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Fluorophore-Conjugated Holliday Junctions for Generating Super-Bright Antibodies and Antibody Fragments

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Abstract: *The site-specific modification of proteins with fluorophores can render a protein fluorescent without compromising its function. To avoid self-quenching from 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-catalyzed reaction. These fluorescent Holliday junctions improve fluorescence yields for both single-domain and full-sized antibodies without deleterious effects on antigen binding.*

Fluorescent versions of various biomolecules[1] are important tools for studying their structure and function. To enable detection by flow cytometry and fluorescence microscopy,[2] proteins are commonly labeled with fluorophores with excitation and emission wavelengths longer than those of the endogenous aromatic amino acids Trp, Tyr, and Phe. The intensity of the emitted fluorescent signal limits detection and quantification with commercially available fluorophores, whether attached covalently or non-covalently. The 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 chains.[5] The introduction of non-canonical amino acids or careful placement of the cysteine or lysine residues targeted for modification onto the scaffold can be used to circumvent 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] The use of genetic fusions with a fluorescent protein is a viable alternative, but the presence of the fluorescent protein may 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), which is 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 an 11-residue recognition sequence.[9] Tag-mediated labelling utilizing self-labelling proteins such as the SNAP-, CLIP-, or Halo-tags 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.

Herein, we describe the use of Holliday junctions as semi-rigid DNA-based structures to enable the attachment of multiple fluorescent probes onto a single-chain antibody fragment (VHH) of approximately 15 kDa to overcome self-quenching and improve signal strength (Scheme 1). Because each of the four 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 cytofluorometry. The antigen-binding capacity of VHH7 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 to one another, a transition minimized in the presence of low concentrations of metal ions relative to standard extracellular labeling

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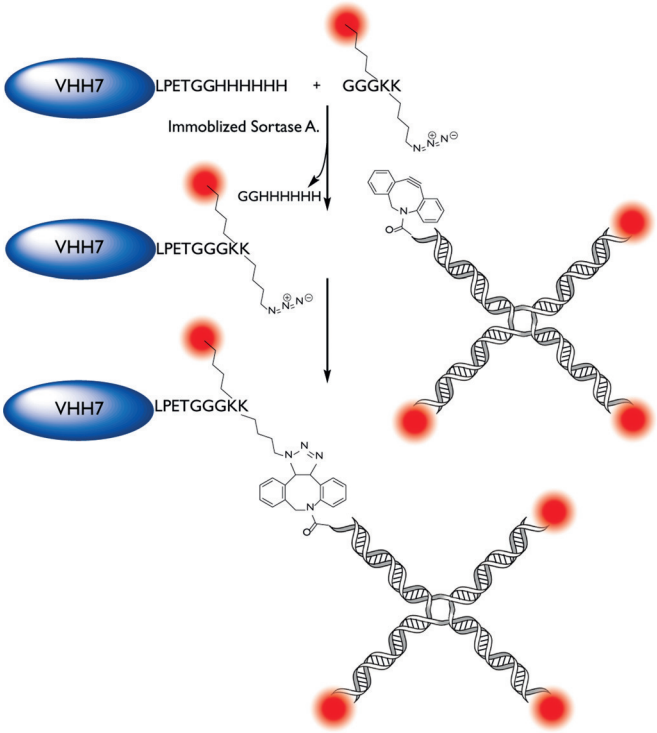

Scheme 1. The single-domain antibody fragment VHH7 is equipped with a C-terminal LPETG motif to enable sortase recognition. A short peptide containing an N-terminal GGG sequence serves as the nucleophile in the sortase-mediated ligation step to install an azide group and a fluorescent moiety (red) onto 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 sequences of the DNA strands lead to the assembly of a Holliday junction. After overnight incubation with the VHH, the DBCO and azide react in a copper-free cycloaddition to yield the desired product.

conditions. The cruciform planar structure is more stable in the absence of high [Mg^{2+}], which would otherwise minimize electrostatic repulsion between phosphate groups.[14] At low [Mg^{2+}], Holliday junctions are cruciform, the angle between two adjacent helical arms being approximately 90°,[15] and so provide a rigid scaffold to attach fluorophores in a manner that avoids self-quenching. Four strands of appropriately complementary 50 bp single-stranded DNA were used to create a Holliday junction (Figure S1 in the Supporting Information). This separates the points of fluorophore attachment by an estimated distance of 12 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 carboxytetra-methylrhodamine (TAMRA) dye or dibenzocyclooctyne (DBCO) handle. 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 C terminus by using 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 first attached one to four fluorophores to the four branches of the Holliday junction without further protein conjugation 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 (FRET) between the TAMRA probes is prevented by the spacing of the fluorophores (Figure 1 A). We measured the fluorescence intensity by using ImageQuant and found an approximately 3.7-fold increase in signal strength for the $(TAMRA)_4$-labeled probe in comparison to single TAMRA labeling (Figure 1 B), which is in good agreement with expectations.

To demonstrate the utility of the multiple-fluorophore probes, we labeled a VHH7 variant[12e] modified to contain a C-terminal LPETGGHHHHHH motif for both purification and sortase-mediated ligations. Sortase A from *S. aureus* recognizes the LPXTG motif and cleaves between the T and G with simultaneous formation of an active thioester intermediate, which is then resolved by a polyglycine nucleophile. We used a nucleophile from 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 into the desired product with a single TAMRA dye and an azide handle for a click ligation. The fluorescent 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

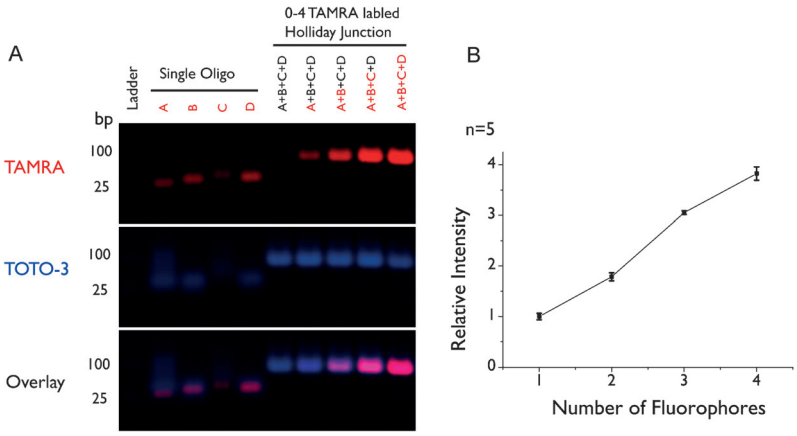

Figure 1. A) Fluorescence scanning (580 nm emission) shows the increase in fluorescence upon the addition of each successive TAMRA moiety to the Holliday junction. The right side of the gel shows a fluorescence scan of the gel stained with the TOTO-3 dye to demonstrate even loading of the various DNA 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 the attachment of additional fluorophores.

(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 (TAMRA-VHH7; Figure 2).

The Holliday-junction-modified VHH7 was tested by flow cytometry analysis 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 chain that is fused at its C terminus to EGFP. Upon the addition of either TAMRA-VHH7 or $TAMRA)_4$-VHH7, the Class II MHC-EGFP positive fraction shifts to yield the expected double-positive population. At all concentrations tested, we observed an approximately 4-fold increase in fluorescence (Figure 3C) for the $(TAMRA)_4$-VHH7 conjugate compared to single-labeled VHH7 (Figure 1, Figure 3B). Therefore, the binding of VHH7 to Class II MHC is not affected by appending the Holliday junction at a position distal from the antigen binding site (Figure 3A). We also examined the performance of labeled VHH7 in confocal microscopy. Class II MHC-EGFP positive B cells were incubated for 30 min 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 TAMRA-VHH7 yielded a faint signal, but $(TAMRA)_4$-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 the imaging of proteins that may be expressed only at low levels.

Full-sized antibodies, unlike VHHs, cannot be expressed in bacteria and are more difficult and expensive to produce. Therefore increasing the fluorescence of full-sized immunoglobulins (Igs) important, especially when using them as directly fluorophore-conjugated staining reagents. Full-sized Igs 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). 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 four Holliday junctions (and a theoretical maximum of 16 moles of fluorophore/mole of Ig).

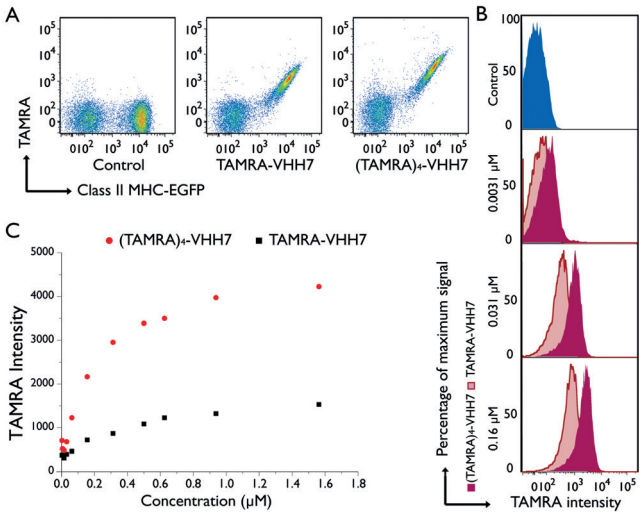

Figure 3. A) Splenocytes isolated from Class II MHC-EGFP knock-in mice received no treatment or were treated with a VHH7 probe containing a single TAMRA dye or the Holliday junction probe with four TAMRA fluorophores and analyzed by flow cytometry. The results show that the specificity of the VHH7 is not affected by the labelled Holliday junction, B) Increasing concentrations of VHH7 give a shift in intensity for singly labelled TAMRA-VHH (pink) and the $(TAMRA)_4$-VHH (maroon). C) Quantitation of the flow cytometry data shows an approximately 4-fold increase in intensity for $(TAMRA)_4$-VHH7 over TAMRA-VHH7.

The reaction of the full sized sortase-ready IgG specific for the surface marker DEC205 proceeded similarly to that of 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 handle and three Alexa Fluor 647 (Alexa647) dye moieties. 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 IgGs with different degrees of labeling; the first contains only the two sortagged Cy5 dye moieties and the second has two Holliday junctions (eight dye moieties).

We used A20 cells, a B cell derivative with moderate levels of surface Dec205. As for VHH7, we saw

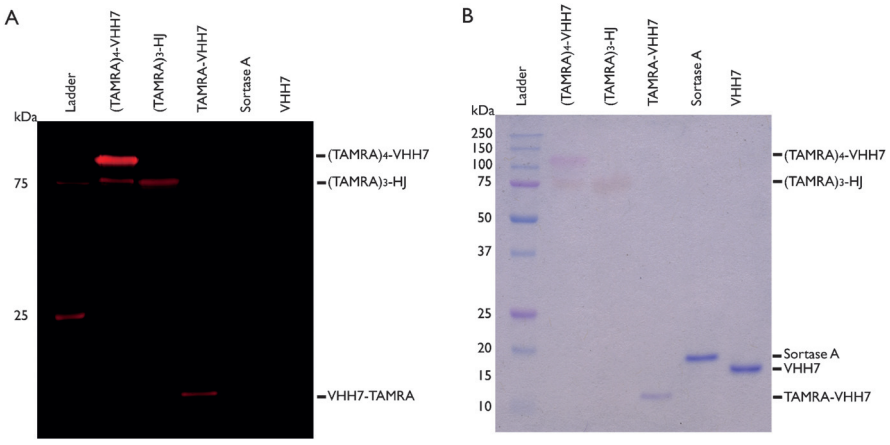

Figure 2. Fluorescence scanning (580 nm emission; A) and the corresponding Coomassie-stained gel (B). Equal amounts of mono-TAMRA-labeled VHH7 and $(TAMRA)_4$-labeled VHH7 were loaded on the gel. The $(TAMRA)_4$-labeled VHH7 shows the expected increase in fluorescence intensity. HJ = Holliday junction.

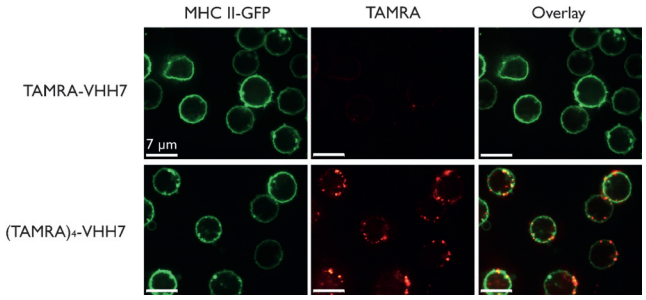

Figure 4. Splenic B cells expressing a Class II MHC-EGFP fusion were incubated with $(TAMRA)_4$-VHH7 or with TAMRA-VHH7. $(TAMRA)_4$-VHH7 shows enhanced fluorescence and reveals co-localization with the EGFP fusion protein.

a strong increase in fluorescence with the Holliday-junction-labeled IgGs. The double Holliday junction probe (eight dye moieties) shows an approximately three-fold increase in fluorescence, which is only slightly less than the expected 4-fold increase, compared to the two-dye antibody, as confirmed by flow cytometry analysis (Figure 5A). The two-dye antibody was difficult to visualize by microscopy but the antibodies labelled with two Holliday junctions could readily be seen (Figure 5B).

The assembly of dye-conjugated Holliday junctions 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 either the reaction between primary amines and NHS-activated fluorophores or cysteine-selective modification with 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 a full size antibody were labeled with the fluorophore-conjugated Holliday junctions. Their binding properties were not altered, as shown by flow-cytometry and immunofluorescence. The Holiday junctions increase fluorophore intensity significantly, a trait especially useful when studying proteins that have low expression levels and may thus 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 applications reported herein, 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 the modification of Holliday junctions is not limited to the installation of fluorophores but could include cytotoxic drugs or their precursors, the production of protein–drug conjugates presents additional opportunities for application.

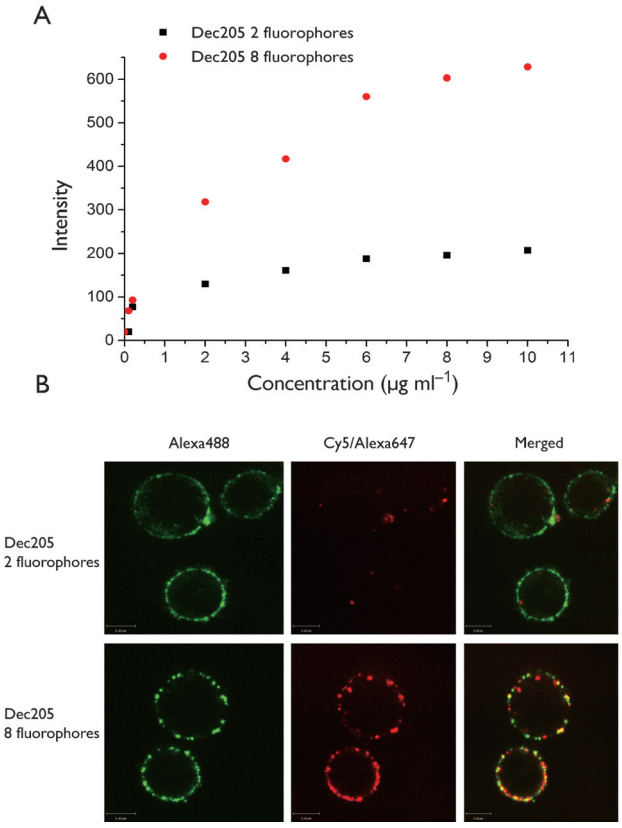

Figure 5. A) Flow cytometry of A20 cells incubated with anti-Dec205 labeled with either two or eight fluorophores. An approximately 3-fold increase in fluorescence was detected for anti-DEC205 modified with eight fluorophores compared to anti-DEC205 labeled with two fluorophores. B) A20 cells were captured on a poly-lysine-coated slide and imaged by confocal microscopy. Cells were co-stained with a commercial Alexa488 anti-DEC205 antibody as reference. As expected, a far brighter signal is detected for anti-DEC205 labeled with eight fluorophores.

Keywords: antibodies · DNA technology · Holiday junctions · imaging agents · immunocytochemistry

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