10-plex HCR spectral imaging and linear unmixing

Spectral imaging and linear unmixing workflow overview

1. **HCR RNA-FISH/IF (see protocols at Molecular Technologies).** Prepare 12 sample types (HCR RNA-FISH and/or HCR IF for a total of 10 RNA and/or protein targets):
   (a) 10-plex sample or multiple replicate samples (1 fluorophore for each of 10 targets): use all 10 HCR probe sets and amplifiers.
   (b) 1-plex reference sample for each of 10 targets: use the corresponding HCR probe set and amplifier for a given target.
   (c) Two unlabeled autofluorescence (AF) samples: omit all HCR probe sets and amplifiers.
2. **AF scan (see Section 1).** Use one unlabeled sample to perform an excitation-emission scan to determine the maximal AF excitation wavelength, which in turn determines a set of optimized detection wavelengths using 4 detectors.
3. **Spectral imaging (see Section 2).** Spectrally image 12 sample types using 11 excitation wavelengths (one optimized for each fluoropohore and one optimized for AF):
   (a) 10-plex sample.
   (b) 1-plex samples (obtain reference sample for each fluorophore).
   (c) Unlabeled sample (obtain reference spectrum for AF).
4. **Linear unmixing (see Section 3).** Use the 11 reference spectra (one per fluorophore and one for AF) to linearly unmix the 10-plex image to obtain 11 unmixed channels (one per fluorophore and one for AF).

Technical support: support@moleculartechnologies.org

1. Protocol for performing autofluorescence excitation-emission scan using Leica Stellaris 8

1. Open the LAS X software (this protocol was developed using version 4.5).
2. At the top of the window, click "Configuration" > "Hardware" > change "Bit Depth" to 16.
3. Click "Acquire" at the top of the window to return to the image acquisition screen.
4. Place one of the autofluorescence samples on the microscope to conduct an excitation-emission scan (also known as a $\Lambda\lambda$ scan) to determine the optimal autofluorescence excitation wavelength.
5. In the left panel, under "Acquisition Mode", change "xyz" to "xy$\Lambda\lambda$".
6. In the left panel, in the "$\Lambda\lambda$: Excitation Emission Scan Settings" sub-panel, click the plus sign in the upper lefthand corner.
7. In the pop-up menu, click "Reset Values to Default".
8. In the pop-up menu, in the following order, use the mouse scroll wheel to set "Excitation Steps" to 18 (this will automatically also set "Excitation Stepsize" to 20 nm), "Detection Steps" to 14, "Detection Bandwidth" to 20 nm, and "Detection Stepsize" to 24 nm. Close the pop-up menu.
9. Under the eyepiece, navigate to the region of the autofluorescence sample with the most intense autofluorescence.
10. Enter "Live" imaging mode, and adjust the laser intensity and/or detector gain so that the highest pixel intensity is approximately 25% of the maximum possible pixel intensity. The laser line and HyD S 1 detector may need to be moved to different wavelengths to see the autofluorescence.
    NOTE: *Keeping the pixel intensities low ensures pixel saturation will not occur during the $\Lambda\lambda$ scan, as pixel saturation would obscure the spectral information.*
11. Click "Start" to begin the excitation-emission scan.
12. When the scan is finished, click on the "LambdaLambda 001" file under "Open projects" in the left panel.
13. Click "Process" at the top of the window.
14. Click "Excitation / Emission Contour Plot" in the left panel.
15. In the right panel, drag the "t" slider so that the sample is visible. The "Λ" slider and pixel intensity slider may also need to be adjusted to make the sample visible.
16. In the right panel, click the Rectangle, Oval, or Polygon button at the top of the screen, and draw a region in the image around the brightest autofluorescence.
17. In the middle panel, click "Apply" at the bottom of the screen. This will display a contour plot.
18. Reposition the crosshairs to the maximum of the contour plot, and make note of the Excitation wavelength displayed at the bottom right of the plot. This wavelength, henceforth denoted as λ_{AF}, will serve as the optimal autofluorescence excitation wavelength.
    NOTE: *Going forward, if the sample preparation protocol remains the same, the optimal autofluorescence excitation wavelength (λ_{AF}) determined here can be used for future batches of experiments with this sample type, and this excitation-emission scan does not need to be repeated for each batch.*

2. Protocol for spectral imaging using Leica Stellaris 8

1. Open the LAS X software (this protocol was developed using version 4.5).
2. At the top of the window, click "Configuration" > "Hardware" > and make sure "Bit Depth" is set to 16.
3. Click "Acquire" at the top of the window to return to the image acquisition screen.
4. Click "Acquisition" in the left panel, and make sure "Acquisition Mode" is set to "xyz".
5. In the middle panel of the software, create 11 Settings. Settings 1-10 are used to image the target fluorophores, while Setting 11 is used to image autofluorescence.
6. Configure Settings 1-10 as follows:

Setting	Laser line (nm)	Detector(s)	Detector wavelengths (nm)	Corresponding fluorophore
1	405	HyD S 1	410–450	Alexa405
2	440	HyD S 1, S 2, S 3	450–475, 475–495, 495–520	Atto425
3	488	HyD S 1, S 2	493–513, 513–533	Alexa488
4	518	HyD S 1, S 2, S 3	523–543, 543–563, 563–583	Alexa514
5	557	HyD S 1, S 2, S 3	566–580, 580–600, 600–620	Alexa546
6	590	HyD S 1, S 2, S 3	600–620, 620–640, 640–660	Alexa594
7	629	HyD S 2, S 3	640–660, 660–680	Atto633
8	686	HyD S 2	696–723	Alexa700
9	755	HyD S 2, S 3	765–780, 780–795	Alexa750
10	790	HyD S 3, R 5	815–830, 835–850	iFluor800

Table S1. Settings 1-10 configurations for spectral imaging.

7. For Setting 11 (autofluorescence), set the laser line to the optimal autofluorescence excitation wavelength (λ_{AF}) determined via the AF excitation-emission scan above.
8. For Setting 11, activate the HyD S 1, S 2, S 3, and X 4 detectors. The detector wavelengths will be determined by λ_{AF}. Configure the detectors for Setting 11 as follows:

Detector	Lower wavelength (nm)	Upper wavelength (nm)
HyD S 1	λ_{AF} + 6	λ_{AF} + 41
HyD S 2	λ_{AF} + 41	λ_{AF} + 76
HyD S 3	λ_{AF} + 76	λ_{AF} + 111
HyD X 4	λ_{AF} + 111	λ_{AF} + 171

Table S2. Setting 11 configuration for spectral imaging. λ_{AF}: optimal autofluorescence excitation wavelength.

For example, if the optimal autofluorescence excitation wavelength (λ_{AF}) was measured to be 459 nm, the Setting 11 detectors would be configured as follows:

- HyD S 1: 465–500 nm
- HyD S 2: 500–535 nm
- HyD S 3: 535–570 nm
- HyD X 4: 570–630 nm

9. Place the 10-plex sample on the microscope.
10. For each of the 11 Settings:

(a) Navigate to the position in the sample that has the maximum intensity for the fluorophore corresponding to that Setting.

(b) For the linear unmixing to perform properly, it is important that no pixels are saturated. Therefore, while using the "Live" mode, set the laser intensity and detector gain(s) for that Setting so that the maximum pixel intensity for each detector is no more than 50% of the maximum possible value. Do not change the laser wavelength or detector wavelengths; the Format, Speed, Zoom, Averaging, and Accumulation settings may be adjusted as needed.
NOTE: *Each detector collects emissions spectra over a range of wavelengths. For some Settings, multiple detectors are utilized to collect a broader range of emissions spectra for a given fluorophore.*

(c) Click "Capture Image", and double-check that the captured image reaches no more than 50% of saturation for all pixels in all detectors. The captured image may then be deleted.

11. As a final check, to ensure that no pixels will become saturated, while in "Live" mode, traverse the entire sample and verify that no pixel intensities exceed 50% of saturation in any of the detectors. Because it is possible that the laser for one Setting can cross-excite a fluorophore corresponding to a neighboring Setting, be sure to check that the chosen laser intensities and detector gains do not result in pixel intensities above 50% of saturation in the detectors of neighboring Settings. Decrease the laser intensities and/or detector gains if any pixel intensities are too high.

12. Now that the laser intensity and detector gain settings are determined for all 11 Settings, do not change the laser intensity or detector gain settings again.

13. Collect a Z-stack for the 10-plex sample by setting the "Begin" and "End" locations for a Z-stack and clicking "Start".

14. One by one, place each of the 10 reference spectrum samples on the microscope. Find the area of the sample with the brightest fluorescence, and collect a single Z-section at that location. Rename each image file to indicate the target name and fluorophore number.
NOTE: *All 11 Settings should still be active when collecting the reference spectrum sample images.*

15. Place the other autofluorescence sample on the microscope. Find the area of the sample with the brightest autofluorescence, and collect a single Z-section at that location. Rename the image file to "autofluorescence".
NOTE: *All 11 Settings should still be active when collecting the autofluorescence sample image.*

3. Protocol for linear unmixing using the LAS X software

1. Click “Process” along the top of the window.
2. Within the “ProcessTools” menu in the left panel, click “Channel Dye Separation” (under “Dye Separation”).
3. Click “Open Projects” at the top of the left panel.
4. One by one, for each reference spectrum file and the autofluorescence image file:
   (a) Click on the image file in the left panel.
   (b) In the right panel, look at the image(s) corresponding to the Setting for that sample, and reposition and resize the circular region selector so that it covers the brightest region of the target (or the brightest region of the autofluorescence for the autofluorescence sample). Avoid including pixels that are outside the brightest region to prevent corruption of the fluorophore spectrum.
   (c) Click “Add” near the bottom of the middle panel. This records a reference spectrum for the fluorophore.
5. Click “Save Matrix” in the middle panel, give the matrix a descriptive name, and click “Save”.
6. Click on the 10-plex image file in the left panel.
7. Click “ProcessTools” at the top of the left panel.
8. Click “Automatic Dye Separation” in the left panel.
9. Within the middle panel, under “Method”, click the “Manual” circle, which allows the matrix to be loaded.
10. Enter “11” for “Fluorescent Dyes” in the middle panel.
    NOTE: *Rescale should be left as “All Channels”.*
11. Click on the dividing line between the right and middle panels of the software and drag it all the way to the right (thereby making the panel with the 26 channels of images as small as possible). This reveals a button at the bottom of the middle panel labeled “Load”. Click the “Load” button.
12. Navigate to the saved matrix file, click on the matrix file, and click “Open”.
13. Click the “Apply” button located to the right of the “Load” button.
14. Click the “Apply” button at the bottom of the screen to unmix the 10-plex image.
15. To view the unmixed image, click “Acquire” at the top of the window. In the left panel, the 11-channel unmixed image (one channel per target plus one channel for autofluorescence) will have appeared with “DyeSep” added near the end of the file name.
16. Save the project.

Citation Notes

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- **10-plex HCR spectral imaging**
  HCR spectral imaging of any combination of 10 RNA and/or protein targets with 1-step quantitative HCR signal amplification for all targets simultaneously (Schulte *et al.*, 2024a).
  Software: HCR Imaging Python module including Dot Detection 2.0 and Unmix 1.0 packages.

- **HCR protein:protein complex imaging**
  Multiplexed, quantitative, high-resolution imaging of protein:protein complexes with/without HCR IF and/or HCR RNA-FISH with simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets (Schulte *et al.*, 2024b).

- **HCR RNA-FISH/IF**
  A unified approach to multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free HCR signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf *et al.*, 2021).

- **HCR IF**
  Multiplexed, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf *et al.*, 2021).

- **HCR RNA-FISH (v3.0)**
  Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes:

  qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context;

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput single-cell analysis;

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in an anatomical context.

  Protocols for v3.0 in diverse organisms are adapted from the zoo paper.
  Software: Dot Analysis 1.0 package.

- **Quantitative HCR (qHCR) imaging with subcellular resolution**
  Analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos. The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi *et al.*, 2018).
  Software: Read-out/Read-in 1.0 package.

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections

- **Digitial HCR (dHCR) imaging with single-molecule resolution**
  Digital mRNA absolute quantitation with single-molecule resolution in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**
  Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  2nd generation HCR RNA-FISH technology (v2.0) using DNA probes and DNA HCR amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi *et al.*, 2014).

- **HCR RNA-FISH (v1.0)**
  1st generation HCR RNA-FISH technology (v1.0) using RNA probes and RNA HCR amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi *et al.*, 2010).

- **HCR mechanism**
  The hybridization chain reaction (HCR) mechanism enables isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

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