

## Directions for Use

### Collagen Hybridizing Peptide

5-FAM, CY3 AND BIOTIN

Catalog Number 5264, 5265 and 5276

#### Background

Collagen is the most abundant protein in mammals. It is the major structural component of almost all organs and tissues, providing the framework for cell attachment and growth. Programmed collagen degradation occurs during tissue development, homeostasis and repair. However, excessive collagen degradation is implicated in a variety of diseases, such as cancer, inflammation, and fibrosis [1].

The triple helix is the hallmark protein structure of collagen. During tissue remodeling, the triple helical collagen molecules are degraded by specific proteases (e.g., MMP or cathepsin K) and become unfolded at body temperature. The Collagen Hybridizing Peptide (CHP) is a synthetic peptide that can specifically bind to such denatured collagen strands through hydrogen bonding, both in histology [2], *in vivo* [3], and *in vitro* (3D cell culture) [4]. By sharing the structural motif and the Gly-X-Y repeating sequence of natural collagen, CHP has a strong capability to hybridize with denatured collagen strands, in a fashion that is similar to a DNA fragment annealing to its complimentary DNA strand during PCR [1,2,3]. CHP is an extremely specific probe for unfolded collagen molecules: it has negligible affinity to intact collagen molecules due to the lack of binding sites; it is also inert towards non-specific binding because of its neutral and hydrophilic nature [5].

Collagen is the major building block of all load-bearing tissues including tendon, ligament, cornea, cartilage and bone. It was recently found that unfolding of the collagen triple helix can occur during mechanical damage to connective tissues [6], and that CHP can specifically detect and localize such mechanically unfolded collagen molecules *in situ* [6], enabling understanding of the mechanical behavior and damage mechanism of these tissues at the molecular level.

Collagen is also one of the most widely used natural scaffold materials for regenerative medicine. The process of harvesting native extracellular matrix (ECM) by removing cells from animal tissues (i.e., decellularization) may alter the collagen structure and negatively affect the mechanical property and regenerative capacity of the ECM materials. CHP enables assessment of the structural integrity of collagen molecules within these processed matrices [7], and can facilitate optimization of the decellularization protocols.

Additionally, CHP can be used in several biochemical assays, such as in-gel Western blot [5], for identification and quantification of collagen content in a biological sample.

CHP conjugates from Advanced BioMatrix are labeled with 5-FAM (F-CHP, Catalog No. 5264-60UG) / sulfo-Cyanine3 (R-CHP, Catalog No. 5276-60UG) for fluorescence detection, or biotin (B-CHP, Catalog No. 5265-UG) for avidin/streptavidin-mediated detection.

#### Applications

Immunofluorescence, immunohistochemistry, cell imaging (2D or 3D), SDS-PAGE (in-gel Western blot)

#### Descriptions:

Name	Collagen Hybridizing Peptide, 5-FAM Conjugate	Collagen Hybridizing Peptide, Cy3 Conjugate	Collagen Hybridizing Peptide, Biotin Conjugate
Catalog No.	5264-UG	5276-UG	5265-UG
Acronym	F-CHP	R-CHP	B-CHP
Specialty	Straight-forward fluorescence detection in green	Straight-forward fluorescence detection in red	Flexible avidin / streptavidin mediated detection options based on needs, allowing non-green fluorescence and HRP methods to avoid background and enhance signal
Formula	$C_{135}H_{175}N_{31}O_{45}$	$C_{144}H_{198}N_{33}O_{46}S_2$	$C_{124}H_{181}N_{31}O_{39}S$
Molecular weight	2952.01 g/mol	3191.44 g/mol	2762.01 g/mol
Ex/Em	494 nm / 512 nm	548 nm / 563 nm	N/A
Synonym	collagen mimetic peptide, CMP		
Solubility	water, aqueous buffers		
Shipping	Shipped as powder at ambient temperature. Store at -20 °C upon arrival and until use.		
Storage	-20 °C as powder for long term storage; 4 °C after reconstitution in water, no need to aliquot and freeze. For F-CHP and R-CHP, protect from light.		

## Precautions and Disclaimer

These products are for research and development (R&D) purposes only and are not intended for human or other uses. Please consult the Material Safety Data Sheets for information regarding hazards and safe handling practices.

## Features of CHP reagents

- More informative, reliable and convenient than zymography, DQ collagen, SHG, and TEM
- High affinity and unparalleled specificity to collagen with essentially no nonspecific binding
- Applicable to denatured collagens of all subtypes and from all species; binding relying on collagen's secondary structure instead of specific epitopes
- Suitable for both frozen and paraffin-embedded sections with no need for antigen retrieval
- A non-antibody approach with no species restrictions, compatible for co-staining with any antibody
- Small size (2% of IgG by MW) enabling facile tissue penetration during whole tissue staining (with no need for sectioning)
- Stable in solution under 4 °C, eliminating the need to aliquot for storage

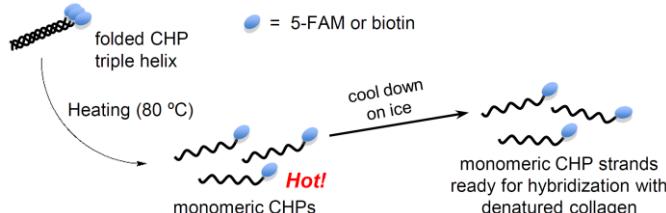
## Protocols

**(A) Sample reconstitution and handling:** Dissolve the 0.3 mg of peptide powder (F-CHP, R-CHP, or B-CHP) in 1 mL of pure water or phosphate-buffered saline (1x PBS), vortex well and centrifuge, to prepare a stock solution containing approximately 100 µM of CHP. Store the stock solution at 4 °C. Dilute the stock solution to assay dependent concentrations upon use.

For the 60 µg products, dissolve the powder in 400 µL water or PBS to get a stock solution with a CHP concentration of 50 µM. For the 30 µg samples, dissolve in 200 µL water or PBS for a stock solution with the CHP concentration of 50 µM.

**Special steps:** CHP tends to slowly self-assemble into CHP triple helices in solution during storage. Such CHP trimers have no driving force to hybridize with unfolded collagen strands. Therefore, the trimeric CHP must be dissociated to monomers by heating prior to use. Since the trimerization of CHP takes hours to occur at low µM concentrations, the heat-dissociated CHP can stay as active monomer strands that are available for hybridization with unfolded collagen. A common protocol is heating the CHP solution (after diluting to the desired concentration) to 80 °C in a water bath, and quickly quenching it to room temperature followed by immediate application to

target collagen substrates, as described below in detail. A heating block and an ice-water bath may be needed in most



applications (not provided).

**Caution:** turn off the heating device after the experiment to avoid accidents.

### (B) For staining histology slides

Note: for tissues that are strongly auto-fluorescent in green, we recommend using B-CHP or R-CHP instead of F-CHP, which allows colorimetric detection, or fluorescence detection in non-green channels. Details of protocol (B) is discussed in details in ref [2].

1. Removing the embedding OCT compound or paraffin with standard procedures.
2. Since CHP has low non-specific binding to tissue, blocking with serum or BSA can be omitted. However, when co-staining samples with CHP and antibodies, we recommend blocking the tissue slides with 10% serum or 5% BSA. (For certain tissue types, e.g., kidney, it may be necessary to block endogenous biotin using a standard kit for B-CHP staining.)
3. Dilute the CHP stock solution in a PBS buffer. A concentration of 20 µM is recommended for initial trials. [Note: the optimal CHP concentration (usually 5-30 µM) is sample dependent.] Depending on the sample size, one tissue section may require 20 to 200 µL of diluted CHP solution.
4. Using a heating block or a water bath with temperature control, heat the dilute CHP solution in a sealed microtube at 80 °C for 5 min. (There is no need to heat the CHP stock solution.)
5. To avoid thermal damage to the tissue sample, after heating, immediately immerse the CHP microtube in an ice-water bath for 15-90 s to quench the solution to room temperature. The required cooling time depends on the solution volume. The microtube can be quickly centrifuged to collect condensation in the tube. Subsequently pipet the solution onto each tissue sample quickly. Achieving minimal deadtime (~1-3 min) is encouraged for this step. (Note: other staining agents, such as a primary antibody, can be diluted into the quenched CHP solution for co-staining.)
6. Incubate the tissues with the staining solution at 4 °C for 2 h. For optimal results, overnight incubation is recommended.

7. After staining, wash the tissue slides in PBS for 5 min three times at room temperature.
8. Samples stained with F-CHP and R-CHP can be analyzed with a standard fluorescence microscope using the GFP and RFP channel/filters. For tissues treated with B-CHP, the collagen-bound CHP can be detected by an avidin/streptavidin-mediated method. [Note: To detect B-CHP, we recommend incubating the tissue samples with 0.005 mg/mL of a streptavidin conjugate (e.g., AlexaFluor dyes labeled streptavidin) in a PBS solution containing 1% BSA for 1 h at room temperature. To detect the co-stained primary antibody, a labeled secondary antibody can be either diluted into the streptavidin solution (for B-CHP co-staining), or added to the slides directly after dilution in a PBS solution containing 1% BSA (for co-staining with F-CHP or R-CHP).]

### (C) Imaging pericellular collagen proteolysis in 3D collagen culture

Note: please check ref [4] for details. The detailed protocols can be modified for specific cell types and assays.

1. To create cell-embedded 3D collagen matrix, rat tail type I collagen dissolved in an acetic acid solution (4 mg/mL) is mixed on ice with 0.34 N NaOH and 10×PBS in an 8:1:1 volume ratio, and  $1 \times 10^5$  cells are added into the neutralized collagen solution. Pour the thoroughly mixed cell-matrix mixture (150  $\mu$ L/well) onto an 8-well chambered cover glass, and incubate at 37 °C for 1 h to ensure full gelation.
2. At specified time points during culture, fix the cell embedding 3D collagen matrix with 4% paraformaldehyde in a PBS solution for 4 h. Subsequently, wash the fixed gels with PBS for 2 h with the buffer changed every 30 min.
3. Prepare a PBS solution containing 5  $\mu$ M of F-CHP / R-CHP in a microtube. Heat the solution for 5 min in an 80 °C water bath and immediate incubate it in an ice/water bath (for 20–90 s depending on volume) to quench the hot solution to room temperature. The quenched solution is immediately added onto the fixed cell-matrix constructs (150  $\mu$ L/well). When needed, primary antibody can be quickly diluted into and mixed with the quenched CHP solution before dispensing for co-staining.
4. Stain the cell-embedded collagen gels with the F-CHP / R-CHP solution without pre-blocking overnight at 4 °C.
5. Following staining, wash the gels with four rounds of 30 min PBS rinsing under gentle shaking.
6. Stain the cell nuclei and actin (if needed) by Hoechst 33258 and phalloidin-TRITC respectively for 30 min at room temperature. Detect the co-staining primary antibodies with the labeled 2<sup>nd</sup> antibodies (e.g., with 1:100 to 1:200 dilution) for over 3 h at room temperature, followed by four rounds of 30 min wash in PBS.

7. The stained gels can be imaged with a confocal fluorescent microscope with a 20x or 60x objective at a distance of 10–40  $\mu$ m from the bottom cover glass.

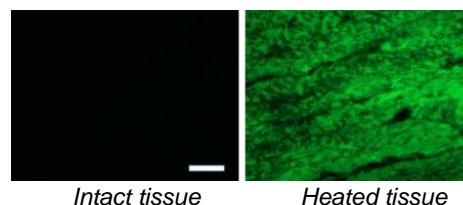
### (D) For in-gel Western blot

Note: Details of protocol (D) is discussed in details in ref [5].

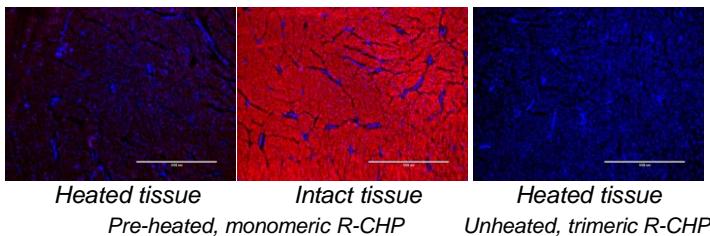
1. Heat collagen proteins at 70 °C in an SDS buffer and resolve the protein bands by an SDS-PAGE gel (e.g., 4-12% bis-tris gel). It is recommended to add 0.5-2  $\mu$ g of collagen into each protein lane for strong signals.
2. After electrophoresis, wash the PAGE gel by deionized water for 5 min three times to remove the remaining SDS. Fixation of protein bands is not required. An area of interest can be cut out of the gel to reduce CHP usage in staining.
3. Prepare a dilute solution of 1-6  $\mu$ M F-CHP / R-CHP from the stock. Heat the dilute solution (1-5 mL) at 80 °C for 5-10 min, and immediately add it onto the cropped PAGE gel to soak it in CHP solution.
4. At room temperature, stain the gel in F-CHP / R-CHP solution in a small staining chamber under gentle shaking for 3 h in dark, followed by washing with water at least three times (0.5 h each time) to remove unbound CHP.
5. The stained collagen bands can be visualized using a fluorescence gel imager/scanner. The gel can be further stained with coomassie brilliant blue.

### Proof-of-concept results

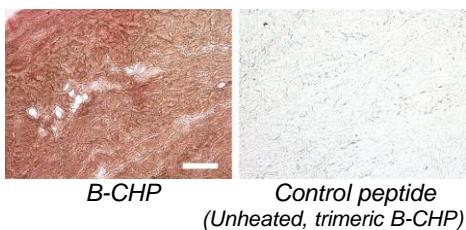
A. Fluorescence micrographs of porcine ligament cryosections stained with F-CHP (15  $\mu$ M, overnight 4 °C incubation). The sample shown in the right panel was treated with hot water to purposefully denature collagen. Significant fluorescent signals from the collagen-bound F-CHP can be detected in the denatured sample, whereas no signals can be seen in the intact section, demonstrating high specificity of CHP for denatured collagen strands. Both samples were stained and imaged under the same conditions. Scale bar: 200  $\mu$ m.



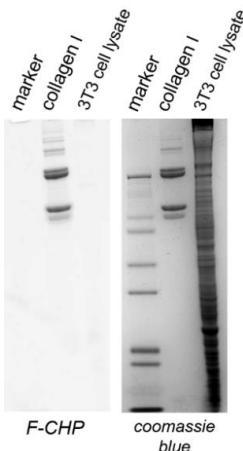
**B.** Sections of frozen porcine ligament showed drastic differences of R-CHP binding between an intact (middle) and a heat-denatured tissue sample (left). Comparing to the pre-heated, monomeric R-CHP (left), the triple-helical R-CHP used without the pre-heating step (right) exhibited dramatic decrease in binding. Scale bar: 1000 µm. Blue: cell nuclei stained by DAPI.



**C.** Following *heat-mediated antigen retrieval*, the complete collagen content in a formalin-fixed (FFPE) section of porcine ligament is visualized non-fluorescently via immunohistochemistry using B-CHP, which is further detected by horseradish peroxidase (HRP) conjugate of NeutrAvidin. Scale bar: 200 µm.



**D.** CHP can specifically detect collagen bands directly in SDS-PAGE gels (in-gel Western blot). After washing off the SDS content, an SDS-PAGE gel containing collagen I (1 µg) and a lysate of 3T3 fibroblast was stained by F-CHP (left, imaged by a fluorescence gel imager,  $\lambda_{ex} = 488$  nm). The same gel was further stained by coomassie blue and photographed (right). The absence of fluorescent bands in the lysate and molecular marker lanes in the left image shows remarkable specificity of CHP for collagen strand binding.



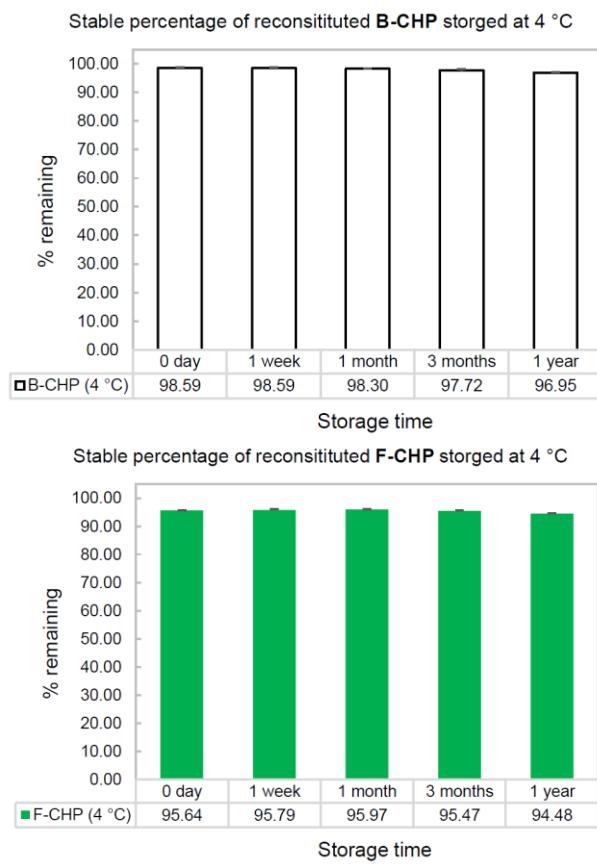
## References

- [1] Targeting and mimicking collagens via triple helical peptide assemblies. *Current Opinion in Chemical Biology*, **2013**, 17, 968–975.
- [2] *In situ* imaging of tissue remodeling with collagen hybridizing peptides. *ACS Nano*, **2017**, 11, 9825–9835.
- [3] Targeting collagen strands by photo-triggered triple-helix hybridization. *Proceedings of the National Academy of Sciences of the United States of America*, **2012**, 109, 14767–14772.
- [4] Visualizing collagen proteolysis by peptide hybridization: From 3D cell culture to *in vivo* imaging. *Biomaterials*, **2018**, 183, 67–76.
- [5] Direct detection of collagenous proteins by fluorescently labeled collagen mimetic peptides. *Bioconjugate Chemistry*, **2013**, 24, 9–16.
- [6] Molecular level detection and localization of mechanical damage in collagen enabled by collagen hybridizing peptides. *Nature Communications*, **2017**, 8, 14913, doi: 10.1038/ncomms14913.
- [7] Molecular assessment of collagen denaturation in decellularized tissues using a collagen hybridizing peptide. *Acta Biomaterialia*, **2017**, 53, 268–278.

## Frequently asked questions

1. How stable are the CHP products in solution at 4 °C? Can the CHP reagents be aliquoted and stored at -20 °C or -80 °C after reconstitution? Why it is recommended to store the powder at -20 °C before reconstitution?

The peptide is highly stable in solution during storage. Here are the stability profiles of the two reagents stored in an aqueous solution at 4 °C. As can be seen, the purity barely decreases following one year storage in solution.



However, if the user wishes, all CHP products can be aliquoted and stored at -20 °C or -80 °C. In fact, there may not be a need to aliquot. The peptides are stable and do not denature or degrade after multiple freeze-thaw cycles. We recommend -20 °C as the storage conditions of the powder just to ensure the products have the highest quality when they are used in the first experiment. We understand that the products are sometimes stored for months upon arrival before they are opened.

2. This peptide has only one label molecule in one peptide molecule. Is this correct? If so, do you control the length of the peptide in production? If the CHP products are a mixture of GXY peptides of different lengths, accurate calculation of molar in one vial would be difficult.

Correct, the CHP probes have only one labeling moiety in one peptide molecule. The peptides are chemically synthesized on solid phase. They are not made by recombinant methods. As such, we accurately control the exact length of the peptides in production. All CHP molecules in a unit vial and in all batches are exactly the same (e.g., same length, same molecular weight). We verify every batch of our products closely with HPLC and MALDI MS.

3. Does the probe need to be heated up prior to use each time or only when reconstituting? If the un-dilute stock solution is exposed to multiple heat-cool dissociation procedure, will the CHP still be stable?

Yes. CHP needs to be heated up prior to use EACH TIME. Once it is cooled down and stored at 4 °C for a while in solution, it will gradually re-assemble into the trimeric form. After years of working with CHP, we found no limit of its heating/cooling cycles. The peptide is very stable both chemically and physically. However, we do NOT recommend users to heat up the stock solution. It is not necessary. The recommended procedure is that a user only aliquot out the needed amount from the stock solution (e.g., 10 µL, 50 µM), and dilute it to the required concentration (e.g., 50 µL, 10 µM) with PBS. Subsequently, the stock solution should be returned to 4 °C storage, and ONLY the diluted solution is heated and active for the experiment. In this way, the stock solution is not going to be heated and cooled down for multiple cycles. Only the diluted solution will be heated once.

4. In step 5 for staining protocol (B), if the dead time for dispensing is over 3 min, will something wrong happen?

If the dead-time for dispensing is over 3 min, the experiment will still work well. The heat-dissociated CHP strands take hours to re-assemble. A short delay of a few minutes is not going to cause a problem. But please do not wait for hours to use after heating. We recommend including a normal control tissue slide in a histology study, so that the pathological slide of interest are stained together with the normal control with the same dead time. The two will be directly comparable.

However, please note that the single strand CHP still re-assembles after heating (at a very slow speed in the diluted solution), so to achieve maximal binding, we still recommend users to minimize the cooling and dispensing dead time.

5. Why do you perform incubation at 4 °C (e.g., step 6 in protocol B)? If this step is performed at room temperature (RT) or 37 °C, what will happen?

We recommend performing staining incubation at 4 °C for these reasons:

- (1) CHP's affinity to denatured collagen is stronger at 4 °C than at RT and 37 °C. The user can perform binding at RT or 37 °C, but colder temperature will achieve a higher level of binding.
- (2) CHP is often used in immunostaining of frozen tissue slides. These unfixed tissue sections need to be maintained at 4 °C during staining.

6. *The step 6 in protocol B mentions optimal staining with overnight incubation, but 2 h minimum. How different are the stains after 2 h incubation vs overnight?*

The answer to this question is published in ref [2], Supporting information, **Fig. S2**.

Link:

[https://pubs.acs.org/doi/suppl/10.1021/acsnano.7b03150/suppl\\_file/nm7b03150\\_si\\_001.pdf](https://pubs.acs.org/doi/suppl/10.1021/acsnano.7b03150/suppl_file/nm7b03150_si_001.pdf)

7. *What kind of fixative or fixation technique is recommended? Will the staining result depend on fixation?*

As described in detail in ref [2], the staining result does not depend on fixation in our tests. Fixation is not necessary for CHP staining. However, if fixing is required for other reasons, the tissue can be chemically fixed before CHP staining. For fluorescent imaging (e.g., when using F-CHP or R-CHP), we recommend using **formaldehyde**, instead of paraformaldehyde or glutaraldehyde, to fix the tissue, because the formaldehyde fixed tissues have much less auto-fluorescence, especially in the FITC fluorescence channel. If the user has to use paraformaldehyde or glutaraldehyde, to overcome the possible autofluorescence issue, we recommend staining the tissue with B-CHP and detecting the CHP binding with a streptavidin conjugate that is labeled with a red / near-infrared dye (e.g. AlexaFluor647) or is labeled with an enzyme (e.g. HRP for non-fluorescence detection).

8. *How to co-stain a slide with CHP and an antibody? If a slide is pre-stained by immunohistochemistry, can it be stained again by CHP?*

- A tissue slide can be readily co-stained with CHP and an antibody together. The primary antibody can be directly diluted into the CHP solution after the CHP solution is heated and cooled down to room temperature. As such, the slides are co-stained with CHP and the antibody together overnight at 4 °C. The antibody can be detected by labeled 2<sup>nd</sup> antibody following incubation. The protocol describing the co-staining steps are briefly mentioned in protocol (B) and (C). Detailed examples and protocols are reported in ref [2] and [4].
- We have not tested whether the CHP will still work or not if the samples have been pre-stained by immunohistochemistry. It may depend on what the exact immunohistochemistry is and what detection method is used to visualize CHP binding.

- If conditions allow, we recommend using un-stained slides for CHP imaging analysis to get the most reliable results.

9. *If antigen retrieval is necessary for immunohistochemistry, may these treatment influence CHP staining?*

Our tests indicate that the CHP staining is drastically enhanced after the tissue is treated by the heat-mediated antigen retrieval (AR) process (unpublished). We believe that the AR treatment can denature all the collagen molecules in the tissue, and the CHP staining following AR simply reveals the total collagen content in the tissue. If AR is necessary for immunohistochemistry, we recommend performing CHP staining on a separate, neighboring slide that is not treated with AR. This way, the CHP staining is only detecting the naturally degraded or denatured collagen.

10. *Would it be better to use the biotin-labeled CHP (B-CHP) to enhance amplification and sensitivity?*

In many cases, we found that all F-CHP, R-CHP, and B-CHP work very well. When higher sensitivity is indeed needed, we found that B-CHP, detected by a fluorescently labeled streptavidin (in the red or far red emission region, such as AlexaFluor647, 555 etc.), gives the best result, due to the multiple fluorophores conjugated to each streptavidin molecule. Horseradish peroxidase (HRP) is another way to enhance the signal, but sometimes there could be some background stain.

11. *Can you recommend a negative control for my histology experiment? How do I know the detected signal is truly due to CHP hybridizing with the unfolded collagen?*

You can verify whether the binding is truly due to CHP-collagen hybridization or non-specific binding by using a CHP solution *without* the pre-heating step. As described in the protocol above, the CHP single strands have a strong propensity to assemble into the trimeric form in solution at 4 °C. Without the pre-heating step, such folded, trimeric CHP probe has no driving force to bind denatured collagen chains; meanwhile, it has the same chemical nature of CHP, making it a great negative control.

If the sample stained with pre-heated CHP following the normal procedure (e.g., protocol B or C) shows positive signal, whereas a matching sample stained with the same CHP solution only without the pre-heating step (negative control) shows no or significantly reduced CHP signal, the detected CHP binding in the positive sample can be considered truly due to CHP-collagen hybridization and not due to non-specific binding. **Fig. C** in the proof-of-concept results section (page 3 of this document) provides an example. More examples are reported in ref [2], supporting information **Fig. S9**:

[https://pubs.acs.org/doi/suppl/10.1021/acsnano.7b03150/suppl\\_file/nm7b03150\\_si\\_001.pdf](https://pubs.acs.org/doi/suppl/10.1021/acsnano.7b03150/suppl_file/nm7b03150_si_001.pdf)



To ensure CHP is fully self-assembled into the trimeric form, make the CHP stock solution with a higher than 50  $\mu$ M concentration (ideally 100-150  $\mu$ M), store it at 4 °C for at least 2 days, and directly dilute it in cold PBS buffer without heating before using.

Please note that it is not surprising to detect some background fluorescence in normal tissue. This is either because of CHP binding to the low content of degraded collagen in native tissue (caused by the endogenous collagen remodeling) or auto-fluorescence from the tissue.