaction (such as EDTA), Ni-NTA agarose (Qiagen) was equilibrated with the binding buffer. An amount of resin providing a capacity of at least twice the amount of labeled protein was added to the sample. The suspension was incubated at 4 °C on the horizontal shaker for 4 h, or until the supernatant had turned colorless. The beads were then washed with at least 10 bed volumes coupling buffer containing 15−20 mM imidazole to remove any unspecifically bound proteins. Finally, the labeled protein was eluted either with 250 mM imidazole in coupling buffer or with a buffer of pH < 6.0.

Biochemical Analysis. The quality and quantity of labeled protein after each step and at the end of the labeling procedure was controlled by RP-HPLC analysis (see Figure 5 for one example, HuR12, and Supporting Information for detailed experimental procedures and data). The quality of the labeled protein was further assessed by denaturing, reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, see Figure 6 for internally labeled HuR12). Quantification of the labeled protein was carried out by UV/vis analysis and the effect of the labeling on the protein folding was assessed by CD-spectroscopy (see Supporting Information for detailed experimental data). Finally, the purity and 1:1 stoichiometry of the labeling approach was controlled by HPLC/ESI-MS analysis (see Figure 5 for HuR12 and Supporting Information for detailed experimental data).

Fluorescence Intensity Multiple Distribution Analysis (FIMDA). FIMDA measurements were recorded in 96 well glass bottom microtiter plates (Whatman) on an Evotec PickoScreen 03 confocal instrument in the 1 dimension fluorescence intensity distribution analysis (1D-FIDA) mode at ambient temperature (constant at 23.5 °C).[56] The Olympus inverted microscope IX70 based instrument was equipped with two fluorescence detectors and a dichroic mirror in the fluorescence excitation path. A HeNe laser (λ = 543 nm, laser power = 478 μW) was used for fluorescence excitation. The laser power at PS03 was measured using a power meter (Field Master, Coherent) right at the backaperture of the objective via a beamsplitter (4%). The actual power at the sample is derived via previously performed calibration measurements, i.e., the transmission loss through the objective is determined via a standard optical power meter with large detection area of 1 cm^2. For TMR detection, a 560DRLP dichroic beamsplitter and a 590DF60 bandpass filter were used in the fluorescence excitation path. The excitation laser light was blocked from the optical detection by an interference barrier filter with OD = 5. A 0.5 nM solution of TMR in assay buffer was used for the adjustment of the confocal pinhole (70 μm) and the optical fibers. The confocal volume was positioned at 150 μm above the glass bottom by longitudinal adjustment of the objective.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures, chemical structures, analytical data, CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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