Mapping mRNA and protein expression with high signal-to-background in diverse organisms

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Time comes into it.
Say it. Say it.
The universe is made of stories,
not of atoms.

- Muriel Rukeyser

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Abstract

In situ hybridization (ISH) techniques allow for the study of the nucleic acid expression within whole biological samples. The quality of probes for ISH dictates how accurate and bright the signal is for the experiment; however, there is currently not a systematic way to determine what the best probe set would be. In response to this, we have developed a framework to optimize an ISH probe set to achieve the greatest signal-to-background ratio. As methods like ISH help obtain more information about biological processes, there is a growing desire to simultaneously analyze various targets within the same sample to examine these complex genetic interactions. To facilitate this, a novel amplification technique called hybridization chain reaction (HCR) has allowed for the *in situ* detection of multiple target mRNAs concurrently in zebrafish embryos. We have now expanded this technology further by adapting HCR amplification for ISH to other model organisms, particularly, whole mount *Drosophila melanogaster* embryos and formalin-fixed paraffin-embedded human tissue sections. Beyond looking at mRNA, immunohistochemistry (IHC) provides another tool to understand biological systems by analyzing protein expression patterns. The ability to easily look at both mRNAs and proteins in the same sample offers significant advantages as each provides unique information, but current methods are technically difficult and labor intensive. In response, we have engineered a scheme to use HCR to amplify signal for IHC. We then used this advancement to develop a straightforward protocol using HCR amplification for simultaneous detection of multiple proteins and mRNAs with a high signal-to-background ratio.

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Chapter 1

Introduction

Although all the cells in a single organism contain the same genetic information, it is the variation in the expression of these genes that dictates numerous processes during development and growth [1, 2]. In situ hybridization (ISH) is a technique that is crucial to understanding the mRNA landscape in an organism during these various steps [3–8]. ISH detects the position and quantity of a target mRNA in an intact organism. Immunohistochemistry (IHC) is an alternate technique to examine biological phenomena by tracking protein expression *in situ*. This allows for the analysis of translational, instead of transcriptional, controls in organisms. A combination of both ISH and IHC techniques in the same sample can thus prove to be a very powerful tool to understanding the biology of an organism.

ISH utilizes nucleic acid probes that are complementary to the RNA target. Early versions of ISH used radioactively labelled probes to visualize where they bound within the sample [3, 9–11]. Later iterations of this method avoided using hazardous radioactive materials and instead adopted fluorescent dyes or colorimetric depositions to image samples. One of the first alternative methods was to directly label probes with excitable dyes, which allows for use of fluorescent microscopy; however, there were issues with low sensitivity [12–15]. This issue was addressed by using a large number of fluorescently labeled short probes that bind to individual mRNA transcripts, but this approach still did not provide sufficient signal amplification for thicker or more complex tissues and embryos [16].

Later ISH techniques developed indirect means to amplify probe signal by utilizing phosphatase and perioxidase enzymatic reactions, where in the presence of a substrate, a colored product is deposited in the probe's proximity. Probes are modified with haptens or biotin and later bound to antibodies or avidin molecules conjugated to corresponding enzymes [17–19]. Alternatively, fluorescent dyes can be conjugated to these antibodies or avidin molecules, which provides greater signal than direct methods [20, 21]. Other amplification schemes have been developed to incorporate both enzymatic and florescent methods by using Tyramide Signal Amplification (TSA), where a peroxidase reaction attaches modified tyramide molecules in close proximity to the enzyme. These

modifications can include fluorescent dyes or haptens, which are then bound by a fluorescent avidin or antibody [22, 23]. These deposition amplification methods provide good sensitivity, and along with ease of use through commercial kits prove to be an appealing technique [24, 25].

Signal amplification is not the only parameter that needs to be considered for a successful ISH experiment. The autofluorescence of a sample and the quality of the probes are two other variables that need to be optimized to detect a target with a high signal-to-background ratio. Methods to reduce autofluorescence include chemical treatments (such as copper sulfate, Sudan Black B, or sodium borohydride) and light exposure treatments [26–28]. Probe quality determines if it binds with a high affinity and high specificity to the target; however, if optimization is necessary, it is generally performed in an ad hoc manner. When the probe set does not yield enough signal, the common solutions are to add more probes to the set, change the probe length, or adjust the probe concentration [16, 29, 30]. At present, there is no systematic method to analyze a probe set and logically determine what the correct course of action should be to improve signal or decrease background. Consequently, a structured approach to probe optimization to maximize the signal-to-background ratio would be very useful for ISH experiments.

The ability to analyze the expression pattern of various genes in parallel would allow for a greater understanding of how these different biological parts interact with one another. ISH can currently detect multiple distinct mRNA species in the same sample using sequential probing and signal amplification [31, 32]. However, these methods for multiplexed detection are time-intensive and suffer from sample degradation since each probe is detected one at a time as the deposition reactions are not orthogonal [33, 34]. Alternatively, in an attempt to use orthogonal schemes, biologists have combined radioactive and colorimetric methods to achieve dual detection of mRNAs, but there is difficulty in imaging and the technique is limited in scope [35, 36]. Thus, a single orthogonal amplification method that can simultaneously detect various mRNA species would be very advantageous in characterizing the complex biological circuits in an organism.

IHC uses antibody proteins to bind to their target antigen protein *in situ* and use different visualization methods to amplify signal. Initial IHC efforts used fluorescently labelled primary antibodies to detect protein targets [37]. However, there were background issues with high autofluorescence, and indirect detection methods were developed using fluorescently conjugated secondary antibodies that bind specifically to primary antibodies [38, 39]. Quantum dots linked to antibodies have also been used as a more sensitive fluorescent label resistant to photobleaching [40, 41]. As was the case with ISH, enzymatic approaches were later incorporated to amplify signal. This was accomplished by attaching peroxidases and phosphatases to antibodies and using deposition reactions in proximity to the bound antibody to visualize signal through a light microscope [42–44]. Later, TSA was adopted to immunostaining as an amplification technique to combine fluorescent and enzymatic methods [45–47].

Detection of two proteins simultaneously was accomplished by using the orthogonal chemistries of a peroxidase and a phosphatase; however, there is difficulty in assessing cellular colocalization of the two targets [48–50]. Additionally, as with ISH, a sequential chromogenic staining scheme can be done, but the protocol is time-intensive and has issues with sample degradation and color overlap [51–54]. Although these protocols can label more than three targets, chromogenic methods are practically limited to no more than two targets in the same sample [55]. Here, fluorescence has the distinct advantage for multiplexed detection of protein targets. Fluorescent dyes can be distinguished using their unique spectra using various filters and mirrors on microscopes, and in the event of spectral overlap, unmixing can be used to discriminate the dyes [56–58]. Fluorescently labeled secondary antibodies are limited by the lack of diversity of host species of primary antibodies and are less sensitive than enzymatic methods [55, 59]. When enzymatic processes involving fluorescent tyramide molecules are used for greater signal amplification, serial labelling is required, which is labor intensive and can suffer from sample degradation. As was the case with ISH, a straightforward technique to simultaneously detect various protein targets would be greatly beneficial in understanding the complex biological networks.

Parallel detection of mRNAs and proteins in the same sample provides for a significant amount of information, but it is time-consuming and non-trivial to combine the two techniques. This protocol would require either completely different detection schemes for the ISH and IHC or sequential staining. Early methods used a combination of radioactive ISH and either colorimetric or fluorescent IHC [60–62]. More recently, serial amplification using TSA with fluorescent dyes has been used to detect both mRNAs and proteins [63]. However, these techniques are labor intensive and not robust due to loss of target from the repeated labeling processes.

The Pierce group at Caltech has developed an alternate amplification technique that easily allows for multiplexed visualization of varied targets in biological samples (figure 1.1). This method uses a process called hybridization chain reaction (HCR), which is a polymerization reaction that converts nucleic acid hairpins into long nicked double stranded polymers [64]. The reaction involves two metastable hairpins (H1 and H2) that remain separate in the absence of a single stranded initiator sequence (I1). When the initiator is introduced, it triggers a polymerization reaction by hybridizing with H1 and opening the hairpin. The exposed sequence from H1 then hybridizes with and opens H2, which can then hybridize with another H1. This process repeats and results in a long polymer tethered to an initiator. The hairpin monomers can be chemically modified with fluorescent dyes, which allows for the polymers to be visualized using techniques like fluorescent microscopy. HCR is a programmable reaction with sequence specificity that permits for easy design of orthogonal systems. This programmability makes HCR an excellent amplification system for the multiplexed detection of mRNAs and proteins *in situ*

HCR has been adopted to amplify signal in multiplexed ISH experiments. Choi et al. demon-

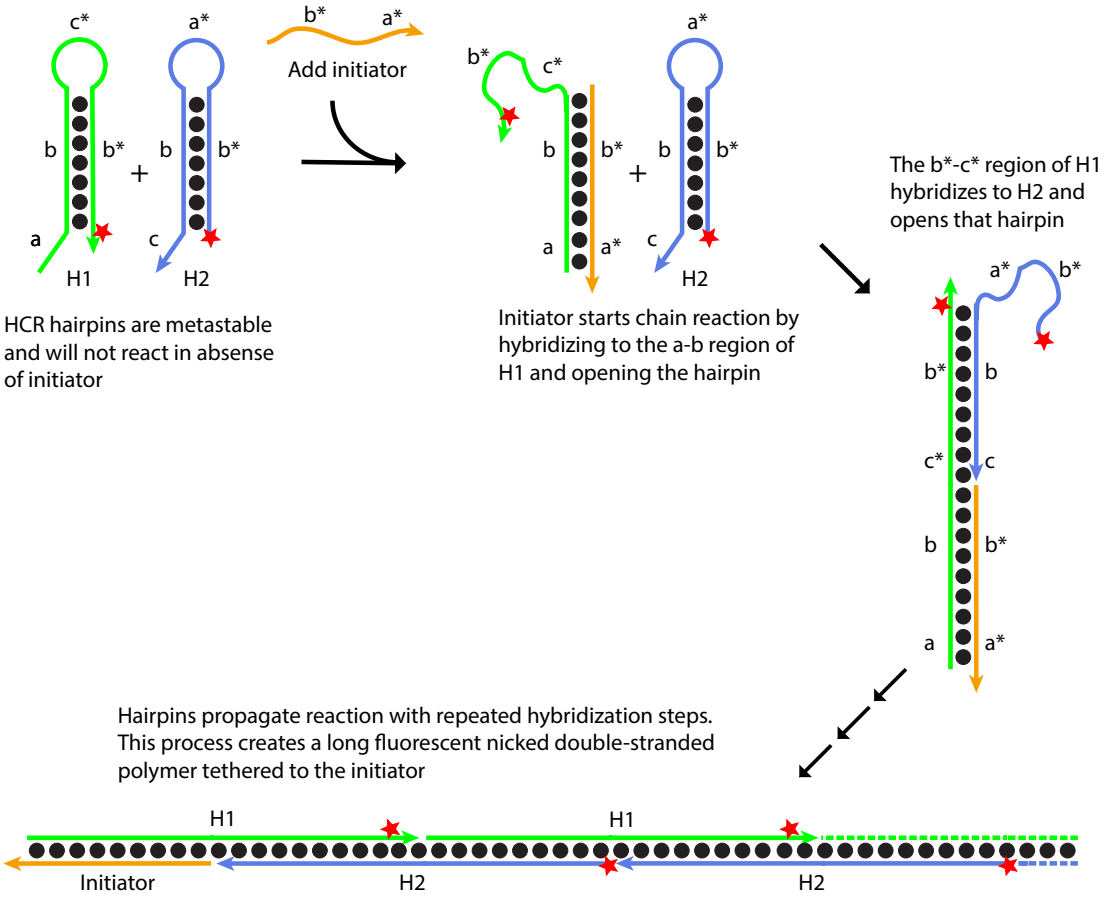

Figure 1.1: Diagram of hybridization chain reaction mechanism. Hairpins H1 and H2 are metastable in the absence of an initiator. When initiator is introduced, it nucleates a reaction where the hairpins assemble into a polymer. The initiator hybridizes with the toehold of H1 and starts a branch migration that exposes the c*-b* region of H1. This exposed single-stranded region then hybridizes with and opens up H2, revealing the b*-a* region of H2, which is the same sequence as the initiator. As a result, the process repeats with H1 and H2 alternatively hybridizing to the leading edge of the polymer creating a long nicked double-stranded polymer that is tethered to the initiator. Since the hairpins are labeled with fluorescent dyes, the polymer can be visualized with techniques such as fluorescent microscopy. Red stars represent fluorescent dyes that are covalently linked to each hairpin monomer.

strated a five-color ISH in whole mount zebrafish embryos using RNA HCR systems [30]. The probes for each target were attached to an initiator that amplified a uniquely dyed HCR system. Other advantages of HCR are signal specificity and sample penetration: the HCR polymers are attached to the target via the probe which prevent signal from diffusing, and HCR monomers are relatively small and only create long polymers once inside the sample in proximity to the target. New DNA HCR systems have been developed that are less expensive, provide greater signal and are more stable than the previous generations RNA HCR system [65]. New applications of HCR amplification, such as to antibody staining, would be beneficial for the study of biology due to the many advantages of HCR, most notably multiplexing. This work will expand the purview of HCR into new methods and model organisms.

Chapter 2 addresses the need for a systematic way to approach probe optimization in ISH experiments. In particular, a method is developed using HCR to analyze individual probes to determine which ones comprise the optimal probe set to maximize the signal-to-background ratio.

Chapter 3 expands the HCR-ISH method into organisms other than zebrafish, specifically whole mount *Drosophila* embryos and formalin-fixed paraffin-embedded tissue sections. These experiments demonstrate the detection of multiple mRNAs in the same sample.

Chapter 4 shows how HCR is adapted to amplify signal for immunohistochemistry. In addition to multiplexing, the signal from HCR-immunohistochemistry (HCR-IHC) is shown to be quantitative. Finally, HCR-ISH and HCR-IHC methods are combined to simultaneously detect multiple mRNAs and proteins with a high signal-to-background ratio in a zebrafish sample.

Chapter 2

Analysis and optimization of signal-to-background for in situ hybridization experiments

2.1 Introduction

An in situ hybridization experiment is an involved and complex protocol in which one tries to maximize signal while keeping background low. Various steps can be optimized, such as sample preparation, probe design and binding and amplification methods [29, 66]. In particular, probe design is a tricky portion of the protocol to improve, as most scientists use brute force to find a probe set that works well. There have been attempts at rational probe design, but there are still no robust methods for design [67–70]. Thus, a systematic way to analyze the quality of a probe to determine the optimal probe set would be extremely valuable.

The quality of probes dictates the presence and specificity of signal. Two common issues with probes are that they do not bind to the target sequence well or bind non-specifically to other parts of the sample. The traditional mindset when there is insufficient signal is that either the probe cannot access the target or the background is too high. The typical solution to this problem is to add more probes [16, 30]. When there is non-specific binding, the action is to increase stringency or alter washes. However, in both cases, it would be simpler to analyze each probe and determine if it would be beneficial or detrimental to the experiment instead of adjusting the protocol or blindly increasing the probe set.

This chapter will present a method to evaluate each individual probe and determine the optimal probe set. The process ascertains if a certain probe is binding well, poorly, or non-specifically. This analysis based on the individual probe data reveals predictions on which subset of probes will yield the highest signal-to-background ratio. This approach will help debunk the conventional wisdom that more is always better with respect to probes, as it will be shown that one extra bad probe can be

very deleterious to a probe set. Demonstration of this process will be performed in cell culture using HCR as an amplification technique. Performing HCR-ISH experiments in cultured cells provides a robust and high-throughput means to obtain data due to ease of sample preparation and data collection via cytometry. The overall goal is to predict the optimal probe set based on properties of individual probes, and subsequently, to confirm the validity of these predictions.

2.2 Characterization of background and signal

This section will define each of the components that comprise the total fluorescence in an ISH experiment. Background is typically attributed to either the native autofluorescence (AF) of the sample or to off-target probe binding [70, 71]. In actuality, what scientists consider to be non-specific binding can be divided further into two separate contributions. The first is called non-specific detection (NSD), where the probe binds to an off-target and causes signal in incorrect areas where there is no target. The second is called non-specific amplification (NSA), where the amplification system causes signal in areas where there is neither any probe nor target. Specifically for HCR-ISH, NSA would occur either where the hairpin monomers get trapped in the sample or where an HCR polymer arises in the absence of an initiator and causes false signal. The final contribution is signal (SIG), which is fluorescence that arises from detecting the correct target. So when looking at the total fluorescence of an ISH sample, one is looking at four total contributions: autofluorescence (AF), non-specific amplification (NSA), non-specific detection (NSD), and signal (SIG) (equation 2.1).

$$\text{Total Fluorescence} = \text{AF} + \text{NSA} + \text{NSD} + \text{SIG} \tag{2.1}$$

2.3 Cell HCR in situ hybridization experimental design

Using ISH experiments in HEK293 wild-type cells and in HEK293 cells expressing an exogenous gene, the various background contributions could be teased out from the total fluorescent signal for each probe. An exogenous gene is targeted here to provide an easy means to obtain the NSD component by using the wild type cell line. The exogenous gene used in this set of experiments was green fluorescent protein (GFP) expressed under a constitutive promoter. Eight different 50-nucleotide probes for HCR were designed to target GFP. Four separate sets of tests will be used on each probe to extract out the individual contributions of each type of background and signal for an individual probe (Figure 2.1). They are as follows:

- Experiment (1) AF: a mock HCR-ISH is performed where both probes and HCR amplifiers are omitted from the procedure, while all other steps remain the same. The only fluorescence from

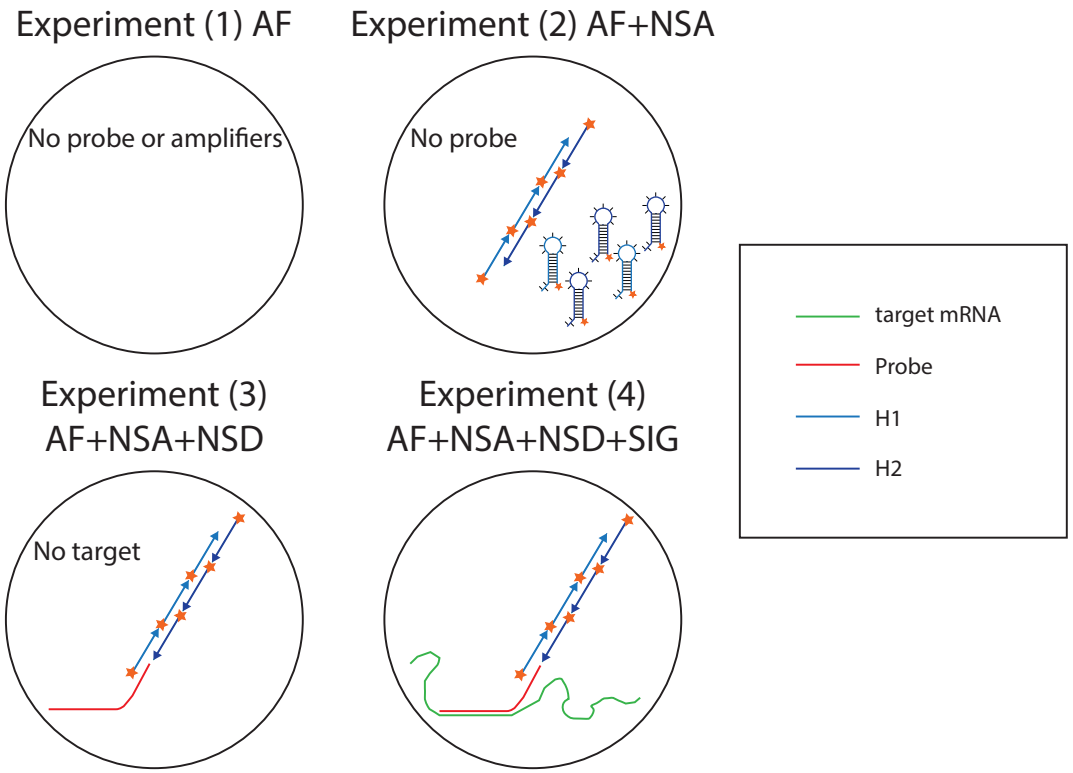

Figure 2.1: Experimental design for probe characterization. The graphical representation of the described experiments is presented here. (1) contains no probes nor amplifiers, so any signal is native autofluorescence. (2) contains only amplifiers, so the signal is either autofluorescence or trapped amplifier in monomer or polymer form. (3) contains probes and amplifiers in the absence of target, so the signal is either autofluorescence, trapped amplifier, or amplifier polymerized in the presence of non-specific probe binding. (4) contains true signal, as well as all types of background seen from the first three experiments.

this sample is the autofluorescence from the sample, since there are no amplifiers to obfuscate this result.

- Experiment (2) AF + NSA: a HCR-ISH is performed where only the probes are omitted from the full procedure. As a result, there are no initiators within the sample, so all polymers created are the result of erroneous triggering or off-target hairpin binding, which represents the NSA term. This sample also includes the AF term.

- Experiment (3) AF + NSA + NSD: a HCR-ISH is performed on the wild-type cell line that lacks the transgene. Since the probe has no correct target to bind to, any additional signal seen compared to experiment (2) is due to probes binding to a non-target site and triggering HCR polymers. This sample also includes the AF and NSA term.

- Experiment (4) AF + NSA + NSD + SIG: a HCR-ISH is performed on a cell line that expresses the transgene. The signal from this sample includes real signal and all types of background: AF, NSA, and NSD.

With each ascending experiment, an additional contribution to total fluorescence is obtained. For instance, experiment (1) gives the AF contribution, and subsequently experiment (2) gives both the AF and the NSA contributions. To simplify the analysis, average fluorescence values for each experimental replicate were utilized. Using averages allows for simple arithmetic to calculate the various backgrounds and signal components as shown below.

- $\overline{\text{AF}}$ = <Experiment 1>
- $\overline{\text{NSA}}$ = <Experiment 2> – <Experiment 1>
- $\overline{\text{NSD}}$ = <Experiment 3> – <Experiment 2>
- $\overline{\text{SIG}}$ = <Experiment 4> – <Experiment 3>

This analysis was performed for each individual probe, and the results are shown in figure 2.2. The uncertainty for each experiment is characterized by the standard deviation across the replicates for that experiment. The uncertainty for each individual component is calculated by propagating uncertainties as described in supplementary information.

2.4 Probe analysis and prediction

With knowledge of all the components that contribute to total signal, the quality of a probe can be established. The signal-to-background term will be used to analyze the quality of a probe. A value of zero indicates no signal, and a value of one indicates that a difference between signal and

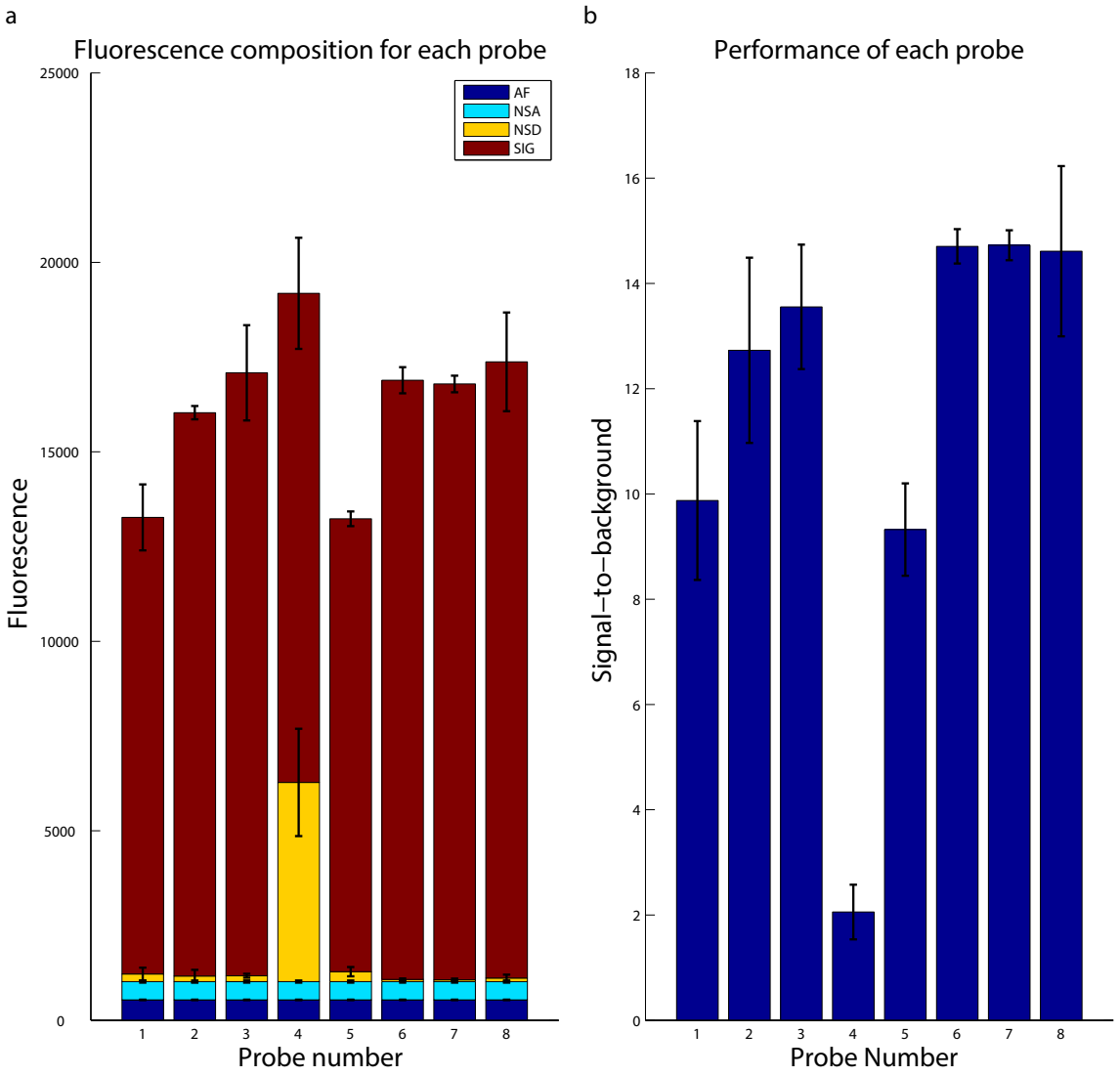

Figure 2.2: Fluorescence composition and performance of each probe. (a) Each of the background components and the signal component are shown for each of the eight individual GFP probes using HCR-ISH experiments on HEK-293 cells. (b) The signal-to-background value is calculated from equation 4.1 for each of the eight individual GFP probes using the calculated component values. All cell experiments were performed in duplicate counting 50,000 cells each time and gated by forward and side scatter to ensure correct counting of cells.

background cannot be ascertained. A higher signal-to-background value for a probe means that it is easier to discern signal from background, and thus it is a better probe than one with a lower signal-to-background value. This value can be calculated for probe n as follows:

$$\text{Signal to Background} = \frac{SIG_n}{(AF + NSA + NSD_n)} \tag{2.2}$$

The signal-to-background values for each of the eight GFP probes was calculated (figure 2.2). This signal-to-background analysis is superior to simply looking at total signal values as it delves deeper into the performance of the probe. For instance, probe 4 is seen to be the brightest probe in terms of absolute signal, but in actuality has the lowest signal-to-background value as a large portion of that signal is background. This illustrates the assumption that brighter is not always better.

With the component and signal-to-background values for each probe, it follows that predictions can be made on the optimal probe set to be used for this experiment. The subset of probes that will yield the highest signal to background ratio can be calculated by first sorting the probes by highest to lowest in terms of signal-to-background values. From this ranking, we computed the signal-to-background for the one best probe, two best probes, three best probes, and so on using:

$$\text{Signal to Background} = \frac{\sum_{n=1}^{N} SIG_n}{(AF + NSA + \sum_{n=1}^{N} NSD_n)} \tag{2.3}$$

Equation 2.3 calculates the overall signal-to-background for a probe set that consists of the x best probes. The probe set size with the maximum signal-to-background ratio is predicted to be the optimal probe set. The predicted signal-to-background values for the best probe sets of size 1 to N probes are shown in figure 2.3. The predictions reveal that the six best probes will have the best signal-to-background ratio.

2.5 Confirmation of prediction and optimal probe set determination

In order to test the predictions from figure 2.3.a, these probe sets were experimentally tested (figure 2.3.b). The relative performance of the different probe sets is well predicted with the 6-probe set outperforming the 8-probe set. Additionally, these findings demonstrate that this protocol can successfully predict not only qualitatively, but also quantitatively, the optimal probe set and its corresponding signal-to-background value using data obtained from individual probes.

A look at the histograms for the 6-probe and 8-probe data shows that the overall signal remains

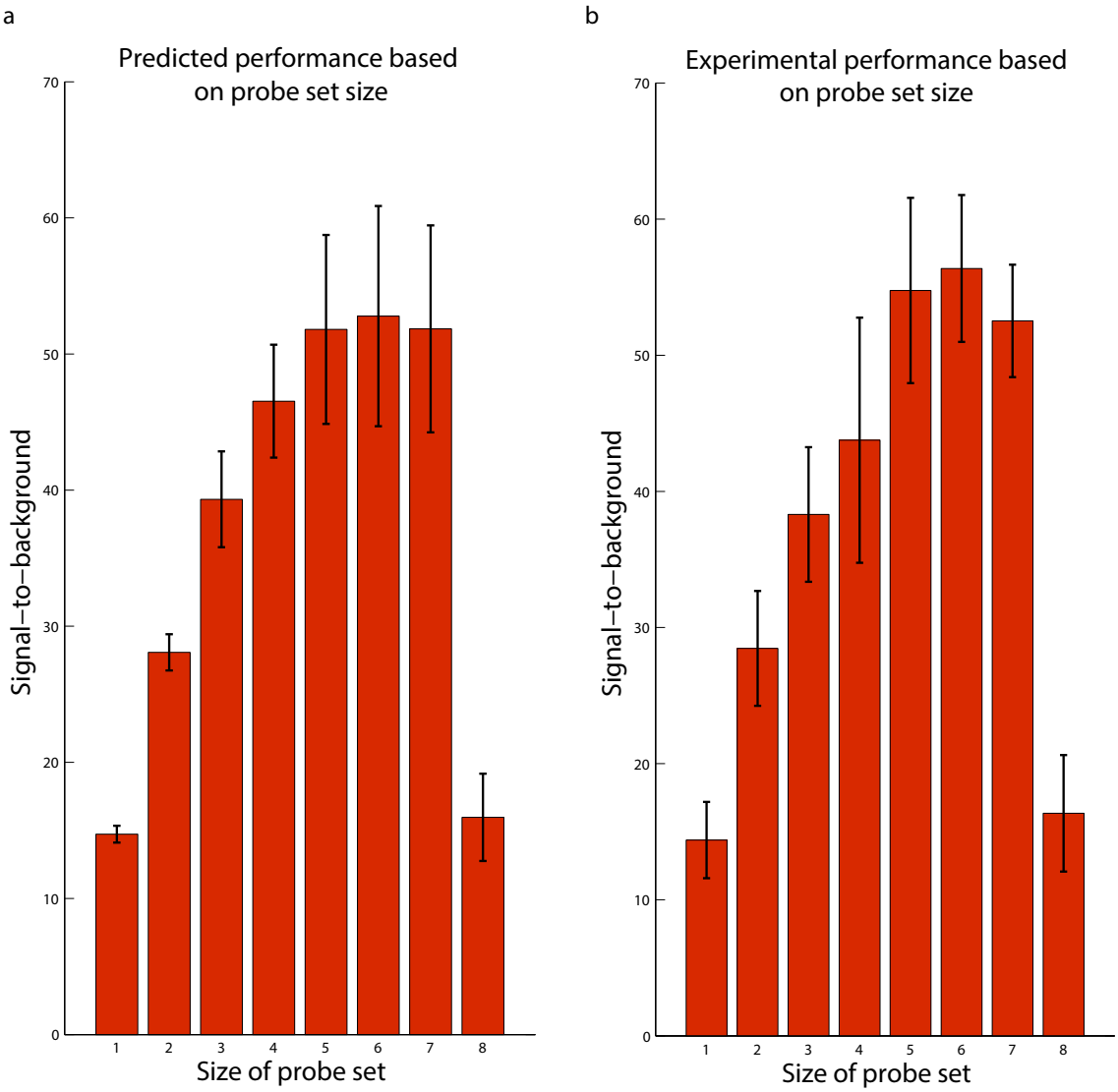

Figure 2.3: Predicted and experimental signal-to-background values based on probe set size. (a) The signal-to-background value is estimated for probe sets containing the N best probes, where 1 $\leq$ N $\leq$ 8. Calculations were performed using equation 2.3. (b) The signal-to-background value is experimentally determined for probe sets containing the N best probes, where 1 $\leq$ N $\leq$ 8. The experimental values nearly match the predicted values from (a), which demonstrates the success of this approach.

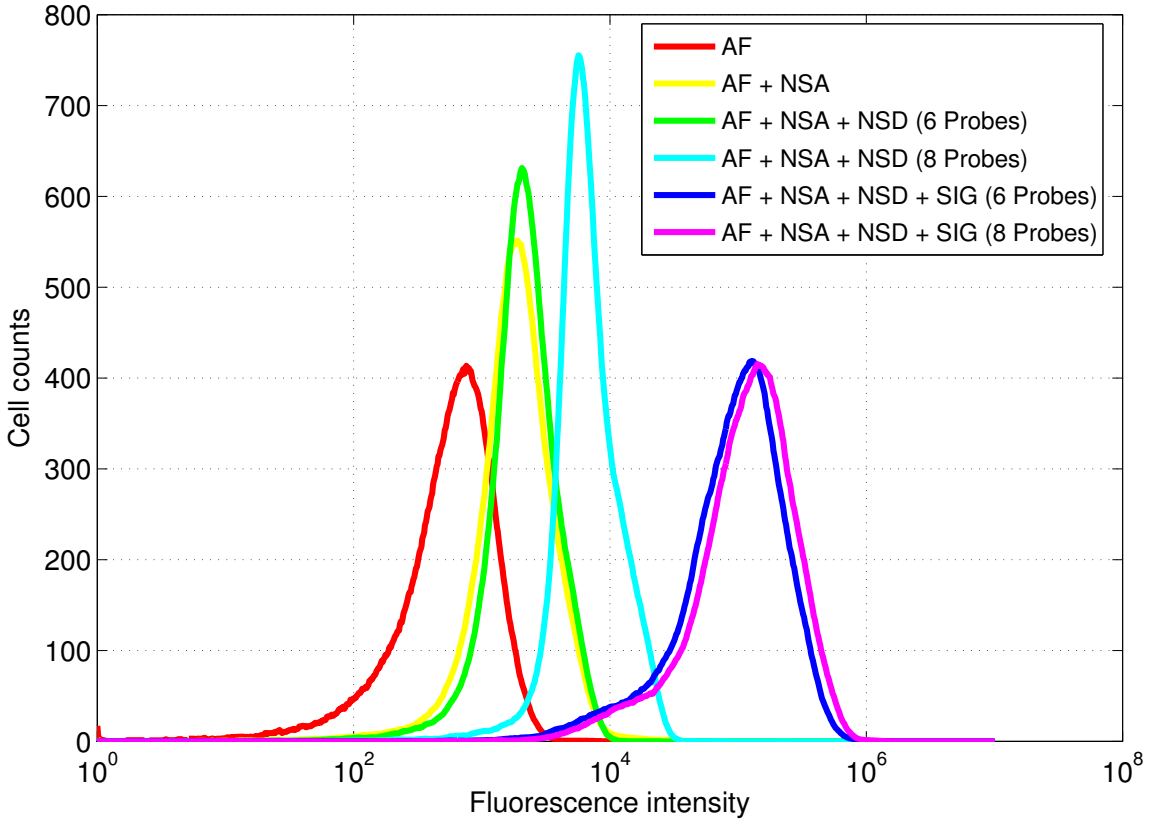

Figure 2.4: Comparison of optimal probe set to full probe set. Histogram analysis comparing the full probe set to the optimal probe set (6 best probes). Total fluorescence remains nearly the same, while the background falls dramatically.

the same with two fewer probes, but NSD decreases significantly (figure 2.4). Removal of the two bad probes causes the probe set to have background that consists primarily of AF and NSA, which means this set of probes did not contribute substantially to background binding.

2.6 Conclusion

This probe optimization method has revealed two lessons. The first discredits the idea that more is better. These studies show that one probe can have nearly the same signal-to-background ratio as eight probes when the latter probe set is polluted by even one bad probe. In this case, removing two probes from the set of eight probes more than tripled the signal-to-background ratio. Thus, the answer to probe optimization may not be adding more probes, but rather removing the bad probes. The second lesson is that analyzing individual probes can provide predictive power to obtaining the greatest signal-to-background ratio in an experiment. The caveat is that cooperativity between probes binding to the same target could undermine the quantitative nature of the optimization. Using this systematic approach, we have been able to demonstrate a rational method for probe optimization for ISH experiments.

Please refer to Appendix A for supplementary information pertaining to this chapter.

Chapter 3

A zoo of mRNA expression

3.1 Introduction

Genetic circuits within an organism consist of highly complex and interactive networks that dictate various biological processes, and understanding how these mechanisms work makes up a large portion of experimental biology [1, 2]. Being able to analyze multiple genes simultaneously would thus be very informative. *In situ* hybridization (ISH) provides an easy tool to analyze the spatial organization of gene expression [3, 9]. However, multiplexed ISH using catalytic or TSA methods require serial amplification, which can result in sample degradation or insufficient signal [33, 34, 72].

Amplification using HCR provides a straight-forward means to perform multiplexed ISH. Additional advantages of HCR for ISH include high sensitivity, deep sample penetration, and signal localization. HCR has been shown to be highly effective for multiplexed ISH experiments in whole-mount zebrafish embryos [30, 65]. It would be beneficial to expand this technology into other model organisms and systems. To this end, this chapter will demonstrate the adaption of HCR-ISH to whole mount *Drosophila melanogaster* embryos and formalin-fixed, paraffin embedded human tissue sections.

3.2 *Drosophila melanogaster* whole mount embryos

Fruit flies (*Drosophila melanogaster*) are a classical model organism that were used to study many of the early genetic networks [73–75]. Chromogenic and fluorescent ISH have been used to study gene expression patterns in flies [4, 76–78]. Simultaneous detection of mRNA has also performed in flies; however, these methods suffer from low signal-to-background or loss of target due to sequential staining [79, 80]. Using HCR as an amplification method provides a solution to both of these issues.

In order to adapt HCR for ISH of fly embryos, the protocols for handling embryos from traditional *Drosophila* ISH and the protocols for probe binding and amplification from zebrafish HCR-ISH were combined. Fixation, permeabilization, and dehydration/rehydration of the embryos were adapted

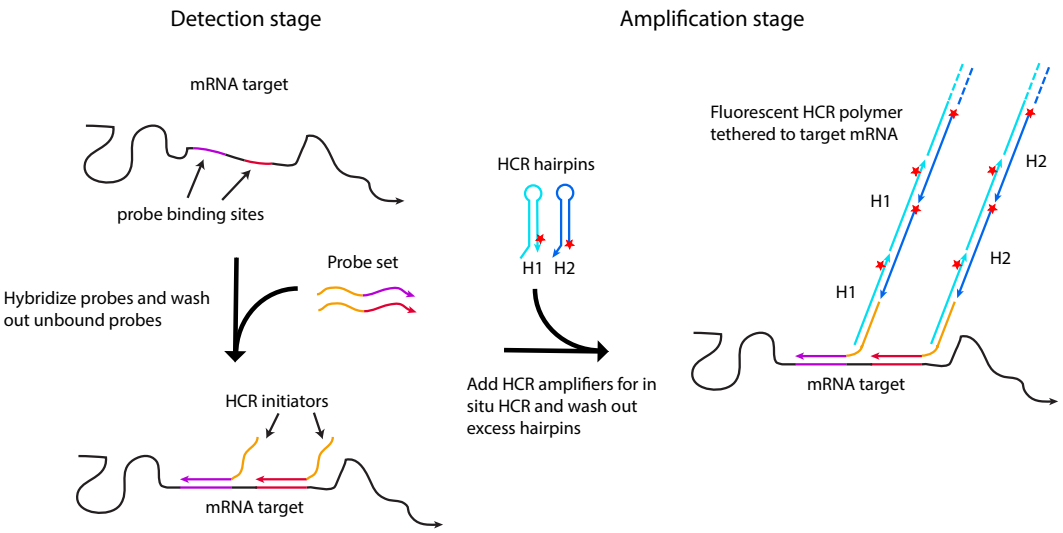

Figure 3.1: Diagram of HCR *in situ* hybridization experiment. In the detection stage, probes containing HCR initiators are hybridized to their mRNA target and excess probes are washed away. Fluorescently labeled hairpins H1 and H2 are added next and polymerize in the presence of initiator. The result is long, nicked double-stranded polymers tethered to the mRNA target via the probes.

from fluorescent ISH experiments of flies [80]. Probe hybridization and HCR hairpin amplification were adapted from the zebrafish HCR-ISH protocol from Choi *et al.* 2014 [65]. DNA probes and amplifiers from the new generation of HCR were used as they provide greater signal than the previous generation's RNA system.

After initial studies to study single genes, experiments were done to simultaneously detect four different genes. The genes for the four color study were selected to be spatially distinct and varied in expression levels [81, 82]. The four chosen genes were *even-skipped* (*eve*), *short gastrulation* (*sog*), *cap'n'collar* (*cnc*), and *caudal* (*cad*). Images of this ISH experiment were taken with fluorescent confocal microscopy using four spectrally different channels that each correspond to a single target, shown in figure 3.2. Each of the four targets showed very good specificity and localization that was corroborated from chromogenic in situ studies [81]. Of particular interest, the expression patterns from HCR-ISH for *eve* and *sog* clearly show the nuclear boundaries in the syncytium during early fly development. This type of resolution cannot be attained with traditional chromogenic ISH methods as the signal becomes diffuse due to the mechanism of enzymatic deposition [80, 83, 84]. However, with HCR amplification, the signal is tethered directly to the probe, allowing for greater signal specificity.

Targets were detected with signal-to-background ratios ranging from 2 to over 20 (table 3.1). These studies were done in triplicate and further analysis was done to reiterate the high signal and low background using histograms that compared pixel intensities between areas of signal to areas

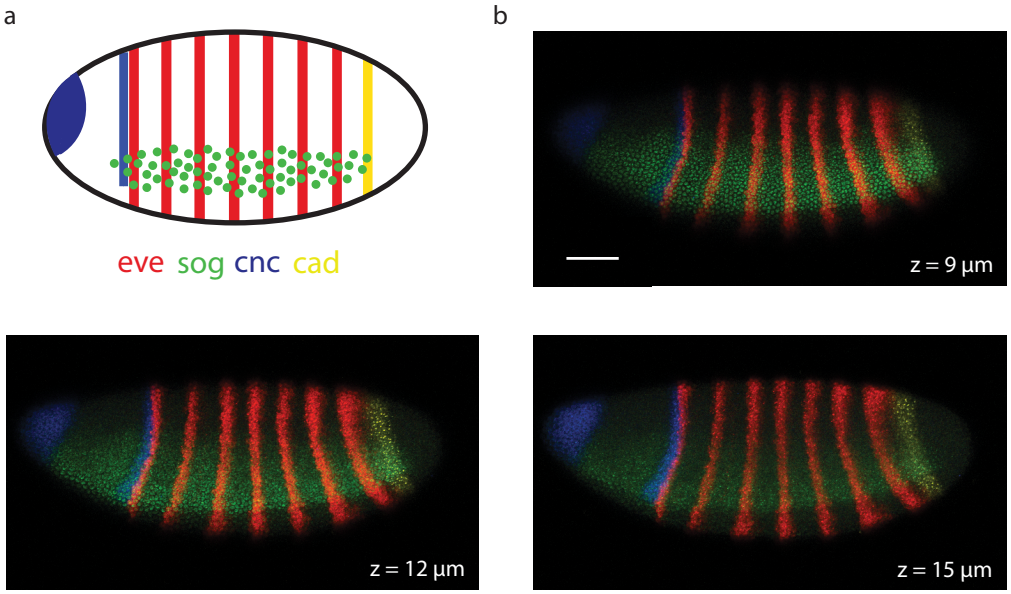

Figure 3.2: Multiplexed mRNA detection in whole mount *Drosophila melanogaster* embryos. Four different gene products in *Drosophila melanogaster* embryos were simultaneously detected using HCR-ISH. (a) Expression atlas for four target mRNAs: *even-skipped* (*eve*), *short gastrulation* (*sog*), *cap'n'collar* (*cnc*), and *caudal* (*cad*). (b) Fluorescent confocal microscopy image of fixed *D. melanogaster* embryo on three planes showing expression pattern of four different mRNAs using HCR *in situ* hybridization. Embryos are stage 4-6, and the scale bar is 50 μm.

Table 3.1: Signal-to-background values for HCR-ISH experiments in whole mount *Drosophila melanogaster* embryos

gene	**signal-to-background**
cad	2.0 ± 0.7
sog	7.2 ± 1.8
cnc	4.2 ± 1.3
eve	21.3 ± 5.4

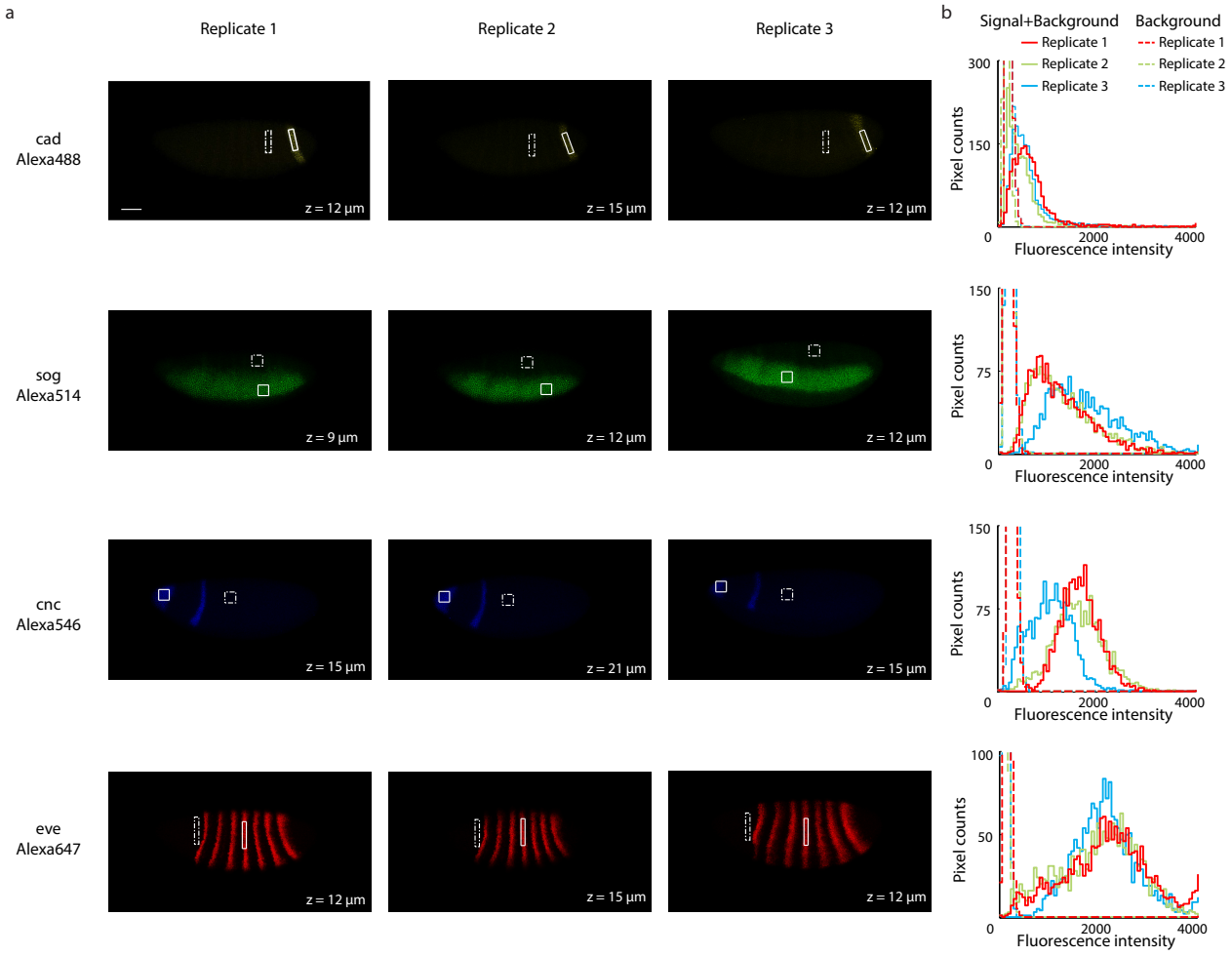

Figure 3.3: Performance of multiplexed ISH using HCR in whole mount *Drosophila melanogaster* embryos. (a) Confocal microscopy images showing the triplicate studies of the four mRNA targets in fly embryos using spectrally distinct fluorescent channels. Representative image slices are shown for each of the replicate samples. (b) Pixel intensity histograms derived from values within a rectangular subsection of signal (solid line box) and of background (dashed line box) for each sample and target. Embryos are stage 4-6, and the scale bar is 50 μm.

of background (figure 3.3). For each of the targets except *cad*, a clear line can be drawn on the histogram to threshold a background pixel from a signal pixel. *cad* presents a different case because its expression pattern is very punctate, so the signal boxes actually include numerous background pixels. As a result, this inability to have a clear delineation between signal and background on the histogram comes as a shortcoming of the analysis, and not of the technique. Overall, HCR successfully detected the expression of various genes in whole-mount fly embryos with a high signal-to-background ratio.

3.3 Formalin-fixed, paraffin embedded human tissue sections

Studying human biology using formalin-fixed, paraffin embedded (FFPE) human tissue sections is very advantageous due to the ease of obtaining a large number of stable samples for examination. Chromogenic and fluorescent ISH have been used to study gene expression patterns in human tissue sections [85–90]. Simultaneous detection of mRNAs has been done using chromogenic and RNAscope methods [91–93]. However, chromogenic methods are limited to two colors due to lack of orthogonal methods or loss of target with serial labeling, and RNAscope is limited by poor sample penetration and loss of target due to great number of washes [94]. HCR as an amplification technique would circumvent these problems due to its natural orthogonality and excellent sample penetration due to using small molecular parts.

In order to adapt HCR for ISH of FFPE tissue sections, certain steps in slide preparation had to be optimized. Sans optimization, these sections exhibited significant autofluorescence and had issues with trapping of HCR amplifier hairpins. Two key steps were isolated to alleviate these background issues. For autofluorescence, using fresh deparaffination solution, such as xylenes or Histo-clear, for every individual experiment was crucial, as any residual paraffin on the slide caused considerable background. Reused deparaffination solution was not as effective as fresh solution at removing paraffin from the slides. For non-specific amplification (NSA) associated with hairpin trapping, the sample was acetylated using acetic anhydride to block positively charged sites with the negatively charged acetyl groups. These positively charged areas were binding to the negatively charged phosphate backbone of the DNA hairpins, so permanently blocking these sites decreased NSA background substantially. Probe hybridization and HCR hairpin amplification were done in humidified chambers using the same buffers and component concentrations as the zebrafish protocol [65]. The major difference between the zebrafish and human tissue protocol is that the probe wash steps for the latter used less stringent conditions by gradually reducing the formamide concentration, otherwise signal was reduced drastically.

The tissue type used for these studies was normal human breast tissue. After optimization of the protocol for these FFPE sections, probe sets for the three separate targets were designed and tested.

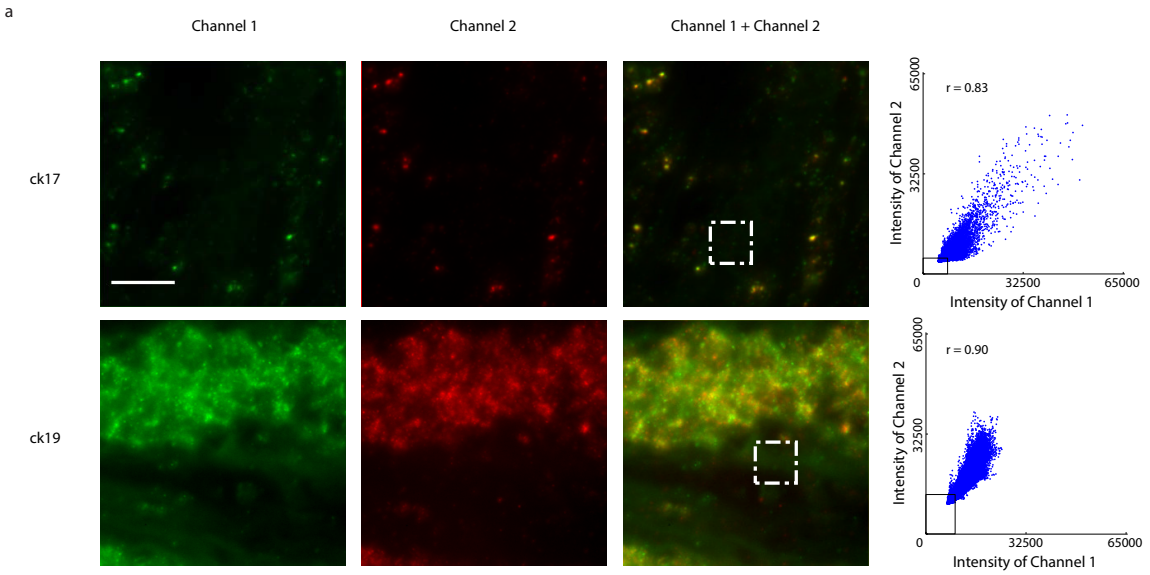

Figure 3.4: Redundant detection of mRNA targets using HCR in formalin-fixed, paraffin-embedded human breast tissue sections. Two separate probe sets (created by dividing a full probe set into two) targeting the same mRNA (either *ck17* or *ck19*) are used to simultaneously detect the target using orthogonal HCR systems with different dyes. Images from each channel and a merged image are shown for each target. The intensities from both channels (channel 1 and channel 2) for all pixels in the image are plotted against each other. A background box (dotted line) is selected. The average intensity of this box plus two standard deviations is used as a threshold to exclude background pixels. A linear correlation in this plot demonstrates that the full probe set is correctly detecting the intended target. The Pearson correlation coefficient is high for both targets, which suggests that the probes are binding the correct target. Tissue sections are from normal human breast tissue, and the scale bar is 10 μm.

The targets were textitcytokeratin 17 (*ck17*), *cytokeratin 19* (*ck19*), and *18S* rRNA (*18S*). These two particular cytokeratin genes were chosen because their expression patterns were predicted to be spatially distinct. Using immunohistochemical and qPCR data from the literature, the expected expression patterns for the targets were as follows: *ck17* in basal epithelial cells, *ck19* in luminal epithelial cells, and *18S* rRNA in both basal and epithelial cells (figure 3.5a) [95–98]. In order to confirm that the probe sets were detecting the correct target, a redundant detection scheme was used (figure 3.4). In this experiment, the probe set for a single mRNA target is split into two separate sets, each with the ability to amplify a different HCR system labeled with a unique dye. These two probe sets are then used to simultaneously detect the target, and the sample is imaged in the two fluorescent channels that correspond with each probe set. The fluorescent intensities from each channel for every pixel in the image are plotted against each other. A rectangular area of background is selected to calculate the average background intensity. Any pixels with intensities that fall below the average background plus two standard deviations value are omitted from the analysis. The correlation between the two channels is calculated, and a linear relationship indicates that the full probe set (combining the two different subsets) is correctly detecting the target. The Pearson correlation coefficient is high for both *ck17* and *ck19*, consistent with each probe set detecting its intended target.

After confirming the probe sets using redundant detection methods, experiments were carried out to simultaneously detect the three different genes (figure 3.5). The expression patterns for *ck17* and *ck19* confirm the immunostaining literature data as they label only basal epithelial and luminal epithelial cells respectively. From the images, the expression patterns for each of these two cytokeratins are mutually exclusive, which indicates that these probe sets can be used to identify specific cell types. Additionally, these targets along with other cytokeratins can be used in future ISH assays as they are markers in many breast cancers [96, 97]. And as with the case with fly embryos, detection of these mRNAs in human tissues sections had a high signal-to-background ratio, ranging from 3 to 7 (table 3.2). This fact is especially significant because these sample types contain very complex tissues with substantial autofluorescence.

Triplicate studies were performed for *ck17* and *ck19* in order to recapitulate the high signal-to-background ratios. For each target and each replicate, pixel intensities from three signal boxes and three background boxes were compared using histogram analysis (figure 3.6). A line can be easily drawn to divide the signal curve from the background curve for both targets, which mean a signal pixel can be clearly distinguished from a background pixel. These ISH studies show that HCR amplification is a viable and highly advantageous method to visualize mRNA expression in FFPE human tissue sections.

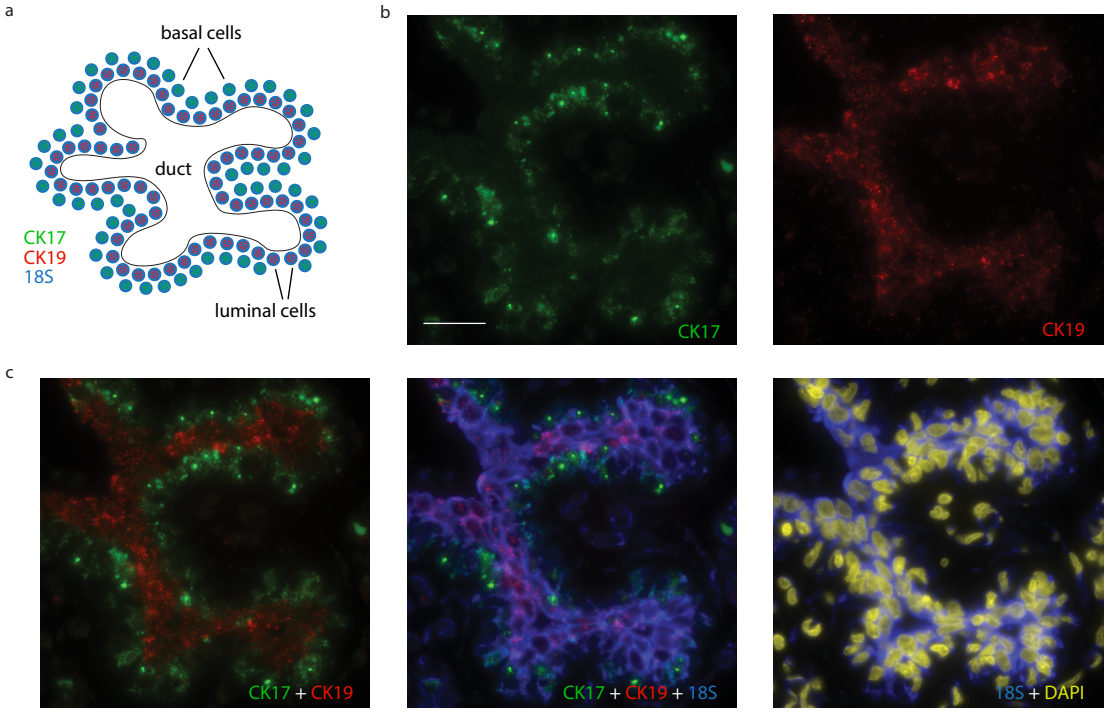

Figure 3.5: Multiplexed mRNA detection in formalin-fixed, paraffin-embedded human breast tissue sections. Three different gene products in FFPE human breast tissue sections were simultaneously detected using HCR-ISH. (a) Expression atlas for three target mRNAs: *cytokeratin 17* (*ck17*), *cytokeratin 19* (*ck19*), *18S* rRNA (*18S*). *ck17* is expressed in the basal epithelial cells, *ck19* is expressed in the luminal epithelial cells, and *18S* rRNA is expressed ubquitiously. (b) Fluorescent microscopy image of formalin-fixed, paraffin-embedded human breast tissue sections showing expression pattern of three different mRNAs using HCR *in situ* hybridization. Additionally, nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI), shown in yellow. The tissue sections are from normal human breast tissue and cut to 4 μm in thickness. The scale bar is 25 μm.

Table 3.2: Signal-to-background values for HCR-ISH experiments formalin-fixed, paraffin-embedded human breast tissue sections

gene	**signal-to-background**
ck17	3.1 ± 0.9
ck19	7.1 ± 2.3

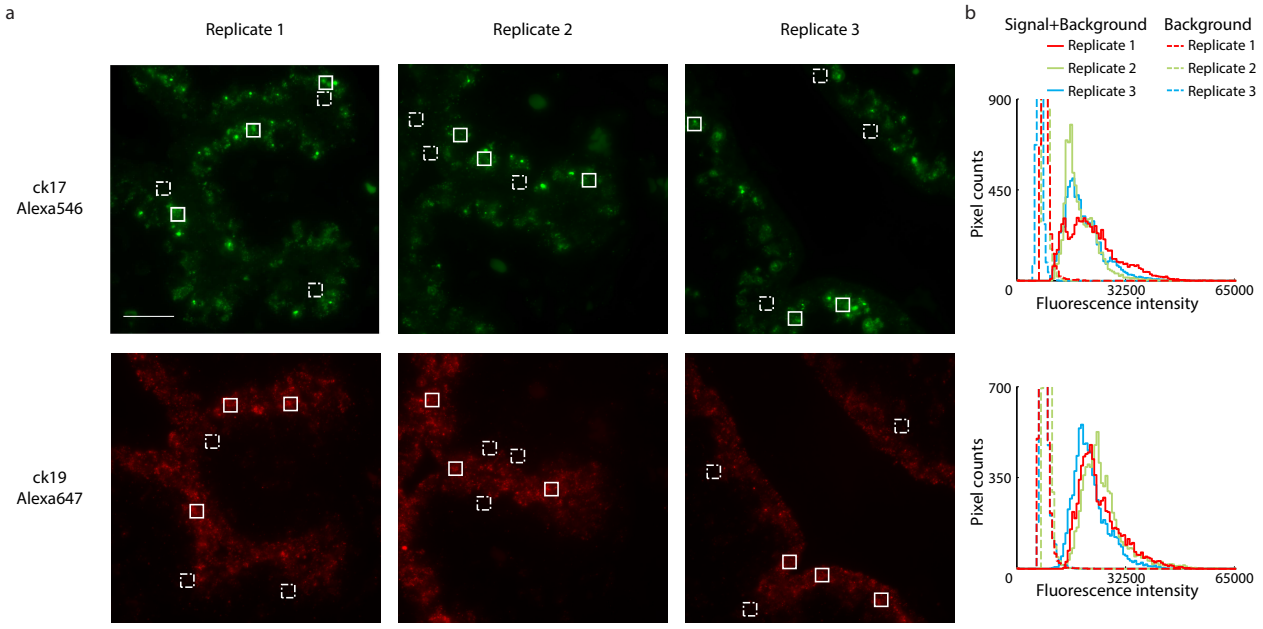

Figure 3.6: Performance of multiplexed ISH using HCR in formalin-fixed, paraffin-embedded human breast tissue sections. (a) Fluorescent microscopy images showing triplicate studies of the three mRNA targets in human tissue sections using spectrally distinct fluorescent channels. (b) Pixel intensity histograms derived from values within three combined rectangular subsections of signal (solid line box) and of background (dashed line box) for each sample and target. Tissue sections are from normal human breast, and the scale bar is 25 μm.

3.4 Conclusion

In this section, ISH using HCR as an amplification method has been expanded into new model organisms, specifically whole mount *Drosophila melanogaster* embryos and formalin-fixed, paraffin embedded human tissue sections. In both of these sample types, it was shown that multiple mRNAs can be simultaneously detected with a high signal-to-background ratio.

Please refer to Appendix B for supplementary information pertaining to this chapter.

Chapter 4

HCR amplification for multiplexed mapping of proteins and mRNAs

4.1 Introduction

Studying protein expression patterns is fundamental to understanding a variety of biological processes. In particular, immunohistochemistry (IHC) is utilized in a variety of academic and clinical diagnostic assays towards this purpose [55, 99, 100]. Chromogenic and fluorescent tyramide amplification techniques in conjunction with antibody detection have allowed for analyzing more than one protein target in a sample; however, these methods require serial labeling for multiplexing [55].

Even though there is a systemic connection between a specific genes mRNA and protein, there is not always a correlation between the expression levels between the two biological macromolecules [101–105]. There have been attempts to study both in the same sample, but these protocols again require serial labeling, which is labor intensive [55, 63]. Consequently, developing a simple and robust method to detect both mRNAs and proteins simultaneously and quantitatively would provide a substantial amount of information in understanding numerous biological pathways.

As was discussed in chapter 3, amplification using HCR may similarly allow for a solution to current concerns with multiplexed IHC and dual IHC-ISH approaches, most specifically, a simple scheme for orthogonal amplifiers [30, 64, 65]. This chapter will demonstrate the adaptation of HCR as an amplification tool for IHC in whole mount zebrafish embryos and formalin-fixed, paraffin embedded human tissue sections. Additionally, a scheme for simultaneous detection using HCR of two expressed genes and their two corresponding proteins in whole mount zebrafish embryos is demonstrated.

4.2 Adapting HCR to immunostaining

In order to use HCR as an amplification tool for IHC, a method needed to be determined to conjugate a nucleic acid initiator to a protein antibody. The chosen scheme was to chemically modify each of the components and join the two via a covalent hydrazone bond (Figure 4.1). This conjugation method has already been used in a variety of ways to connect molecules to antibodies [106, 107]. Also, a hydrazone bond is very stable in neutral pH conditions, which makes it excellent for use in IHC protocols [108, 109]. This reaction between aldehyde and hydrazide functional groups to form hydrazone moieties is also highly efficient and robust unlike other conjugation methods, such as sulfhydrl-maleimide chemistries [110]. Another advantage is the commercial availability of the various materials, including a full conjugation kit from SoluLink.

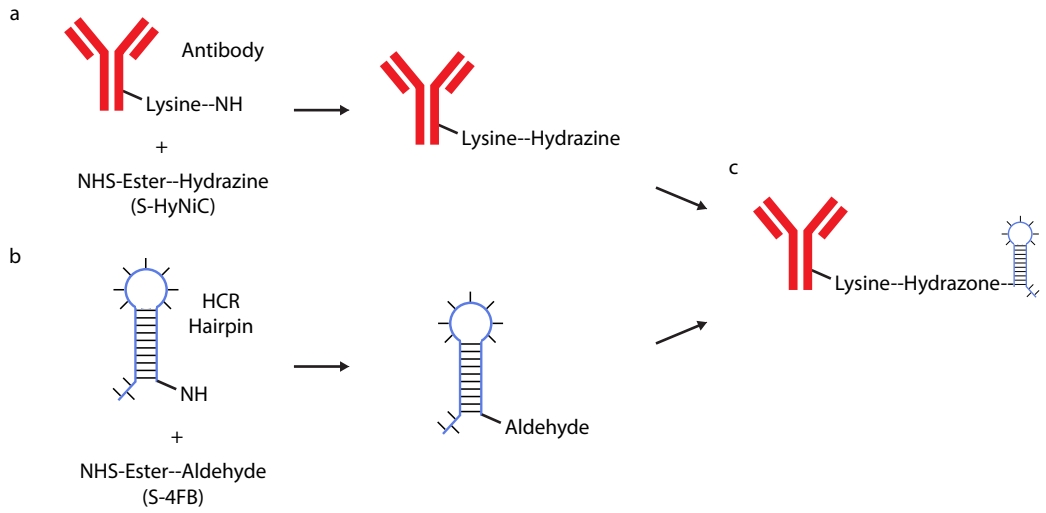

Figure 4.1: Antibody-initiator conjugation scheme. The attachment method for the initiator to the antibody is done by functionalizing each of the individual components with chemical groups that can later form a covalent bond with each other. Note that the next section will explain why a hairpin initiator, instead of a single stranded initiator, will be used in this method. (a) The heterobifunctional linker S-HyNiC is reacted with the amine group of lysine residues within the antibody. This functionalizes the antibody with a hydrazide group. (b) The heterobifunctional linker S-4FB is reacted with the amine group of the initiator, which it had been synthesized with. This functionalizes the nucleic acid with an aldehyde group. (c) The two functionalized components are incubated together in the presence of an aniline catalyst that promotes the formation of a hydrazone bond that covalently links the two components together.

In this scheme, the nucleic acid to be attached to the antibody is synthesized with a terminal free amine and is reacted with N-succinimidyl-4-formylbenzamide (S-4FB). S-4FB is a heterobifunctional linker that contains an ester group and an aldehyde group, and the ester group reacts with amine from the oligonucleotide to functionalize it with an aldehyde group. The antibody is then reacted with succinimidyl-6-hydrazino-nicotinamide (S-HyNiC). S-HyNic is a heterobifunctional linker that

contains an ester group and a hydrazide group, and the ester group reacts with amine from lysine residues on the antibody to functionalize it with a hydrazide group. The nucleic acid and antibodies are then incubated together in the presence of an aniline catalyst, which leads to their covalent linkage via a hydrazone bond. The bond is then confirmed by checking the absorbance at 360 nm, the wavelength at which the bond maximally absorbs.

Most current antibody staining methods involve an indirect approach, where a modified secondary antibody is used to detect the primary antibody that is bound to the protein target. This approach was adopted for HCR antibody staining method as well, which means that the initiator will be conjugated to secondary antibodies. This scheme is advantageous because of the greater amplification that comes from multiple secondary antibodies binding to a single primary antibody, and the fact that a single type of secondary antibody can be used for a variety of different primary antibodies coming from the same species. However, if further modularity is desired, initiators can be conjugated to primary antibodies, but it comes at the cost of some signal amplification. We encountered some issues with decreased binding affinity if the primary antibody is conjugated with nucleic acids because the antibody's epitope recognition site may become compromised, so greater care is needed with the chemical modification of primary antibodies than of secondary antibodies.

4.3 Initiator optimization for use with HCR immunostaining

A straightforward approach to adapting HCR for IHC is to attach the single stranded DNA initiator to the secondary antibody, and then amplify the signal by using fluorescently labeled HCR hairpins. When this was tested in zebrafish embryos, proper signal was seen, but there was also significant background in various parts of the embryo, including in the yolk extension and the embryo surface (Figure 4.2.a). Initial thoughts for this non-specific binding were that the antibody conjugate was too large and that the charge of the nucleic acid made the conjugate sticky. Various approaches were taken in an attempt to alleviate this non-specific binding, such as blocking sticky sites, using more stringent washes, or increased permeabilization to allow bigger molecules to move more freely and not get trapped. The blocking methods included using nucleic acid mimics, such as heparin and salmon sperm DNA, and using chemical modifications, such as using acetic anhydride to neutralize positively charged sites that may bind nucleic acids. The wash stringency was altered by increasing wash times and adding detergents, such as Triton X-100 and sodium dodecyl sulfate. The permeabilization methods included using proteases, such as proteinase K and collagenase. However, none of these initial strategies succeeded, and a different mechanism for this non-specific binding needed to be determined.

Through previous experiences in Pierce group and discussions with collaborators, we then hypothesized that non-specific base-pairing from single-stranded initiators on the secondary antibodies is

causing the background binding in the sample, and not the negatively charged nucleic acid backbone or the size of the antibody conjugate. In order to address this hypothesis, a double-stranded HCR initiator system was developed (Figure 4.3). If the initiator is double-stranded, then the nucleotides are sequestered by other bases in the same molecule, thus preventing the initiator from binding non-specifically. In this system, one of the HCR hairpins (H1) is covalently attached to the antibody. In order to activate the hairpin so that it can polymerize HCR hairpins, free single-stranded initiator (I1) is added to the sample after the antibody has been bound to the target. The initiator binds to and opens up the hairpin, exposing a sequence on the hairpin that can initiate polymerization of HCR hairpins. We hypothesize that even though some free initiators may bind non-specifically in the sample, they are rendered inactive because their binding to the sample sequesters some of the base-pairs required for HCR polymer initiation. This was not the case with single-stranded initiator conjugated to the antibody because there were multiple initiators covalently linked to the antibody. As a result, even if some of the initiators bound to the sample, there are other unbound initiators on that same antibody that can then initiate HCR polymerization, causing the non-specific signal. When this hairpin initiator system was tested for immunostaining in zebrafish embryos, the results were excellent with high signal and low background (Figure 4.2.b). In particular, the non-specific binding to the yolk extension and to the embryo surface was eradicated.

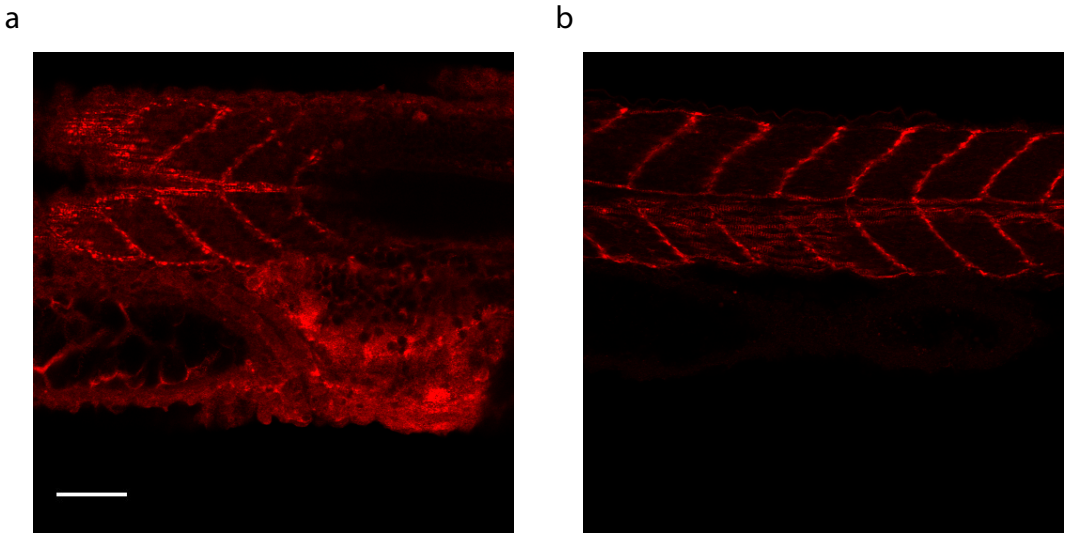

Figure 4.2: Single-stranded initiator vs. hairpin initiator. Fluorescent microscopy images showing detection of Desmin protein in whole mount zebrafish embryos using two types of initiators. (a) A single stranded initiator. (b) An HCR hairpin that was later opened using a single stranded DNA, exposing an initiator used for HCR amplification. The embryos are 27hpf, and the scale bar is 50 μm.

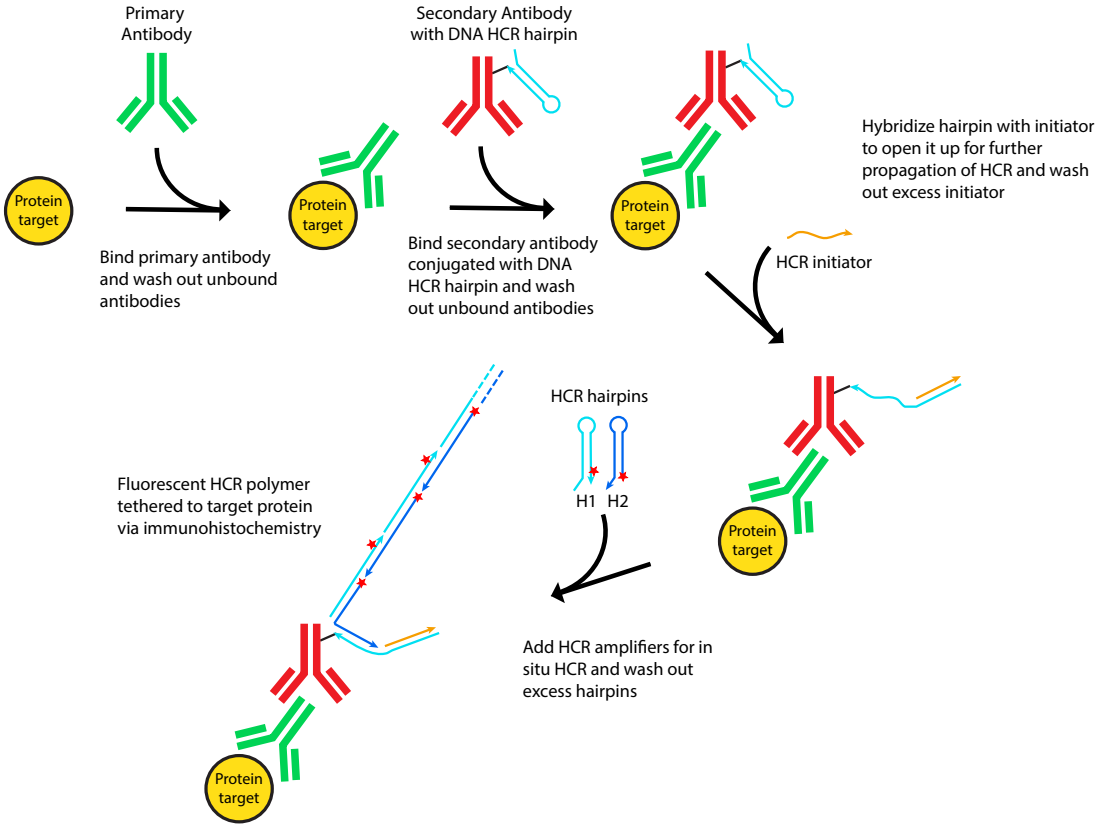

Figure 4.3: Schematic of HCR-immunohistochemistry experiment. The protein target is first detected using a primary antibody. Next, a secondary antibody conjugated with HCR hairpins binds to the primary antibody. Then the HCR hairpin is opened by hybridizing to a free single-stranded initiator that has been added. The exposed single-stranded sequence can subsequently hybridize to another hairpin and nucleate a hybridization chain reaction. Fluorescently labeled hairpins are introduced and form polymers that are tethered to the hairpin on the secondary antibody. The final product consists of a fluorescently labeled HCR polymer attached to the target protein via antibody intermediaries.

4.4 Comparing immunostaining amplification methods in whole-mount zebrafish embryos and formalin-fixed, paraffin-embedded human tissue sections

Once the protocol was optimized for using HCR amplification in antibody staining, its performance was compared to an existing amplification method. The traditional amplification system chosen for this comparison was fluorescently labeled secondary antibodies (direct label). This system was chosen over enzymatic methods as it is currently the best way to simultaneously detect multiple targets without using serial labeling. These two amplification methods were compared in two sample settings: whole-mount zebrafish embryos and formalin-fixed, paraffin-embedded human breast tissue sections.

4.4.1 Whole-mount zebrafish embryos

The protein target chosen for zebrafish embryos was Desmin, and confocal microscopy was used to take images of these stained embryos. Image analysis involved drawing boxes around an area of signal and an area of background, and then extracting the pixel intensities from each of these boxes. The data from each box was averaged to simplify data analysis, as was done in chapter 2. Representative images from this experiment are shown in figure 4.4. The images for the direct labeled embryos were taken under two microscope setting conditions — one that was optimized for HCR amplification and one that was optimized for direct label amplification. The former setting allows for direct comparison between the two methods, and the latter setting confirms that expression is present because the signal is too faint with the other setting. Table 4.1 shows the values for average signal pixel intensity and signal-to-background ratios under HCR amplification imaging settings for both methods. HCR amplification is approximately 20 times brighter than direct label amplification. However, the signal-to-background ratios were approximately the same for both methods at about 30. This indicates two points. The first is that the autofluorescence of the sample and the background caused by direct label amplification are very low, which leads to the high signal-to-background ratio even though signal is relatively dim. The second point is that HCR amplification is causing significant background.

To determine which step in the HCR protocol is causing non-specific amplification, a modified version of the approach outlined in chapter 2 was applied to this method. Using a specific set of controls, each of the background components can be teased out. The components are slightly different than in the HCR *in situ* hybridization case from chapter 2 since there are more pieces required for HCR immunohistochemistry. In addition to autofluorescence (AF) and non-specific amplification (NSA), there are three different non-specific detection (NSD) components. The three

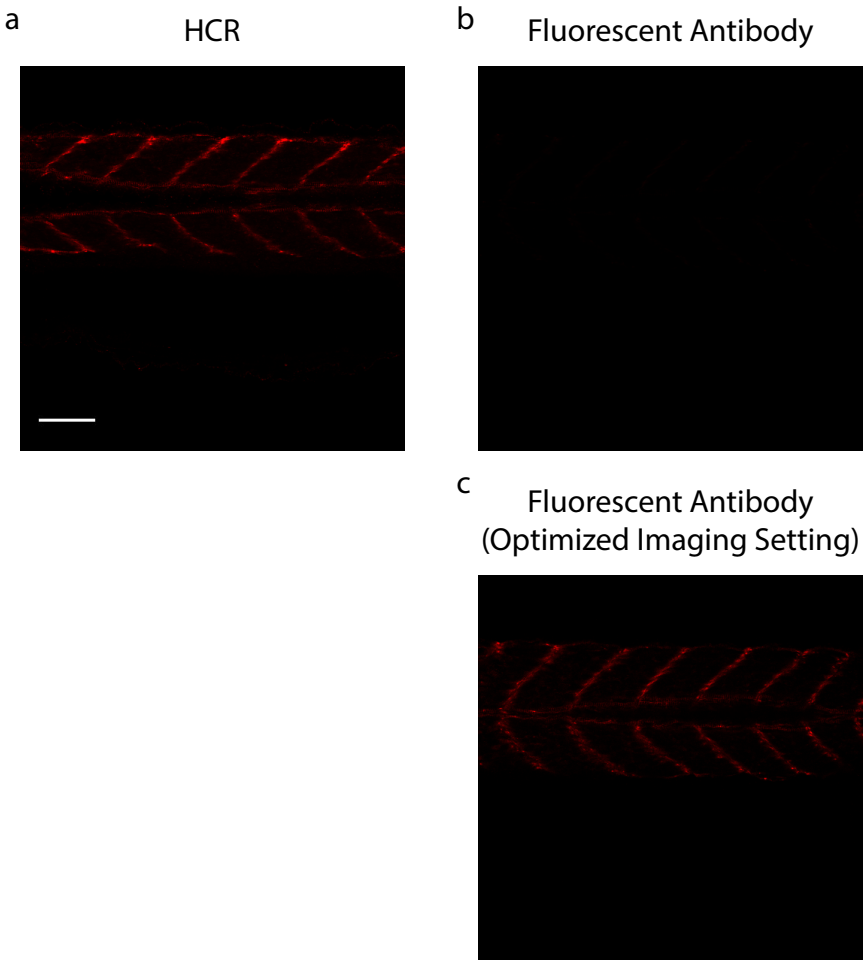

Figure 4.4: Comparison of immunostaining amplification methods in whole-mount zebrafish embryos. Fluorescent confocal microscopy images of zebrafish embryos that have been immunostained to target Desmin with two different amplification schemes: (a) secondary antibodies conjugated to hairpin initiators tethered to fluorescently labeled HCR polymer and (b) fluorescently labeled secondary antibodies. (c) Image of (b) using optimized microscope settings for direct label amplification. The embryos are 27hpf, and the scale bar is 50 μm.

Table 4.1: Average signal intensity and signal-to-background values for two immunostaining amplification schemes in whole-mount zebrafish embryos.

Amplification Method	**Average Signal Intensity**	**Signal-to-background**
HCR Antibody	1230 ± 40	37 ± 14
Fluorescent Antibody	59 ± 30	30 ± 10

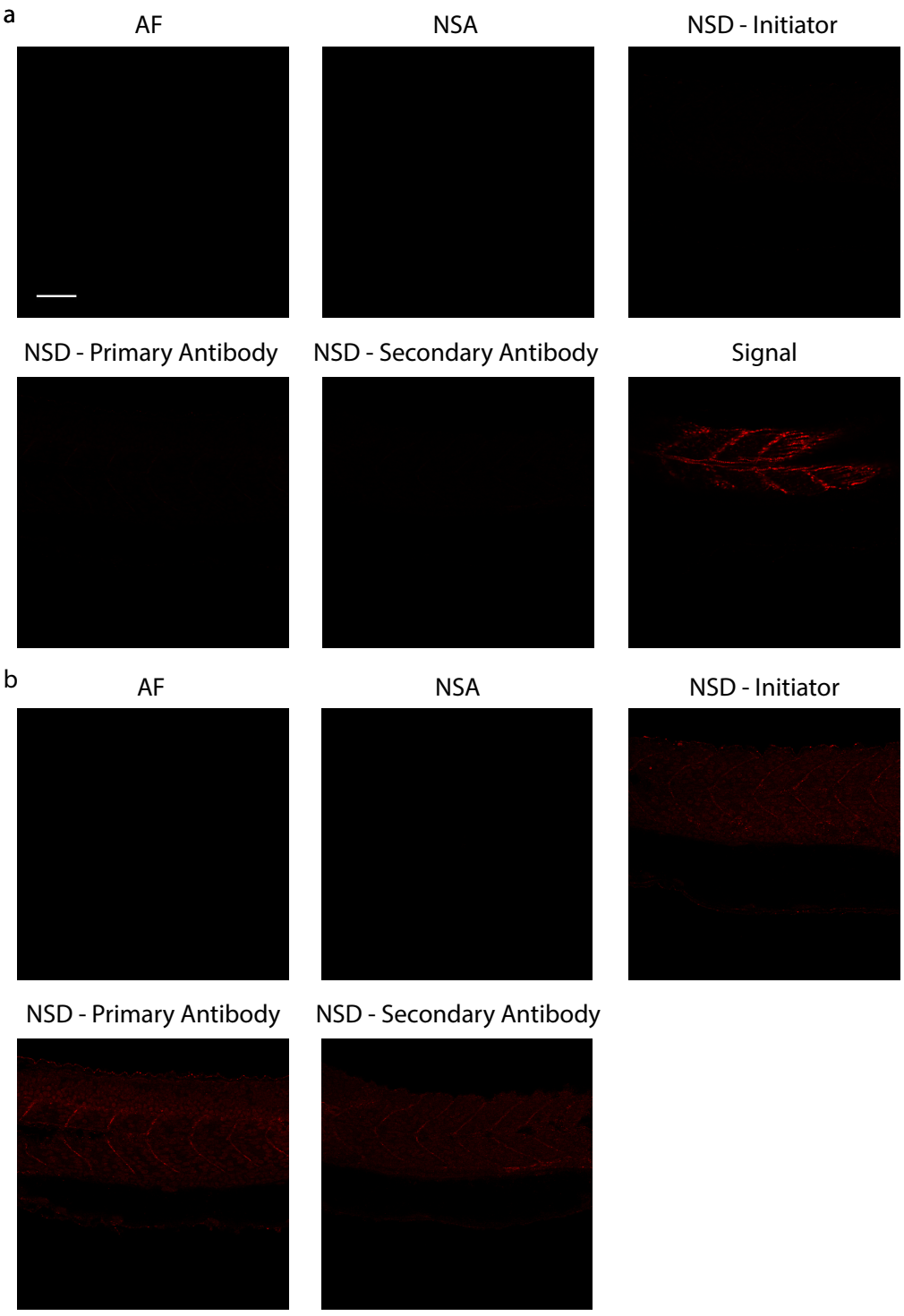

Figure 4.5: Background analysis for HCR immunostaining in zebrafish embryos. (a) Fluorescent confocal microscopy images for control experiments to determine the various contributions to background of HCR amplification in zebrafish embryos. The types of background investigated are AF, NSA, NSD-initiator, NSD-secondary antibody, and NSD-primary antibody. A signal image for the full experiment with signal is shown to demonstrate that the experimental materials are working. (b) The contrast is adjusted for the background images to make clear which step in HCR increases background. The embryos all express a Desmin-Citrine fusion protein, except for the NSD-primary antibody experiment, which are wild-type embryos. Embryos are fixed at 27hpf, and the scale bar is 50 μm.

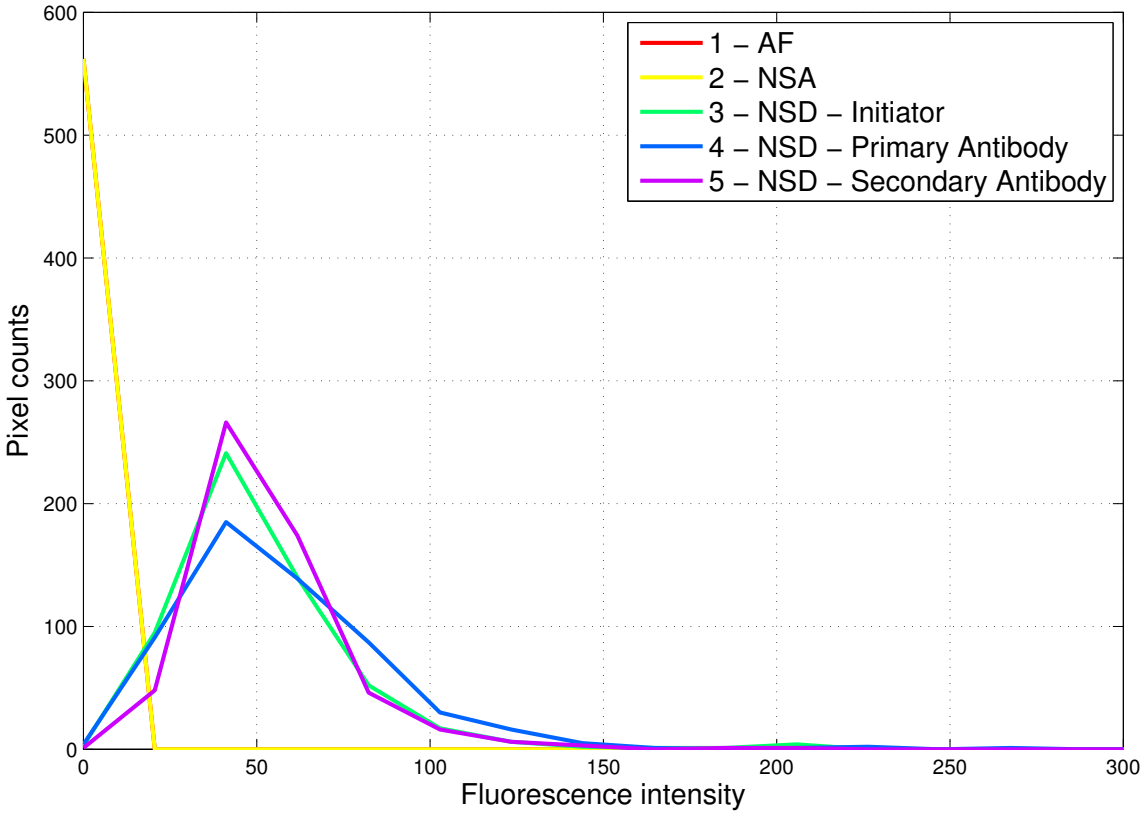

Figure 4.6: Histogram for background analysis for HCR immunostaining in zebrafish embryos. Pixel intensity histograms derived from a box within a representative zebrafish embryo for each control experiment from figure 4.5.

types of NSD are initiator (NSD_I), primary antibody ($NSD_{1\circ}$), and secondary antibody ($NSD_{2\circ}$). NSD-initiator is caused by the free initiator binding to the sample and causing amplification. $NSD_{1\circ}$ and $NSD_{2\circ}$ are respectively caused by the primary antibody or the secondary antibody binding to an off target and causing amplification. The signal-to-background ratio would be calculated as follows:

$$\text{Signal to Background} = \frac{\text{SIG}}{(\text{AF} + \text{NSA} + \text{NSD}_I + \text{NSD}_{1\circ} + \text{NSD}_{2\circ})} \tag{4.1}$$

The target protein chosen for this analysis is Citrine that is expressed as a fusion with Desmin. An anti-GFP antibody can be used to target Citrine, which was used in this case. In this set of experiments, one cumulatively detects an additional form of background with each subsequent experiment. From this information, one can deduce which components are causing the most background. Representative images from this set of experiments and a histogram analysis are shown in figure 4.5. Average intensity values for each of the control experiments is shown in table 4.2. The data indicates that the majority of background is coming from the NSD-initiator component, and that AF and NSA are minimal. This means that the assumption that free initiator becomes inactive when incorrectly bound to the sample is not completely correct. Some bound initiators are still able to initiate an HCR reaction, and even though this is a small fraction, it is significant enough to detrimentally affect signal-to-background. Consequently, this is an area where further optimization would greatly enhance this amplification technique.

Table 4.2: Average pixel intensity values for the cumulative background components of HCR amplification for immunostaining of whole-mount zebrafish embryos.

Background Component	**Average Pixel Intensity**
AF	0.04 ± 0.02
AF + NSA	0.07 ± 0.02
AF + NSA + NSD_I	50 ± 13
AF + NSA + NSD_I + $NSD_{1\circ}$	63 ± 21
AF + NSA + NSD_I + $NSD_{1\circ}$ + $NSD_{2\circ}$	54 ± 20
AF + NSA + NSD_I + $NSD_{1\circ}$ + $NSD_{2\circ}$ + SIG	1500 ± 300

4.4.2 Formalin-fixed, paraffin-embedded human tissue sections

In the zebrafish embryo case, HCR amplification did not help as significantly as hoped due to the very low autofluorescence of the system, which meant that the extra amplification from HCR was not necessary. However, in the human tissue section case, HCR amplification might be more beneficial due to the higher autofluorescence in the sample. Even though there is some added background due to HCR-IHC, the benefits of greater amplification are more pronounced in the case that AF is

greater than NSA and NSD.

The protein target chosen for human tissue sections was Cytokeratin 19. Image analysis was done in the same manner as with the zebrafish embryo staining in the previous section. Representative images from this experiment are shown in figure 4.7. In addition to the images for staining, an additional image for autofluorescence was included. Like before, images for the direct labeled embryos were taken under two microscope setting conditions – one that was optimized for HCR amplification and one that was optimized for direct label amplification. The HCR amplification image setting had a lower laser power and gain than the direct label amplification image setting. This indicates that the total fluorescence from HCR amplification is greater than the direct label method.

Table 4.3 shows the values for average pixel intensity for autofluorescence and both amplification methods, as well as, the signal-to-background ratios for each amplification method. HCR amplification is approximately 4 times brighter than direct label amplification. As was predicted, HCR amplification helped improve the signal-to-background ratio since autofluorescence represented a large portion of the background. Specifically, HCR had a signal-to-background ratio of approximately 6, which is a 2-fold better ratio than the direct label method. Thus, HCR amplification for immunostaining is useful in samples with high autofluorescence.

4.5 Immunostaining using HCR amplification is quantitative

HCR *in situ* hybridization has been shown to be a quantitative tool [111]. This means that in a HCR-ISH experiment, if a voxel is twice as bright as another pixel, then it has approximately twice as much RNA molecules as that second pixel. This relative quantitation would be highly useful in IHC experiments as well. In this section, a redundant detection approach is taken to determine if immunostaining using HCR is also quantitative. When the same target is detected using two sets of reporters, and there is a linear relationship between the pixel intensities from each channel, this indicates not only that the correct target is being detected, but also that quantitative claims can be made.

In this set of experiments, two different systems were used. In the first, the Desmin protein was detected in wild-type zebrafish embryos using an anti-Desmin rabbit antibody. In the second, the citrine protein was detected in ct122a zebrafish embryos using an anti-GFP chicken antibody. For each system, two batches of secondary antibody were conjugated to two different HCR initiators, where one batch had one type and the other half had the other type. Each initiator system was amplified using a spectrally distinct orthogonal HCR amplifier. Images from each fluorescent channel were taken and analyzed (figure 4.8). As was done for the redundant detection studies in chapter 3, the pixel intensities from both channels in a subset of the image are plotted against each other. In both cases, there is a high Pearson correlation coefficient with values of over 0.95, which

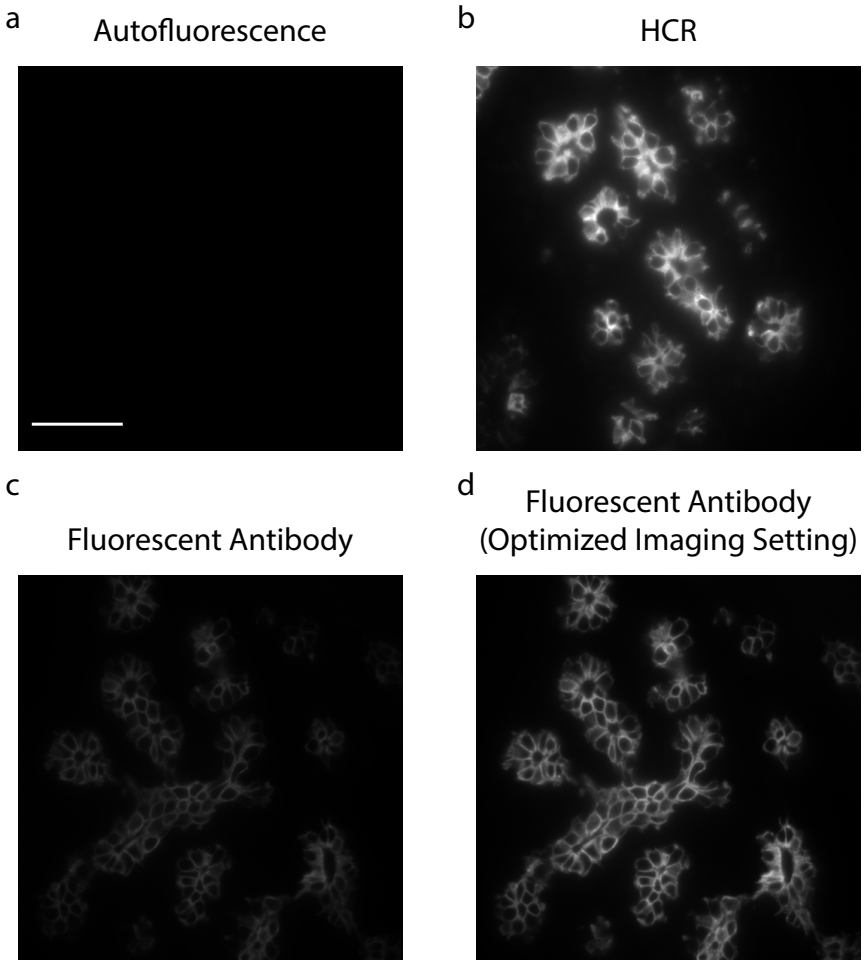

Figure 4.7: Comparison of immunostaining amplification methods in formalin-fixed, paraffin-embedded human tissue sections. (a) Fluorescent microscopy image of untreated human breast tissue sections to analyze autofluorescence. (b,c) Fluorescent microscopy images of untreated human breast tissue sections that have been immunostained to target Cytokeratin 19 with two different amplification schemes: (b) secondary antibodies conjugated to hairpin initiators tethered to fluorescently labeled HCR polymer and (c) fluorescently labeled secondary antibodies. (d) Image of (c) using optimized microscope settings for direct label amplification. The sections are 3 μm thick, and the scale bar is 50 μm.

Table 4.3: Average signal intensity and signal-to-background values for autofluorescence and two immunostaining amplification schemes in formalin-fixed, paraffin-embedded human tissue sections. Studies are done in triplicate

Sample	**Average Signal Intensity**	**Signal-to-background**
Autofluorescence	3081 ± 4	–
HCR Antibody	39300 ± 2900	5.8 ± 0.7
Fluorescent Antibody	9700 ± 1100	2.5 ± 0.3

indicates a linear relationship. This strong correlation indicates that the secondary antibody and HCR amplification are all quantitative because combined they have a linear relationship. It seems a reasonable assumption that the primary antibody quantitatively binds to the protein target, in which case these studies show that HCR-IHC is a quantitative method.

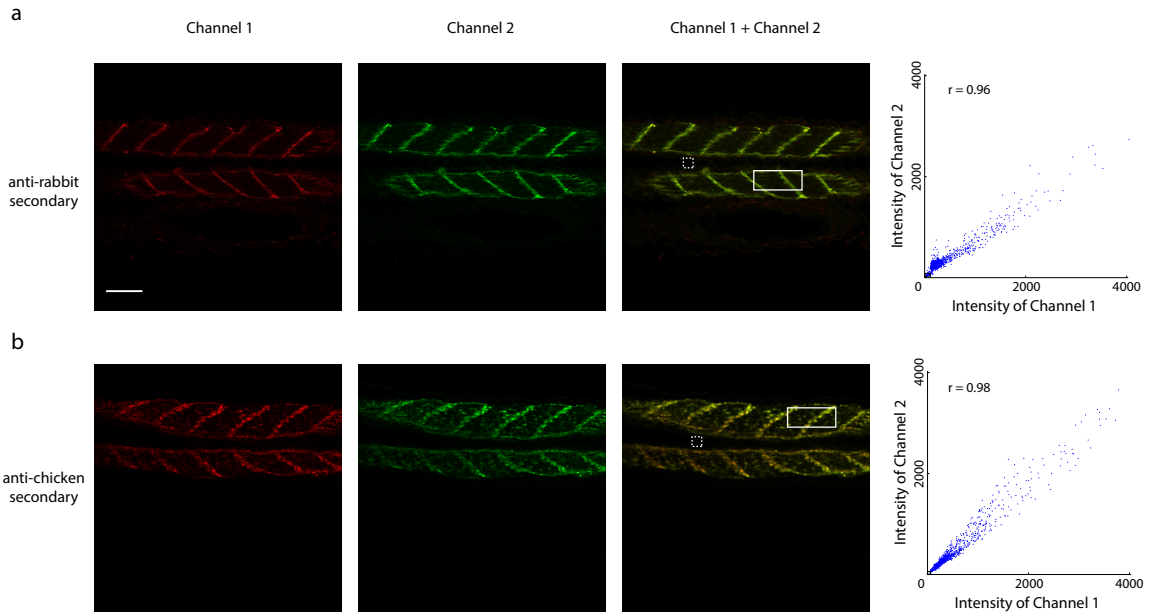

Figure 4.8: Redundant detection of proteins using HCR amplification in whole-mount zebrafish embryos. The primary antibodies are bound to their targets in zebrafish embryos. Next, the corresponding secondary antibodies are added, half labeled with one HCR initiator and the other half labeled with another HCR initiator. Each HCR system is then amplified using a spectrally distinct HCR amplifier. Confocal images from each channel and a merged image are shown for each target. The intensities from both channels (channel 1 and channel 2) for all pixels in the image are plotted against each other. A background box (dotted line) is selected. The average intensity of this box plus two standard deviations is used as a threshold to exclude background pixels. A linear correlation indicates that the signal is relatively quantitative. The correlation coefficient is very strong for both targets, which implies that HCR-IHC is quantitative. Two separate targets were tested to validate findings: (a) line: wild-type, target: Desmin, primary antibody: rabbit anti-Desmin, secondary antibody: donkey anti-rabbit (b) line: ct-122a, target: Citrine, primary antibody: chicken anti-GFP, secondary antibody: goat anti-chicken. Embryos are fixed at 27hpf, each voxel is 2×2 pixels, and the scale bar is 50 μm.

4.6 Simultaneous *in situ* detection of mRNA and proteins

Now that HCR amplification has been used for the detection of either mRNAs using ISH or proteins using IHC, it follows that one should be able to detect both types of targets in the same sample using HCR. A combined method was developed to ensure that the targets remained intact and accessible and that the reagents were compatible. In this protocol, the proteins are detected first

using antibodies, and then the sample is fixed using formaldehyde. Next, probes to detect mRNAs are hybridized to their targets. Lastly, HCR is used to concurrently amplify the signal for each of the detection methods and targets. This method was tested in whole-mount zebrafish embryos.

the HCR hairpin is opened by hybridizing to a free single-stranded initiator that has been added. The exposed single-stranded sequence can subsequently hybridize to another hairpin and nucleate a hybridization chain reaction. Fluorescently labeled hairpins are introduced and form polymers that are tethered to the hairpin on the secondary antibody. The final product consists of a fluorescently labeled HCR polymer attached to the target protein via antibody

The targets were chosen to be two mRNAs and their expressed proteins, specifically *desmin* and the transgene *fetal liver kinase — enhanced green fluorescent protein.* Figure 4.10 shows the images for the simultaneous detection of two mRNAs and their corresponding proteins. This protocol worked extremely well as each of the four targets had a signal-to-background ratio of at least 14 (table 4.4). Additionally, there is a clear delineation between signal and background for each target, as seen on the histogram analysis in figure 4.11. This demonstration indicates that HCR is a strong tool for the multiplexed detection of various types of targets.

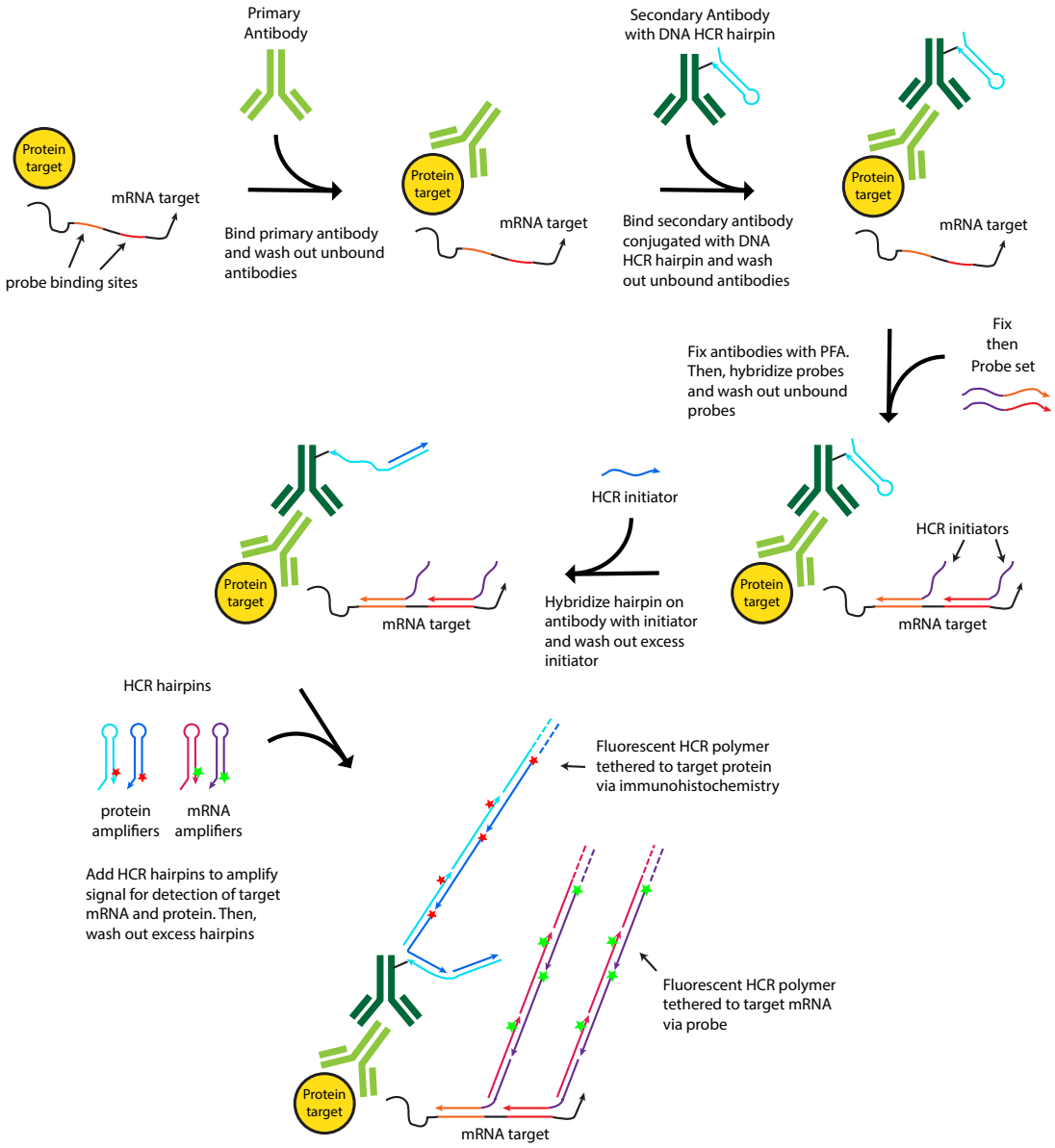

Figure 4.9: Schematic of combined HCR-IHC and HCR-ISH method. The protein target is first detected using a primary antibody, and then secondary antibodies conjugated with HCR hairpins binds to the primary antibody. The sample is fixed using paraformaldehyde to ensure the antibodies remain bound during the probe hybridization steps. Next, probes containing HCR initiators are hybridized to their mRNA target. Free initiator is added to open the hairpin on the secondary antibody to reveal the initiator sequence used for amplification. The initiator for ISH and the initiator for IHC use spectrally distinct orthogonal amplifiers. Fluorescently labeled hairpins are introduced and form long, nicked double-stranded polymers that are either tethered to the probe or the secondary antibody.

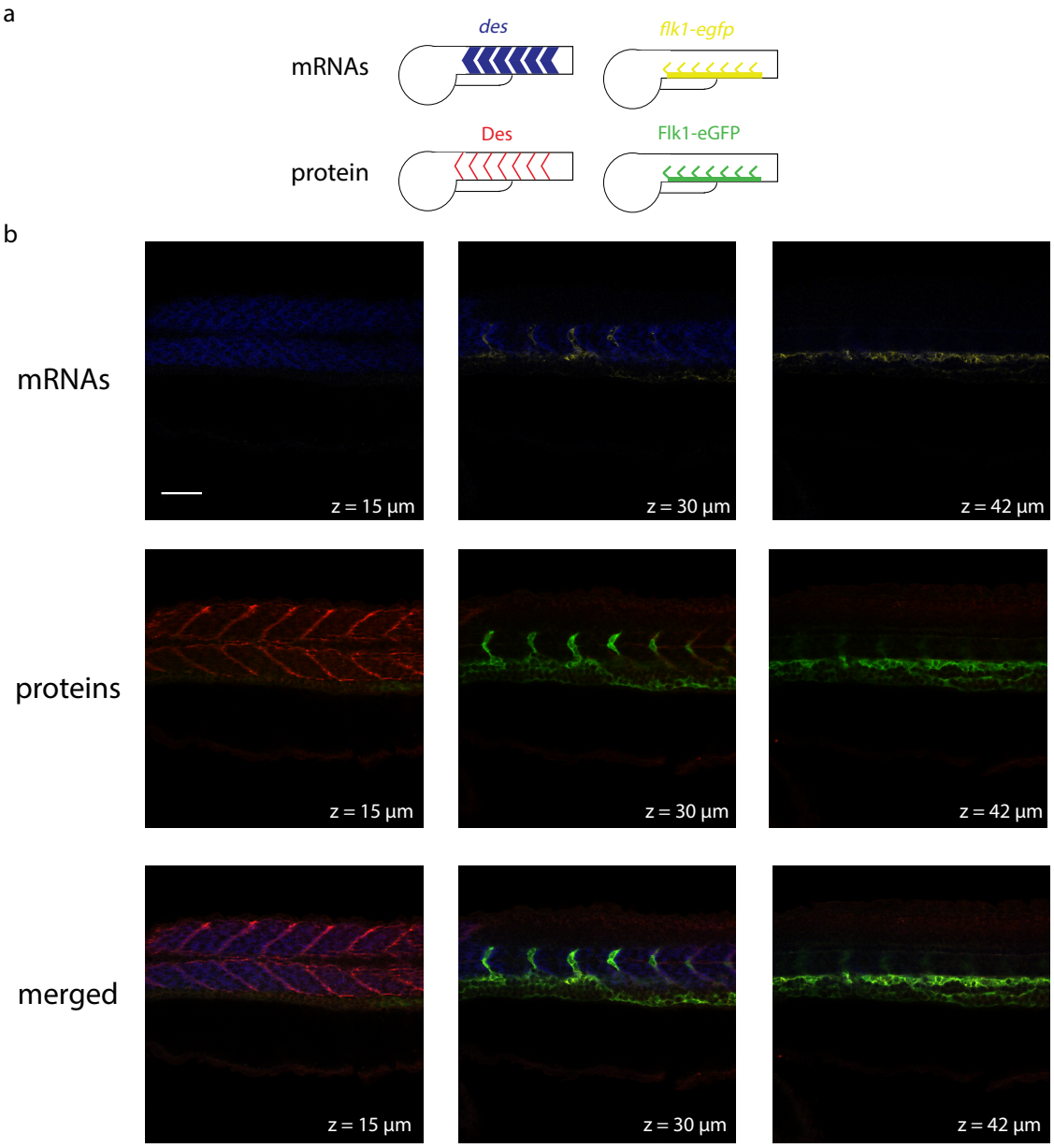

Figure 4.10: Multiplexed detection of mRNAs and proteins in whole mount zebrafish embryos. Two different gene products and their corresponding proteins were simulatenously detected using a combined HCR-ISH and HCR-IHC protocol. (a) Expression atlas for two target mRNAs and two target proteins: *fetal liver kinase — enhanced green fluorescent protein* (*flk1-egfp*/Flk1-eGFP) and *desmin* (*des*/Des). (b) Fluorescent confocal microscopy image of fixed zebrafish embryo on three planes showing expression pattern of two different mRNAs and two different protein targets using HCR *in situ* hybridization and HCR immunohistochemistry. Embryos express the transgene *flk1-egfp* and are fixed at 27hpf. The scale bar is 50 μm.

Table 4.4: Signal-to-background values for HCR-ISH experiments in whole mount *drosophila melanogaster* embryos

mRNA or protein	**signal-to-background**
desmin mRNA	14.6 ± 7.8
Desmin protein	14.0 ± 5.1
flk1-egfp mRNA	19.0 ± 9.2
Flk1-eGFP protein	17.3 ± 6.1

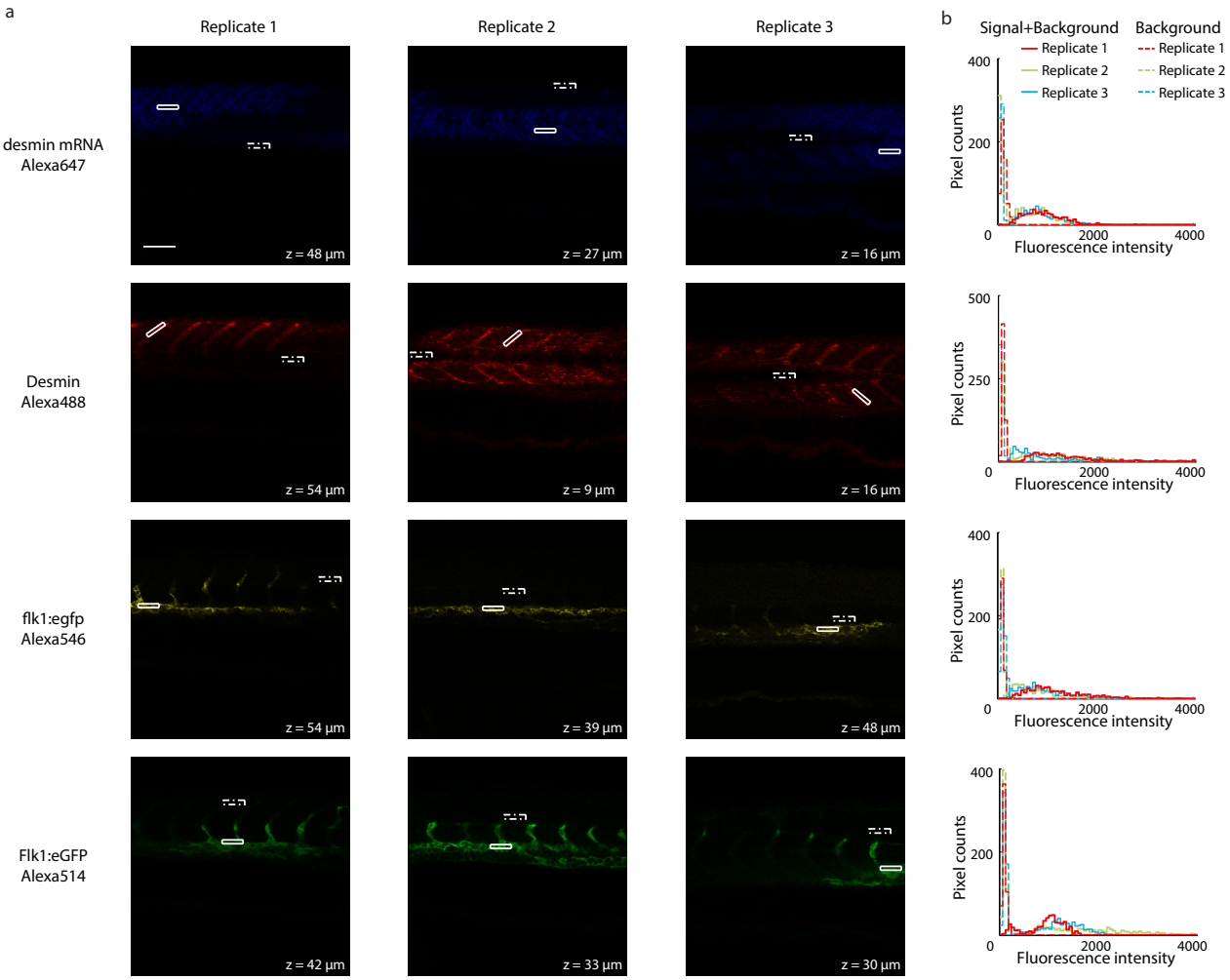

Figure 4.11: Performance of multiplexed ISH and IHC using HCR in whole mount zebrafish embryos. (a) Confocal microscopy images showing the triplicate studies of the two mRNA targets and their two corresponding proteins in zebrafish embryos using spectrally distinct fluorescent channels. Representative image slices are shown for each of the replicate samples. (b) Pixel intensity histograms derived from values within a rectangular subsection of signal (solid line box) and of background (dashed line box) for each sample and target. Embryos are fixed at 27hpf, and the scale bar is 50 μm.

4.7 Conclusion

In this chapter, HCR has been used as an amplification tool for immunohistochemistry. The signal from HCR-IHC is significantly greater than with using directly fluorescently labeled secondary antibodies; however, there is a drawback with added non-specific background from using HCR. As a result, this method would be best served in sample types with high autofluorescence. Consequently, there is room for improvement in this technique by determining how to reduce binding of the free initiator non-specifically within the sample. In future work, this background may be addressed by using a slightly altered hairpin initiator. One suggestion is to use hairpin H1 with a different toehold sequence, called H1′. In this case, the inititator that is used to open the hairpin conjugated to the antibody is unable to initiate HCR polymerization on its own, while the opened hairpin can still nucleate hairpin assembly. Another proposed improvement is to change the conjugation chemistry so that the 5′-end of the hairpin is attached to the antibody instead of the 3′-end. This modification would add another stacking interaction to the HCR polymer and makes the initiation step even more thermodynamically favorable, which results in longer polymers and greater signal. It was also demonstrated that HCR immunostaining is quantitative, which is an extra benefit on top of the gains of amplification and multiplexing. And finally, mRNAs and proteins were detected concurrently in the same sample using HCR to amplify signal for both ISH and IHC. This work has demonstrated that HCR can be used to detect mRNAs and proteins with a high signal-to-background in various organisms and sample types.

Please refer to Appendix C for supplementary information pertaining to this chapter.

Chapter 5

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Appendix A

Supplementary Information for Chapter 2

A.1 Protocols for cell culture

This section contains the protocols and reagents needed to perform HCR-ISH experiments on cultured cells. The protocol is adapted from Choi et al. 2010 [30]. In addition, this section includes the equations used to calculate the fluorescent components and signal-to-background values and their uncertainties for individual probes and probe sets.

A.1.1 Sample preparation protocol

For the probe optimization studies in chapter 2, a HEK293 cell line that expresses d2eGFP constitutively was used.

1. Grow cells on 10 cm plate to approximately 90-95% confluency in High-Glucose DMEM with 10% Fetal Bovine Serum media at 37°C with 5% CO_2.
2. Harvest cells by first aspirating media and rinsing cells with warmed Dulbecco's phosphate-buffered saline (DPBS).[1]
3. Remove DPBS, add 3 mL of warmed 0.25% trypsin-EDTA and incubate at 37°C for 5 min.
4. Add 3mL warmed Low-Glucose DMEM media and spin down cells in 15 mL conical and aspirate liquid.[2]
5. Resuspend pellet in 10 mL warmed DPBS and spin down cells.
6. Fix cells by resuspending pellet in 3 mL 8% formaldehyde and incubate at room temperature for 5 min. Then, spin down cells and aspirate liquid.

[1] All warmed solutions and media are at 37°C.
[2] All spin centrifugations done at 1,500 RCF.

7. Resuspend pellet in 10 mL warmed DPBS, spin down cells, and aspirate liquid. Repeat once.
8. Resuspend pellet in 70% ethanol and incubate at 4°C overnight to permeabilize cells.

A.1.2 In situ HCR protocol

Detection stage

1. For each sample, transfer 100,000-200,000 cells to a 1.5 mL eppendorf tube, spin down, and aspirate.[1]
2. Resuspend pellet in 1 mL of 50% hybridization buffer (HB) warmed to 55°C and prehybridize cells at 55°C for 10 min.
3. Prepare probe solution by adding 2 pmol of each probe into 100 μL of 50/
4. Spin down and resuspend pellet with probe solution and incubate at 55°C overnight.
5. To wash out excess probes, resuspend pellet in 1 mL of 50% and incubate at 55°C for 30 min. Repeat once.

Amplification stage

1. While washing out excess probes, prepare hairpin solution by snap-cooling hairpins by heating to 95°C for 1.5 min and place on ice for 20 seconds. Then leave at room temperature for 30 min in darkness. Add 6 pmol of each hairpin into 100 μL of 40/
2. Spin down and resuspend pellet with 500 μL of 40% HB and incubate at 45°C for 10 minutes
3. Resuspend pellet with hairpin solution and incubate at 45°C overnight
4. To wash out excess hairpins, the following washes are performed using 1 mL of solution and incubating at 45°C for 10 minutes:
   (a) 75% of 40% HB and 25% of 2× SSC
   (b) 50% of 40% HB and 50% of 2× SSC
   (c) 25% of 40% HB and 75% of 2× SSC
   (d) 2× SSC
5. Resuspend in 1 mL of 2× SSC and store at 4°C

[1] For each incubation and wash step going forward, centrifuge at 1,500 RCF and aspirate liquid.

A.1.3 Buffer recipes for cell culture RNA HCR

50% Hybridization buffer (50% HB)	For 40 mL of solution
50% formamide	20 mL formamide
2× sodium chloride sodium citrate (SSC)	4 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
500 μg/mL tRNA	200 μL of 100 mg/mL tRNA
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

40% Hybridization buffer (40% HB)	For 40 mL of solution
40% formamide	16 mL formamide
2× sodium chloride sodium citrate (SSC)	4 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
500 μg/mL tRNA	200 μL of 100 mg/mL tRNA
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

2× SSCT	For 40 mL of solution
2× sodium chloride sodium citrate (SSC)	4 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

A.1.4 Probe and amplifier sequences

Probe sequences

For the probe optimization studies in chapter 2, the target *gfp* mRNA is detected with a probe set consisting of eight single initiator RNA probes. This probe set initiates the RNA HCR amplifier A1. Sequences are listed 5′ to 3′.

Target mRNA: **green fluorescent protein (*gfp*)**
Amplifier: **RNA HCR A1**
Fluorophore: **Alexa Fluor 647**

Probe	Initiator	Spacer	Probe Sequence
1	CCgAATACAAAgCATCAACgACTAgA	AAAAA	CCTCgCCggACACgCTgAACTTgTggCCgTTTACgTCgCCgTCCAgCTCg
2	CCgAATACAAAgCATCAACgACTAgA	AAAAA	gCCAgggCACgggCAgCTTgCCggTggTgCAgATgAACTTCAgggTCAgC
3	CCgAATACAAAgCATCAACgACTAgA	AAAAA	TAggTCAgggTggTCACgAgggTgggCCAgggCACgggCAgCTTgCCggT
4	CCgAATACAAAgCATCAACgACTAgA	AAAAA	TCTTgTAgTTgCCgTCgTCCTTgAAgAAgATggTgCgCTCCTggACgTAg
5	CCgAATACAAAgCATCAACgACTAgA	AAAAA	gTTgTggCTgTTgTAgTTgTACTCCAgCTTgTgCCCCAggATgTTgCCgT
6	CCgAATACAAAgCATCAACgACTAgA	AAAAA	TCgCgCTTCTCgTTggggTCTTTgCTCAgggCggACTgggTgCTCAggTA
7	CCgAATACAAAgCATCAACgACTAgA	AAAAA	CACgAACTCCAgCAggACCATgTgATCgCgCTTCTCgTTggggTCTTTgC
8	CCgAATACAAAgCATCAACgACTAgA	AAAAA	CTCCACCTCCggCgggAAgCCATggCTAAgCTTCTTgTACAgCTCgTCCA

Amplifier sequences

Initiator and hairpin sequences for the RNA HCR amplifiers used in this chapter.

`/5′-dye-C12/`: 5′ Alexa Fluor modification with a C12 spacer
`/C9-dye-3′/`: 3′ Alexa Fluor modification with a C9 spacer

RNA HCR A1

I1	CCgAAUACAAAgCAUCAACgACUAgA
H1	UCUAgUCgUUgAUgCUUUgUAUUCggCgACAgAUAACCgAAUACAAAgCAUC /C9-dye-3'/
H2	/5'-dye-C12/ CCgAAUACAAAgCAUCAACgACUAgAgAUgCUUUgUAUUCggUUAUCUgUCg

A.1.5 Reagents and supplies

High-Glucose DMEM (ThermoFisher Scientific Cat. # 11995-065)

Dulbecco's phosphate-buffered saline (DPBS) (ThermoFisher Scientific Cat. # 14190-250)

Fetal Bovine Serum (ThermoFisher Scientific Cat. # 16140-071)

16Ethanol, 200 proof, Anhydrous (VWR Cat. # 71002-422)

Formamide (Deionized) (Ambion Cat. # AM9342)

20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)

50% Tween 20 (Life Technologies Cat. # 00-3005)

Heparin (Sigma Cat. # H3393)

50× Denhardt's solution (Life Technologies Cat. # 750018)

Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)

tRNA from baker's yeast (Roche Cat. # 10109509001)

A.2 Calculations of fluorescent components, signal-to-background ratios, and error analysis

A.2.1 Calculation of Fluorescent Components

Fluorescent components

- Autofluorescence (AF)
- Non-Specific Amplification (NSA)
- Non-Specific Detection (NSD)
- Signal (SIG)

Using the average values of the experimental replicates outlined in figure 2.1, each of the components are calculated as follows:

Information from each experiment

- <Experiment 1> = AF
- <Experiment 2> = AF + NSA
- <Experiment 3> = AF + NSA + NSD = Background (BACK)
- <Experiment 4> = AF + NSA + NSD + SIG

Average component calculations

- $\overline{\text{AF}}$ = <Experiment 1>
- $\overline{\text{NSA}}$ = <Experiment 2> – <Experiment 1>
- $\overline{\text{NSD}}$ = <Experiment 3> – <Experiment 2>
- $\overline{\text{SIG}}$ = <Experiment 4> – <Experiment 3>

The uncertainty for each experiment is the standard deviation for the replicates of that experiment. The uncertainty for each of the average components were calculated as follows[1]:

- $\sigma_{\mathrm{AF}} \leq \sigma_{experiment\ 1}$
- $\sigma_{\mathrm{NSA}} \leq \sqrt{{\sigma_{experiment\ 2}}^2 + {\sigma_{experiment\ 1}}^2}$
- $\sigma_{\mathrm{NSD}} \leq \sqrt{{\sigma_{experiment\ 3}}^2 + {\sigma_{experiment\ 2}}^2}$
- $\sigma_{\mathrm{SIG}} \leq \sqrt{{\sigma_{experiment\ 4}}^2 + {\sigma_{experiment\ 3}}^2}$

A.2.2 Calculation of signal-to-background

The signal-to-background ratio and error for each probe was calculated as follows:

$$\text{Signal-to-Background} = \frac{\mathrm{SIG}}{\mathrm{BACK}}$$

$$\text{Uncertainty} \leq \text{Signal-to-Background}\sqrt{\left(\frac{\sigma_{SIG}}{\mathrm{SIG}}\right)^2 + \left(\frac{\sigma_{BACK}}{\mathrm{BACK}}\right)^2}$$

[1]The calculated upper bounds on uncertainty use the assumption that the correlation between the variables is non-negative.

Appendix B

Supplementary Information for Chapter 3

This section contains the protocols and reagents needed to perform HCR-ISH experiments on whole-mount fly embryos and formalin-fixed, paraffin-embedded tissue sections using the next generation DNA HCR amplifiers. The HCR protocol is adapted from Choi et al. 2014 [30]. The fly embryo ISH protocol is adapted from Kosman et al. 2004 [80].

B.1 Protocols for whole-mount fruit fly embryos (*Drosophila melanogaster*)

B.1.1 Sample preparation protocol

1. Collect Drosophila embryos and incubate with yeast paste (food source) until they reach stage 4-6 (approximately 3 h).
2. Rinse embryos into a mesh basket and wash excess yeast paste using DI H_2O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorinate embryos.
4. Rinse the basket with DI H_2O.
5. Transfer embryos to a scintillation vial containing 8 mL of 4.5% formaldehyde fixation solution.
6. Shake the vial for 25 min on a shaker.[1]
7. Remove the bottom liquid phase in the vial.
8. Add 8 mL of methanol (MeOH) and shake the vial hard for 1 min. Devitellinized embryos will sink to the bottom of the vial.

[1] Shaking does not have to be very vigorous. However, embryos must move continuously within the heptane-fixative interphase to be exposed uniformly to the solvent and fixative.

9. Remove all liquid and rinse 2 times in MeOH to remove debris.

10. Store embryos in 1 mL of MeOH at −20°C before use.

B.1.2 Buffer recipes for sample preparation

4.5% formaldehyde fixation solution	For 8 mL of solution
4.5% formaldehyde	975 μL of 37% formaldehyde
0.5× PBS	400 μL of 10× PBS
25 mM EGTA	76 mg of EGTA
50% heptane	4 mL of heptane

B.1.3 Multiplexed in situ HCR protocol

Detection stage

1. For each sample, transfer 50 μL of embryos (using a cut pipet tip) to a 1.5 mL eppendorf tube.
2. Rinse embryos 4 times by quickly adding 1 mL of ethanol (EtOH), inverting the tube, and aspirating the supernatant.
3. Add 500 μL of EtOH and 250 μL of xylene and invert the tube.
4. Add an additional 250 μL of xylene and invert the tube.
5. Add another 250 μL of xylene again and invert the tube. The tube should now contain 500 μL of EtOH and 750 μL of xylene.
6. Rock the tube at room temperature for 45–60 min. Longer rocking time is acceptable.
7. Aspirate the supernatant.
8. Rinse embryos once and wash 3 × 5 min with EtOH.[2]
9. Rinse embryos once and wash 2 × 5 min with MeOH.
10. Wash with 50% MeOH / 50% PBST for 5 min.
11. Wash 1 × 10 min and 2 × 5 min with PBST.
12. Rock embryos in 1 mL of 20 mg/mL proteinase K solution at room temperature for 7 min.
13. Rinse embryos 2 times and wash 2 × 5 min with PBST.
14. Rock embryos in 4% formaldehyde post-fixation solution at room temperature for 25 min.
15. Rinse embryos and wash 5 × 5 min with PBST.
16. Pre-hybridize with 100 μL of probe hybridization buffer for 2 h at 65°C.
17. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 45°C.
18. Remove the pre-hybridization solution and add the probe solution.
19. Incubate embryos overnight (12–16 h) at 45°C.
20. Remove excess probes by washing with probe wash buffer at 45°C:

[2] All washes have a volume of 1 mL unless specified. All washes before pre-hybridization (step 16) are done with rocking.

(a) 2 × 5 min

(b) 2 × 30 min

Wash solutions should be pre-heated to 45°C before use.

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 10 min at room temperature.
2. Prepare 6 pmol of each fluorescently labeled hairpin by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95°C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 h) at room temperature.
6. Remove excess hairpins by washing with 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

B.1.4 Sample mounting for microscopy

1. Place 25 μL of embryos on a 25 mm × 75 mm glass slide.
2. Add 1–2 drops of Prolong Gold antifade reagent mounting medium onto the embryos and stir to mix using a pipette tip.
3. Place a 22 mm × 22 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used to acquire images.

B.1.5 Buffer recipes for in situ HCR

Proteinase K solution	For 2 mL of solution
4 μg/mL proteinase K	0.4 μL of 20 mg/mL proteinase K
	Fill up to 2 mL with PBST

4% formaldehyde post-fixation solution	For 2 mL of solution
4% formaldehyde	216 μL of 37% formaldehyde
	Fill up to 2 mL with PBST

Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

B.1.6 Reagents and supplies

Baker's yeast (VWR Cat. # IC10140001)

37% formaldehyde (Fisher Scientific Cat. # F79-4)

10× PBS (Life Technologies Cat.# AM9625)

Ethylene glycol tetra acetic acid (EGTA) (Sigma Cat. # E4378)

Heptane, HPLC-grade (EMD Millipore Cat. # HX0080-6)

Methanol (Mallinckrodt Chemicals Cat. # 3016-16)

Ethanol, 200 proof (VWR Cat. # V1001G)

Xylene (Mallinckrodt Chemicals Cat. # 8668-02) 20 mg/mL proteinase K solution (Life Technologies Cat. # 25530-049)

Formamide (Deionized) (Ambion Cat. # AM9342)

20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)

Heparin (Sigma Cat. # H3393)

50% Tween 20 (Life Technologies Cat. # 00-3005)

50× Denhardt's solution (Life Technologies Cat. # 750018)

Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)

25 mm × 75 mm glass slide (VWR Cat. # 48300-025)

22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)

Prolong Gold antifade reagent hard mounting medium (Life Technologies # P36936)

B.2 Protocols for FFPE human tissue sections (*Homo sapiens sapiens*)

B.2.1 Multiplexed in situ HCR protocol

Detection stage

1. Deparaffinize FFPE tissue by immersing slide in Histo-Clear II for 3 × 5 min.[1]
2. Rehydrate with a series of graded EtOH washes for 3 min at room temperature:
   (a) 100% EtOH
   (b) 100% EtOH
   (c) 95% EtOH
   (d) 70% EtOH
   (e) RNase-free H_2O
   (f) RNase-free H_2O.
3. Incubate in 1× TBS for 5 min.
4. Immerse slide in 10 μg/mL of proteinase K solution for 40 min at 37°C.[2]
5. Wash slide 2 × 3 min at room temperature in TBST.
6. Immerse slide in 0.2 N HCl for 20 min at room temperature.
7. Incubate slides in 5× SSCT for 5 min.
8. Place slide in an RNase-free staining glass trough with a stir bar.
9. Add 200 mL of 0.1 M triethanolamine-HCl at pH 8.0.
10. Add 500 μL of acetic anhydride **slowly and carefully** with constant stirring.
11. Turn off stirrer when the acetic anhydride is dispersed and allow the slide to incubate for 10 min.
12. Incubate slide in 5× SSCT for 5 min.
13. Pre-warm two humidified chambers with one at 45°C and the other one at 65°C.
14. Dry slide by blotting edges on a Kimwipe.

[1] Each 50 mL Falcon tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. A larger number of slides could be processed together using a Coplin jar.

[2] We recommend this step to be formed in a falcon tube placed inside a 37°C water bath to minimize temperature fluctuation.

15. Add 200 μL of probe hybridization buffer on top of the tissue sample.
16. Pre-hybridize for 10 min inside the 65°C humidified chamber.
17. Prepare probe solution by adding 0.2 pmol of each probe (1 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 65°C.
18. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
19. Add 50–100 μL of the probe solution on top of the tissue sample.[3]
20. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in the 45°C humidified chamber.
21. Immerse slide in 2 × SSC with 0.1% SDS at room temperature to float off coverslip.
22. Remove excess probes by incubating slide at 45°C in:
    (a) 75% of probe wash buffer / 25% 5× SSCT for 15 min
    (b) 50% of probe wash buffer / 50% 5× SSCT for 15 min
    (c) 25% of probe wash buffer / 75% 5× SSCT for 15 min
    (d) 100% 5× SSCT for 15 min

    Wash solutions should be pre-heated to 45°C before use.
23. Immerse slide in 5× SSCT for 5 min at room temperature.

Amplification stage

1. Dry slide by blotting edges on a Kimwipe.
2. Add 200 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 2 h at room temperature.
3. Prepare 6 pmol of each fluorescently labeled hairpin by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95°Cfor 90 seconds and cool to room temperature in a dark drawer for 30 min).
4. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.

[3] Amount of probe solution depends on the size of the coverslip.

6. Add 50–100 μL of the hairpin solution on top of the tissue sample.[4]
7. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in a humidified chamber at room temperature.
8. Immerse slide in 5 × SSCT at room temperature to float off coverslip.
9. Remove excess hairpins by incubating slide in 5 × SSCT at room temperature for:
   (a) 2 × 30 min
   (b) 1 × 5 min

B.2.2 Sample mounting for microscopy

1. Dry slide by blotting edges on a Kimwipe.
2. Add 50 μL of SlowFade Gold mounting medium on top of human tissue section.
3. Place a 22 mm × 40 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss Axio Observer inverted light microscope equipped with an Plan-Apochromat 63×/1.4 Oil Ph3 M27 objective was used to acquire images.

[4] Amount of hairpin solution depends on the size of the coverslip.

B.2.3 Buffer recipes for in situ HCR

Proteinase K solution	For 1 mL of solution
10 μg/mL proteinase K	15 μL of 20 mg/mL proteinase K
	Fill up to 30 mL with 1× TBS
0.2 N HCl	For 30 mL of solution
0.2 N HCl	500 μL of 37% ($\approx$ 12 N) HCl
	Fill up to 30 mL with ultrapure H_2O
0.1 M Triethanolamine-HCl	For 200 mL of solution
0.1 M triethanolamine	2.67 mL of 7.5 mM triethanolamine
Adjust pH to 8.0	350 μL 37% HCl
	Fill up to 200 mL with ultrapure H_2O
TBST	For 50 mL of solution
1× TBS	5 mL of 10× TBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O
Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
100 μg/mL salmon sperm DNA	400 μL of 10 mg/mL salmon sperm DNA
	Fill up to 40 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

B.2.4 Reagents and supplies

FFPE human normal breast tissue section (Pantomics Cat. # BRE01 Block # F091926B08)

Histo-Clear II (National Diagnostics Cat. # HS-202)

Ethanol, 200 proof (VWR Cat. # V1001G)

10× Tris-buffered saline solution (TBS) (Research Products International Cat. # T60075)

20 mg/mL Proteinase K (Life Technologies Cat. # AM2546)

10% Tween 20 Solution (Bio-Rad Cat. # 161-0781)

Hydrochloric Acid (HCl) (EMD Millipore Cat. # HX0603-75)

Triethanolamine (Acros Organics Cat. # AC42163-1000)

Acetic anhydride (Mallinckrodt Chemicals Cat. # 2420-04) Formamide (Deionized) (Ambion Cat. # AM9342)

20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)

Heparin (Sigma Cat. # H3393)

50× Denhardt's solution (Life Technologies Cat. # 750018)

Dextran sulfate, mol. wt. > 500,000 (Sigma Cat. # D6001)

UltraPure salmon sperm DNA solution (Life Technologies Cat. #15632-011)

22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)

SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

B.3 Probe and Amplifier Sequences

B.3.1 *D. melanogaster* Probes

For the fly studies of Figures 3.3 and 3.4, each of four target mRNAs is detected with a different probe set. Each probe set contains four to five 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) [112]. Spatial and temporal expression information were obtained from FlyBase [81].

Target mRNA: **short gastrulation (*sog*)**
Amplifier: **DNA HCR B1**
Fluorophore: **Alexa Fluor 514**

Initiator I1	**Spacer**	**Probe Sequence**	**Spacer**	**Initiator I2**
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	ATCACggTggAACTCgTCgCCAgACACCgCAgATCTTgCCCgTggCATTC	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	TCCgTCTgCAgggCCgTgTATTTTggCCACCTTTCCggCCAgAgCCAACT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CTggggATCCgTTTTgCCATCgggAAggTggTgCCTCCAgTAgCgAACTg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCgACgACAgCTCCAggACATTgATCTCggCAgAgggTTTgCgCACACgT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gCTCgggTCCACgATgTgTCCCTggATgCgCAgATgTgggTACTTCTTgg	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **even skipped (*eve*)**

Amplifier: **DNA HCR B3**

Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggCggTCgTgATgggCATggTggCTCTCCATgTTgTAggTTCggTATCCg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTgCCgTACTTgggTggCCAAgAggTCCACAACCAggggCTTCTggTCCA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCgAgCCgCggCTgCCgTTCAAggAgTTATCCggACTTggATAggCATTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TCCAAgCCgACCCAgCTggTCACgggTgAAggCggTgCgATAgCggCgTA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggCCAgTTCgCAAgCgACggggACgggACACgTAgTTCTCCTTgTAgAAC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **cap-n-collar (*cnc*)**

Amplifier: **DNA HCR B2**

Fluorophore: **Alexa Fluor 546**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ATCTggTCCAATTTgCgTTTCCTgCCAATTCTgggCAgCgACCTTgTTCT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gTCCATgggCAggTTAATgATgTCCggCACTgAAATgggTATgTTCAAgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCgCTTTTCATCgCgTgTCAgATgCTCTTCCTCCAAgTTggCTgCTgTTT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTgATAggAgTAgCCgggATTAgTgATgCggATACggTgACATATCggAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **caudal (*cad*)**

Amplifier: **DNA HCR B5**

Fluorophore: **Alexa Fluor 488**

Initiator I1	**Spacer**	**Probe Sequence**	**Spacer**	**Initiator I2**
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	gTTgTTgTTgTTgTTggTTgTTgTTggCgACggCACTCAAATggTgTgCC	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TgCTgCATCAgCTggTgggCCgAACgTgggCACATTCTgAACgAAgTTAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CCAAATTggCgggCACTgTgCTTTTgTgTgTAgggCAgTgTgTTgTAgTA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	AAgTCCACATCgCCCAggAACTggTggTTCggCggCgTgTggTggTAgTT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	TTggTTgTggTTgTgCTggATAggCgggCTTCTTCATCCAgTCgAAgTAT	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

B.3.2 Human Tissue Section probes

For the human studies of Figures 3.6 and 4.1, each of three target rRNA or mRNAs is detected with a different probe set. Each probe set contains one to eight 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) [112]. Spatial and temporal expression information were obtained from the literature [95–98].

Target mRNA: **keratin 17 (*KRT17*)**
Amplifier: **DNA HCR B2**
Fluorophore: **Alexa Fluor 546**

Initiator I1	**Spacer**	**Probe Sequence**	**Spacer**	**Initiator I2**
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TgCCggggTTCgCCCgCTTCCTTTATAggCCACCAAgTgggCgTAgCgAT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCAggCACACAggAgAAgggCTggAgAggAgAggggCCCCAAgTTgTgTA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CCAgCAgATCCCAgCCTgCAggAgCCggCACCCAggCCgCCAgACAgCCg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	AAgCTgTAgCAgCTggAgTAgCTgCTACCCCCgAgggTgCTgCCCAggCC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TTCCACAATggTACgCACCTgACgggTggTCACCggTTCTTTCTTgTACT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CggCTgCCTCCCTgCCTCCTgggTggCCggCCggggTAgCTgAgTCCTCA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTgAAgCAgggggCTgAggCTggAgAggCCggAgACTgTggggCAgATgg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCTgAgTCAACAAgCTTTATTgTCATCAggCAAggAAgCATggggAAggg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

Target mRNA: **keratin 19 (*KRT19*)**

Amplifier: **DNA HCR B1**

Fluorophore: **Alexa Fluor 647**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gAgCACggACggAgCAACCCTggTCTCAgAAgCTgCgATTCgCgggAggA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	CCTCCgAAggACgACgTggCCgACgACTggCgATAgCTgTAggAAgTCAT	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	ATATT	gTAggCCCCCgAggAggACgAggACACAAAgCgggCggAggACACggATA	ATATA	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg

Target mRNA: **18S ribosomal RNA (*RNA18S5*)**

Amplifier: **DNA HCR B5**

Fluorophore: **Alexa Fluor 488**

Initiator I1	Spacer	Probe Sequence	Spacer	Initiator I2
CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	AAAAA	CggAATTAACCAgACAAATCgCTCCACCAACTAAgAACggCCATgCACCA	ATTTT	CACTTCATATCACTCACTCCCAATCTCTATCTACCC

B.3.3 DNA HCR amplifier sequences

Initiator and hairpin sequences for the five DNA HCR amplifiers used in the current chapter. Sequences are from Choi et al. 2014 [65].

`/5′-dye-C12/:` 5′ Alexa Fluor modification with a C12 spacer
`/C9-dye-3′/:` 3′ Alexa Fluor modification with a C9 spacer

DNA HCR B1

I1	gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg
I2	gCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg
H1	CgTAAAggAAgACTCTTCCCgTTTgCTgCCCTCCTCgCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg /C9-dye-3'/
H2	/5'-dye-C12/ gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACgCTCTTCCCgTTTgCTgCCCTCCTCAAgAAAgAATgC

DNA HCR B2

I1	CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC
I2	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
H1	ggCggTTTACTggATgATTgATgAggATTTACgAggAgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC /C9-dye-3'/
H2	/5'-dye-C12/ CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCCgATgATTgATgAggATTTACgAggATggACTgAgCT

DNA HCR B3

I1	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
I2	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
H1	CgggTTAAAgTTgAgTggAgATATAgAggCAgggACAAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC /C9-dye-3'/
H2	/5'-dye-C12/ gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCggAgTggAgATATAgAggCAgggACggATTAgACTTT

DNA HCR B4

I1	CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC
I2	CACATTTACAgACCTCAACCTACCTCCAACTCTCAC
H1	gAAgCgAATATggTgAgAgTTggAggTAggTTgAggCACATTTACAgACCTCAACCTACCTCCAACTCTCAC /C9-dye-3'/
H2	/5'-dye-C12/ CCTCAACCTACCTCCAACTCTCACCATATTCgCTTCgTgAgAgTTggAggTAggTTgAggTCTgTAAATgTg

DNA HCR B5

I1	CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT
I2	CACTTCATATCACTCACTCCCAATCTCTATCTACCC
H1	ATTggATTTgTAgggTAgATAgAgATTgggAgTgAgCACTTCATATCACTCACTCCCAATCTCTATCTACCC /C9-dye-3'/
H2	/5'-dye-C12/ CTCACTCCCAATCTCTATCTACCCTACAAATCCAATgggTAgATAgAgATTgggAgTgAgTgATATgAAgTg

Appendix C

Supplementary Information for Chapter 4

C.1 Methods

C.1.1 Protocol for antibody conjugation with DNA

To conjugate a DNA initiator or hairpin to an antibody, order the DNA oligonucleotide from IDT with an amine modification and have it HPLC purified. Obtain at least 25 OD_{260} of the DNA strand. Also use secondary antibodies that are completely unconjugated.

Modification of amino-oligonucleotide with S-4FB

1. Dissolve DNA to 0.5 $OD_{260}/\mu L$ with ultrapure H_2O.
2. Desalt and buffer exchange the DNA using Vivaspin 500 centrifugal filter with 5 kilodalton (kDa) molecular weight cutoff (MWCO). [1]
   (a) Add 450 μL buffer A to 50 μL of DNA solution and place 500 μL of new solution into centrifugal filter, and spin at 14K RCF for 10 min.
   (b) Discard flow through and raise volume of DNA solution back to 500 μL with buffer A, and spin at 14K RCF for 10 min. Repeat two times.
   (c) Pipette out solution after spins, and bring volume to 52 μL with Buffer A
3. Measure concentration of DNA solution using Nanodrop spectrophotometer using 10 mm path-length. Dilute solution 1:200 by adding 398 μL of H_2O to 2 μL of DNA, and measure the A_{260} using 2 μL. Divide A_{260} value by 5 to obtain concentration in units of $OD_{260}/\mu L$. Multiply by

[1] Ensure molecular weight cutoff is small enough to not lose the DNA.

the remaining amount of solution in microliters to determine units of OD_{260}, and this number should be above 15. If not, obtain more oligonucleotide.

4. Resupend 1 mg of N-succinimidyl-4-formylbenzamide (S-4FB) in 40 μL of anhydrous dimethylformamide (DMF). Vortex or pipette up and down repeatedly until S-4FB is completely dissolved.

5. Determine the amount of S-4FB needed to react with 50 μL of amine modified DNA solution by performing the following calculations [2]:

   - Nanomoles of amino modified DNA per microliter = [OD_{260}/μL] * [nmol/OD_{260}]
   - Amount of S-4FB in μL = [nmol of DNA/μL of DNA solution] * [50 μL solution] * [1 mol of DNA / 1×10^9 nmol of DNA] * [247,200 mg S-4FB / mol S-4FB] * [40 μL S-4FB solution / 1 mg S-4FB] * [20 mol S-4FB / 1 mol DNA]

6. Mix 50 μL of DNA solution with 25 μL of DMF and the amount of dissolved S-4FB calculated in the previous step. Incubate at room temperature for 2 h.

7. Desalt and buffer exchange the DNA to remove excess S-4FB using Vivaspin 500 centrifugal filter with 5 kilodalton (kDa) molecular weight cutoff (MWCO).

   (a) Add buffer C to DNA-S-4FB reaction until it reaches 500 μL and place in centrifugal filter, and spin at 14K RCF for 10 min.

   (b) Discard flow through and raise volume of DNA solution back to 500 μL with buffer C, and spin at 14K RCF for 10 min. Repeat two times.

   (c) Pipette out solution after spins, and bring volume to 50 μL with Buffer C

8. Measure molar substitution ratio (MSR) of 4FB-DNA using 2-HP. The MSR is the number of S-4FB molecules that have attached to each DNA molecule. This number should be approximately 1.

   (a) Prepare a blank solution by adding 2 L H_2O to 18 L 2-HP solution. For the sample, mix 2 L 4FB-DNA to 18 L 2-HP solution. Incubate both at 37°C for 1 h.

   (b) Measure A_{260} and A_{360} using Nanodrop spectrophotometer using 1 mm pathlength. If A_{260} is significantly higher than 1, then dilute sample 1 in 10 using H_2O and remeasure. Multiply that result by 10 to obtain the A_{260} of the original solution.

   (c) Calculate MSR as follows: [A_{360} / A_{260}] * [ϵ_{260} of DNA molecule / 24500)[3]

9. 4FB modified DNA oligonucleotide can be stored at at 4°C for up to a year

[2] Assume 20 fold molar excess of S-4FB over DNA is needed to complete reaction.
[3] ϵ_{360} of S-4FB is approximately 24,500.

Modification of antibody with S-HyNiC

1. Desalt and buffer exchange 100 μL of antibody using 0.5 mL Thermo Scientific Zeba Spin Desalting Columns with a 40 kDa MWCO.

   (a) Remove the bottom of the column and loosen cap. Spin column at 1,500 RCF for 1 min to remove storage buffer.

   (b) Discard flow through and add 300 μL buffer A to top of column resin, and spin at 1500 RCF for 1 min. Repeat twice.

   (c) Change collection tube to new 2.0 mL eppendorf tube. Add 100 μL of antibody to top of column resin and spin at 1500 RCF to collect desalted antibody in Buffer A.

2. Measure concentration of antibody solution using Nanodrop spectrophotometer using protein A_{280} module. Then dilute antibody to 1 mg/mL using Buffer A.

3. Resupend 1 mg of succinimidyl-6-hydrazino-nicotinamide (S-HyNiC) in 35 μL of anhydrous dimethylformamide (DMF). Vortex or pipette up and down repeatedly until S-HyNiC is completely dissolved.

4. Add 2 μL of S-HyNiC to 100 μL of 1 mg/mL antibody in Buffer A and incubate at room temperature for 2 h.

5. Desalt and buffer exchange the S-HyNiC conjugated antibody to remove excess S-HyNiC using 0.5 mL Thermo Scientific Zeba Spin Desalting Columns with a 40 kDa MWCO.

   (a) Remove the bottom of the column and loosen cap. Spin column at 1,500 RCF for 1 min to remove storage buffer.

   (b) Discard flow through and add 300 μL buffer C to top of column resin, and spin at 1500 RCF for 1 min. Repeat twice.

   (c) Change collection tube to new 2.0 mL eppendorf tube. Add antibody and S-HyNiC reaction to top of column resin and spin at 1500 RCF to collect desalted modified antibody in Buffer C.

Conjugation of antibody to DNA

1. Determine amount of 4FB-DNA to be conjugated with modified antibody. Assume average molecular weight of antibody (IgG or IgY) is 150 kDa and that a 5-fold excess of 4FB-DNA over antibody is needed to complete the reaction. Calculate as follows:

   - Amount of 4FB-DNA in μL = [μg of antibody / 150000 μg/μmol] * [1000 nmol / 1 μmol] * [5 / MSR] * [1 / 4FB-DNA concentration in mM (nmol/μL)]

2. Mix antibody with the amount of 4FB-DNA calculated in the previous step. Add 1/9 the total volume of 10X Turbolink Catalyst Buffer and pipette up and down to mix. Incubate at room temperature for 2 h.

3. Desalt and buffer exchange antibody conjugate to remove excess free 4FB-DNA using 0.5 mL Amicon Ultra Centrifugal Filters with a 100 kDa MWCO. In addition, this step also removes any unconjugated antibody [110].

   (a) Raise volume of conjugation reaction to 500 μL with 1$\times$ PBS and add to column. Spin at 14000 RCF for 5 min.

   (b) Discard flow through, raise volume to 500 μL with 1$\times$ PBS and spin at 14000 RCF for 5 min. Repeat 2 times.

   (c) Turn column over in new collection tube and spin at 1000 RCF for 2 min to collect purified conjugated antibody. Raise volume of conjugated antibody to 50 μL with 1$\times$ PBS.

4. Determine concentration of conjugated antibody using BCA assay.

   (a) Make BCA working solution by mixing 49 μL of reagent A with 1 μL of reagent B for each reaction.

   (b) Mix 2.5 μL of antibody conjugate with 50 μL of BCA working solution and incubate at 37°C for 30 min.

   (c) In addition to the antibody conjugate sample, also make a standard curve using various concentrations of a Bovine Gamma Globulin standard.

   (d) Cool BCA reactions to room temperature and measure concentration on Nanodrop using BCA module.

   (e) Dilute antibody to 1 mg/mL (or to the concentration desired for storage).

C.1.2 Buffer recipes for antibody conjugation with DNA

Buffer A	For 50 mL of solution
150 mM NaCl	2.5 mL of 3M NaCl
100 mM Na_2HPO_4	5 mL of 1M Na_2HPO_4
	pH to 7.4 using HCl and NaOH
	Fill up to 50 mL with H_2O

Buffer C	For 50 mL of solution
150 mM NaCl	2.5 mL of 3M NaCl
100 mM Na_2HPO_4	5 mL of 1M Na_2HPO_4
	pH to 6.0 using HCl and NaOH
	Fill up to 50 mL with H_2O

0.5M 2-HP Solution	For 50 mL of solution
500 mM 2-hydrazinopyridine-2HCl (2-HP)	91 μL of 50 mg/mL 2-hydrazinopyridine-2HCl
100 mM MES Buffer, pH 5.0	50 mL of 100 mM MES Buffer, pH 5.0

C.1.3 Protocols for HCR-IHC and combined HCR-ISH / HCR-IHC

C.1.3.1 Whole-mount zebrafish embryos

Sample preparation protocol

1. Collect embryos and incubate at 28°C in a petri dish with egg H_2O until they reach approximately 24 h post-fertilization (hpf).
2. Dechorionate embryos using two pairs of sharp tweezers under a dissecting scope.
3. Transfer embryos (27 hpf) to a 2 mL eppendorf tube and remove excess egg H_2O. Wash once with 1 mL of 1× PBS.
4. Fix embryos in 1 mL of 4% paraformaldehyde (PFA) for 24 h at at 4°C.[1]
5. Wash embryos 3 × 5 min with 1 mL of 1× PBS to stop the fixation. Fixed embryos can be stored at 4°C.
6. Dehydrate and permeabilize embryos with a series of methanol (MeOH) washes at 1 mL each:
   (a) 100% MeOH for 4 × 10 min.
   (b) 100% MeOH for 1 × 50 min.
7. Rehydrate with a series of washes with increasing 1× phosphate-buffered saline + 0.1% Tween 20 (1× PBST) concentration at 1 mL for 5 min each:
   (a) 75% MeOH / 25% 1× PBST
   (b) 50% MeOH / 50% 1× PBST
   (c) 25% MeOH / 75% 1× PBST
   (d) 5 × 100% 1× PBST
8. Store embryos at 4°C for up to a month before use.

[1] Use PFA that has been freshly made that day and cooled to 4°C to reduce autofluorescence.

HCR-IHC protocol

1. For each sample, transfer 6–10 embryos (using a cut pipet tip) to a 2.0 mL eppendorf tube.
2. Wash with 1 mL 1× PBST. [1]
3. Incubate with 1 mL of 10 μg/mL of proteinase K for 4 min.
4. Rinse twice with 1× PBS, then two 5 min washes with 1 mL 1 × PBST.
5. Fix with 1 mL of 4% PFA for 20 min.
6. Rinse twice with 1× PBS, then five 5 min washes with 1 mL 1 × PBST.
7. Block with 1× PBDT for 30 min.
8. Mix primary antibody with 250 μL 1× PBDT and add to embryos. Incubate either at room temperature for 2 hours or 4°C overnight.[2]
9. Wash five times with 1× PBDT for 5 min.
10. Mix 1.25 μL secondary antibody with 250 μL 1× PBDT and add to embryos. Incubate at room temperature for 1.5 h.
11. Wash five times with 1× PBDT for 5 min.
12. Block nucleic acid binding sites by incubating with 1 mL Initiator Binding Solution for 10 min.
13. Incubate with 10 μM free initiator in Initiator Binding Solution for 30 min.
14. Wash three times with 5× SSCT for 5 min.
15. Pre-amplify embryos with 250 μL of amplification buffer for 30 min at room temperature.
16. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95°C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
17. Prepare hairpin solution by adding all snap-cooled hairpins to 250 μL of amplification buffer at room temperature.
18. Remove the pre-amplification solution and add the hairpin solution.
19. Incubate the embryos overnight (12–16 h) at room temperature.
20. Remove excess hairpins by washing with 5× SSCT at room temperature:

[1] For every wash step, aspirate liquid out of tube and add next wash.
[2] Antibody dilution should be optimized for each different primary antibody.

(a) 2 × 5 min

(b) 2 × 30 min

(c) 1 × 5 min

Sample mounting for microscopy

1. Place 50 μL of 3% methyl cellulose to a 25 mm × 75 mm glass slide between two sets of scotch tape 7 pieces thick.

2. Add embryos and use fine hair to manipulate embryos.

3. Place a 22 mm × 22 mm No. 1 coverslip on top.

4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used to acquire images.

Combined HCR-IHC and HCR-ISH protocol

1. Perform HCR-IHC protocol up to step 11
2. Fix with 1 mL of 4% PFA for 20 min.
3. Rinse twice with 1× PBS, then five 5 min washes with 1 mL 1 × PBS. item Pre-hybridize with 500 μL of probe hybridization buffer for 30 min at 65°C.
4. Prepare probe solution by adding 0.5 pmol of each probe (0.5 μL of 1 μM stock per probe) to 250 μL of probe hybridization buffer at 45°C.
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate embryos 4 h at 45°C.
7. Remove excess probes by washing with probe wash buffer at 45°C:
   (a) 2 × 5 min
   (b) 2 × 30 min
8. Block nucleic acid binding sites by incubating with 1 mL Initiator Binding Solution for 10 min.
9. Incubate with 10 μM free initiator in Initiator Binding Solution for 30 min.
10. Wash three times with 5× SSCT for 5 min.
11. Pre-amplify embryos with 250 μL of amplification buffer for 30 min at room temperature.
12. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (heat at 95°C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
13. Prepare hairpin solution by adding all snap-cooled hairpins to 250 μL of amplification buffer at room temperature.
14. Remove the pre-amplification solution and add the hairpin solution.
15. Incubate the embryos overnight (12–16 h) at room temperature.
16. Remove excess hairpins by washing with 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min
17. Image the same as HCR-IHC protocol.

C.1.3.2 Human tissue sections

HCR-IHC protocol

1. Deparaffinize FFPE tissue by immersing slide in Histo-Clear II for 3 × 5 min.[1]
2. Rehydrate with a series of graded EtOH washes for 3 min at room temperature:
   (a) 100% EtOH
   (b) 100% EtOH
   (c) 95% EtOH
   (d) 70% EtOH
   (e) RNase-free H_2O
   (f) RNase-free H_2O
3. Dry slide by blotting edges on a Kimwipe.
4. Block sample by adding 100 μL of 1× PBDT on top of the tissue sample.
5. Prepare primary antibody solution by mixing 100 μL 1× PBDT to a proper dilution of primary antibody.[2]
6. Remove the 1× PBDT and drain excess buffer on slide by blotting edges on a Kimwipe.
7. Add 100 μL of the antibody solution on top of the tissue sample.
8. Place a coverslip on the tissue sample and incubate either at room temperature for 2 hours or 4°C overnight in a humidified chamber.
9. Immerse slide in 1× PBST at room temperature to float off coverslip.
10. Remove excess antibodies by washing slides twice in 1× PBST for 10 min.
11. Prepare secondary antibody solution by mixing 100 μL 1× PBDT with 0.5 μL secondary antibody.
12. Add 100 μL of the antibody solution on top of the tissue sample.
13. Place a coverslip on the tissue sample and incubate at room temperature for 30 min – 1 h in a humidified chamber.
14. Immerse slide in 1× PBST at room temperature to float off coverslip.

[1] Each 50 mL Falcon tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. A larger number of slides could be processed together using a Coplin jar.

[2] Antibody dilution should be optimized for each different primary antibody.

15. Remove excess antibodies by washing slides twice in 1× PBST for 10 min.
16. Block sample by adding 100 μL of Initiator Binding Buffer on top of the tissue sample.
17. Prepare initiator solution by mixing 100 μL Initiator Binding Buffer with 1 μL of 1 μM initiator.
18. Remove the Initiator Binding Buffer and drain excess buffer on slide by blotting edges on a Kimwipe.
19. Add 100 μL of the initiator solution on top of the tissue sample.
20. Place a coverslip on the tissue sample and incubate at room temperature for 30 min in a humidified chamber.
21. Immerse slide in 5× SSCT at room temperature to float off coverslip.
22. Remove excess initiators by washing slides twice in 5× SSCT for 10 min.
23. Dry slide by blotting edges on a Kimwipe.
24. Add 100 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 30 min at room temperature.
25. Prepare 12 pmol of each fluorescently labeled hairpin by snap cooling 4 μL of 3 μM stock in hairpin storage buffer (heat at 95°Cfor 90 seconds and cool to room temperature in a dark drawer for 30 min).
26. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
27. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
28. Add 100 μL of the hairpin solution on top of the tissue sample.
29. Place a coverslip on the tissue sample and incubate either 6 h or overnight (12–16 h) in a humidified chamber at room temperature.
30. Immerse slide in 5 × SSCT at room temperature to float off coverslip.
31. Remove excess hairpins by incubating slide in 5 × SSCT at room temperature for:
    (a) 2 × 30 min
    (b) 1 × 5 min

Sample mounting for microscopy

1. Dry slide by blotting edges on a Kimwipe.
2. Add 50 μL of SlowFade Gold mounting medium on top of human tissue section.
3. Place a 22 mm $\times$ 40 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss Axio Observer inverted light microscope equipped with an Plan-Apochromat 63$\times$/1.4 Oil Ph3 M27 objective was used to acquire images.

C.1.4 Buffer recipes for HCR-IHC and HCR-ISH

1× PBS	For 50 mL of solution
1× phosphate buffered saline (PBS)	5 mL of 10× PBS
	Fill up to 50 mL with ultrapure H_2O

1× PBST	For 50 mL of solution
1× phosphate buffered saline (PBS)	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

1× PBDT	For 50 mL of solution
1× phosphate buffered saline (PBS)	5 mL of 10× PBS
1% bovine serum albumin (BSA)	0.5 g of BSA
1% DMSO	500 μL of DMSO
0.1% Triton X-100	500 μL of 10% Triton X-100
	Fill up to 50 mL with ultrapure H_2O

Initiator Binding Buffer	For 50 mL of solution
5× SSC	12.5 mL of 20× SSC
1% bovine serum albumin (BSA)	0.5 g of BSA
100 μg/mL salmon sperm DNA	500 μL of 10 mg/mL salmon sperm DNA
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

5× SSCT	For 50 mL of solution
5× sodium chloride sodium citrate (SSC)	12.5 mL of 20× SSC
0.1% Tween 20	500 μL of 10% Tween 20
	fill up to 50 mL with ultrapure H_2O

Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

C.1.5 Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)

FFPE human normal breast tissue section (Pantomics Cat. # BRE01 Block # F091926B08)

Histo-Clear II (National Diagnostics Cat. # HS-202)

Methanol (Mallinckrodt Chemicals Cat. # 3016-16)

Ethanol, 200 proof (VWR Cat. # V1001G)

20 mg/mL Proteinase K (Life Technologies Cat. # AM2546)

Albumin Bovine, Fraction V (MP Biosciences Cat. #0216006925)

UltraPure salmon sperm DNA solution (Life Technologies Cat. #15632-011)

Formamide (Deionized) (Ambion Cat. # AM9342)

20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)

10× Tris-buffered saline solution (TBS) (Research Products International Cat. # T60075)

10× Phosphate-buffered saline solution (PBS) (Ambion Cat. # AM9625)

50% Tween 20 (Life Technologies Cat. # 00-3005)

10% Tween 20 Solution (Bio-Rad Cat. # 161-0781)

Dimethyl sulfoxide (DMSO) (Sigma Cat. # D2650)

Triton X-100 (Sigma Cat. # T8787)

Heparin (Sigma Cat. # H3393)

50× Denhardt's solution (Life Technologies Cat. # 750018)

Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)

tRNA from baker's yeast (Roche Cat. # 10109509001)

25 mm × 75 mm glass slide (VWR Cat. # 48300-025)

22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)

22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)

Methyl cellulose (Sigma Cat. # M0387)

SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

Vivaspin 500 Centrifugal Concentrators, 5,000 MWCO (Sartorius Cat. # VS0111)

Zeba 0.5 mL Spin Desalting Columns, 40K MWCO (ThermoFisher Scientific Cat. # 87766)

Amicon Ultra-0.5 Centrifugal Filter Unit, NMWL 100,000kDa (EMD Millipore Cat. # UFC510024)

Sulfo-4FB Crosslinker 1mg (VWR Cat. # 48366-067)

S-HyNiC Crosslinker 1mg (VWR Cat. # 95041-960)

Anhydrous DMF (VWR Cat. # 95042-018)

Invitrogen GFP Tag Antibody, Molecular Probes (Life Technologies Cat. # A10262)

Rabbit polyclonal to Desmin (Abcam Cat. # ab86083)

Mouse monoclonal [RCK108] to Cytokeratin 19 (Abcam Cat. # ab9221)

AffiniPure Donkey Anti-Rabbit IgG (Jackson Immuno Research Labs Cat. # NC9908878)

Alexa Fluor 647-AffiniPure Donkey Anti-Rabbit IgG (Jackson Immuno Research Labs Cat. # NC0254454)

AffiniPure Goat Anti-Chicken IgY (Jackson Immuno Research Labs Cat. # NC0174565)

Pierce Goat anti-Mouse IgG1 Cross Adsorbed Secondary Antibody (Life Technologies Cat. #31236)

Alexa Fluor 647 Goat Anti-Mouse IgG1 (Life Technologies Cat. #A21240)

C.2 Probe and Amplifier Sequences

C.2.1 Zebrafish probes

For the zebrafish studies of Figures 4.10 and 2.1, two target mRNAs are detected with a different probe set. Each probe set contains six 2-initiator DNA probes. Within a given probe set, each DNA probe initiates the same DNA HCR amplifier. Sequences are listed 5′ to 3′. Target sequences were obtained from the National Center for Biotechnology Information (NCBI) [112].

Target mRNA: **desmin (*des*)**

Amplifier: **DNA HCR B3**

Fluorophore: **Alexa Fluor 647**

Initiator I1	**Spacer**	**Probe Sequence**	**Spacer**	**Initiator I2**
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gTTCTTCTgCTTgTCggCCATgATATAgACgTTgTggCTgTTgTAgTTgT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCAgCTCgATgCggTTCACCAgggTgTCgCCCTCgAACTTCACCTCggC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ACgCTgCCgTCCTCgATgTTgTggCggATCTTgAAgTTCACCTTgATgCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCgggTCTTgTAgTTgCCgTCgTCCTTgAAgAAgATggTgCgCTCCTggA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgTAgCCTTCgggCATggCggACTTgAAgAAgTCgTgCTgCTTCATgTgg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCggTCACgAACTCCAgCAggACCATgTgATCgCgCTTCTCgTTggggTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

Target mRNA: **transgene(fetal liver kinase 1:green fluorescent protein) (*tg(flk1:gfp)*)**

Amplifier: **DNA HCR B2**

Fluorophore: **Alexa Fluor 546**

Initiator I1	**Spacer**	**Probe Sequence**	**Spacer**	**Initiator I2**
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TTggCTTCTCTgAgAgCCTCgTTATTCTTgTTCACTgCCTggTTCAAATC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	TCTCgCAggTgTAggACTggAgCTggTgACggAACTgCATggTCTCCTgC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	ggTTTggACATgTCCATTTggATCTgCACCTgACTCTCCTgCATCTggTT	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTTCgTgAAgACCCTCgATACgTCTTTCCAggTCCAgCCTggCCAgAgTg	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	gCAgCATCgACATCAgCTCTgAAAgCAgAAAggTTgTTTTCAgCTTCCTC	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC
CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	AAAAA	CTCACTCATTTgCCTCCTCAgAgACTCATTggTgCCCTTgAgAgAgTCAA	AAAAA	AgCTCAgTCCATCCTCgTAAATCCTCATCAATCATC

C.2.2 DNA HCR amplifier sequences

Initiator and hairpin sequences for the three DNA HCR amplifiers using for the adaptation of HCR to IHC protocols [65]. The hairpin H1 is modified with a 9-carbon spacer and 3' amine for conjugation purposes. The initiator I1 is the free initiator used to open the hairpin attached to antibody for amplification.

DNA HCR B1

I1	gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg
H1	CgTAAAggAAgACTCTTCCCgTTTgCTgCCCTCCTCgCATTCTTTCTTgAggAgggCAgCAAACgggAAgAg /C9-amine-3'/

DNA HCR B4

I1	CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC
H1	gAAgCgAATATggTgAgAgTTggAggTAggTTgAggCACATTTACAgACCTCAACCTACCTCCAACTCTCAC /C9-amine-3'/

DNA HCR B5

I1	CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT
H1	ATTggATTTgTAgggTAgATAgAgATTgggAgTgAgCACTTCATATCACTCACTCCCAATCTCTATCTACCC /C9-amine-3'/

C.3 IHC experimental details

The dilutions used for the various antibodies are as follows:

- rabbit anti-Desmin: 1:200
- chicken anti-GFP: 1:200
- mouse anti-Cytokeratin 19: 1:100
- all secondary antibodies: 1:200

The details for each figure's experiments are shown below.

Figure 4.2.a

Fish line: wild-type

Target: Desmin

Primary Antibody: rabbit anti-Desmin

Secondary Antibody: donkey anti-rabbit

Initiator: B4-I1

Fluorophore: Alexa Fluor 647

Figure 4.2.b

Fish line: wild-type

Target: Desmin

Primary Antibody: rabbit anti-Desmin

Secondary Antibody: donkey anti-rabbit

Initiator hairpin: B4-H1

Fluorophore: Alexa Fluor 647

Figure 4.4

Fish line: wild-type

Target: Desmin

Primary Antibody: rabbit anti-Desmin

Secondary Antibody: donkey anti-rabbit

Initiator hairpin: B4-H1

Fluorophore: Alexa Fluor 647

Figure 4.5

Fish line: ct122a

Target: Citrine-Desmin fusion

Primary Antibody: chicken anti-GFP

Secondary Antibody: goat anti-chicken

Initiator hairpin: B4-H1

Fluorophore: Alexa Fluor 647

Figure 4.7

FFPE Tissue: Normal Human Breast

Target: Cytokeratin 19

Primary Antibody: mouse monoclonal anti-Ck19

Secondary Antibody: goat anti-mouse

Initiator hairpin: B4-H1

Fluorophore: Alexa Fluor 647

Figure 4.8.a

Fish line: wild-type

Target: Desmin

Primary Antibody: rabbit anti-Desmin

Secondary Antibody: donkey anti-rabbit

Initiator hairpin (1): B4-H1

Fluorophore (1): Alexa Fluor 647

Initiator hairpin (2): B5-H1

Fluorophore (1): Alexa Fluor 546

Figure 4.8.b

Fish line: ct122a

Target: Citrine-Desmin fusion

Primary Antibody: chicken anti-GFP

Secondary Antibody: goat anti-chicken

Initiator hairpin (1): B4-H1

Fluorophore (1): Alexa Fluor 647

Initiator hairpin (2): B5-H1

Fluorophore (1): Alexa Fluor 546

Figure 4.10 and Figure 4.11

Fish line: tg(flk1:gfp)

Target (1): Flk1-GFP fusion

Primary Antibody: chicken anti-GFP

Secondary Antibody: goat anti-chicken

Initiator hairpin: B1-H1

Fluorophore: Alexa Fluor 514

Target (2): Desmin

Primary Antibody: rabbit anti-Desmin

Secondary Antibody: donkey anti-rabbit

Initiator hairpin: B5-H1

Fluorophore: Alexa Fluor 488