Nanomolar affinity protein trans-splicing monitored in real-time by fluorophore–quencher pairs†

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High background originating from non-reacted, ‘always-on’ fluororescent probes remains a key unsolved problem in life science since washing procedures are not easily applicable. Covalent labeling approaches with simultaneous activation of fluorescence are advantageous to increase sensitivity and to reduce background signal. Here, we combined high-affinity protein trans-splicing with fluorophore/quencher pairs for online detection of covalent N-terminal ‘traceless’ protein labeling under physiological conditions in cellular environments. Substantial fluorescence enhancement at nanomolar probe concentrations was achieved.

Site-specific introduction of synthetic probes into proteins by genetic, metabolic, or enzyme-mediated ligation allows to visualize, to track, and to manipulate proteins even in their native environment. Notably, the steadily growing repertoire of bio-orthogonal reactions for site-specific protein modification substantially promoted the analysis of protein folding, conformational dynamics, and protein interactions. In contrast to the genetic fusion of auto-fluorescent proteins, bio-orthogonal ligation methods permit to pinpoint bright and stable synthetic fluorophores with high photon yields to proteins *in vitro* as well as *in vivo*. The most prominent labeling methods are based on tagging approaches, where the protein of interest (POI) is genetically fused to a peptide tag (His-tag, LAP-tag, etc.) or small protein (e.g. SNAP-tag) for chemo-selective attachment of the reporter.† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6cc08862g

Especially ligation methods, which exhibit fluorogenic properties such as the FLAsH/ReAsH technology, the SNAP, BL, TMP, as well as the PYP-tag, are particularly powerful for protein labeling in a complex biological environment. Thus, extensive washing procedures are bypassed to eliminate the excess of non-reacted ‘always-on’ fluorescent probes prior to imaging. Moreover, synthetic probes often accumulate in sub-cellular organelles when washing is not applicable. In this regard, a high-affinity, fast covalent labeling process with simultaneous activation of fluorescence would be of great advantage to increase sensitivity and to reduce background signal for visualization. However, all recent technologies are limited by their relatively large size (> 15 kDa), compromising protein function, localization, or dynamics of the target protein. In addition, high quantities (µM) of the fluorogenic probe are required for efficient protein labeling and is hitherto not accomplished at nanomolar concentrations.

A promising ligation approach for covalent protein labeling is intein-mediated protein trans-splicing (PTS). Here, the autocatalytic intein domain is split into two fragments, IntN and IntC, which re-associate to mediate the trans-splicing reaction, resulting in a covalent linkage between the flanking exteins, ExtN and ExtC. The incorporation of a quencher into IntN could turn off the fluorescence of a dye-modified ExtN part, thereby rendering an unreacted F (ExtN-IntN) non-fluorescent. After PTS, the fluorophore is transferred to the POI, whereby a concomitant fluorescence dequenching takes place and fluorescence is restored. So far, this approach was only applied for C-terminal protein labeling at µM concentrations using the Ssp DnaE and Npu DnaE intein with fluorescein (FAM) or rhodamine/QSY as fluorophore/quencher (F/Q) pair. However, the respective synthetic intein fragments (> 40 aa), FAM as molecular probe, and the applied concentrations (µM) are inconsistent with an aspired minimalistic approach to incorporate photo-stable fluorophores. Aiming at site-specific protein modifications, we recently advanced the smallest nanomolar affinity split intein based on the Ssp DnaB M86 (14 aa). Here, we use high-affinity PTS for the N-terminal chemo-selective introduction of a blue-absorbing (FAM/Dabcyl) as well as red-absorbing (sCy5/BHQ-3) F/Q pair with superior photo-physical properties (Scheme 1). This enables the real-time monitoring of reporter transfer to the POI by PTS. Moreover, it offers high fluorescence turn-on protein modification at nM probe concentration.

All F fragments were synthesized by fluorenylmethoxycarbonyl (Fmoc) based solid-phase peptide synthesis (SPPS) with a C-terminal lysine for the incorporation of the respective quencher and...
Scheme 1. Nanomolar affinity protein trans-splicing monitored by fluorophore/quencher pairs. F/Q-modified synthetic IN is rendered fluorescently dormant, whereas fluorescence is restored upon trans-splicing. To span a broad visible spectrum, FAM/Dabcyl as well as sCy5/BHQ-3 were incorporated into IN. Based on the high-affinity interaction pair Ni-trisNTA/His-tag, N-terminal protein modifications are possible down to the sub-nanomolar range. CSGDSLISA, residue 1–11 of Ssp DnaB M86 mini-intein.

First, we determined the degree of fluorescence quenching of both F/Q pairs. As expected, Dabcyl as well as BHQ-3 efficiently quenched the fluorescence of FAM or sCy5 at the N terminus of IN, respectively. The distance between fluorophore and quencher was estimated to be ~30 Å, which is in a good range for efficient quenching (Fig. S4, ESI†). Fluorescence quenching in the µM as well as nM range was slightly more effective with scy5/BHQ-3 (94% at 1 µM, 96% at 10 nM) than with FAM/Dabcyl (92% at 1 µM, 91% at 10 nM). Quenching efficiencies were determined as percentage loss of emission intensity in comparison to an IN fragment lacking the quencher moiety. As benchmark for effective fluorescence quenching, we digested the F/Q-modified IN with proteinase K to determine the maximal fluorescence intensities of the respective IN fragments. For both F/Q-modified IN constructs, the obtained maximum intensity values were identical to the intensity magnitude of fluorophore-labeled IN. Real-time monitoring of PTS by fluorescence release was followed at 1 µM of F/Q-modified IN and 4 µM of HI–T for 1 h at 25 °C, additionally corroborated by SDS-PAGE in-gel fluorescence analysis (Fig. 1). After 1 h of PTS, an 11-fold increase in fluorescence intensity was received for FAM–Dabcyl, whereas scy5–sCy5/BHQ-3 resulted in a 13-fold fluorescence enhancement. In the presence of trisNTA, fluorescence recovery was slightly decreased with FAM/Dabcyl (~8%) and significantly diminished with scy5/BHQ-3 as F/Q pair (~20%). Notably, in the presence of Ni-trisNTA, we gained a slightly higher dequenching efficiency in the µM range (Fig. 1 and Table S3, ESI†), most likely based on photo-induced electron transfer (PIT). Fluorescence recovery was corrected for the amount of N-terminal cleavage of the respective F/Q-modified IN with HI–T for both F/Q pairs (FAM/Dabcyl and scy5/BHQ-3). Proteinase K treatment served as a control (ctrl) for absolute fluorescence release. FAM/sCy5-labeled IN (~1 µM) or IN–Ni-trisNTA (nM) lacking the quencher moiety were used as positive control for PTS.

With regard to the F/Q pair and the Ni-trisNTA moiety position in the small IN, we were concerned about the impact of both F/Q pairs on the affinity between the intein fragments. We therefore determined the equilibrium dissociation constant Kd by microscale thermophoresis (MST), using the residual fluorescence of the quenched IN and splice-inactive HI–T (hereafter referred to as HI–T(N154A,S+1A,H73A)). A Kd of 425 ± 35 nM was determined for HI–T(N154A,S+1A,H73A) and FAM–Dabcyl,
whereas a slightly lower affinity was revealed in the case of FAM-I^4-Dabcyl-trisNTA (650 ± 55 nM). In the presence of Ni(II)-loaded trisNTA, a 50-fold increased affinity was observed ($K_D = 14 ± 1$ nM; F^4-Ni-trisNTA). Notably, virtually identical $K_D$ values were obtained for the red-shifted sCy5/BHQ-3 pair, emphasizing that both F/Q pairs have no significant negative impact on $I^4/I^C$ affinity in comparison to fluorophore-labeled $I^N$ (Table S3 and Fig. S6, ESI†). In line with the dramatic increase in affinity triggered by the Ni-trisNTA/His-tag interaction, enhancement in fluorescence (11-fold) was exclusively promoted in the nM range in the presence of the small recognition element (Fig. 1). Worth mentioning, lacking the minimalistic lock-and-key element, no fluorescence dequenching was detected.

Since the onset of fluorescence is a direct indicator for successful PTS, the fluorescence increase was directly used as a scale bar for the trans-splicing yield. PTS yields between $I^C$ and F/Q-modified $I^N$ fragments were comparable to those obtained with non-modified $I^N$ (Table S3, ESI†). Moreover, nearly identical yields were obtained for both F/Q-equipped $I^N$-Ni-trisNTA constructs at 10nM compared to 1 μM. This emphasizes that our approach produces fluorescently labeled proteins in the nM range with more than 50% yield, which is superior to undirected, non-guided PTS at very low probe concentrations. By fluorescence increase during PTS, kinetic parameters were determined via fluorescence spectroscopy. Notably, correction for N-terminal cleavage was performed. All F/Q-modified $I^N$ resulted in fast PTS with identical $k_{on}$ values (11–14 min) in the absence as well as presence of the minimal recognition element (Table S3 and Fig. S7, ESI†) and are not affected by the concentration range (μM down to nM). Strikingly, the presence of the F/Q pair had no impact on trans-splicing kinetics.

To gain further insights into trans-splicing kinetics, $k_{on}$ values for $I^N/I^C$ fragment association were determined by fluorescence anisotropy with $H^C_{I^N/154A,S+1A,H73A}T$ (Fig. S8, ESI†). We observed $k_{on} = 0.068 ± 0.005 \cdot 10^{-3}$ M$^{-1}$ s$^{-1}$ and 0.061 ± 0.003 $10^{-3}$ M$^{-1}$ s$^{-1}$ for FAM/Dabcyl- and sCy5/BHQ-3-modified $I^N$, respectively. This is in agreement with $k_{on} = 0.054 ± 0.003 \cdot 10^{-3}$ M$^{-1}$ s$^{-1}$ for unmodified $I^N$ and $I^TH$.[28] For FAM/ Dabcyl- and sCy5/BHQ-3-modified $I^N$/Ni-trisNTA, $k_{on} = 75.2 ± 6.2$ M$^{-1}$ s$^{-1}$ and 74.3 ± 5.8 M$^{-1}$ s$^{-1}$ were observed at μM concentrations. A similar $k_{on}$ was measured in the nM range (Fig. S8, ESI†), which is in the same regime as the $k_{on}$ between Ni-trisNTA and a His$_6$-tagged protein.[31] In the presence of Ni-trisNTA, intein fragment association is controlled by the minimalistic lock-and-key element and 10,000-fold accelerated.

Although we observed extremely fast $k_{on}$ kinetics for fragment association, the overall trans-splicing reaction ($k_{PTS}$, $t_{1/2}$) is not accelerated (Fig. S8 and S9, ESI†). However, the ultra-fast pre-assembly of inteins will help to overcome degradation in cellular systems and will gain relevance when very low probe concentrations are required.

Encouraged by these results, we aimed at real-time monitoring of PTS in crowded cellular environments. Hence, we performed trans-splicing in cell lysates of human cervical cancer (HeLa) cells with 1 μM/10 nM of F/Q-modified $I^N$ fragments and 4 μM/40 nM of Hi$^C$. Because of superior photo-physical properties, we focused on sCy5/BHQ-3. PTS was followed in real-time by fluorescence dequenching using 1 μM of $I^N$, $I^N$/Ni-trisNTA, or $I^N$-Ni-trisNTA, respectively, as well as by SDS-PAGE in-gel fluorescence (Fig. 2 and Fig. S10, ESI†). At μM concentrations, all $I^N$ constructs resulted in nearly identical reaction kinetics ($t_{1/2} = 10–11$ min; $k_{PTS} = 1.7–1.9 \cdot 10^{-3}$ s$^{-1}$; Table 1), hence confirming that our approach is undisturbed by the crowded cellular environment. Despite that, all values are in very good accordance with data reported for unmodified $I^N$ and $I^TH$.[30] In contrast, real-time monitoring of PTS in the nM range was only achieved if promoted by the Ni-trisNTA/His-tag interaction (Fig. 2).

Again, after 1 h, SP formation is very similar (70–75%) with $I^N$ in the absence or presence of cell lysate and only moderately

Table 1 Interaction analysis and kinetics of high-affinity PTS in cellular environment. Equilibrium dissociation constants $K_D$ were determined by MST with $H^C_{I^N/154A,S+1A,H73A}T$ in human cell lysates. Pseudo first-order rates $k_{PTS}$ and $t_{1/2}$ values were derived from fluorescence dequenching, using 1 μM of $I^N$ and 4 μM of Hi$^C$. In the nanomolar range, $k_{PTS}$ and $t_{1/2}$ were analyzed at 10 nM of respective $I^N$ and 40 nM of Hi$^C$ (italics)

<table>
<thead>
<tr>
<th>$I^N$ fragment</th>
<th>$K_D$ (nM)</th>
<th>$k_{PTS}$ ($10^{-3}$ s$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
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<tbody>
<tr>
<td>$I^N$</td>
<td>450 ± 65</td>
<td>1.8 ± 0.4</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>$I^N$/trisNTA</td>
<td>610 ± 100</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>$I^N$/Ni-trisNTA</td>
<td>25 ± 9</td>
<td>1.9 ± 0.3</td>
<td>2.0 ± 0.2</td>
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<tr>
<td></td>
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<td>2.0 ± 0.5</td>
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decreased with I\(^{13}\)-Ni-trisNTA (59–63\%). However, when the concentration of I\(^{13}\) or I\(^{15}\)-trisNTA was reduced by two orders of magnitude (10 nM), no fluorescence dequenching and thus no trans-splicing were observed (Fig. S10, ESI\(^+\)). This, in turn, demonstrates that in a cellular environment PTS was drastically enhanced (>10-fold) by the presence of the high-affinity lock-and-key element. Additionally, covalent SP formation at nM concentrations was confirmed by in-gel fluorescence analysis. Notably, unspecific protein labeling by F/Q-modified I\(^{15}\) fragments in the absence of HICT was not detected in crowded cell environment. Detailed analysis of reaction kinetics at nM concentrations revealed \(t_{1/2} \approx 10\) min and pseudo first-order rate constants \(k_{\text{PTS}} \approx 1.8 \times 10^{-3}\) s\(^{-1}\) (Table 1), demonstrating that the kinetics are in the same range as at \(\mu\)M concentrations. Nevertheless, slightly lower SP formation (49–53\%) was observed. More important, the crowded environment of human cell lysates had no influence on reaction kinetics of trans-splicing since all values are similar to those of the corresponding unmodified I\(^{15}\).\(^{10}\) In addition, \(K_D\) values for the intein fragments in the absence or presence of cell lysates (Table 1 and Table S3, ESI\(^+\)) are comparable to those of unmodified I\(^{15}\).

This leads to the conclusion that the C-terminal incorporation of a fluorescence quencher, even in combination with the multivalent metal-chelator head, has no impact on the PTS reaction kinetics. It has to be emphasized that our F/Q approach combined with the high-affinity lock-and-key element excels itself when ultra-low concentrations are required and even if PTS is performed in crowded environments.

In summary, we herein described for the first time a fluorescence quenched ultra-small high-affinity split intein system for real-time monitoring of N-terminal protein labeling by trans-splicing. We demonstrated that the simultaneous incorporation of a fluorophore and the respective quencher into I\(^{15}\) leads to a strong quenched state, whereas, upon PTS, a strong fluorescence increase was observed. Enhancement of the I\(^{15}\)\(^{15}\) affinities by the multivalent metal-chelator trisNTA enabled PTS at nM concentrations under physiological conditions as well as in complex human cell lysates. trans-Splicing kinetics of the intein fragments are neither affected by the F/Q incorporation nor by the trisNTA moiety. The ultra-small fluorogenic high-affinity split intein system is an unprecedented example for real-time monitoring of the trans-splicing reaction in cell-like environments as well as for protein labeling with fluorogenic probes at nM concentration. The fluorescence increase and single-digit-background fluorescence of our high-contrast fluorogenic probe will help to solve the problem of fluorescence background of non-reacted probes under no-wash conditions. Since the fluorescence turn-on is efficiently promoted in cell lysates, current progress is directed towards in vivo applications. In combination with high-throughput intracellular delivery via cell squeezing,\(^{2,36}\) our approach will extend the range of protein modifications and open up new opportunities for cellular applications.

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