10-Plex HCR™ Gold Spectral Imaging User Guide

This User Guide enables 10-Plex HCR™ Gold spectral imaging for robust imaging of 10 RNA and/or protein targets with 1-step quantitative HCR™ Gold signal amplification for all 10 targets simultaneously. 10-plex HCR™ Gold spectral imaging has the following key properties:

- No repeated imaging
  - Suitable even for whole-mounts and delicate specimens that are not amenable to repeated imaging
  - No repeated staining, imaging, stripping, or registration
- Standardized ingredients for robust performance
  - HCR™ Gold Amplifiers: orthogonal 10-set
  - Labels: optimized 10-set
  - Excitation wavelengths: optimized 10-set
  - Detection wavelengths: optimized 10-set
- Quantitative in all 10 channels
  - All 10 channels enable analog RNA or protein relative quantitation with subcellular resolution
  - All 10 channels enable digital RNA absolute quantitation with single-molecule resolution

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10-Plex HCR™ Gold Spectral Imaging Workflow

Below is a general overview of the steps involved in 10-Plex HCR™ Gold spectral imaging. See Supplementary Section S2.1 of Schulte et al., 2024 for a detailed protocol.

HCR™ Gold RNA-FISH/IF

Follow the protocols in the appropriate HCR™ Gold User Guides:

- HCR™ Gold RNA-FISH User Guide
- HCR™ Gold IF User Guide
- HCR™ Gold RNA-FISH/IF User Guide

to perform HCR™ Gold RNA-FISH and/or IF including:

- Sample preparation
- Antibody binding (for protein targets)
- Probe hybridization (for RNA targets)
- Amplification

on 12 sample types:

- 10-plex sample or multiple replicate samples (1 label for each of 10 targets): use all 10 HCR™ Gold Probes and Amplifiers.
- 1-plex reference sample for each of 10 targets: use the corresponding HCR™ Gold Probe and Amplifier for a given target.
- Two unlabeled autofluorescence (AF) samples: omit all HCR™ Gold Probes and Amplifiers.

NOTE: *When assigning targets to Ch1-Ch10, we recommend assigning higher-expression targets to channels that employ labels with emissions spectra strongly overlapping sample autofluorescence (e.g., Ch1-Ch3 for whole-mount zebrafish embryos and fresh-frozen mouse brain sections) or with emissions spectra for which the microscope detectors are less sensitive (e.g., Ch10 in the near-IR wavelength range).*

AF Scan

Use one unlabeled sample to perform an excitation-emission scan to determine the maximal AF excitation wavelength (λ_{AF}), which in turn determines the optimized detection wavelengths for AF (see Table 1).

NOTE: *approximately 30–45 sec of microscope time*

Spectral Imaging

Spectrally image 12 sample types using 11 excitation wavelengths in succession (one optimized for each label and one optimized for AF; see Table 1):

- 10-plex sample.
- 1-plex samples (obtain reference sample for each label).
- Unlabeled sample (obtain reference spectrum for AF).

NOTE: *approximately 30–45 sec of microscope time per optical section*

Linear Unmixing

Use the 11 reference spectra (one per label and one for AF) to linearly unmix the 10-plex image to obtain 11 unmixed channels (one per label and one for AF).

NOTE: *approximately 5-10 sec of computational time*

Table 1. Excitation and detection wavelengths for 10-plex HCR™ Gold spectral imaging.

Channel	Excitation wavelength (nm)	Detector wavelengths (nm)	Label
1	405	410–450	405
2	440	450–520	425
3	488	493–533	488
4	518	523–583	514
5	557	566–620	546
6	590	600–660	594
7	629	640–680	633
8	686	696–723	700
9	755	765–795	750
10	790	815–850	800
AF	λ_{AF}	$(\lambda_{AF} + 6)$–$(\lambda_{AF} + 171)$	–

Frequently Asked Questions (FAQ)

What comes in an HCR™ Gold RNA-FISH kit?

- HCR™ HiFi Probe
- HCR™ HiFi Probe Hybridization Buffer
- HCR™ HiFi Probe Wash Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

What comes in an HCR™ Gold IF kit?

- HCR™ 2° Antibody Probe
- HCR™ Antibody Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

Can I order a subset of these components?

- Yes. You can order kits containing any subset of the above components.

What HCR™ Gold Amplifiers are available for multiplexing?

- For multiplex experiments, choose a different HCR™ Gold Amplifier (X1, X2, . . . , X10) and label (405, 425, 488, 514, 546, 594, 633, 647, 700, 750, 800) for each target that will be imaged in the same sample (for example, amplifier X3-647 for target 1, amplifier X2-546 for target 2, . . .).
- We recommend labels (488, 546, 647, 750) for robust 4-plex bandpass imaging.
- We recommend labels (405, 425, 488, 514, 546, 594, 633, 700, 750, 800) for robust 10-plex spectral imaging.
- For flexibility, amplifiers X1, X2, and X3 are available with multiple label options (488, 546, 647), while for expanded multiplexing, amplifiers X4–X10 are each available with one label option.
- We recommend using a longer-wavelength label (e.g., 647 or 750) for targets that are more difficult to detect (e.g., low-expression target and/or short target sequence), as autofluorescence tends to be higher in shorter-wavelength channels (e.g., 405 or 488).

What do I order for a multiplex HCR™ Gold RNA-FISH/IF experiment?

- Order one HCR™ Gold RNA-FISH kit for each target RNA
- Order one HCR™ Gold IF kit for each target protein

- Example 4-Plex Experiment (2 RNAs, 2 proteins):
  - HCR™ Gold RNA-FISH kit for target RNA1:
    - HCR™ HiFi Probe: target RNA1 for use with amplifier X3
    - HCR™ HiFi Probe Hybridization Buffer (for use with all RNA-FISH kits)
    - HCR™ HiFi Probe Wash Buffer (for use with all RNA-FISH kits)
    - HCR™ Gold Amplifier: X3 with label 647
    - HCR™ Gold Amplifier Buffer (for use with all RNA-FISH and IF kits)
    - HCR™ Gold Amplifier Wash Buffer (for use with all RNA-FISH and IF kits)
  - HCR™ Gold RNA-FISH kit for target RNA2:
    - HCR™ HiFi Probe: target RNA2 for use with amplifier X4
    - HCR™ Gold Amplifier: X4 with label 750
  - HCR™ Gold IF kit for target Protein1:
    - 1° antibody: Rabbit Anti-Protein1 (your own 1° antibody)
    - HCR™ 2° Antibody Probe: Donkey Anti-Rabbit for use with amplifier X1
    - HCR™ Antibody Buffer (for use with all IF kits)
    - HCR™ Gold Amplifier: X1 with label 488
  - HCR™ Gold IF kit for target Protein2:
    - 1° antibody: Mouse Anti-Protein2 (your own 1° antibody)
    - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X2
    - HCR™ Gold Amplifier: X2 with label 546

Are HCR™ Gold Amplifiers interchangeable between HCR™ Gold RNA-FISH kits and HCR™ Gold IF kits?

- Yes! The HCR™ Gold Amplifiers (488, 546, 647) are interchangeable between HCR™ Gold RNA-FISH and HCR™ Gold IF kits. Likewise, HCR™ Gold Amplifier Buffer and HCR™ Gold Amplifier Wash Buffer are interchangeable between HCR™ Gold RNA-FISH and HCR™ Gold IF kits.

HCR™ Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **10-Plex HCR™ Spectral Imaging**
  HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).
- **HCR™ RNA-FISH/IF**
  HCR™ RNA-FISH/IF enables a unified approach to multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf et al., 2021).
- **HCR™ IF**
  HCR™ IF enables multiplex, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).
- **HCR™ RNA-FISH**
  - Third-generation HCR™ RNA-FISH (v3.0) enables multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018).
  - Second-generation HCR™ RNA-FISH (v2.0) using DNA HCR™ Probes and DNA HCR™ Amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi et al., 2014).
  - First-generation HCR™ RNA-FISH (v1.0) using RNA HCR™ Probes and RNA HCR™ Amplifiers: multiplex mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).
- **Subcellular Quantitative RNA and Protein Imaging**
  HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).
- **Single-Molecule Quantitative RNA Imaging**
  HCR™ RNA-FISH enables digital RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016, Choi et al., 2018).
- **Read-Out/Read-In Analysis Framework**
  The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).
- **Protocols in Diverse Sample Types**
  Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper (Choi et al., 2016):

  - bacteria in suspension
  - FFPE human tissue sections
  - generic sample in solution
  - generic sample on a slide
  - mammalian cells on a slide
  - mammalian cells in suspension
  - whole-mount chicken embryos
  - whole-mount fruit fly embryos
  - whole-mount mouse embryos
  - whole-mount nematode larvae
  - whole-mount sea urchin embryos
  - whole-mount zebrafish embryos and larvae

- **HCR™ RNA Flow Cytometry**
  HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines (Choi et al., 2018).

- **HCR™ Northern Blots**
  HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol (Schwarzkopf & Pierce, 2016).

- **HCR™ Amplifiers**
  HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).