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Tips and tricks

Note: this is just an informative document with general recommendations.
Please contact support@olink.com should you have any queries.

Document last reviewed 2011-11-17

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Outline

1. Experiment
2. Sample analysis and storage
3. Image analysis and presenting data
4. Probemaker

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1. EXPERIMENT

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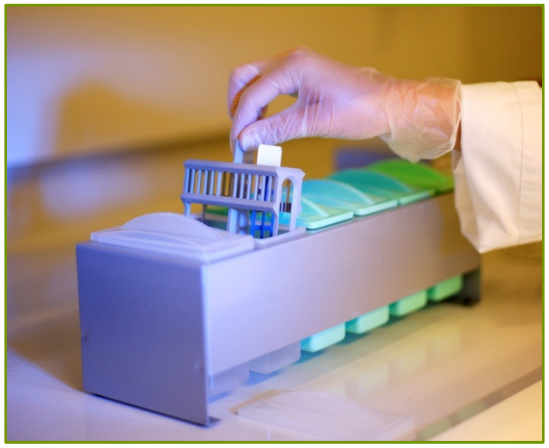
Sample and antibodies

- Sample fixed on glass slides.
- Optimized:
  - Pretreatment.
  - Blocking.
  - Primary antibodies, diluent, titer.



- Protocol: own or take a working one and optimize.
  E.g. DAKO Education Guide, IHC world, Nordiq, Abcam etc.

- Antibodies:
  - affinity purified polyclonal
  - Monoclonal (avoid ascites)

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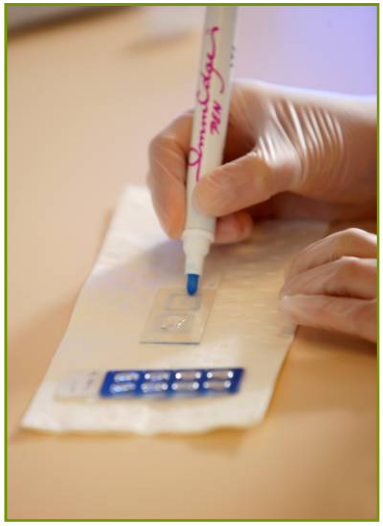
Duolink assay: equipment needed

- Grease pen



- Humidity chamber

- Freeze block for enzymes

- Incubator 37 ℃

- Staining jar and shaker

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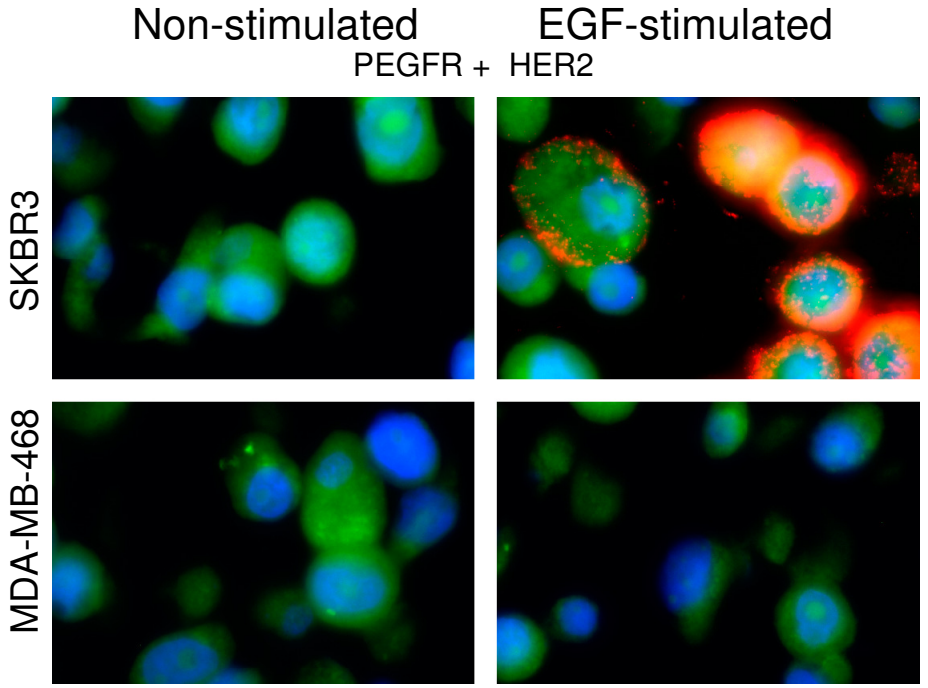
Biological controls

Best controls and recommended to be included.

- Positive control:
  - Known protein interaction or modification.
  - Overexpression, stimulation.
- Negative controls:
  - Irrelevant protein (e.g. non-interacting, present in different compartment).
  - Sample not expressing target protein or down-regulated/repressed, silencing, mutation etc.



Lower HER2 in MDA cells

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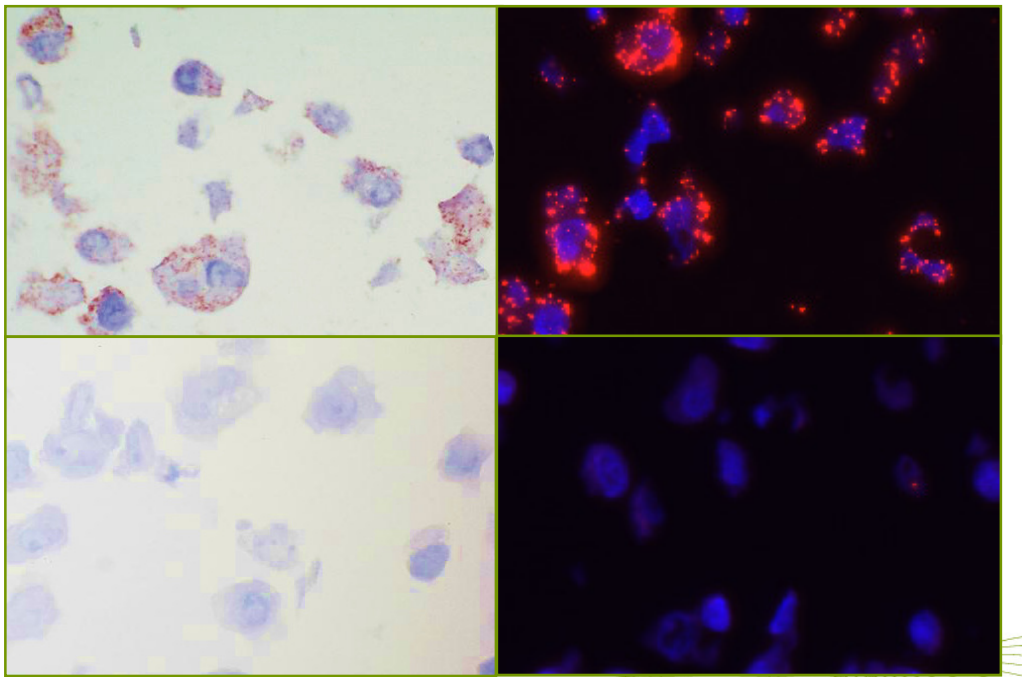
Duolink assay: technical controls

Technical negative controls:

- Leave one primary antibody out.
  - Information about unspecifc binding of primary antibodies.
  - Helpful to find an optimal titer.
- Leave both primary antibodies out.
  - Information about non specificity of PLA probes.
- Do not use unspecific sera!

Duolink II Control Kit:

- Useful to check assay conditions in your lab.
- Positive control of Detection Reagent.

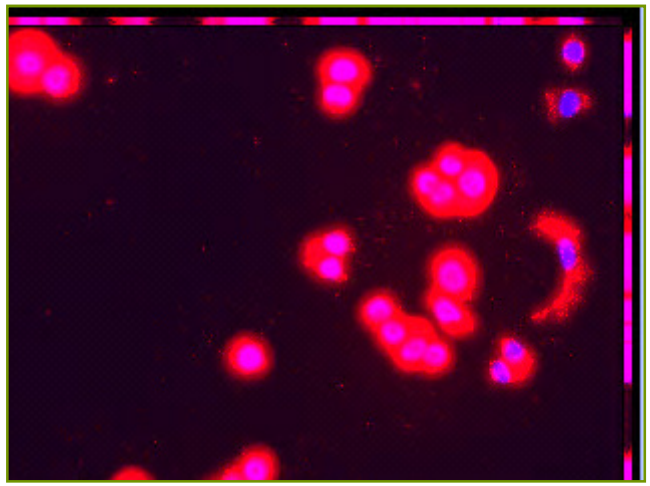


Above: positive control; Below: negative control.
Left: Brightfield; Right: Fluorescence.

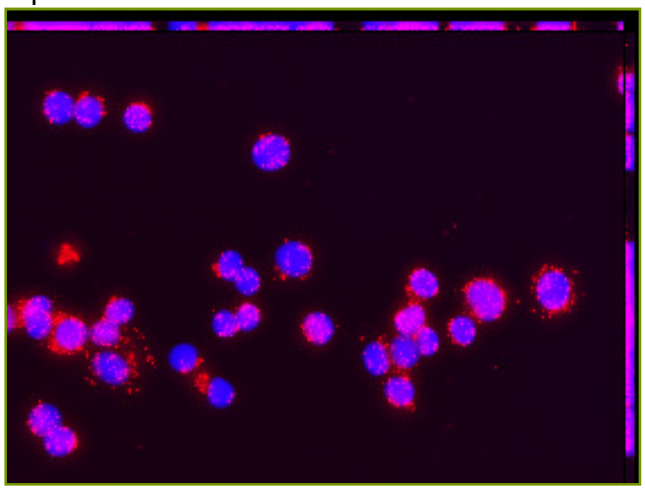
Common parameters to consider:

- Check antibody titer to obtain specific and distinct staining.

High titer



Optimized titer



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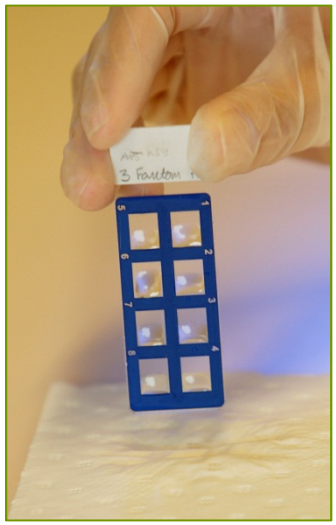
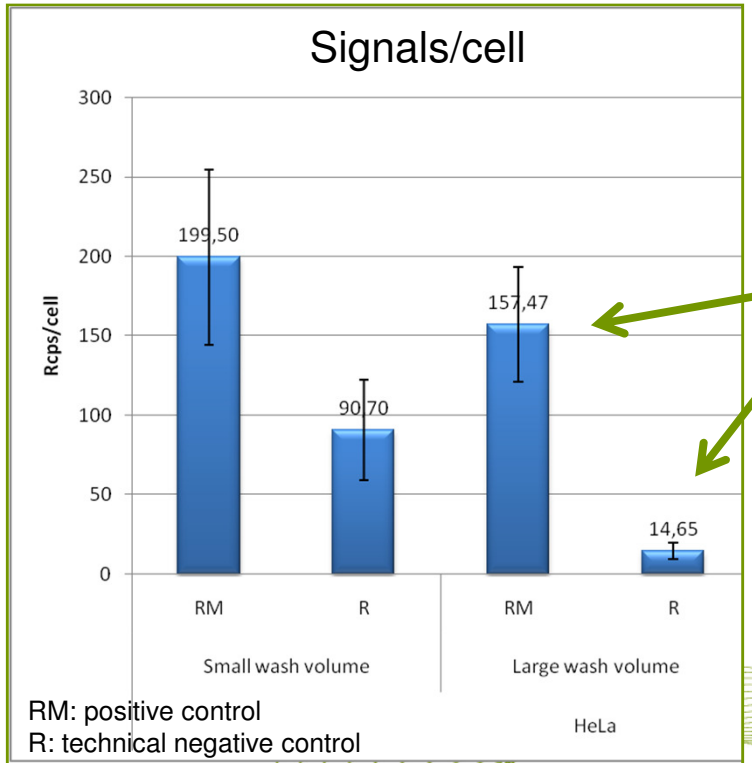
Common parameters to consider:

- Use appropriate blocking agent. Dilute PLA probes in a buffer containing the blocking agent.
- Never let your sample dry out during the Duolink assay.
- Gently tap-off excess solutions.
- Wash in at least 70 mL of wash buffers.
- Use Wash Buffers A and B where specified.

Gentle tap-off





Better signal-to-noise ratio with proper wash volume

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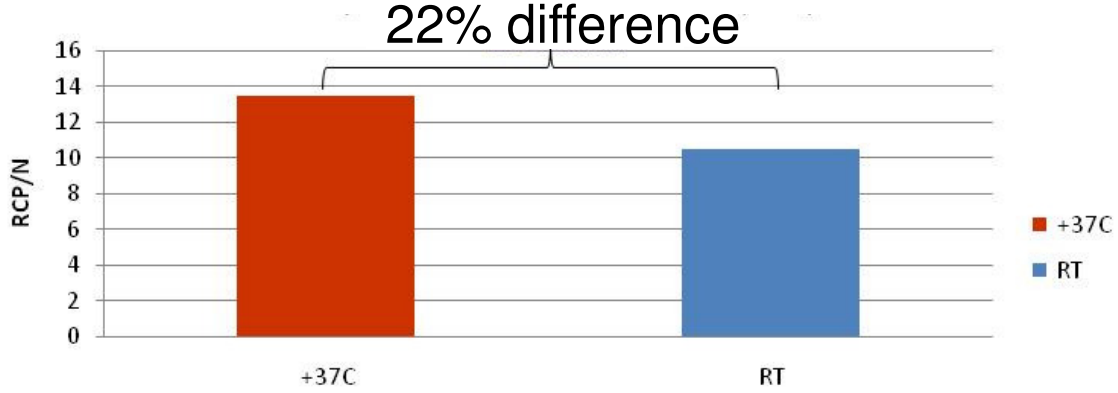
Common parameters to consider:

- Incubation temperature:

Keep humidity chamber preheated inside incubator

Incubator always at 37 ℃



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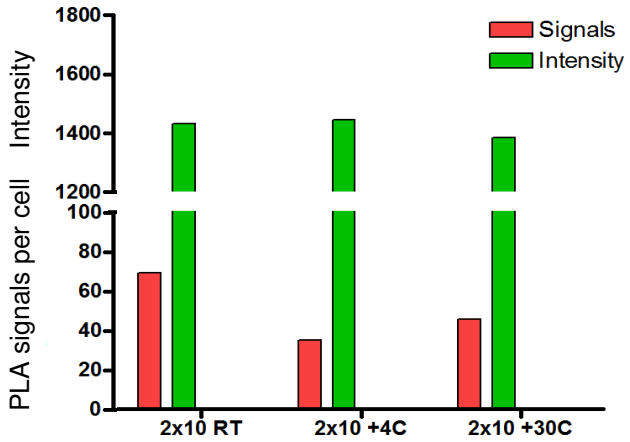
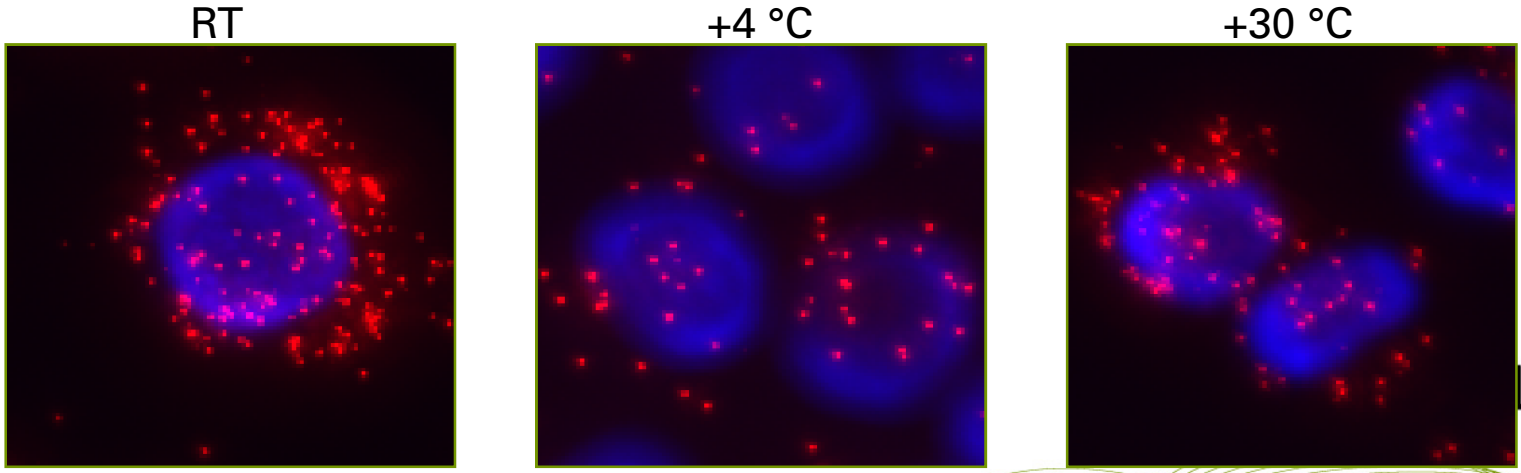
Common parameters to consider:

- Wash temperature:
  - Bring wash buffers to room temperature.
  - Perform wash at room temperature.





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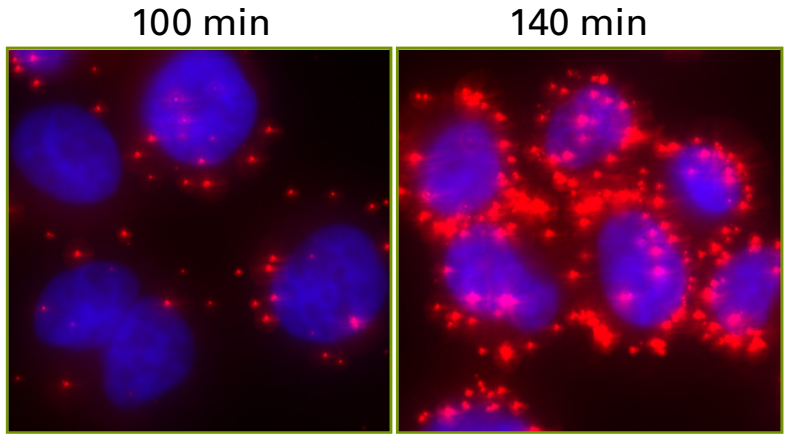
Common parameters to consider:

- Amplify for 100 min to avoid coalescent signals.



- Thaw reagents but use freeze block for Ligase and Polymerase.

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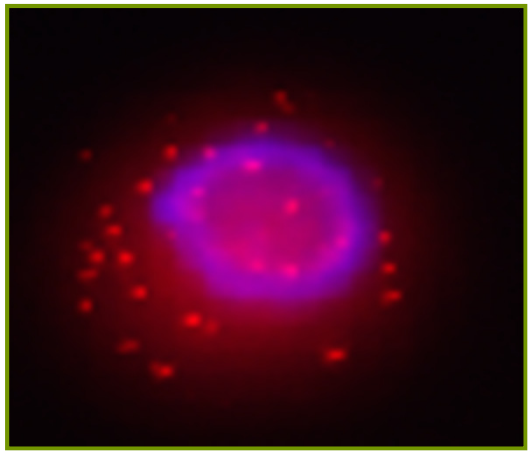
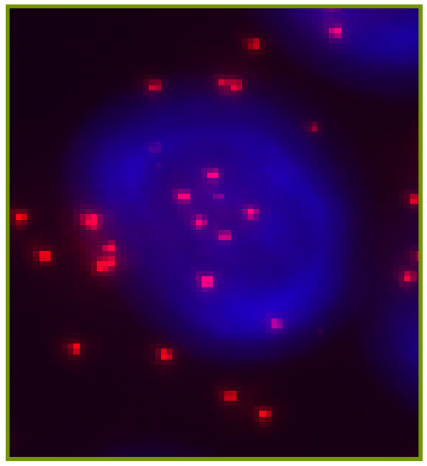
Image interpretation: common autofluorescence interference.

- Red nuclei artifact:



No artifact:



- Use Wash B and Duolink II Mounting Medium with DAPI.
- Do not overexpose

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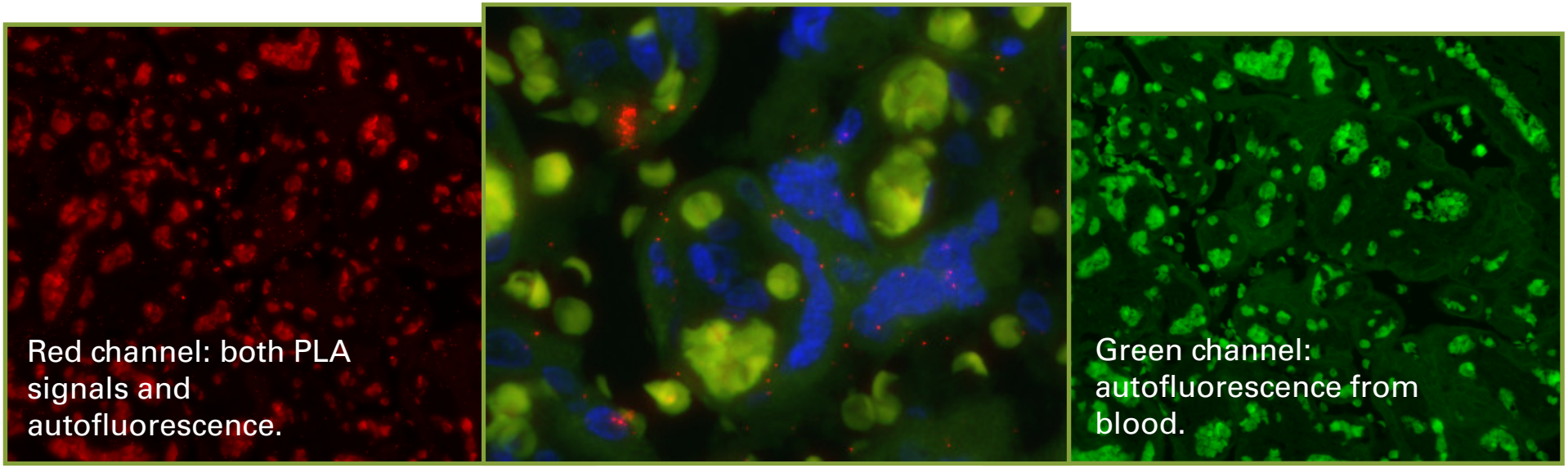
Image interpretation: common autofluorescence interference.

- Autofluorescence:
  - Inherent to sample.
  - Can be observed in green channel.
  - Alternative: use FarRed / Brightfield.



Superimposed image with three color channels: PLA signals can be distinguished from autofluorescence background.

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2. SAMPLE ANALYSIS & STORAGE

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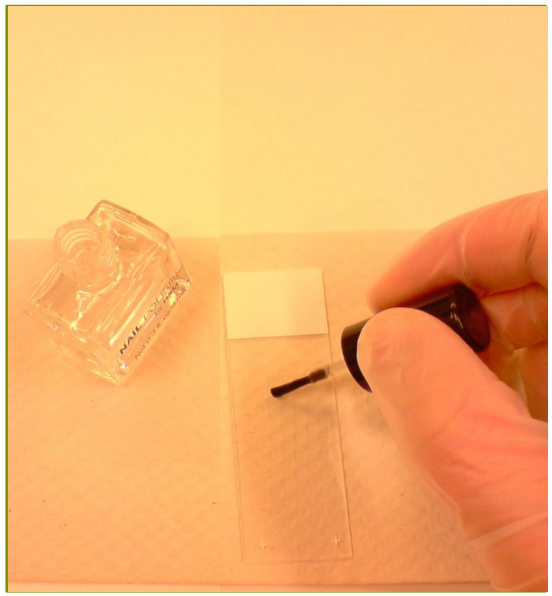
Mounting and storage

- Duolink Brightfield Mounting Medium: non-aqueous, xylene based.
- Duolink II Mounting Medium with DAPI: aqueous, contains anti-fade and DAPI for Fluorescence application.

Storage: O/N in dark at RT before mounting

<4 days in dark at 4 ℃ after mounting



Seal and freeze at -20℃, for months after mounting

Notes:

- We have tested many other media and PLA signals fade away with many of them.
- Add a few drops and press out any excess media.

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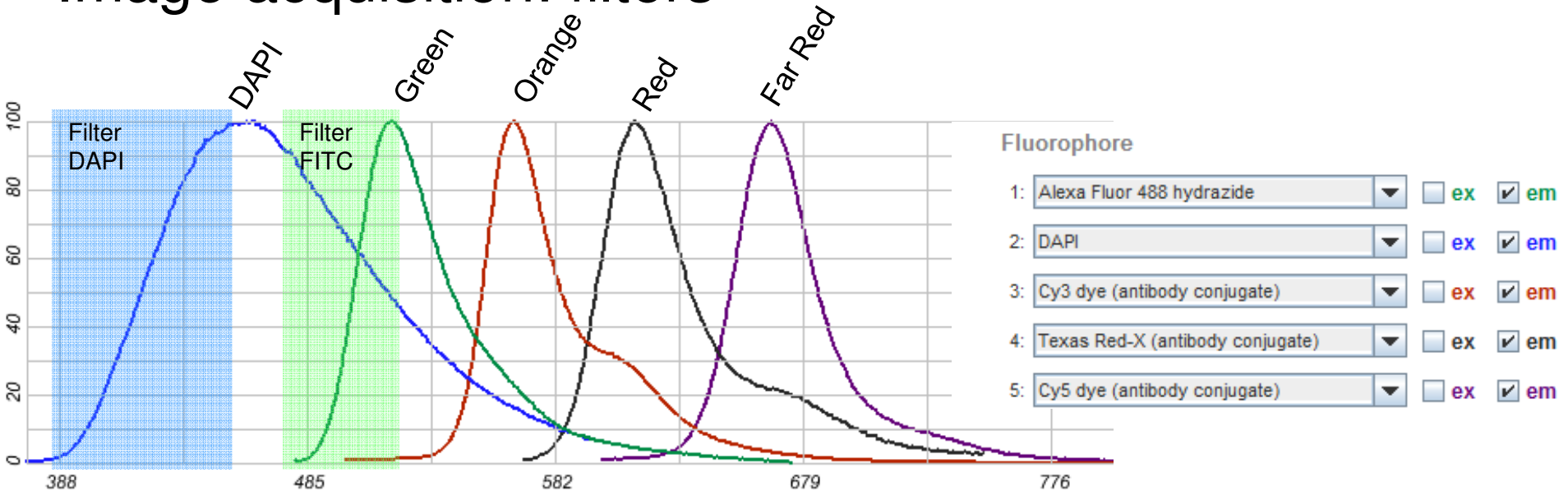
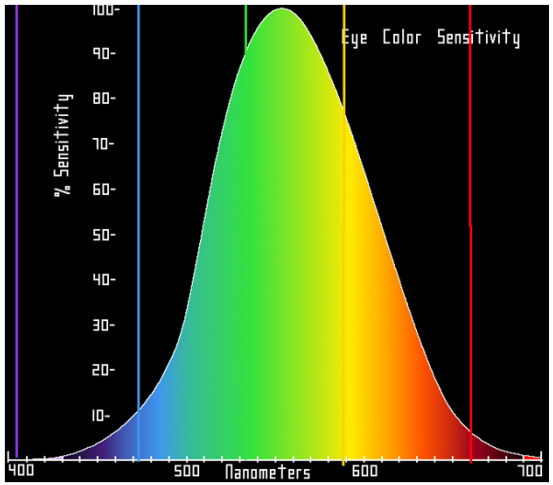
Image acquisition: filters





Relative brightness sensitivity of the human visual system as a function of wavelength

- FarRed:
  - Focus on cell nuclei plane.
  - Obtain z-stacks if necessary.

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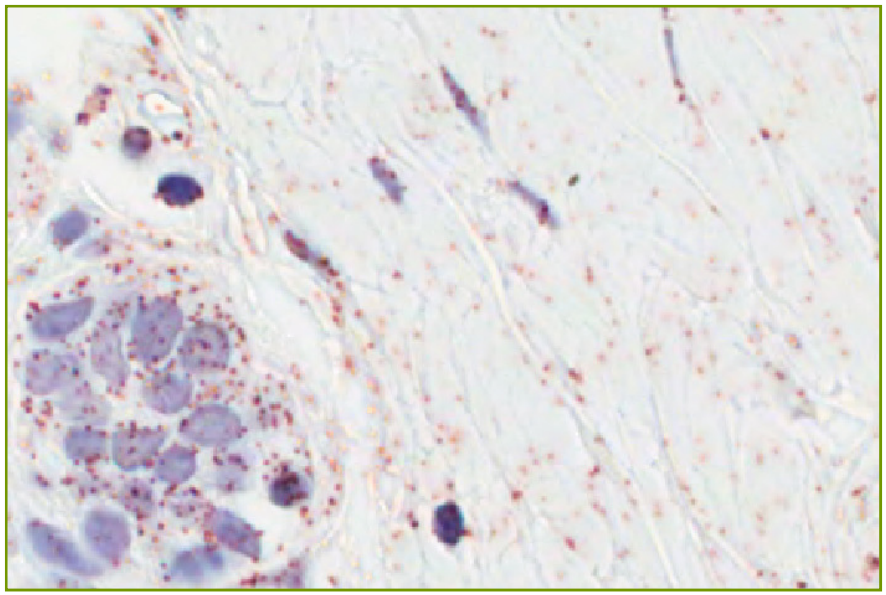
Image acquisition: parameters

- Brightfield application:
  - Good contrast.
  - White background/unstained tissue.

Correct background



Dark background

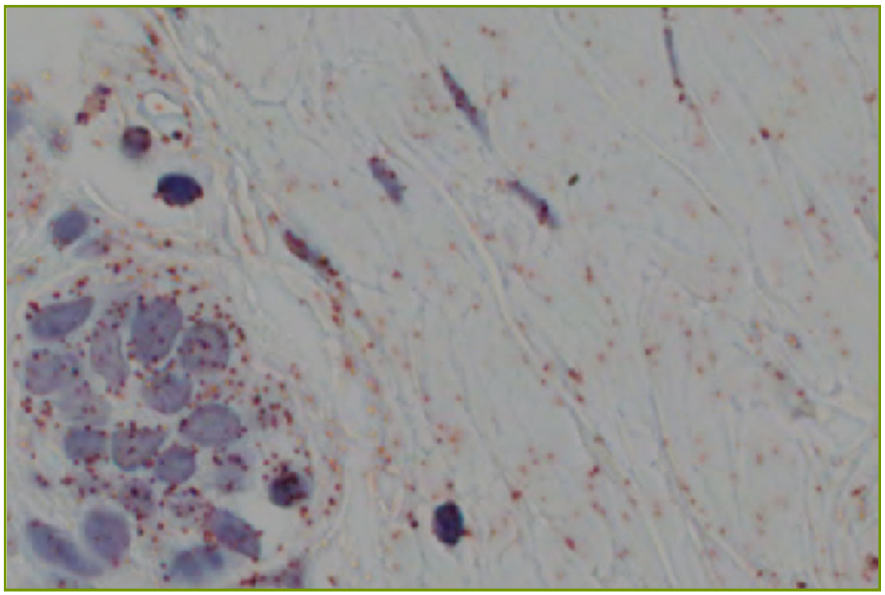


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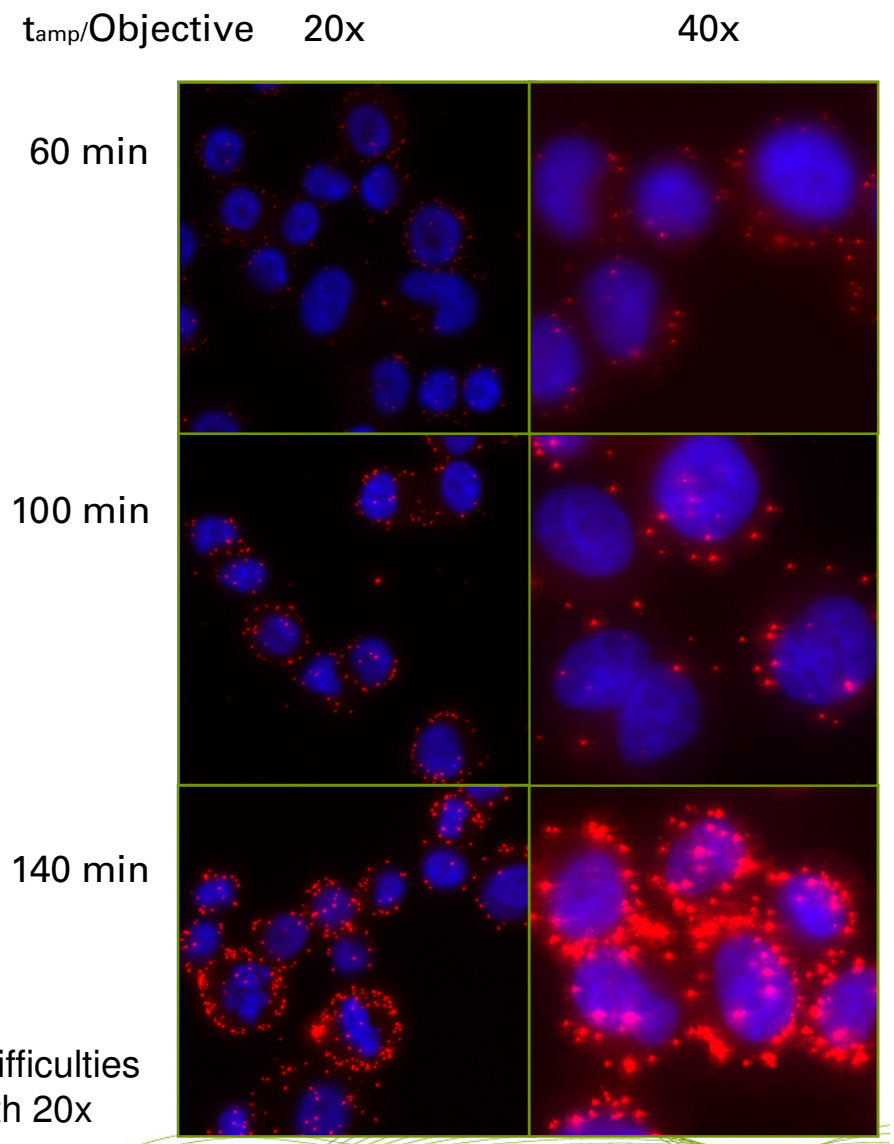
Image acquisition: parameters

- Fluorescence application:
  - 20x or 40x objective.
  - Do not overexpose.
    - Signals can coalesce.
    - Can give rise to autofluorescence.
  - Z-stacks if possible



Tip:
Look with 40x if difficulties finding signals with 20x

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3. IMAGE ANALYSIS & PRESENTING DATA

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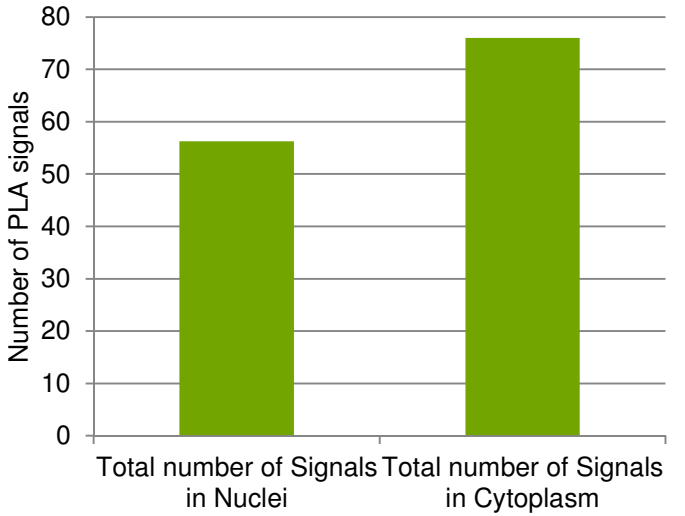
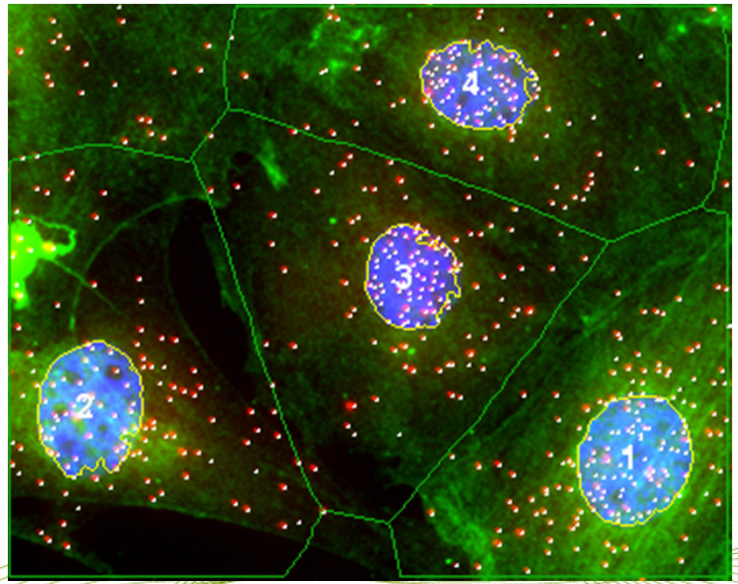
Quantification

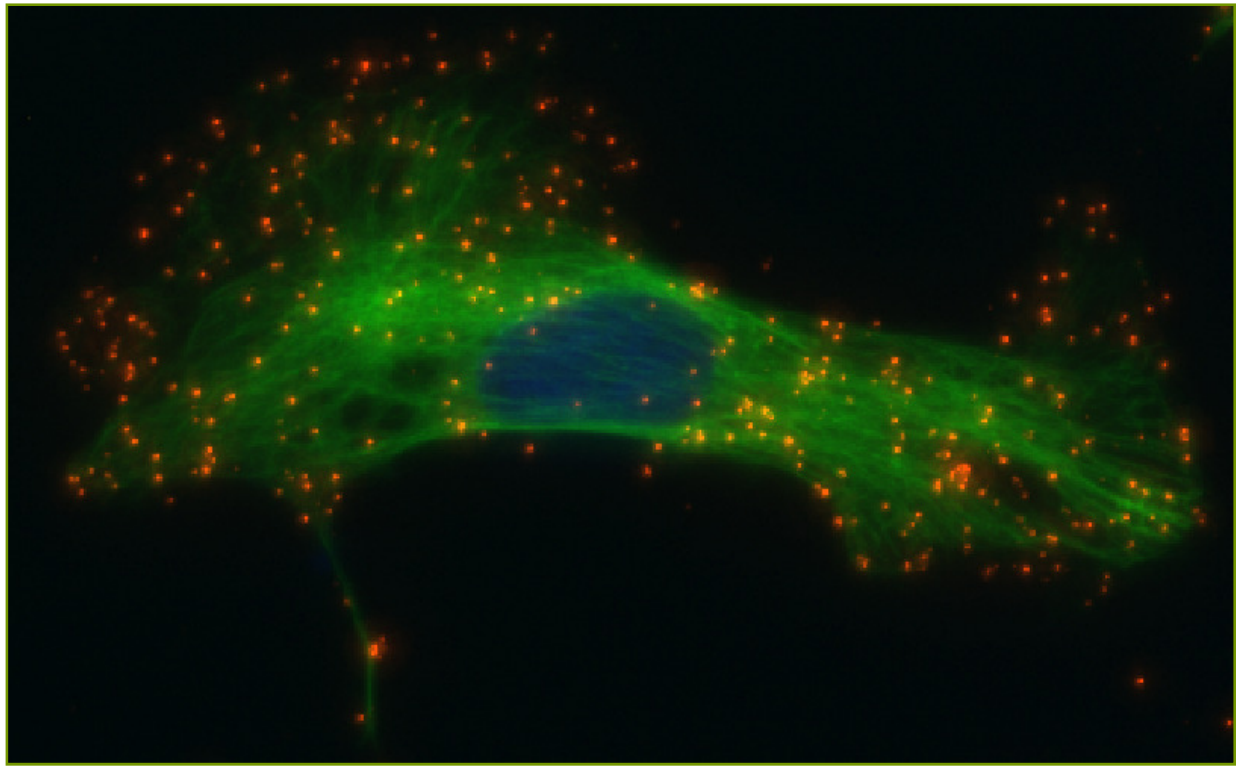
- Compare only samples that have been run in parallel.
  - Images taken with same acquisition parameters under same session.
- Use 20x or 40x.
  - 63x or 100x nice images for publication but not worth quantifying.
- Quantification is relative, e.g.:
  - Positive vs. negative controls.
  - Signals in nuclei vs. signals in cytoplasm.





Counterstaining

- Add counterstaining after Wash buffer B step in the protocol, wash and mount.



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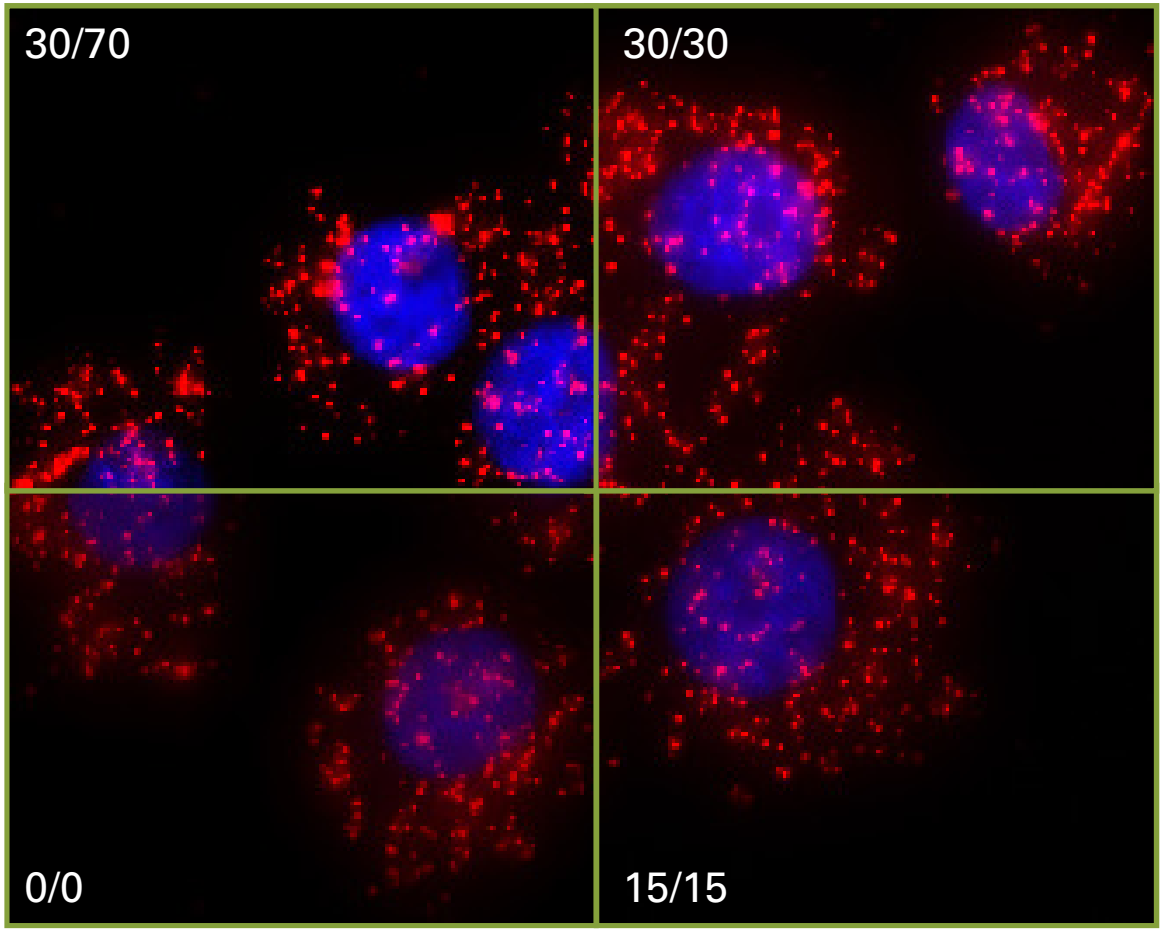
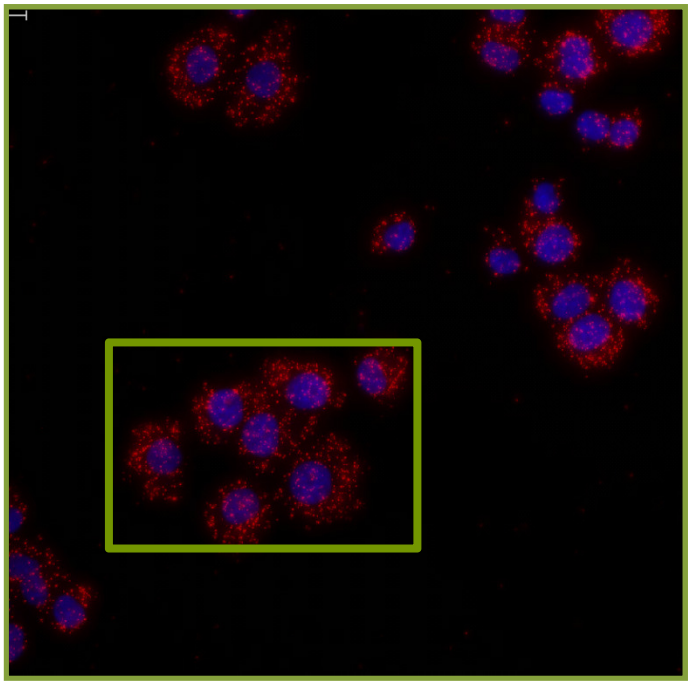
Presenting data

- Zoom in and scale up.
- Increase brightness and contrast for presentations and publications.



Example on how increasing brightness/contrast (numerical values above) can be useful when presenting data.



Zoom in on a region of interest (above) and scale up (left).

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4. PROBEMAKER

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Probemaker: antibody requirements

- Good quality antibodies:
  - Affinity purified polyclonals.
  - Non-ascites monoclonals.
  - Non-modified (e.g. no biotinylation).
  - Stock 1 mg/mL.
  - Stock buffer additive free, ideally PBS. Pretreatment:

Buffer exchange	Affinity purification
G25, G50	Protein A, G
Small molecules: e.g. azide, Tris	Macromolecules: e.g. gelatin, BSA

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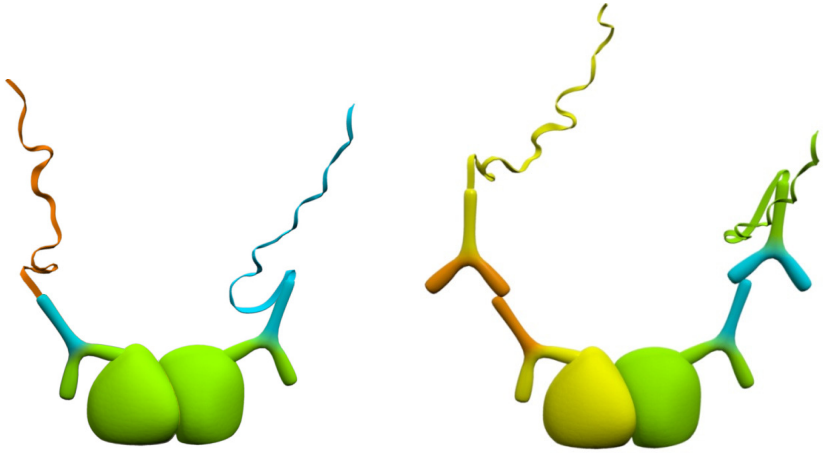
Probemaker: assay

- Concentration of conjugated antibody:

Primary: higher concentration than for IHC/IF.



Secondary: start with concentration as for IHC/IF.

- Dilution of conjugated antibody:
  - Custom solutions: blocking agents + Assay Reagent.
  - Duolink II solutions: PLA probe Diluent.

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Contact for support

▶You are very welcome to contact us for further support requests at: support@olink.com or +46 18 444 3970.

▶When contacting us, please, provide the following information:

- Description of your assay.
- Images of your results.
- Controls (positive/negative, biological/technical) that have been performed (if any).
- Previous IF/IHC results (if any).

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