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Holliday junctions generate super-bright antibodies and antibody fragments in sortase-catalyzed reactions

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Abstract

Site-specific modification of proteins with fluorophores can render a protein fluorescent without compromising its function. To avoid self-quenching of multiple fluorophores installed in close proximity, we used Holliday junctions to label proteins site-specifically. Holliday junctions enable modification with multiple fluorophores at reasonably precise spacing. We designed a Holliday junction with three of its four arms modified with a fluorophore of choice and the remaining arm equipped with a dibenzocyclooctyne substituent to render it reactive with an azide-modified, fluorescent single domain antibody fragment or an intact immunoglobulin, produced in a sortase-

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catalyzed reaction. We conclude that fluorescent Holliday junctions improve fluorescence yields for both single domain and full-sized antibodies, without deleterious effects on antigen binding.

Keywords

single domain antibody; antibody labeling; sortase; immunocytochemistry

Fluorescent versions of various biomolecules^[1] are important tools to study their structure and function. To enable detection by flow cytometry and fluorescence microscopy,^[2] proteins are commonly labeled with chromophores having excitation and emission wavelengths longer than those of their endogenous aromatic amino acids, Trp, Tyr or Phe. The intensity of the emitted fluorescent signal limits detection and quantification of commercially available fluorophores, whether attached covalently or non-covalently. Brightness of fluorescein- or rhodamine-labeled proteins does not necessarily increase proportionally with the extent of labeling.^[3] In fact, fluorescence yield often decreases, because fluorophores self-quench when present in high local concentrations.^[4] Moreover, chemical labeling techniques are not particularly specific, because they can modify any accessible free lysine or cysteine side chain.^[5] Introduction of non-canonical amino acids, or careful placement of the cysteine or lysine residues targeted for modification onto the scaffold also circumvents specificity issues in labeling. The presence of multiple fluorophores installed more or less randomly complicates detailed characterization of the target of interest and may also compromise its function.^[6] Finally, the use of genetic fusions with a fluorescent protein is a viable alternative, but the presence of the fluorescent protein may again affect the function of its fusion partner.^[7]

Site-specific labeling methods for the installation of fluorescent probes on proteins include formylglycine-generating enzyme (FGE), used to append an aldehyde tag onto a specific pentapeptide sequence, which may then react with aminooxy-linked fluorophores,^[8] or biotin ligase, which may be used to attach biotin or biotin derivatives onto a 11-residue recognition sequence.^[9] Tag-mediated labelling utilizing self-labelling proteins as the SNAP-, CLIP-, or the Halo-tag may be used to attach exogenously supplied fluorophores.^[10] Intein-mediated protein ligation (IPL) creates a C-terminal thioester that can be ligated to a short fluorescently labeled peptide.^[11] Sortase-mediated modifications have also been used for site-specific labeling.^[12] Regardless, self-quenching interferes with fluorescence yields when multiple fluorophores are installed in close proximity. The challenge therefore remains to increase signal intensity compared to that of a single fluorophore, and to do so site-specifically.

Here we describe the use of Holliday junctions as semi-rigid DNA-based structures to enable attachment of multiple fluorescent probes onto a ~15 kDa single chain antibody fragment (VHH) to overcome self-quenching and improve signal strength (Scheme 1). Because each of the 4 oligonucleotides that participate in the formation of the Holliday junction is unique and is synthesized separately, each arm can be fixed with respect to length and the substituent of choice. The structure of the protein-DNA conjugate allows positioning of the fluorophores at a distance sufficient to avoid quenching. We demonstrate an almost

linear increase in fluorescence intensity by gel electrophoresis of the substrate-DNA conjugate in comparison with single labeled substrate. We confirmed the intensity shift of a DNA-labeled single domain antibody fragment (VHH7) specific for Class II MHC products by cytofluorimetry. VHH7's antigen binding capacity was not affected by installation of the Holliday Junction. This technology is applicable to any protein of interest and extends the utility of sortase-mediated ligations.

Holliday junctions are highly negatively charged polyelectrolytes that can undergo a two-state-like isomerization transition in the presence of metal ions such as Mg^{2+} ^[13]. This metal ion-induced transition may place fluorophores installed at the extremities in close proximity of one another, a transition minimized in the presence of low concentrations of metal ions, as pertains to standard extracellular labeling conditions. The cruciform planar structure is more stable in the absence of high $[Mg^{2+}]$ which would otherwise minimize electrostatic repulsion between phosphates.^[14] At low $[Mg^{2+}]$, Holliday junctions are cruciform, the angle between two adjacent helical arms being $\sim 90^\circ$ ^[15], and so provide a rigid scaffold to attach fluorophores in a manner that avoids self-quenching. Four strands of appropriately complementary 50bp single stranded DNA were used to create a Holliday junction (Figure S1). This separates the points of fluorophore attachment by an estimated distance of 24 nM. We modified the sequence^[16] for each branch with an AT-rich stretch to avoid quenching by G residues near the 5' point of fluorophore attachment. The 5' end of each DNA strand was modified to contain an amine handle, which was then reacted with an N-hydroxysuccinimide (NHS)-activated carboxytetramethylrhodamine (TAMRA) or NHS-activated dibenzocyclooctyne (DBCO). After assembly into a Holliday junction, each dye and the DBCO handle will thus be positioned away from each other. Separately we installed a short peptide containing a fourth TAMRA dye and an azide functionality at the protein's C-terminus, using a sortase-mediated ligation. The Holliday junction and protein were then joined via these "click" handles to yield a product with four fluorophores attached to VHH7, the protein of interest. We attached one to four fluorophores on the four branches of the Holliday junction to compare the signal intensity by *in situ* gel scanning after electrophoretic separation. The observed proportional increase in signal intensity confirms that Förster resonance energy transfer between each of the TAMRA probes is prevented by the spacing of the fluorophores (Figure 1A). We measured the fluorescent intensity using ImageQuant and found a ~ 3.7 fold increase in signal strength for the (TAMRA)₄ labeled probe in comparison to TAMRA labeling (Figure 1B) in good agreement with expectations.

To demonstrate the utility of the multiple fluorophore probes we labeled VHH7, ^[12e] modified to contain a C-terminal LPETGGHHHHH motif for both purification and for sortase-mediated ligations. Sortase A from *S. aureus* recognizes the LPXTG motif, cleaves between the T and G with simultaneous formation of an active thioester intermediate, which is then resolved by a poly-glycine nucleophile. We used a nucleophile of the sequence GGGK(TAMRA)K(azide) (Figure S2). We used a mutant sortase with increased activity and Ca^{2+} independence^[17]. We achieved full conversion of VHH7 to the desired product with a single TAMRA dye and an azide handle for a "click" ligation. The Holliday Junction was produced by labeling the 5' end of the three strands with a TAMRA dye. The fourth strand was labeled at the 5' position with a dibenzocyclooctyl (DBCO) handle for a copper-

free strain-promoted cycloaddition. The four individual strands were then hybridized to obtain the Holliday structure. Upon incubation at 4 °C overnight with the GGGK(TAMRA)K(azide)-modified VHH7, the two “click” handles reacted to form the protein-DNA hybrid. As observed in the DNA-only Holliday junctions, the DNA-protein hybrid likewise demonstrates the expected increase in fluorescence intensity compared to the single fluorophore labeled protein (VHH7-TAMRA) (Figure 2).

Holliday junction-modified VHH7 by flow cytometry of splenocytes from homozygous Class II MHC-EGFP knock in mice, in which all Class II MHC-expressing cells (e.g. B cells and dendritic cells) display an intact Class II MHC β -chain, fused at its C-terminus with EGFP. Upon addition of either VHH7-TAMRA or the VHH7-Holliday junction, the Class II MHC-EGFP positive fraction shifts to yield the expected double positive population. At all concentrations tested we observed a ~4-fold increase in fluorescence (Figure 3C) for the VHH7-Holliday junction adduct, compared to single labeled VHH7 (Figure 1, 3B). Therefore, binding of VHH7 is not affected by appending the Holliday junction at a position distal from the antigen binding site (Figure 3A). We also examined performance of labeled VHH7 in confocal microscopy. Class II MHC-EGFP⁺ B cells were incubated for 30 minutes at 4 °C with either VHH7 containing a single TAMRA or with (TAMRA)₄-labeled VHH7 and then examined at ambient temperature at identical instrument settings. Single labeled VHH7-TAMRA yielded a faint signal, but (TAMRA)₄-labeled VHH7 produced a much improved image, showing co-localization for surface and internalized Class II MHC-EGFP (Figure 4). This example illustrates the utility of this labeling method, especially for imaging of proteins that may be expressed only at low levels.

Full-sized antibodies, unlike VHH's, cannot be expressed in bacteria and are more difficult and expensive to produce. Therefore increased fluorescence of full-sized IgGs is of importance, especially when using them as directly fluorophoreconjugated staining reagents. Full sized IgG's also provide an opportunity to install at least two LPXTG sortase recognition sites, one at each C terminus of the two identical heavy chains (HC). Although it might be possible to also modify the C-terminus of the light chains with an LPXTG motif^[18] and so obtain the possibility of installing 4 Holliday junctions (and a theoretical maximum of 16 moles of fluorophore/mole of IgG).

The reaction of the full sized sortase-ready IgG specific for the surface marker DEC205 proceeded similarly as seen for VHH7. First two Gly3 peptides containing an azide and a Cy5 dye were sortagged onto each IgG HC. We then performed a “click” reaction to install a Holliday Junction containing a DBCO and three Alexa647 dyes. Cy5 and Alexa647 have similar excitation and emission properties and for the purpose of this experiment are considered interchangeable. We generated two types of anti DEC205 IgG with different degrees of labeling; the first contains only the two sortagged Cy5 dyes, the second has two Holliday Junctions (eight dyes).

We used A20 cells as a B cell derivative with moderate levels of surface Dec205. As for VHH7, we saw a strong increase in fluorescence with the Holliday junction-labeled IgG's. The double Holliday junction probe (eight dyes) shows an approximately three-fold increase in fluorescence – only slightly less than the expected 4-fold increase, compared to the two-

dye labeled antibody, confirmed by FACS analysis (Fig 5A). The two-dye labeled antibody was difficult to visualize by microscopy but double Holliday junction-labeled antibodies could readily be seen (Fig. 5B).

The Holliday junction-dye assembly is a simple and useful means of enhancing fluorescent signal detection, especially if applied to a quantitative and site-specific labeling strategy, as is the case for sortase-catalyzed reactions. Common antibody labeling approaches mostly exploit a reaction between primary amines and NHS-activated fluorophores, or a cysteine-selective modification using maleimide derivatives. In this situation, the site(s) at which the label is introduced may vary from molecule to molecule. For every preparation, the coupling efficiency between dye and protein must be determined empirically. Even then, the number and position of fluorophores on the labeled protein is likely to be a heterogeneous mixture. We position multiple fluorophores at the ends of Holliday junctions such that self-quenching is avoided, while simultaneously boosting signal intensity. Both a single domain antibody fragment (VHH) and full size antibody were labeled with Holliday junctions. Their binding properties were not altered as shown by flow-cytometry and immunofluorescence. The Holliday junctions increase fluorophore intensity significantly, a trait especially useful when studying proteins that have low expression levels and that may be difficult to visualize by conventional labeling techniques. However, repeated in vivo introduction of any such modified product, will require a detailed assessment of immunogenicity. For the in vitro application reported here, this is obviously not an issue. It will be interesting to explore the possibility of adding sortase recognition sites to both the heavy and light chains of an IgG molecule: one could thus add four Holliday junctions and install 16 moles of fluorophore per mole of immunoglobulin. Since modification of Holliday junctions is not limited to the installation of fluorophores, but could include cytotoxic drugs or their precursors, the production of biologicals as protein-drug conjugates presents additional opportunities for application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

The single domain antibody fragment, VHH7, is equipped with a C-terminal LPETG motif to enable sortase recognition. A short peptide containing an N-terminal GGG sequence serves as the nucleophile in the sortase-mediated ligation step to install an azide group and a fluorescent moiety onto the VHH. Meanwhile, three sets of ssDNA of unique sequence are reacted with N-hydroxysuccinimide (NHS)-activated TAMRA and a fourth strand is reacted with NHS-activated DBCO. The sequence of each DNA strand allows assembly of a Holliday junction. After overnight incubation with the VHH, the DBCO and azide react in a copper-free “click” cycloaddition to yield the desired product.

Figure 1.

(A) Fluorescent scanning (580 nm emission) shows the increase in fluorescence upon addition of each successive TAMRA moiety to the Holliday Junction. The middle section of the gel shows a fluorescence scan of the gel stained with TOTO-3 dye to demonstrate even loading of the various DNA oligomers. (B) The fluorescence intensity of the singly labeled Holliday junction was set to 1.0 and the intensities of the other adducts were expressed relative to this value to assess 3.8-fold increase upon attachment of additional fluorophores.

Figure 2. Fluorescent scanning (580 nm emission) (shown in A) and the corresponding Coomassie-stained gel (shown in B). Equal amounts of mono TAMRA-labeled VHH7 and (TAMRA)₄-labeled VHH7 were loaded on the gel. The (TAMRA)₄-labeled VHH7 shows the expected increase in fluorescence intensity.

Figure 3.

(A) Splenocytes isolated from Class II MHC-EGFP knock in mice received no treatment or were treated with a VHH7 probe containing a single TAMRA dye or the Holliday Junction probe with four TAMRA fluorophores, demonstrating that specificity of the VHH7 is not affected by the labelled Holliday Junction (B) increasing concentrations of VHH7 show a shift in intensity for single labelled TAMRA VHH-TAMRA (pink) and the (TAMRA)₄-Holliday Junction (maroon). (C) Quantitation of FACS data shows a ~4-fold increase in intensity for (TAMRA)₄-VHH7.

Figure 4.

Splenic B cells expressing a Class II MHC-EGFP fusion were incubated with (TAMRA)₄-VHH7 or with VHH7-TAMRA single TAMRA dye. (TAMRA)₄-VHH7 shows enhanced fluorescence and co-localization with the EGFP fusion protein.

Figure 5.

(A) Flow cytometry of A20 cells incubated with anti-Dec205 labeled with either 2 or 8 fluorophores. A ~3 fold increase in fluorescence was detected for anti-DEC205 modified with 8 fluorophores compared to anti-DEC205 labeled with 2 fluorophores. (B) A20 cells were captured on a poly-lysine coated slide and imaged by confocal microscopy. As expected, a far brighter signal is detected for anti-DEC205 labeled with 8 fluorophores.