Multiplex, quantitative, high-resolution imaging of protein:protein complexes via hybridization chain reaction

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ABSTRACT: Signal amplification based on the mechanism of hybridization chain reaction (HCR) facilitates spatial exploration of gene regulatory networks by enabling multiplex, quantitative, high-resolution imaging of RNA and protein targets. Here, we extend these capabilities to the imaging of protein:protein complexes, using proximity-dependent cooperative probes to conditionally generate a single amplified signal if and only if two target proteins are colocalized within the sample. HCR probes and amplifiers combine to provide automatic background suppression throughout the protocol, ensuring that even if reagents bind nonspecifically in the sample, they will not generate amplified background. We demonstrate protein:protein imaging with high signal-to-background in human cell lines, mouse proT cells, and highly autofluorescent formalin-fixed paraffin-embedded (FFPE) human breast tissue sections. Further, we demonstrate multiplex imaging of 3 different protein:protein complexes simultaneously and validate that HCR enables accurate and precise relative quantitation of protein:protein complexes with subcellular resolution in an anatomical context. Moreover, we establish a unified framework for simultaneous multiplex, quantitative, high-resolution imaging of RNA, protein, and protein:protein targets, with 1-step, isothermal, enzyme-free HCR signal amplification performed for all target classes simultaneously.

KEYWORDS: Protein:protein complex imaging, hybridization chain reaction (HCR), quantitative HCR (qHCR) imaging, mouse proT cells, formalin-fixed paraffin-embedded (FFPE) human breast tissue sections

INTRODUCTION

Methods for imaging molecular complexes have been comparatively less explored than methods for imaging RNA and protein targets, yet represent an important frontier for spatial exploration of the interactome. Generating one signal conditional on the proximity of two molecules provides a sub-diffraction-limit readout, in contrast to independent imaging of the same two molecules with two signals. Protein:protein complexes play central roles in diverse cellular processes including transcription, translation, signaling, development, and disease. To date, imaging of protein:protein complexes has predominantly been performed using proximity ligation assays (PLA) that exploit enzyme-mediated ligation and rolling circle amplification, leading to challenges with both false-negatives (formation of non-circular ligation products) and false-positives (background evident in technical controls that omit one reaction component), as well as issues with cost and variable enzyme activity. Alternatively, to avoid the use of enzymes, a proximity-based HCR approach has been developed that uses a kinetic trigger mechanism to desequester an HCR initiator if two probes are bound to proximal target proteins; this approach has so far been limited to 1-plex applications.

Over the course of nearly two decades, we have developed simple and robust HCR RNA in situ hybridization (RNA-ISH) and immunohistochemistry (IHC) methods that enable biologists, drug developers, and pathologists to perform multiplex, quantitative, high-resolution imaging of RNA and protein targets in highly autofluorescent samples. Here, we sought to use HCR principles to extend these benefits to the imaging of protein:protein complexes. An HCR amplifier consists of two species of kinetically trapped DNA hairpins (h1 and h2) that co-exist metastably in solution, storing the energy to drive conditional self-assembly of an HCR amplification polymer upon exposure to a cognate initiator sequence (i1; Figure 1A). Using HCR RNA-ISH, an RNA target is detected using one or more pairs of split-initiator DNA probes, each carrying a fraction of HCR initiator i1 (Figure 1B). Probe pairs that hybridize specifically to proximal binding sites on the target RNA colocalse a full HCR initiator i1 capable of triggering HCR signal amplification. Meanwhile, any individual probes that bind nonspecifically in the sample do not colocalse full HCR initiator i1 and do not trigger HCR. Using HCR IHC, a protein target is detected using an unlabeled primary antibody probe, which in turn is detected by an initiator-labeled secondary antibody probe that carries an HCR initiator i1 capable of triggering HCR signal amplification (Figure 1C).

We hypothesized that the split-initiator concept from HCR RNA-ISH (Figure 1B) could be generalized using the antibody probes of HCR IHC (Figure 1C) to enable simple and robust HCR imaging of protein:protein complexes using a split-initiator antibody probe pair in conjunction with a new proximity probe (Figure 1D). Here, we demonstrate that this combination of proximity-dependent cooperative probes and metastable HCR amplifiers enables multiplex, quantitative, high-resolution imaging of protein:protein complexes, including full compatibility with HCR RNA-ISH and HCR IHC.
**Figure 1.** Applying HCR principles to enable simple and robust imaging of protein:protein complexes. (A) HCR mechanism. Stars denote fluorophores. Arrowhead indicates 3’ end of each strand. (B) HCR RNA-ISH: an RNA target is detected using a pair of split-initiator DNA probes, each carrying a fraction of HCR initiator i1. (C) HCR IHC: a protein target is detected using an unlabeled primary antibody probe and an initiator-labeled secondary antibody probe carrying HCR initiator i1. (D) HCR protein:protein imaging: a protein:protein target complex is detected with a pair of unlabeled primary antibodies, a pair of split-initiator secondary antibodies each carrying a fraction of HCR initiator i1, and a proximity probe.

**Figure 2.** Imaging protein:protein complexes using HCR. (A) Three-stage protocol. Detection stage: unlabeled primary antibody probes bind to protein targets 1 and 2; wash; split-initiator secondary antibody probes p1 and p2 bind to primary antibody probes; wash. Proximity stage: if p1 and p2 are proximal, a proximity probe hybridizes to the proximity domains of p1 and p2 to colocalize full HCR initiator i1. Amplification stage: colocalized full HCR initiator i1 triggers self-assembly of fluorophore-labeled HCR hairpins into a tethered fluorescent amplification polymer; wash. (B) Multiplexing timeline. The same three-stage protocol is used independent of the number of protein:protein target complexes.

p2) each carrying a fraction of HCR initiator i1 and a proximity domain. In the proximity stage, if the two protein targets are colocalized in the sample, the proximity probe is able to hybridize to p1 and p2 to colocalize a full HCR initiator i1 capable of triggering HCR signal amplification. Note that the proximity probe creates a cooperative probe junction (Figure 1D) inspired by the cooperative probe junction created in HCR RNA-ISH (Figure 1B), with the DNA proximity probe taking the place of the RNA target. Any split-initiator probes that bind nonspecifically or to isolated protein targets in the sample can hybridize to the proximity probe, but will not colocalize a full HCR initiator i1 and will not trigger HCR. In the amplification stage, each colocalized full HCR initiator i1 triggers self-assembly of metastable fluorophore-
labeled HCR hairpins (h1 and h2) into a tethered fluorescent amplification polymer to generate an amplified signal at the site of the protein:protein target complex.

**Imaging protein:protein complexes in human cells, mouse proT cells, and FFPE human breast tissue sections.** To evaluate the performance of our split-initiator approach for imaging protein:protein complexes, we compared the fluorescence intensity between three pairs of biological sample types using the same imaging settings for both sample types. Positive samples are expected to form the protein:protein complex of interest; negative samples are expected to have minimal or no formation of the protein:protein complex of interest. For each pair of sample types, we calculate an estimated signal-to-background ratio, using the positive sample type to estimate signal plus background and the negative sample type to estimate background. This approach yields a conservative estimate of performance, as characterizing background in a sample containing little or no protein:protein target complex places an upper bound on background and hence a lower bound on signal-to-background.

First, we compared the fluorescence intensity for the β-catenin:E-cadherin complex in A-431 and HeLa adherent human cell lines. While A-431 cells form the β-catenin:E-cadherin complex at the cell membrane of intercellular junctions, HeLa cells express N-cadherin rather than E-cadherin and therefore lack the β-catenin:E-cadherin complex. As expected, A-431 cells (Figure 3A) display strong signal at intercellular junctions and HeLa cells display no visible staining (Figure 3B), with a signal-to-background ratio of 26 ± 4 between the two cell lines (mean ± SEM for representative regions of N = 3 replicate samples). See Sections S2.2–S2.4 for additional data.

Next, we imaged Scid.adh.2C2 mouse proT cells in search of the RUNX1:PU.1 target complex. The Scid.adh.2C2 cell line has emerged as a useful proT cell line for studying T cell development, with exogenous introduction of PU.1 protein capable of reverting the cell line to an earlier developmental time point, in part via direct or indirect interactions
between PU.1 and other proteins such as RUNX1.32–34 Because the Scid.adh.2C2 cell line does not endogenously express the PU.1 protein,35 Scid.adh.2C2 cells cannot natively form the RUNX1:PU.1 complex. When the Scid.adh.2C2 cell line is retrovirally transduced with PU.1, it is unknown whether PU.1 forms a complex with RUNX1 or interacts less directly.33 Here, imaging the RUNX1:PU.1 target complex, we observe signal in cells retrovirally transduced with a PU.1-containing vector (Figure 3C) and no visible staining for cells retrovirally transduced with an empty vector (Figure 3D), with a signal-to-background ratio of 15 ± 3 between the two experiment types (mean ± SEM for representative regions of N = 3 replicate wells on a slide). These results provide evidence that RUNX1 and PU.1 are spatially colocalized in Scid.adh.2C2 cells and not merely logically linked.

To test performance in highly autofluorescent samples, we detected the β-catenin:E-cadherin complex in normal and pathological FFPE human breast tissue sections. The β-catenin:E-cadherin complex is robustly formed in normal breast epithelial cells, but the expression of and interaction between the β-catenin and E-cadherin proteins is interrupted when breast epithelial cells become cancerous in the invasive lobular carcinoma disease process.35,36 We obtained paired normal and invasive lobular carcinoma FFPE breast tissue sections from the same patient and evaluated them for the β-catenin:E-cadherin complex, observing strong signal in normal breast tissue (Figure 3E) and no visible staining in cancerous tissue (Figure 3F), with a signal-to-background ratio of 30 ± 3 between the two tissue types (mean ± SEM for representative regions of N = 3 replicate sections).

In summary, protein:protein complexes are imaged with high signal-to-background across three different paired sample types, including highly autofluorescent FFPE tissues.

**Multiplex protein:protein imaging.** HCR RNA-ISH and HCR IHC enable straightforward multiplexing for RNA and protein targets to allow multidimensional analyses of gene expression in an anatomical context.20–22,24–26 To likewise enable multiplex imaging of protein:protein complexes, we used NUPACK37,38 to design proximity probes for three orthogonal HCR amplifiers. Figure 4 demonstrates multiplex protein:protein imaging for three target complexes that localize to different compartments of A-431 adherent human cells: cytoskeletal α-tubulin:β-tubulin complex, membranous β-catenin:E-cadherin complex, and nuclear speckle SC35:SON complex. High signal-to-background is observed for all three protein:protein target complexes, with background estimated based on technical control experiments that omit the primary and secondary antibody probes for one protein or the other within a given complex (see Table S1 for details). Multiplexing is straightforward using a three-stage protocol independent of the number of protein:protein target complexes (Figure 2B): all protein targets are detected in parallel, proximity is verified for all protein target pairs in parallel, and amplification is performed for all colocализed full HCR initiators in parallel.

**qHCR imaging: relative quantitation of protein:protein complexes with subcellular resolution.** We have previously demonstrated that HCR imaging enables accurate and precise relative quantitation of both RNA and protein targets with subcellular resolution in an anatomical context, generating an amplified signal that scales approximately linearly with the number of target molecules per imaging voxel.24–26 Here, we validate that the proximity probe and split-initiator antibody probe pair preserve the quantitative nature of HCR imaging for protein:protein target complexes. To test relative quantitation, we detect each protein in the complex with an unlabeled primary antibody probe as usual, and then redundantly detect each primary antibody probe with two batches of split-initiator secondary antibody probes, where each batch interacts with a different proximity probe and triggers a different spectrally distinct HCR amplifier (Figure 5A), yielding a two-channel image (Figure 5B). If HCR signal scales approximately linearly with the number of target protein:protein complexes per voxel, a two-channel scatter plot of normalized voxel intensities will yield a tight linear distribution with zero intercept.25 Consistent with expectation, we observe high accuracy (linearity with zero intercept) and precision (scatter around the line) for subcellular voxels in both cultured human cells (Figure 5C; top) and highly autofluorescent FFPE human breast tissue (Figure 5C; bottom).

**Simultaneous multiplex imaging of protein, protein:protein, and RNA targets.** We have previously shown that HCR RNA-ISH and HCR IHC enable multiplex, quantitative, high-resolution RNA and protein imaging in highly autofluorescent samples.26 Here, we demonstrate compatible multiplex imaging of protein, protein:protein, and RNA targets using initiator-labeled antibody probes for protein targets, proximity probes and split-initiator antibody probe pairs for protein:protein targets, and split-initiator DNA probe pairs for RNA targets, with simultaneous HCR signal amplification for all target classes (Figure 6A). In A-431 adherent human cells, mitochondrial HSP60 protein targets, cytoskeletal α-tubulin:β-tubulin protein:protein target complexes, and nuclear U6 RNA targets are all imaged simultaneously (Figure 6B) with high signal-to-background (see Table S1 for additional details).

**Unified framework for multiplex, quantitative, high-resolution imaging.** We have shown that HCR imaging provides a unified framework for multiplex, quantitative, high-resolution imaging of RNA targets, protein targets, and pro-
Figure 5. qHCR imaging: relative quantitation of protein:protein complexes with subcellular resolution in an anatomical context. (A) Two-channel redundant detection of a protein:protein complex: each target protein is detected by an unlabeled primary antibody probe and two batches of secondary antibody probes that interact with orthogonal proximity probes to colocalize full HCR initiators that trigger orthogonal spectrally distinct HCR amplifiers (Ch1: Alexa546; Ch2: Alexa647). (B) Two-channel confocal images. Top: β-catenin:E-cadherin complex in A-431 cells (0.18x0.18x0.8 µm pixels). Bottom: β-catenin:E-cadherin complex in a 5 µm FFPE normal human breast tissue section (0.57x0.57x3.3 µm pixels). (C) High accuracy and precision for protein:protein relative quantitation in an anatomical context. Highly correlated normalized signal (Pearson correlation coefficient, r) for subcellular voxels in the indicated regions in panel B. Top: 2.0x2.0x0.8 µm voxels. Bottom: 2.0x2.0x3.3 µm voxels. Accuracy: linearity with zero intercept. Precision: scatter around the line. See Section S2.6 for additional data.

**METHODS**

**Probes, amplifiers, and buffers.** Probes, amplifiers, and buffers were obtained from Molecular Technologies, a non-profit academic resource within the Beckman Institute at Caltech. Details on the probes, amplifiers, and buffers for each experiment are displayed in Table S1 for HCR imaging of protein:protein complexes. In Table S2 for HCR RNA-ISH, and in Table S3 for HCR IHC.

**HCR imaging of protein:protein complexes.** HCR imaging of protein:protein complexes, with optional co-detection of protein and RNA targets, was performed in adherent
human cell lines (A-431 or HeLa) using the protocol detailed in Section S1.8. A-431 cells (ATCC, CRL-1555) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose and pyruvate (Gibco, 11995-073) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, F4135). HeLa cells (ATCC, CRM-CCL-2) were cultured in Eagle’s Minimum Essential Medium (EMEM) (ATCC, 30-2003) supplemented with 10% FBS (Sigma-Aldrich, F4135). HCR imaging of protein:protein complexes was performed in Scid.adh.2C2 mouse proT cells13 cultured in RPMI1640 medium (Gibco, 31800022) supplemented with 10% FBS (Sigma-Aldrich, F2042), 1× Penicillin-Streptomycin-Glutamine (Gibco, 10378-016), 0.1 mM sodium pyruvate (Gibco, 11360-070), 1× MEM non-essential amino acids (Gibco, 11140-050), and 50 μM β-mercaptoethanol (Gibco, 21905-023) using the protocol detailed in Section S1.9. HCR imaging of protein:protein complexes was performed in 5 μm FFPE normal human breast tissue sections (Acepix Biosciences, HuN-06-0027) and 5 μm FFPE invasive lobular carcinoma human breast tissue sections (Acepix Biosciences, HuC-06-0101) from the same patient using the protocol detailed in Section S1.10.

**Microscopy.** Confocal microscopy was performed using a Leica Stellaris 8 inverted confocal microscope. All images are displayed without background subtraction. Each channel (except for DAPI) is displayed with 0.01% of pixels saturated across three replicates. Details on the objectives, excitation wavelengths, detectors, and detection wavelengths used for each experiment are displayed in Table S5.

**Image analysis.** Image analysis was performed as detailed in Section S1.7, including: definition of raw pixel intensities; measurement of signal, background, and signal-to-background; and calculation of normalized subcellular voxel intensities for qHCR imaging.

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**ASSOCIATED CONTENT**

Supporting Information
Materials, additional methods, and replicate data.

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**Notes**
The authors declare competing financial interests in the form of patents, pending patent applications, and the startup company Molecular Instruments.
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ABBREVIATIONS USED

FFPE, formalin-fixed paraffin-embedded; HCR, hybridization chain reaction; IHC, immunohistochemistry; ISH, in situ hybridization; PLA, proximity ligation assay; qHCR, quantitative hybridization chain reaction.

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