INSTRUCTIONS FOR USE

MolBoolean™ (MolB00001 - Mouse/Rabbit)

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Abbreviations
    NB: Nota Bene
    RCA: Rolling circle amplification

RCPs: Rolling circle products
FFPE: Formalin fixed paraffin embedded
TMA: Tissue microarray

1. Introduction

MolBoolean™ Mouse/Rabbit (Cat. MolB00001) is a kit for in situ protein proximity analysis in tissue and cells. The MolBoolean assay utilizes anti-mouse and anti-rabbit secondary proximity probes and a proprietary oligonucleotide setup that enables the simultaneous detection of both free and interacting (within a proximity of ~40 nm) fractions for two proteins of interest (protein A, protein B and interaction proteins AB).

It uses two different immunofluorescent detection reporters with maximum emission wavelengths at 590 nm (ATTO565, TxRed filter or similar) and at 664 nm (ATTO647N, Y5 filter or similar), respectively.

1.1 MolBoolean assay steps

The MolBoolean™ assay involves seven steps to detect whether the proteins of interest are interacting or present individually:

Step 1: Proximity probe binding to primary antibodies.

Step 2: Proximity probe arm hybridization to DNA circle oligo.

Step 3: DNA nicking.

Step 4: Tag oligo incorporation.

Step 5: DNA ligation.

Step 6: Rolling Circle Amplification.

Step 7: Detection.

Figure 1 provides a schematic representation of the MolBoolean™ assay steps.

Detection of interacting proteins A (magenta) and B (green).

After binding their respective target proteins, the primary antibodies are recognised by two species-specific proximity probes (each conjugated with an information-containing DNA oligonucleotide (oligo), termed "arm") (step 1).

The probe arms then hybridize to a preformed DNA circle oligo (the "information receiver") (step 2), creating double-stranded DNA that contains two nickase recognition sequences (cyan arrowhead indicates nicking position). These will be cut during the DNA nicking step (step 3).

Next, two "identifier tag" oligos get incorporated into the DNA circle (step 4) followed by DNA ligation (step 5). The DNA circle now contains information (incorporated identifier tags) on the interaction with two proximity probe arms.

Rolling circle amplification (RCA) creates long concatemeric rolling circle products (RCPs) (step 6).

In the last step (step 7), two separate fluorophore-labeled tag-specific detection reporter oligos hybridize to the RCPs, revealing the identities of the incorporated identifier tags, thereby indicating the presence of interacting proteins A/B in the sample. Thus, the detection of RCPs in both fluorescent channels will be indicative of interaction between proteins A and B in the sample.

Detection of individual protein A and B

Free, non-interacting proteins A and B will be detected individually. At these locations, only one probe arm will hybridize to the DNA circle, hence only one nicking site will be revealed, allowing only one identifier tag to become incorporated into the DNA circle. This creates RCPs where only one of the two fluorescent detection reporter oligos can hybridize. Consequently, these RCPs will emit light in only a single fluorescent channel
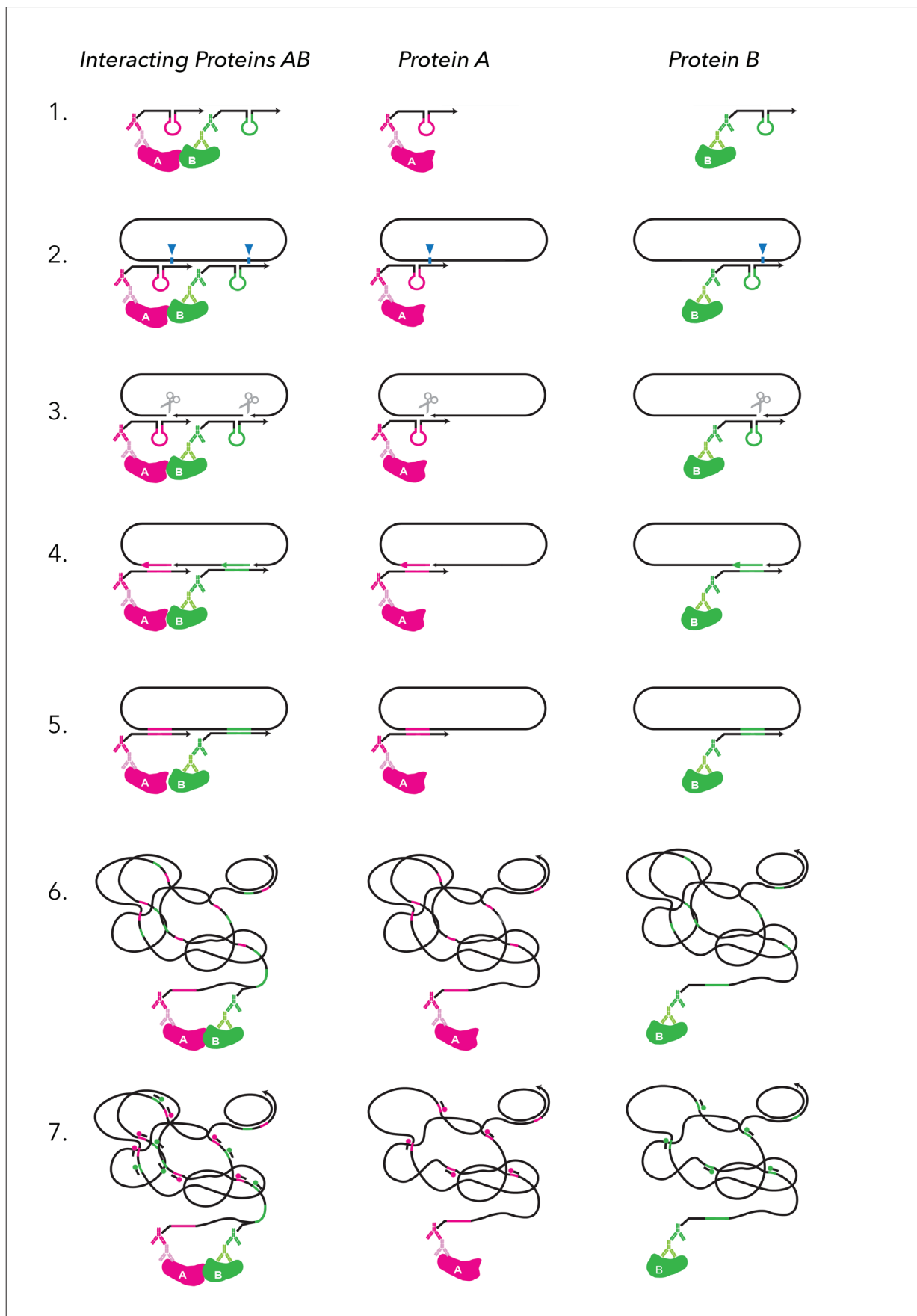
Figure 1. Schematic representation of the MolBoolean principle for detection of interacting (AB) and free (A or B) proteins.

2. Kit Content and Storage

MolBoolean™ Mouse/Rabbit kit enables MolBoolean analysis in fixed cells and in FFPE tissue samples. The kit volume, 4.8 ml total working volume, is enough for approximately 120 assays in cells (40 µl/assay) and 60 assays in tissue (80 µl/assay). A 40 µl assay volume should be enough to cover a sample area of 1 cm^2. Adjust accordingly to match your experimental setup.

The MolBoolean kit contains 15 separate kit tubes (outlined in section 2.1). The kit is shipped on dry ice. Upon receipt, store all the reagents at -20 °C. The Diluent (1x) could also be stored for up to 6 months at 4 °C.

2.1 MolBoolean kit components

Tube number	Tube ID
1	Blocker (4x)
2	Diluent (1x) *
3	Probe A (80x)
4	Probe B (80x)
5	Circle (40x)
6	Additive (40x)
7	Buffer A (10x)
8	Nickase enzyme (80x)
9	Buffer B (8x)
10	Tag oligos (40x)
11	Ligase enzyme (80x)
12	Polymerase enzyme (40x)
13	Buffer C (5x)
14	Detection oligos (40x)
15	Buffer D (10x)

* Immediately prior to use, prepare "Complete diluent" by adding Tween-20 to the Diluent (1x) (tube 2) to a final concentration of 0.1% (v/v) as follows:
- Prepare an 2 % (v/v) Tween-20 intermediate solution by adding 0.2 ml Tween-20 to 10 ml TBS. Mix for 10 min using a glass beaker and magnetic stirrer.
- Dilute the intermediate solution 1 in 20 with Diluent (1x) (e.g. 25 µl of 2 % Tween-20 intermediate to 475 µl Diluent) to get the final Complete Diluent. Store the Complete Diluent at 4°C and use within 1 week.

3. General instructions

- Assay volume depends on the cell or tissue sample size.
- Use best practices when pipetting to minimize reagents consumption.
- Completely defrost all oligonucleotides, buffers and additive tubes at room temperature and vortex well before use. If a precipitate is seen in any of the buffer tubes, they can be heated in the palm of the hand until completely dissolved. Remember to always vortex before use.
- Put all the enzymes, oligonucleotides, buffers or additives straight back to their correct storage condition after use unless otherwise stated in the protocol.
- Vortex carefully and spin-down all enzymes using a bench-top microcentrifuge before use.
- Keep enzymes in a frozen cold block during use.
- Wait to add enzymes until immediately prior to adding the assay solution to the sample.
- Conduct all assay incubation periods in humid chambers (unless otherwise stated).
- All wash steps should be performed at room temperature (unless otherwise stated) in a wash beaker (Coplin jar) with the test slides fully submerged in wash buffer and with gentle agitation.
- Remove excess wash buffer from samples before adding reagents.
- Do not allow slides/samples to dry.
- The detection oligos are light-sensitive reagents. Always keep it protected from light. This should also be taken into consideration during washing after the detection oligo step.
- Incubation times or temperatures other than those specified may give erroneous results.
- All chemicals should be handled with caution.

4. Required Materials and Equipment NOT Provided in the Kit

Material	Requirements/Notes
Two target-specific primary antibodies of choice	One of mouse and the other of rabbit origin
Fixed cell material on test slide	• Cells fixed with 3.7% formaldehyde diluted in Dulbecco's PBS (prepared from 37% formaldehyde stock, Sigma-Aldrich, Cat. 252549) have been tested. *Note: The user should determine the optimal fixation method for their chosen primary antibodies.* • Cells should be cultivated in chamber slides that allow the removal of chamber walls. Cells cultivated on Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (Thermo Fisher, Cat. 154941) have been tested.
Fixed tissue material on test slide	To date, FFPE human TMA material has been tested
Reagents needed for antigen retrieval of tissue sample	According to primary antibody and protein target specification
Deionized water	Sterile filtered or Milli-Q
Wash buffers	• TBS (Tris-buffered saline, pH 7.6 at 22 °C) • 0.2X TBS *(1 litre prepared as follows: 200 ml of TBS + 800 ml of deionized water.* • TBS supplemented with 0.05% Tween-20 (TBST) • High Salt Buffer (HSB); 0.85 M NaCl + 1x TBS *(1 litre prepared as follows: 200 ml of 4.25M NaCl + 100 ml of 10X TBS+700 ml of deionized water).* • HBS supplemented with 0.05% Tween-20 (HSBT)
Permeabilization buffer	0.2% (v/v) Triton X-100 in TBS. As Triton X-100 is viscous and therefore difficult to pipette at small volumes, it is advisable to first prepare a 2% (v/v) Triton X-100 intermediate solution by adding 0.2 ml Triton to 10 ml TBS. Dilute the intermediate solution 1 in 10 with TBS to get the final working solution (0.2% (v/v) (e.g. 1 ml 2% Triton X-100 intermediate solution to 9 ml TBS).
Nuclear marker such as Hoechst 33342 or DAPI	Can be applied during staining or during mounting. *NB:* The nuclear marker will bind to the Rolling Circle Product (RCP). Therefore, it is advisable to use it at the lowest concentration possible. *Suggestion:* dilute Hoechst 33342 1:5000 and 1:1250 for cells and tissue, respectively (diluted in TBS). Apply to the sample for 5 mins (see more details in sections 6.10 & 6.11).
Coverslips	For use with fluorescent microscopy (#1.5 glass thickness)
Mounting medium of choice	Anti-fade mounting medium, e.g. Slow Fade Gold antifade reagent (Thermo Fisher, Cat. S36940)

4. Required Materials and Equipment NOT Provided in the Kit *(Cont.)*

Equipment	Requirements/Notes
Incubator	37°C
Microwave or hot plate	To heat wash buffer in section 6.11
Hydrophobic pen	To retain liquid on test sample and minimize assay volume
Microcentrifuge with slide insert	Used to remove excess liquid from test slides before mounting
Vortex	Common laboratory equipment
Microcentrifuge	Common laboratory equipment
Humidity chamber	Used during assay incubations. • Place a pre-wetted paper at the bottom of a container (e.g. an empty slide storage box). • The container must permit the placement of test slides above, without touching the wet paper. • The container should be sealed during assay incubations. • Ensure that the paper does not dry out at any time.
Freeze block for enzymes	Storage of enzymes during use
Wash beakers (Coplin jars)	Used for wash of test slides
Orbital shaker	Used for wash of test slides
Hydrophobic pen	To retain liquid on test sample and minimize assay volume
Microcentrifuge with slide insert	Used to remove excess liquid from test slides before mounting
Nail polish	Used to seal glass coverslip after mounting
Fluorescence microscope (confocal or widefield)	Equipped with appropriate filters/detectors, lasers/camera, and software for image acquisition

5. Preparation of Cell and Tissue Samples

5.1   Cell Seeding and Cell Fixation

NB 1. This protocol has been optimized for the analysis of the interaction between E-cadherin and β-catenin in MCF7 cells (see section 8.1.1 for MolBoolean example images in cells). It should only be seen as a recommendation and the user should determine the optimal cell culture and fixation method for their targets and primary antibodies.

- Seed MCF7 cells on Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (Thermo Fisher, Cat. 154941) at a density of 40-60.000 cells per well (in 300 µl growth medium).
- Let the cells grow until a 75% cell confluency is observed (approximately 48 hours).
- On the day of fixation, remove the cell medium and wash cells with ice-cold Dulbecco's Phosphate Buffered Saline (DPBS) while test slides are placed on ice.
- Fix cells with pre-chilled 3.7% formaldehyde solution (prepared by diluting 37% formaldehyde stock solution (Sigma-Aldrich, Cat. 252549) 1 in 10 with DPBS) for 15 min while test slides are placed on ice.
    Note: Make sure that the 37% formaldehyde stock is properly mixed before preparation. Invert flask a few times before using it.
- Wash cells three times in DPBS at room temperature.
- Remove the chamber walls and release the test slide using the equipment supplied with the chamber slides.
- Proceed directly to the cell permeabilization step (section 5.3). Alternatively, dry the test slides using a bench-top microcentrifuge with appropriate insert and store at -20°C until use.

5.2   FFPE TMA sectioning, deparaffinization and antigen retrieval

NB 2. This protocol has been optimized for MolBoolean analysis on FFPE human TMAs (see section 8.1.2 for MolBoolean example images in tissue). It should only be seen as a recommendation and the user should determine the optimal tissue preparation methods for their targets and primary antibodies.

Atlas Antibodies' standard protocol is outlined below.
- Cut 4µm thick sections from FFPE human TMAs and bake overnight at 50°C. Once the TMA sections are prepared, store at -20°C until use.

- On the day of use, perform deparaffinization and re-hydration of the slides in xylene and graded ethanol to distilled water. During re-hydration, the 5 min 95% ethanol step contains 0.3% H_2O_2 that blocks endogenous peroxidase.
- Perform antigen retrieval using Heat Induced Epitope Retrieval (HIER) in retrieval buffer pH 6.1 (Dako, Agilent, Santa Clara, CA, USA) using a pressure boiler (Decloaking chamber, Biocare Medical, CA, USA) as heat source. Perform HIER by heating the TMA-slides immersed in retrieval buffer. The pressure boiler machine increases the temperature until 110°C and pressure to 40 psi. Maintain these conditions for 20 min and then slowly cool to 90°C.
- Rinse the slides in distilled water and place in Pierce™ 1X TBS Tween™ 20 Buffer (Thermo Fisher cat no: 28360) until use in step 5.3.

5.3   Cell and Tissue Permeabilization

- Prepare the permeabilization buffer as described in section 4.
- Remove the cell test slides from the freezer (from step 5.1) or use the TMA prepared in step 5.2.
- Add a Hydrophobic Barrier Pen (e.g. ImmEdge Hydrophobic Barrier PAP Pen, Vector Laboratories Cat. H-4000) around the wells of the cell test slide, or around the tissue on the tissue test slides. For the cell slides, let the hydrophobic pen dry off for 1 min prior to adding the permeabilization buffer. For the tissue test slide, immediately cover the TMA with TBS after applying the hydrophobic pen to avoid drying and leave in TBS until ready to apply the permeabilization buffer.
- Apply permeabilization buffer to the samples and incubate in a humid chamber for 15 mins. This is done by adding the permeabilization solution as a bubble directly on top of the cells or tissue. Add roughly 80 µl permeabilization buffer per 1 cm^2 area of cells or tissue.
- Wash slides 2 times 2 min in TBST in a Coplin jar on an orbital shaker.

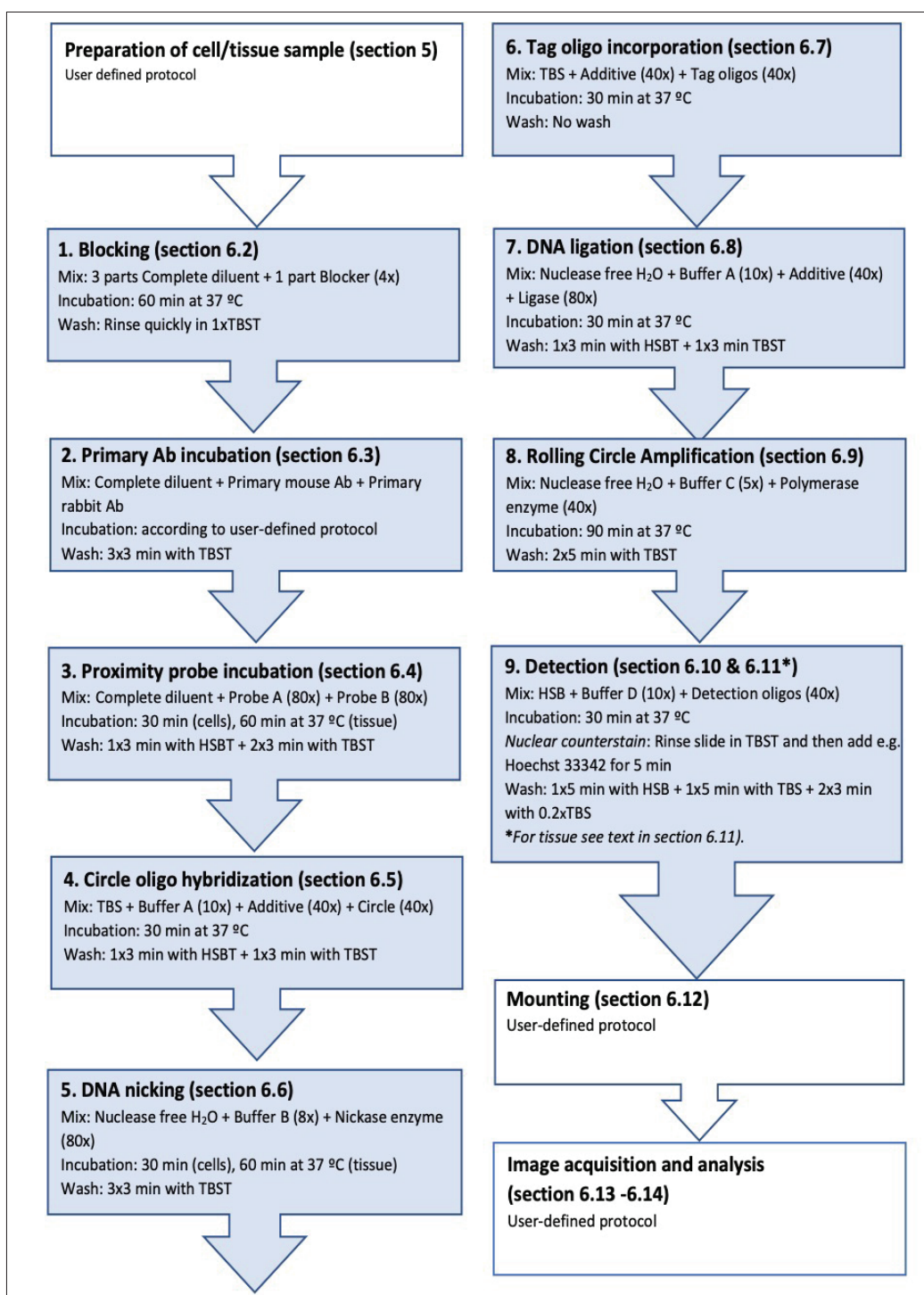## 6. MolBoolean Assay Procedure in Cells and Tissue

NB 3. For the following steps, prepare enough assay volumes to fully cover all the samples. An assay volume of 40 µl is enough to cover a sample area of 1 cm² (one well on a cell test slide, prepared in section 5.1) and 80 µl is enough to cover one TMA (prepared in section 5.2). Adjust accordingly to match the experimental setup.

NB 4. All the wash steps are performed in a Coplin jar with gentle agitation on an orbital shaker.

NB 5. All the assay steps involve applying solutions as a bubble on top of each sample. Always ensure that the entire cell/tissue surface is covered with the liquid, using a pipette tip to spread the liquid from the top of the bubble, taking care not to touch the sample surface.

NB 6. Several steps require that the solutions are pre-prepared prior to assay start. Always read instructions to ensure that solutions are ready in time for subsequent assay steps.

6.1   MolBoolean workflow

Preparation of cell/tissue sample (section 5)
User defined protocol

1. Blocking (section 6.2)
Mix: 3 parts Complete diluent + 1 part Blocker (4x)
Incubation: 60 min at 37 ºC
Wash: Rinse quickly in 1xTBST

2. Primary Ab incubation (section 6.3)
Mix: Complete diluent + Primary mouse Ab + Primary rabbit Ab
Incubation: according to user-defined protocol
Wash: 3x3 min with TBST

3. Proximity probe incubation (section 6.4)
Mix: Complete diluent + Probe A (80x) + Probe B (80x)
Incubation: 30 min (cells), 60 min at 37 ºC (tissue)
Wash: 1x3 min with HSBT + 2x3 min with TBST

4. Circle oligo hybridization (section 6.5)
Mix: TBS + Buffer A (10x) + Additive (40x) + Circle (40x)
Incubation: 30 min at 37 ºC
Wash: 1x3 min with HSBT + 1x3 min with TBST

5. DNA nicking (section 6.6)
Mix: Nuclease free H_2O + Buffer B (8x) + Nickase enzyme (80x)
Incubation: 30 min (cells), 60 min at 37 ºC (tissue)
Wash: 3x3 min with TBST

6. Tag oligo incorporation (section 6.7)
Mix: TBS + Additive (40x) + Tag oligos (40x)
Incubation: 30 min at 37 ºC
Wash: No wash

7. DNA ligation (section 6.8)
Mix: Nuclease free H_2O + Buffer A (10x) + Additive (40x) + Ligase (80x)
Incubation: 30 min at 37 ºC
Wash: 1x3 min with HSBT + 1x3 min TBST

8. Rolling Circle Amplification (section 6.9)
Mix: Nuclease free H_2O + Buffer C (5x) + Polymerase enzyme (40x)
Incubation: 90 min at 37 ºC
Wash: 2x5 min with TBST

9. Detection (section 6.10 & 6.11*)
Mix: HSB + Buffer D (10x) + Detection oligos (40x)
Incubation: 30 min at 37 ºC
Nuclear counterstain: Rinse slide in TBST and then add e.g. Hoechst 33342 for 5 min
Wash: 1x5 min with HSB + 1x5 min with TBS + 2x3 min with 0.2xTBS
For tissue see text in section 6.11).

Mounting (section 6.12)
User-defined protocol

Image acquisition and analysis (section 6.13 -6.14)
User-defined protocol

6.2 Blocking

- Prepare **"Complete Diluent"** according to instructions in section 2.1.
- Prepare blocking solution by combining 1 part of **Blocker (4x) (tube 1)** with 3 parts **Complete Diluent**. Mix by inverting the tube a few times. Keep the remainder of the Complete Diluent at room temperature until use in section 6.3.
- Add blocking solution to the entire sample area.
- Incubate for 1 hour at 37°C in a humidity chamber.
- Rinse slides briefly in TBST before adding the primary antibodies. The primary antibodies should be added within 5 minutes from this step.

6.3 Primary antibody incubation

NB 7. Primary antibodies are not supplied as part of the MolBoolean kit. The user should include their own antibodies, one of rabbit and one of mouse origin. Their specificity and sensitivity should have been validated on relevant test samples.

NB 8. If the primary antibodies are of monoclonal and polyclonal nature, it is recommended to apply the antibodies sequentially, with the monoclonal antibody (mAb) applied prior to the polyclonal antibody (pAb). If so, follow the steps outlined in this section. Sequential primary antibody incubations ensure that the mAb has sufficient access to the epitope on protein A, before being masked by the molecular crowding that could occur when pAbs bind several epitopes on protein B.

NB 9. It is advisable to include "omitting" control samples when validating the MolBoolean results. That is, omitting the primary antibodies one by one (replaced by Complete Diluent), and then perform the MolBoolean assay as usual. These controls will indicate if non-specific background signal is generated in either of the two fluorescent channels.

- Prepare the mAb solution by diluting in **Complete Diluent** to reach the final mAb concentration.
- Apply 32 µl of mAb solution per 1 cm² of cell or tissue area. Incubate the slides in a humid chamber at room temperature for 2 hours.
- During the mAb incubation time, prepare the pAb solution by diluting in **Complete Diluent** to reach a 5x concentrated intermediate pAb solution. E.g. if the final pAb concentration should be 2 µg/ml, then prepare a 10 µg/ml intermediate solution.
- Add the intermediate pAb solution directly to the mAb solution on the sample, achieving a 1 in 5 dilution to reach a final pAb concentration.

E.g., add 8 µl of intermediate pAb solution to 32 µl mAb solution to achieve a final pAb concentration of 2 µg/ml. Mix the combined mAb and pAb solutions by careful pipetting.
- Incubate the slides containing combined mAb and pAb in a humid chamber over night at 4°C. Keep the remaining **Complete Diluent** at 4°C until use in section 6.4.
- Remove the antibody solution by decanting the slide on a paper towel or similar. If several different antibody solutions (e.g. omitting controls) are tested on the same slide, make sure to avoid cross contamination between samples. This could be avoided by removing each sample individually with a pipette tip.
- Wash slides for 3x3 min with TBST.

6.4 Proximity probe incubation

- Pre-heat the **Complete Diluent** for 20 min at 37 °C.
- In the same tube, dilute **Probe A (80x) (tube 3)** and **Probe B (80x) (tube 4)** 1:80 with **Complete Diluent**.
- Add enough of the probe solution to cover the sample area.
- Incubate the slides in a humid chamber for 30 min (cells) or 60 min (tissue) at 37°C.
- Decant the solution and wash slides for 1x 3 min with HSBT and then 2x 3 min with TBST.

6.5 Circle oligo hybridization

NB 10. **Buffer A (10x) (tube 7)** contains Dithiothreitol (DTT) and should only be opened in a fume hood.

NB 11. **Buffer A (10x)** can take a bit longer to defrost. Start defrosting 30 min before use. If a precipitate is seen, heat the tube in the palm of the hand until completely dissolved. Remember to always vortex before use.

NB 12. After use, keep **Additive (40x) (tube 6)** and **Buffer A (10x) (tube 7)** on ice. They will be used again in the Tag oligo incorporation (section 6.7) and DNA ligation steps (section 6.8).

- In the same tube, dilute **Buffer A (10x) (tube 7)** 1:10, **Additive (40x) (tube 6)** 1:40 and **Circle (40x) (tube 5)** 1:40 with TBS.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate the slides in a humid chamber for 30 min at 37°C.
- Decant the solution and wash slides for 1x 3 min with HSBT and 1x 3 min with TBST.

6.6   DNA Nicking

- In the same tube, dilute **Buffer B (8x) (tube 9)** 1:8 and **Nickase enzyme (80x) (tube 8)** 1:80 with Nuclease free H_2O.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate the slides in a humid chamber for 30 min (cells) or 60 min (tissue) at 37°C.
- Decant the solution and wash slides for 3x 3 min with TBST.

6.7   Tag Oligo Incorporation

- In the same tube, dilute **Additive (40x) (tube 6)** 1:40 and **Tag oligos (40x) (tube 10)** 1:40 with TBS.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate the slides in a humid chamber for 30 min at 37°C.
- Do not wash the slides at this stage, simply decant the solution and move straight to DNA ligation step.

6.8   DNA Ligation

NB 13. Buffer A (10x) (tube 7) contains Dithiothreitol (DTT) and should only be opened in a fume hood.

NB 14. Since the previous step (section 6.7) does not involve a wash step, the DNA Ligation assay mixture needs to be ready by the end of the previous incubation. Keep in mind not to add the DNA ligase enzyme component until just before use.

NB 15. Since the previous step (section 6.7) does not involve a wash step containing Tween-20, the ligase assay solution may not spread as easy over the sample surface. Use a pipette tip to spread the liquid from the top of the bubble, taking care not to touch the sample surface.

- In the same tube, dilute **Buffer A (10x) (tube 7)** 1:10, **Additive (40x) (tube 6)** 1:40 and **Ligase enzyme (80x) (tube 11)** 1:80 with Nuclease free H_2O.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate the slides in a humid chamber for 30 min at 37°C.
- Decant the solution and wash slides for 1x 3 min with HSBT and 1x 3 min with TBST.

6.9   Rolling Circle Amplification (RCA)

NB 16. Buffer C (5x) (tube 13) contains Dithiothreitol (DTT) and should only be opened in a fume hood.

NB 17. If a precipitate is seen in **Buffer C (5x) (tube 13)**, heat the tube in the palm of the hand until completely dissolved. Remember to always vortex before use.

- In the same tube, dilute **Buffer C (5x) (tube 13)** 1:5 and **Polymerase enzyme (40x) (tube 12)** 1:40 with Nuclease free H_2O.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate the slides in a humid chamber for 90 min at 37°C.
- Decant the solution and wash slides for 2x 5 min with TBST.

6.10   Detection Cells

NB 18. The detection oligos and nuclear counterstain are light-sensitive reagents. Always keep it protected from light. This should also be taken into consideration during washing after the detection and nuclear counterstain step.

- In the same tube, dilute **Buffer D (10x) (tube 15)** 1:10 and **Detection oligos (40x) (tube 14)** 1:40 with HSB.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate slides in a humid chamber for 30 min at 37°C.
- Nuclear counterstain (e.g. Hoechst 33342): Rinse slide in TBST and then incubate with Hoechst 33342, diluted 1:5000 in TBS, for 5 mins at room temp.
- Decant the solution and wash slides for 1x 5 min with HSB, 1x 5 min with TBS and 2x 3 min with 0,2x TBS.
- Decant excess wash buffer from the slides and dry the slides quickly using a bench-top microcentrifuge with appropriate slide insert.
- Mount with a coverslip (#1.5 glass thickness) using a mounting solution of choice. Seal the edges of the cover glass with nail polish or similar if needed.
- Wait a minimum of 30 min before imaging.

6.11   Detection Tissue

NB 19. The detection oligos and nuclear counterstain are light-sensitive reagents. Always keep it protected from light. This should also be taken into consideration during washing after the detection and nuclear counterstain step.

NB 20. The heating of the HSB and TBS buffers could be performed as follows: Add buffer to Coplin jar and microwave at 750W for 7 seconds (alternatively, use a hotplate to heat buffer). If a higher temperature than 40°C is reached, adjust using relevant room temperature buffer to reach the correct temperature. Add slides to the heated buffer and quickly check that temperature stays at 40°C. The actual wash is then performed on an orbital shaker at room temperature.

- In the same tube, dilute **Buffer D (10x) (tube 15)** 1:10 and **Detection oligos (40x) (tube 14)** 1:40 with HSB.
- Vortex the complete assay mixture gently, spin down.
- Add assay mixture to the entire sample area.
- Incubate slides in a humid chamber for 30 min at 37°C.
- Decant the solution and wash slides for 1x 5 min with HSB (preheated to 40°C), 1x 5 min with TBS (preheated to 40°C).
- Nuclear counterstain (e.g. Hoechst 33342): Rinse slide in room temperature TBST and then incubate with Hoechst 33342, diluted 1:1250 in TBS, for 5 mins at room temp.
- Decant nuclear counter stain solution and wash slides for 2x 3 min with 0.2x TBS.

6.12   Mounting

NB 21. The reagents on the glass slide are light sensitive, keep protected from light.

- Decant excess wash buffer from the slides and dry the slides quickly using a bench-top microcentrifuge with appropriate slide insert.
- Mount with a coverslip (#1.5 glass thickness) using a mounting solution of choice. Seal the edges of the cover glass with nail polish or similar if needed.
- Wait a minimum of 30 min before imaging.

7. Image Acquisition, Processing and Analysis

7.1   Image Acquisition

Image your slides with a widefield or confocal fluorescent microscope with filters/detectors matching the ATTO565 emission spectra (emission 575-620 nm, Texas red filter or similar) for protein A or ATTO647N (657nm-750 nm, Y5 filter or similar) emission spectra for protein B. If a nuclear counter stain was used, then the DAPI filter/detector should also be applied.

For image analysis purpose, images should be acquired with microscopy settings that correspond as close to the Nyquist rate as possible. This essentially means that imaging parameters are set with high enough resolution in x and y. This ensures that images have sufficient spatial resolution, which in turn enables post-image deconvolution and single object segmentation during image analysis.

To achieve the Nyquist rate, it is advisable to image the MolBoolean slides with as high magnification and numerical aperture objective as possible (63x or 100x objective). Preferably, images should be acquired using a confocal microscope, although a widefield fluorescent microscope could also be sufficient.

The necessary microscopy parameters to achieve the Nyquist rate can be calculated using the free calculator on the Scientific Volume Imaging website: *https://svi.nl/NyquistCalculator.* By entering the specific microscope and image parameters for the experiment (microscope type, numerical aperture, maximum excitation and emission wavelength for fluorophores and refractive index of immersion medium), the optimal x and y pixel size will be calculated. Aim to adjust the zoom and scanning settings for the image acquisition to achieve x and y pixel densities that are as close to the Nyquist sampling as possible.

7.2   Image Processing

To help achieving an efficient segmentation of RCPs (explained in section 7.3), it is advisable to perform post-image deconvolution prior to image analysis. Image deconvolution removes the "noise" (Point Spread Function-PSF) around objects in a microscope image, improving their resolution. Image deconvolution can be performed using several image analysis programs, e.g. Huygens deconvolution by Scientific Volume Imaging *(license cost required).*

Several microscope image software includes integrated post-image deconvolution options.

Image deconvolution could also be performed with the freeware ImageJ, using certain deconvolution plugins. The following sections describe one approach to perform image deconvolution in ImageJ. Instructions are included on how to download ImageJ and the necessary plugins (section 7.2.1), and how to use them for image deconvolution (sections 7.2.2 and 7.2.3)

For a visualization of how to perform the deconvolution procedure, watch the tutorial video available on the MolBoolean product landing page (or visit atlasantibodies.com).

7.2.1   Download Image J Software and Plugins

The first time deconvolution is performed, ImageJ needs to be downloaded and installed.

- Download ImageJ from Download (imagej.net). The installation file will be in the form of a .zip file.

- Move the downloaded file to a folder on the computer desktop and then right click and select ´extract all´.

- When prompted, uncheck the box that says 'show extracted files when complete' and then select 'extract all'.

- A new folder with the same name as the downloaded file should appear after a few minutes of extraction. Inside that folder is a folder marked 'ImageJ', containing the 'ImageJ. exe', which will launch the ImageJ application. Pin the ImageJ.exe to a location of choice.

  Next, two Image J plugins will need to be downloaded and installed.

- The first plugin is called 'Parallel Spectral Deconvolution'. Retrieve the plugin as a zip folder (named 'Parallel Spectral Deconvolution file') from the MolBoolean product landing page (or visit atlasantibodies.com).

- Move the downloaded 'parallel_spectral_ deconvolution-1.9-bin.zip' folder to the 'ImageJ' folder (created during the download of the ImageJ software). Then move it to the folder marked 'plugins'.

- Extract the files by right clicking the zip folder and select ´extract all´. Save the extracted files in the folder marked 'plugins'. Ensure that the extracted file folder is given the name 'parallel_ spectral_deconvolution-1.9-bin'. If not, delete the zipped and extracted folders and repeat the procedure. When extraction is complete, delete the original zip file.

- The second plugin is called 'Diffraction PSF 3D'. Retrieve the plugin as a zip folder (named 'Diffraction PSF 3D file') from the MolBoolean product landing page (or visit atlasantibodies.com). This plugin will not need to be extracted. Simply open the zip folder and copy the 'Diffraction _PSF_3D.class' file to the ImageJ>plugins location on the computer. With ImageJ and two plugins downloaded, open the ImageJ.exe application. An interface will appear *(Figure 2)*.

- Select the 'Plugins' menu and then click on 'Install' *(Figure 3A).* A window will open from where files can be selected.

- Find the ImageJ folder and then the Plugins folder inside it (created during the download of imageJ and plugins). Select the folder marked 'parallel_spectral_deconvolution-1.9-bin' and then select from inside it the folder marked 'ParallelSpectralDeconvolution'.

- Finally double click on 'parallel_spectral_ deconvolution-1.9.jar' *(Figure 3B).* After double clicking on this file, one will be prompted to save a copy, click save and the plugin will be installed. To summarize, the file path is: ImageJ>Plugins>parallel_spectral_ deconvolution-1.9-bin>ParallelSpectralDeconv olution>parallel_spectral_deconvolution-1.9.jar

- Repeat this process for 'parallelcolt-0.7.2.jar' *(Figure 3C).* Simply double click the file, it will not need saving. file path: ImageJ>Plugins>parallel_ spectral_deconvolution-1.9-bin>ParallelSpectr alDeconvolution>parallel_ parallelcolt-0.7.2.jar.

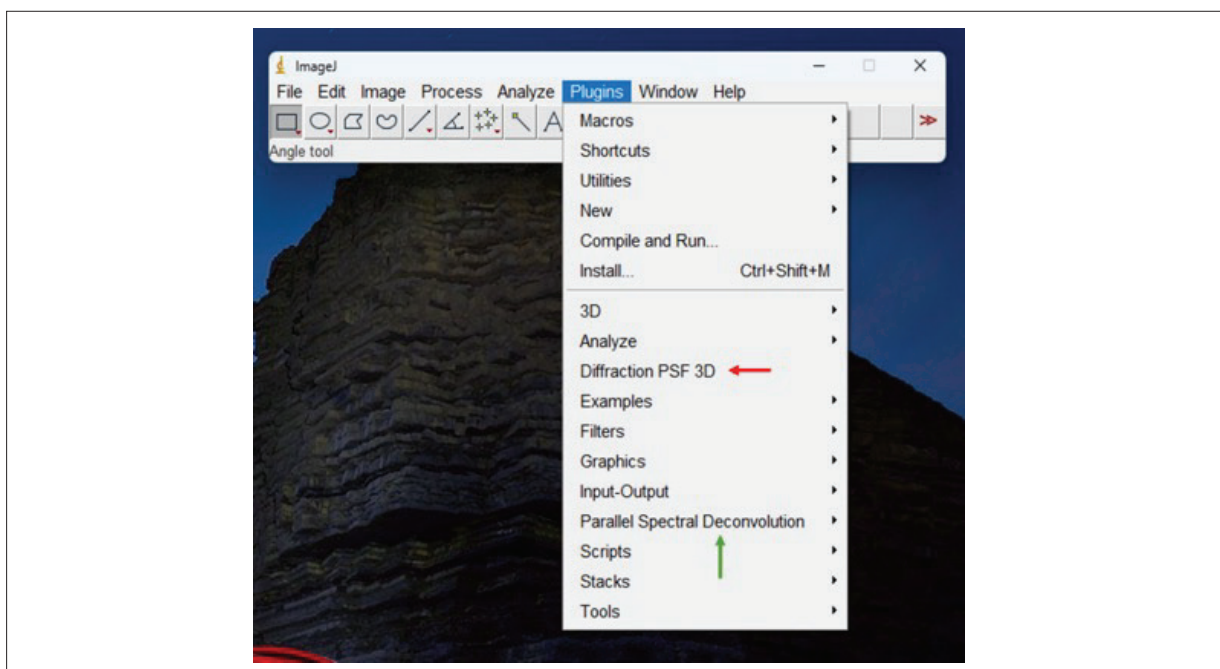- Close ImageJ and open it again to allow installation of plugins to finalize.
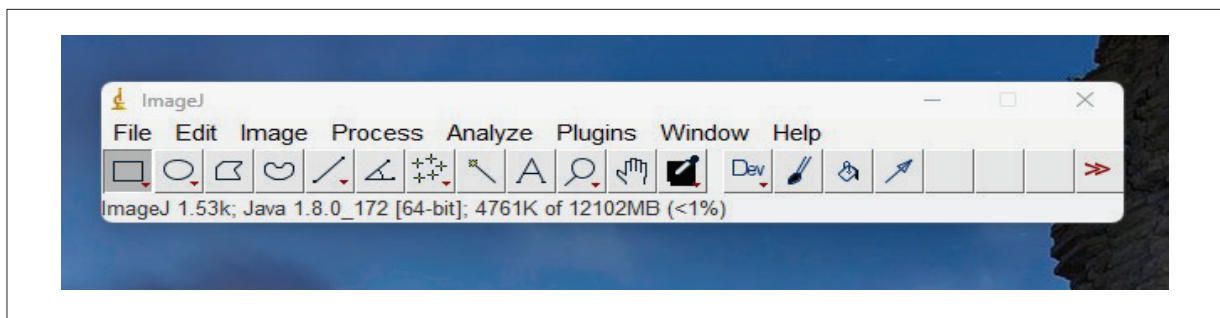
Figure 2. ImageJ interface
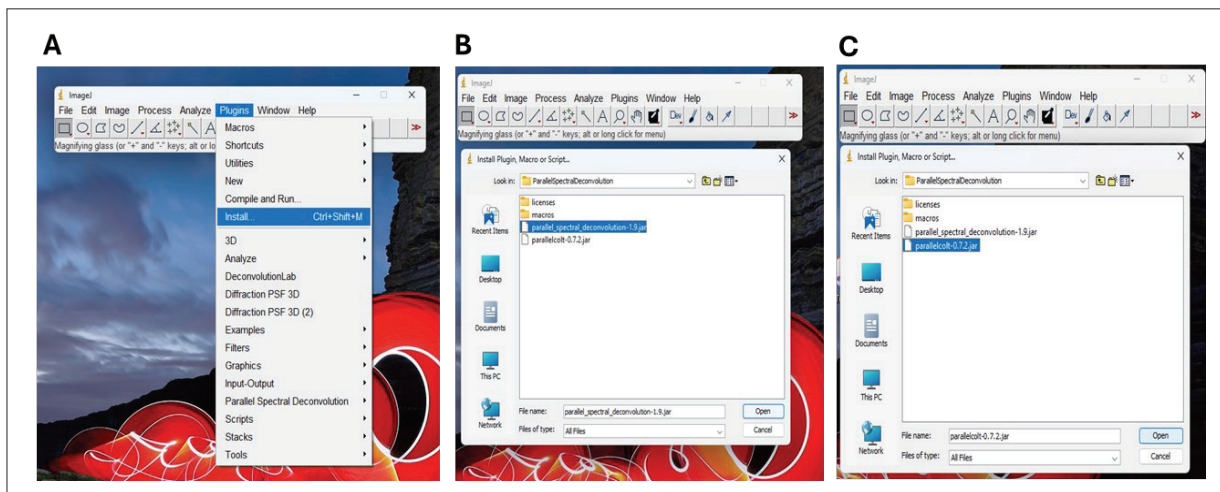
Figure 3. Installing plugins with ImageJ

Two new entries should now be seen in the ImageJ plugins menu as shown in **Figure 4** (red arrow 'Diffraction PSF 3D' and green arrow 'Parallel Spectral Deconvolution').

Figure 4. Plugins menu with installed plugins

7.2.2   Creating a Point Spread Function (PSF)

As mentioned in the introduction of section 7.2, when an object is imaged with a microscope, it will appear slightly "blurred" with a certain "noise" around the edges. This is known as 'the convolution of points by the microscope optics'. The amount of convolution is determined by the point spread function (PSF). It is possible to estimate the theoretical PSF based on the microscope image acquisition settings. Therefore, one can bring back the acquired image closer to the true objects, a process known as deconvolution.

This section describes the generation of a theoretical PSF (will be saved as a TIFF file) using the 'Diffraction PSF 3D plugin'.

A separate PSF image will have to be generated for each fluorescent channel that has been used during image acquisition, meaning that for the MolBoolean analysis, three different PSF images will have to be generated; 'PSF ATTO565', 'PSF ATTO647N' and 'PSF Hoechst 33342' (the nuclear counterstain marker, DAPI can also be used).

Note that the PSF images will only have to be generated once for the same image acquisition settings.

- Open the 'Diffraction PSF 3D' plugin from the ImageJ plugin menu (red arrow in *Figure 4*). A window will appear (pictured in *Figure 5A*). In this window there are 11 input fields that need input to be able to create a PSF. They are listed below:

1. Index for refraction of the media: Refers to the unitless refractive index for whatev:r immersion medium that was used on the microscope objective during imaging. If no immersion medium has been used (dry objective), the immersion medium will be air (the refractive index for air is 1.000), but if viewing through immersion oil or any other substance the index of refraction will have to be located. Often it is printed on the bottle or available online. Refractive index for conventional microscope immersion oils is 1.51.   The example images presented in this protocol used a 63x Plan apochromatic water immersion objective with 1.2 Numerical aperture from Leica microsystems (PL APO 63x/1.2 NA). Therefore, the refractive index for water (1.33) is entered in the input field *(Figure 5)*.

2. Numerical aperture: Numerical aperture should be printed on the objective itself along with the objective's magnification. In our example case, an objective with NA 1.2 was used.

3. Wavelength: This is the maximum emission wavelength for the fluorophore. For ATTO565 this is 590 nm.

4. Longitudinal spherical aberration: Simply enter 0.00 in in this field.

5. Image pixel spacing: If a confocal microscope was used, this is the pixel size for the generated image and should be entered in nanometres. The pixel size can be found in the image properties. In our example, we acquired the image with a pixel size of 0.051 μm. As this should be converted to nm, we enter 51 in the input filed.

If a widefield microscope was used and images acquired with a camera, the pixel size (μm) should be listed either online or in the manual for the camera. Divide the pixel size with the magnification of the objective lens. For example, if the pixel size of your given camera is 6.5 μm, and a 63x lens was used, divide 6.5 μm by 63 to get 0.1 μm. When converted to nanometers, the final pixel size is 100 nm.

6. Slice spacing (z): This number is important during 3D deconvolution but is irrelevant for 2D (simply put 0.00).

Note. This protocol describes the process of 2D-deconvolution. Even if 3D z-stacks have been acquired, deconvolve each slice of the z-stack individually (more details in section 7.2.3).

7. Width: This is the width (x) in pixels of the image that should be deconvolved. In our example the image width was 2400 pixels.

8. Heigh: This is the height (y) in pixels of the image that should be deconvolved. In our example the image height was 2400 pixels.

9. Depth, slices: For 2D images this number is always 1.

10. Normalization: This can simply be left as default 'Sum of pixel values = 1'.

11. Title: The name of the PSF image. Name the PSF image for the ATTO565 channel 'PSF ATTO565'.

- When all the numbers have been entered, click 'ok', then 'ok' again when the small window 'Rayleigh resolution' appears (*Figure 5B*). After a few seconds, a black image will appear containing a small white round-shaped object (red arrow in Figure 5C). This is the theoretical PSF for the ATTO565 fluorescent channel. Save the 'PSF ATTO565' image as a TIFF file in a new folder on the computer named 'PSF'.

- Now repeat the same procedure for the ATTO647N and Hoechst 33342 (or DAPI) fluorescent channels. The only detail that needs changing in the input fields is the 'Wavelength' and the 'Title'. The maximum emission wavelength for ATTO647N is 664 nm and for Hoechst 33342, 461 nm (DAPI, 465 nm). Name these images 'PSF ATTO647N' and 'PSF Hoechst 33342', respectively, and save them in the same folder as the 'PSF ATTO565' image.
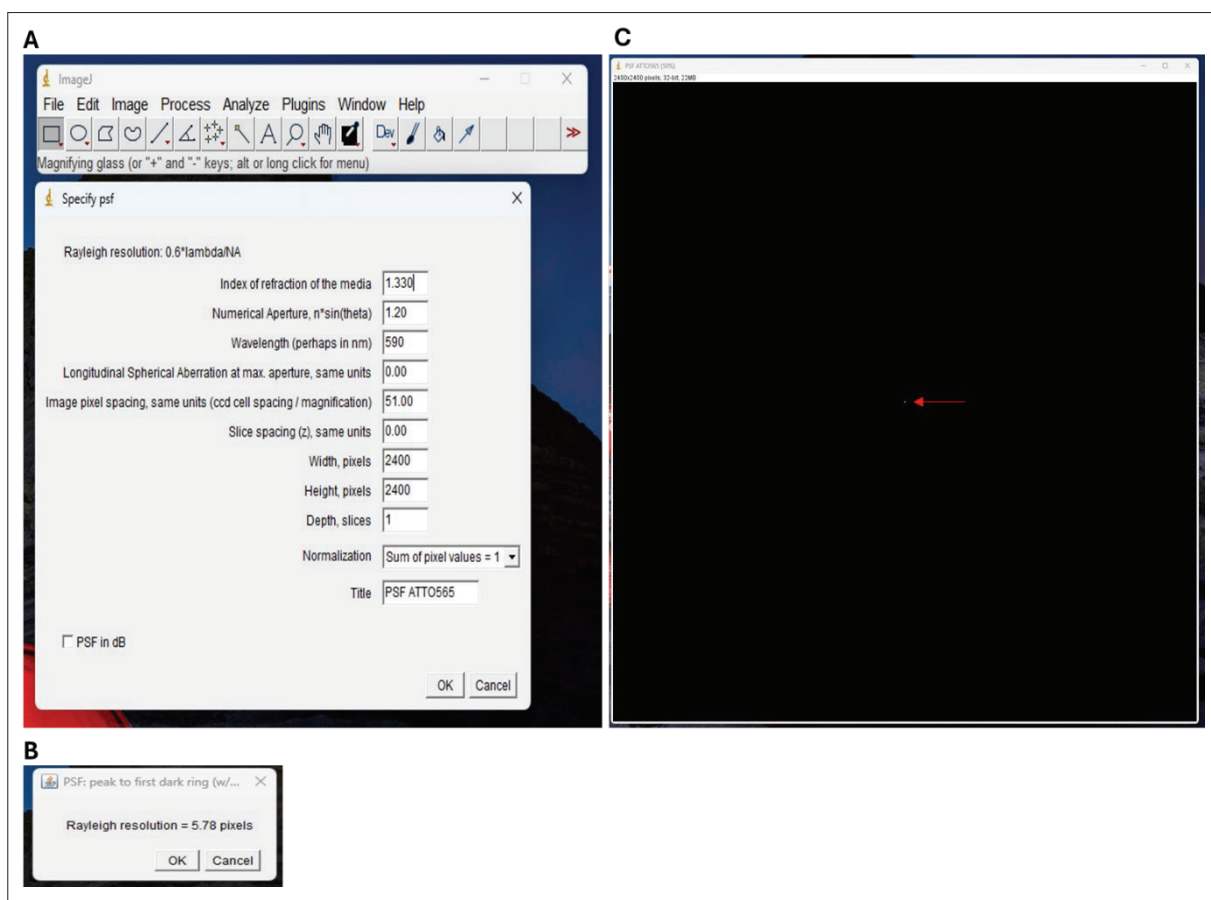
Figure 5. Diffraction PSF 3D Plugin window

7.2.3 2D Deconvolution

This section outlines the process of performing 2D deconvolution, i.e. deconvolution on a single image focal plane (single z slice). If 3D z-stacks have been acquired, it is important to perform individual 2D deconvolution on each z slice so that they can be analysed separately in CellProfiler (section 7.3). The reason being that, if a maximum projection image of the 3D z-stack is used, there is a risk of creating false positive overlapping fluorescent signals (protein interaction) from RCPs located in different z-slices.

- Start by exporting the fluorescent images from your microscope software. Export them as raw grey scale TIFF files (TIFFs). Export three separate TIFFs per image set, representing the Hoechst 33342 (channel 00 (ch00)), ATTO565 (ch01) and ATTO647N (ch02). Save the TIFFs in a new folder on your computer named 'Raw grey scale TIFFs'. If several image sets are to be analyzed, name them image1, image2 and so on.

In the example presented herein, MolBoolean was performed using a mouse anti e-cadherin antibody (detected in the ATTO565 channel, ch01), a rabbit anti β-catenin antibody (detected in the ATTO647N channel, ch02) and the nuclear counter stain Hoechst 33342 was used (detected in the DAPI channel, ch00). See Figure 6 for an illustration of the exported TIFFs. Note that three image sets have been exported (image 1-3), including three channels per image set (ch00, ch01 and ch02).

- Open the'PSF 565' image in ImageJ (File>open>location of the PSF 565.tif)

- Open the three grey scale TIFFs corresponding to the ATTO565 channel (files ending with ch01.tif).

- From the plugins menu select 'Parallel Spectral Deconvolution' (green arrow in **Figure 4**) and then click '2D Spectral Deconvolution". A window with multiple dropdown menus will appear as shown in **Figure 7**.
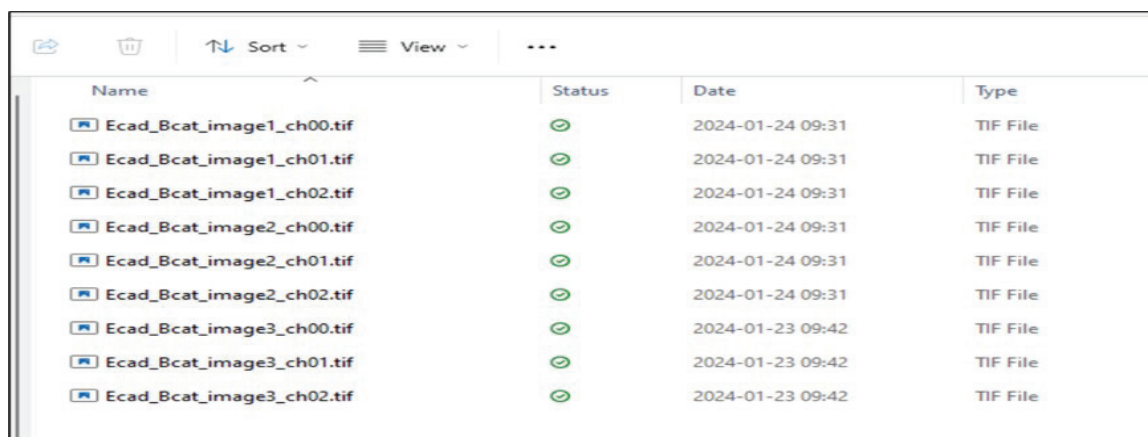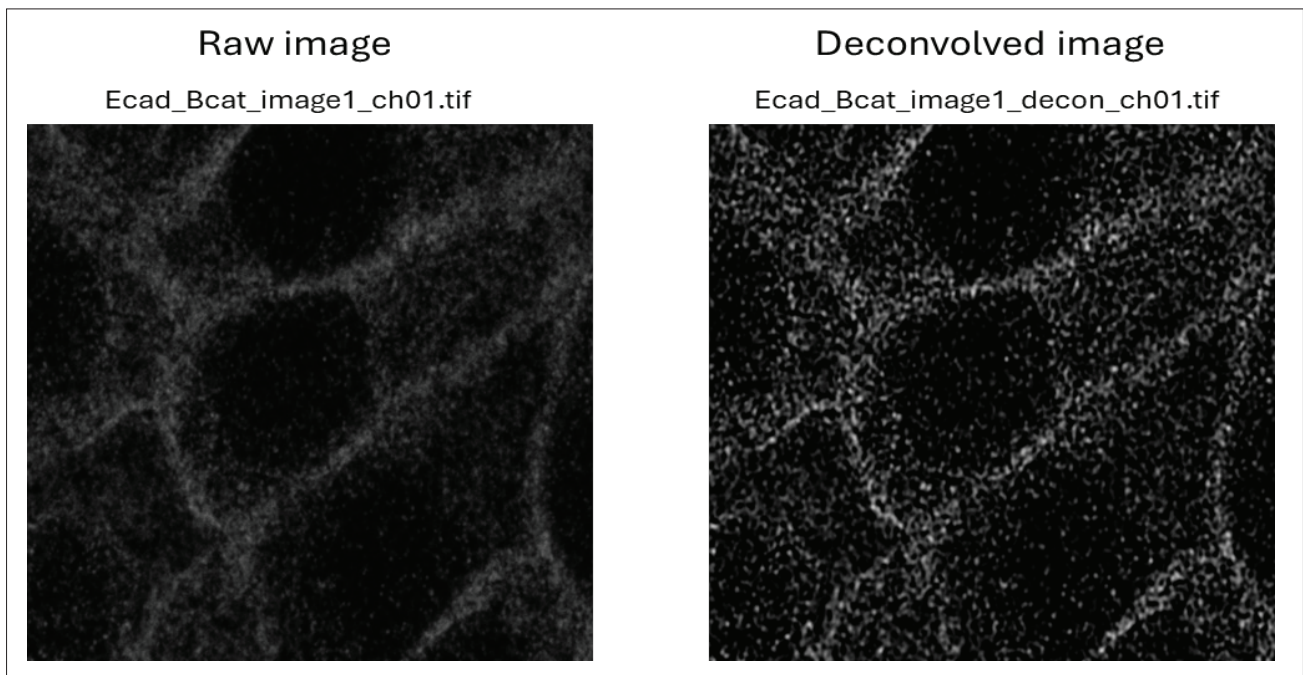

Figure 6. Exported raw grey scale TIFFs
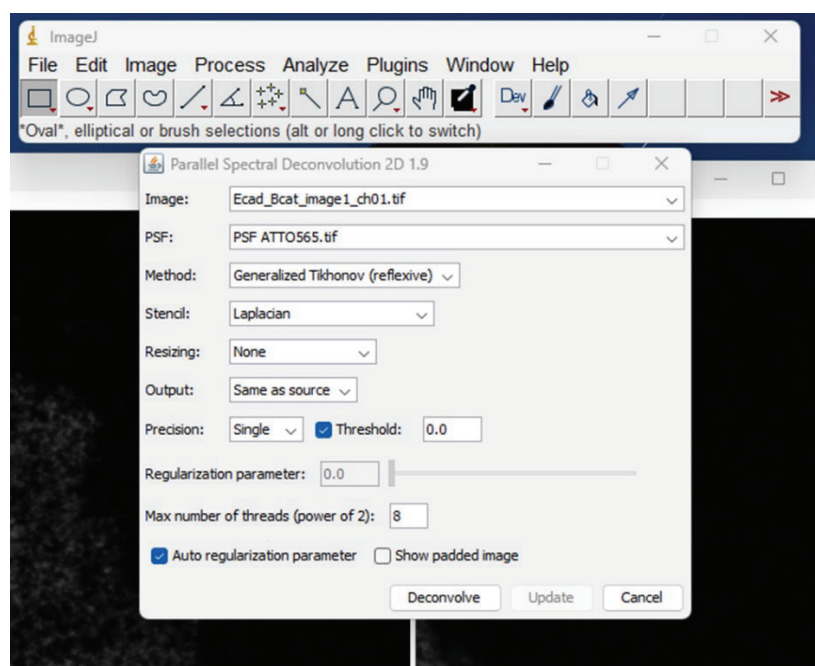
Figure 7. The Parallel Spectral Deconvolution Plugin

Figure 8. Comparison of a raw and deconvolved image.

- In the image drop down menu, select the greyscale image ('Ecad_Bcat_image1_ch01.tif' in Fig.7) and in the PSF dropdown menu, select the PSF image. All settings can be left per default (Fig. 7). Click 'deconvolve'. After a few seconds, a deconvolved image will appear (compare raw and deconvolved image in *Figure 8*).

- Save the deconvolved image as a TIFF file in a new folder on the computer, named 'Deconvolved images'. When saving, use the same file name as the raw images, but add '_decon_'. In the presented example the first image will be called 'Ecad_Bcat_image1_decon_ch01.tif'

- Repeat the same deconvolution procedure for image 2 and 3 for the ATTO565 channel (ch.01.tif).

- Now repeat the same procedure for the three ATTO647N (ch02) images and the three Hoechst 33342 (ch00) images, using the 'PSF ATTO647N' and the 'PSF Hoechst 33342' images, respectively. In the end, one should end up with nine deconvolved images.

7.3 Image Analysis

Image analysis is needed to calculate the fractions of free versus interacting proteins in the MolBoolean results. An important step in the image analysis workflow is to achieve a good segmentation of the rolling circle products (RCPs).

This allows for the specific quantification of the number of RCPs that are detected in only the ATTO565 and ATTO647N (from heron referred to as ATTO647) fluorescent channels, representing free non-interacting fractions for the two analyzed protein targets.

Furthermore, it enables the quantification of RCPs that are detected in both the ATTO565 and ATTO647 fluorescent channels, indicating protein interaction (proximity) in the sample.
Any image analysis software that can achieve such segmentation and quantification can be used to analyse the MolBoolean results.

One image analysis platform that can be used to analyse the MolBoolean results is the freely available Cell Profiler™ image analysis software, developed by the Broad Institute of MIT and Harvard. We have created a Cell Profiler pipeline dedicated to the MolBoolean analysis. The pipeline is available for download on the MolBoolean product landing page (or visit atlasantibodies.com).

For a visualization of how to perform the image analysis procedure in Cell Profiler™, watch our tutorial video available on the MolBoolean product landing page (or visit atlasantibodies.com).

7.3.1 Download Cell Profiler and MolBoolean Pipeline

The first time MolBoolean image analysis is performed one will need to download the CellProfiler™ software.

- Download Cell Profiler™ from this site: https://cellprofiler.org/.
- Pin the CellProfiler.exe to a location of choice.

With Cell Profiler downloaded and installed, the MolBoolean pipeline can be utilized.

- Download the Cell Profiler pipeline named 'MolBoolean image analysis.ccproj' from MolBoolean product landing page (or visit atlasantibodies.com). Save the file locally on the computer.

7.3.2 Performing MolBoolean Image Analysis in Cells

7.3.2.1 Open pipeline and upload images
- Start by clicking the 'MolBoolean image analysis. ccproj' pipeline file. This will open the Cellprofiler interface, and the MolBoolean image analysis pipeline will automatically upload (*Figure 9*). Minimize the 'Welcome to CellProfiler' window.

- In the 'Images' module' (red arrow in *Figure 9A*), add the deconvolved images (saved in section 7.2.3) by 'drag and drop' where indicated. The images should now be visible in the image window, with three separate fluorescent channels per image set (ch00.tif, ch01.tif and ch02.tif) (green arrow in *Figure 9B*).

- Next, the 'Metadata' module gives the option of extracting information from the image file names (i.e, metadata) that will be stored along with the measurements. The metadata to be extracted is defined using a 'Regular expression' input field. As a default, the MolBoolean image analysis pipeline extracts the 'image number' from the file names (see underlined text in *Figure 10*). If other metadata is to be extracted from file names, one needs to update the regular expression field accordingly.

- In the next module 'NamesAndTypes', names are assigned to the image files originating from the three different fluorescent channels. As a default, the images from ATTO565 and ATTO647 channels should contain _ch01 and _ch02, respectively. The nuclear counter stain Hoechst 33342 channel should contain _ch00. If an alternative way is used to differentiate between the fluorescent channels, update the naming criteria accordingly.

- Click update at the bottom of the window (Red arrow in *Figure 11A*). The image files will be sorted according to the assigned naming criteria (green arrow in *Figure 11B*).
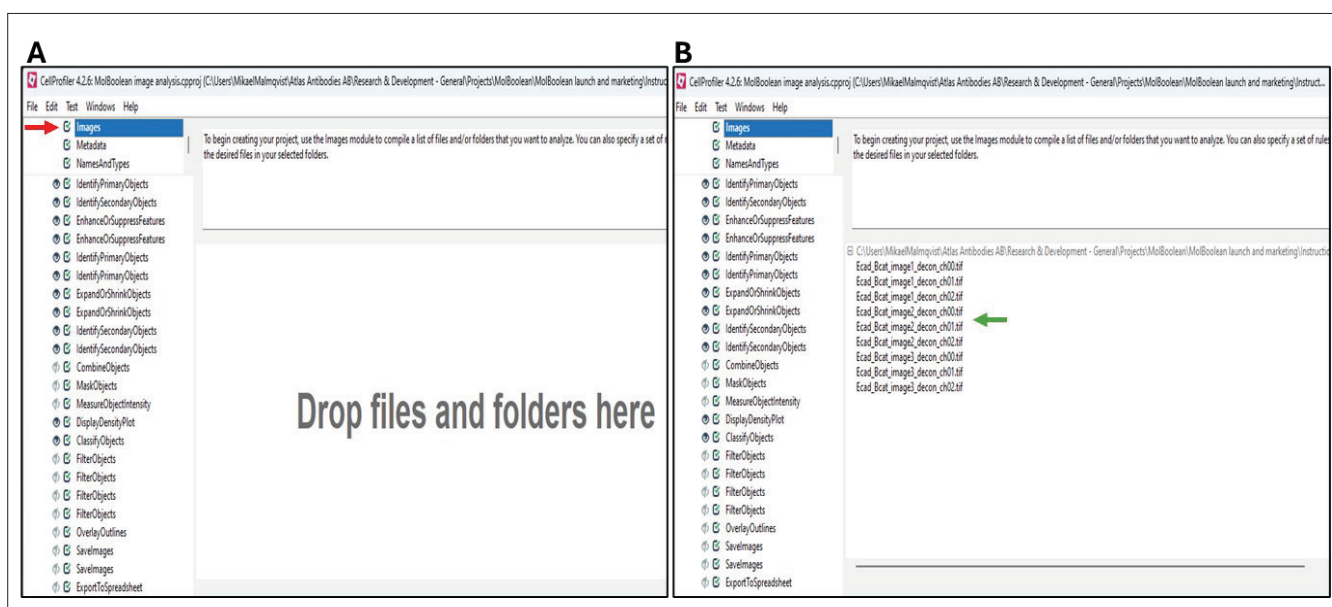
Figure 9. Opening MolBoolean image analysis pipeline and adding image files.
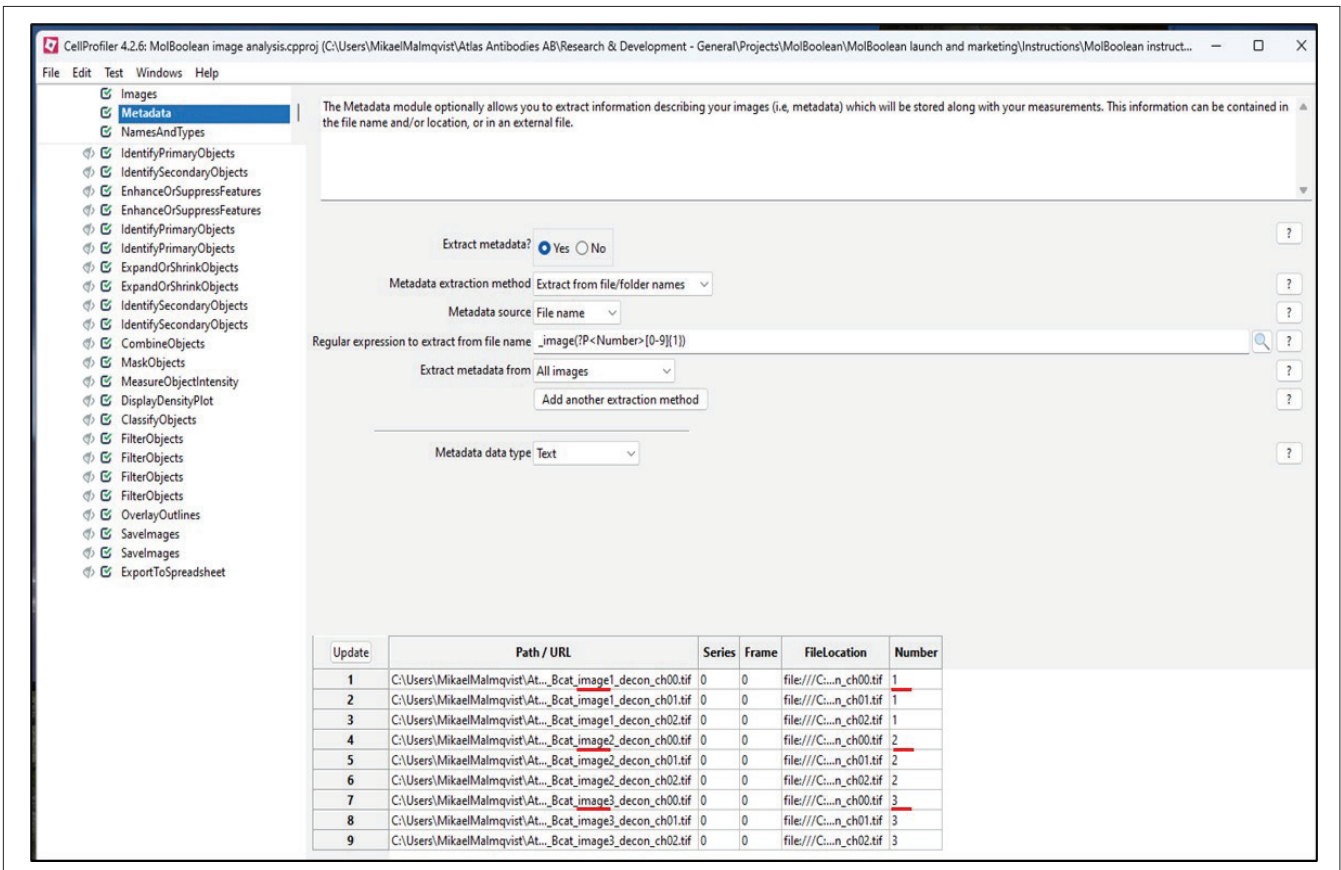
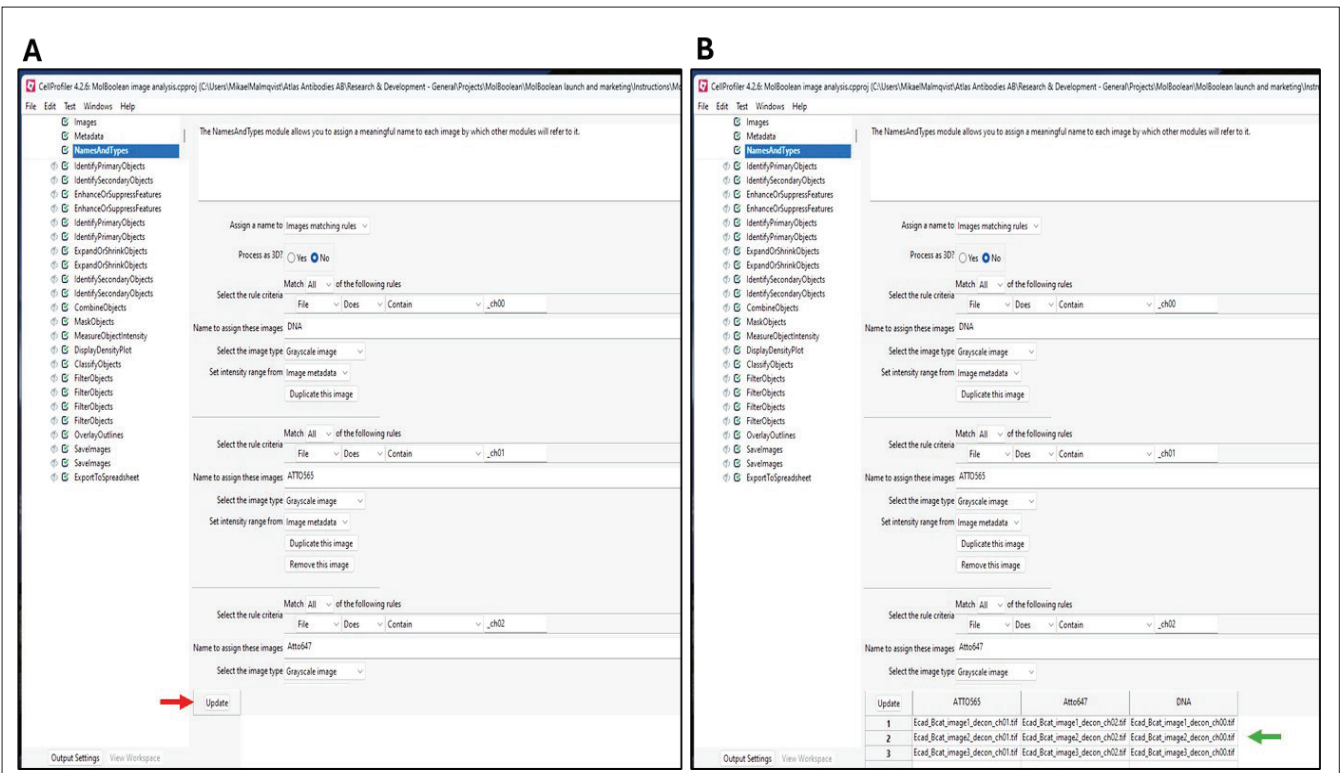**Figure 10.** Extraction of metadata from image files

Figure 11. Assign names to image files.

7.3.2.2 Test the pipeline settings in Test mode

The MolBoolean image analysis pipeline is composed of a series of modules that will ultimately segment and quantify the number of RCPs with fluorescent signal in the ATTO565 and the ATTO647 channels. The modules are pre-loaded with default settings. However, these settings may have to be altered slightly based on the image parameters of the experiment. CellProfiler permits the testing of settings in 'Test mode' before performing the final image analysis.

- Click 'Start Test Mode' at the left bottom corner of the window (see red arrow in *Figure 12A*). The current module is indicated by a green arrowhead (*Figure 12B)*. To visualize the output from a module in test mode, make sure that the module output is activated and visible (click the 'eye' symbol and ensure that the green tick is present, red arrows in *Figure 12B*).

- The modules that should be tested in 'Test mode' are listed below. For a better visualization of how to test the settings, watch our tutorial video available on the [MolBoolean product landing page](#) (or visit atlasantibodies.com).

1. Identify Primary Objects
The aim of this module is to select the number of nuclei in the images. At first, keep the default settings and click 'Step' at the bottom of the window (always try the default settings first) (red arrow in *Figure 12C*). CellProfiler will open a new output window showing the identified nuclei in the image (*Figure 13A*). This window will display the input grey scale image in the top left image field (ch00.tif) and the nuclei outlines in the bottom left image field. The identified nuclei are highlighted in the top right image field and counted in the table '#number of accepted objects'.

If some nuclei from the input image are not selected, adjust the input fields in the 'Typical diameter of objects' (red arrow in *Figure 13B*). Try a few different 'Min' and 'Max' input numbers so that each nucleus is counted only once. One could use the 'Measure length' tool (red arrow in *Figure 13A*) to measure the diameter of the nuclei. One could also use the 'Median diameter' measurement in the table (green arrow in *Figure 13A*) as an indicator.

To avoid picking up sub structures within the same nucleus, try to not set the 'Min' diameter any less than the smallest nuclei in the image.

Once a change has been made in any of the inputs, one will need to click 'step' (red arrow in *Figure 12C*) again and CellProfiler will generate an updated output file.

If the correct number of nuclei are still not identified in the image, try to change the 'Two-class or Three-class thresholding' setting from 'Two classes' to 'Three classes' (red arrow in *Figure 13C*). Try either 'Foreground' or 'Background' option (green arrow in *Figure 13C*).

NB 22. To use a different image set during test mode (i.e. switching from image set 1 to image set 2 or 3), click 'Next Image Set' (green arrow in *Figure 12C)*.

NB 23. It may not be possible to efficiently determine the number of nuclei in the images. For example, it could be difficult in tissue images where the nuclei are often less uniform. Follow instructions in section 7.3.3 for how to analyse such images.

2. Identify Secondary Objects
The aim of this module is to outline the cell borders. It is within these areas that the number of RCPs will later be counted for the respective channels. CellProfiler will generate an output window showing the outlined cell borders (*Figure 14A*).

If some of the cells are missing, try to change the 'Select the method to identify the secondary objects' setting from 'Propagation' to 'Distance-N'. Input the number of pixels by which to expand out from nuclei (see red arrow in *Figure 14B*).

NB 24. Following the comment in ***NB 23,*** if the number of nuclei cannot be determined in 'Identify Primary Objects', then it will not be possible to outline cell borders. See instructions in section 7.3.3 for how to proceed in those cases.

3. Enhance Or Supress Features
This module is performed for both the ATTO565 and ATTO647 channels. It aims to sharpen the RCPs in the images. This module is not necessary if images have been deconvolved sufficiently during image processing (section 7.2). However, for the functionality of downstream modules in the pipeline, it is important to still process the images though this pipeline.

The result window will display the input grey scale image in the left image field and the enhanced image (named 'FilteredATTO565' or 'Filtered ATTO647') (*Figure 15A*) in the right field.

If using deconvolved images, aim to select a 'Feature size' that will generate a 'Filtered' image that is unaltered from the original input image. It is recommended to select a feature size that is the same as the largest RCPs in the image (red arrow in *Figure 15B*).
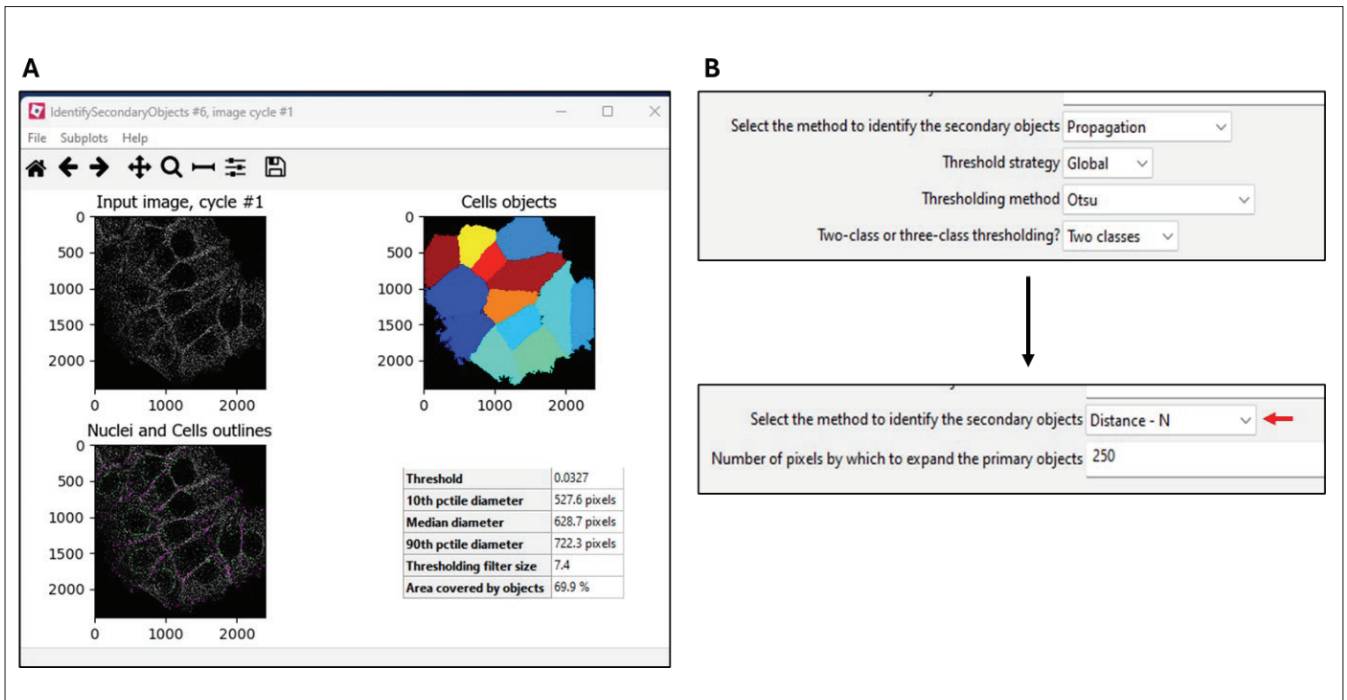
The 'DisplayHistogram' module, described below, will give an overview of the object sizes in the image. Once the size of the largest RCPs has been determined, one could go back to this module again and adjust the size accordingly.
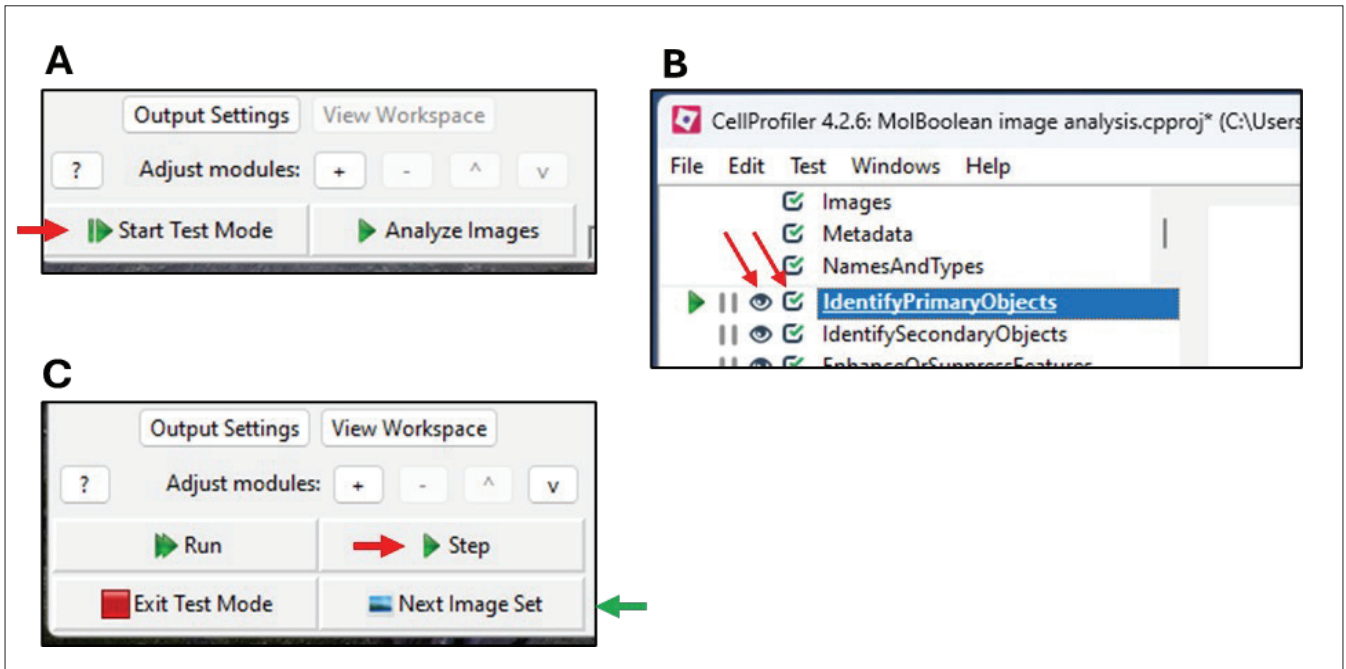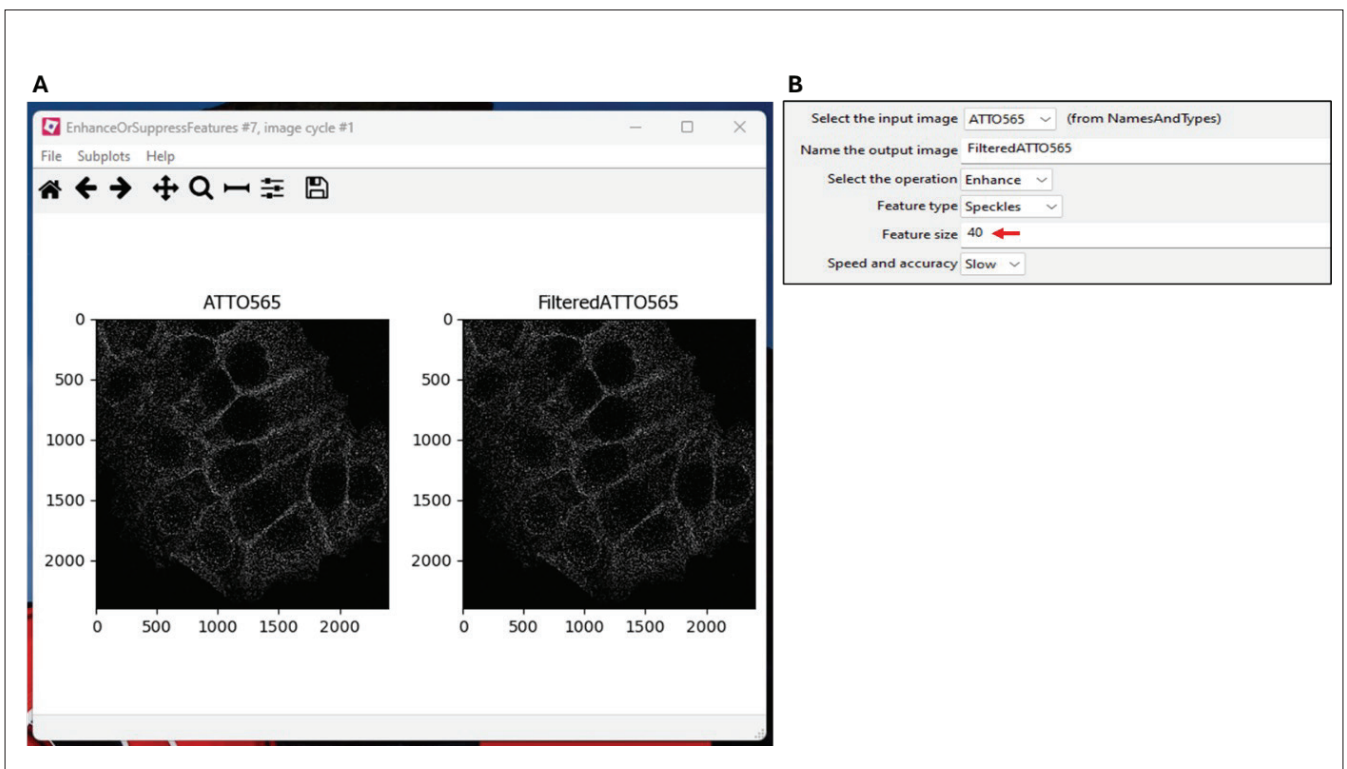

Figure 12. Start the Test Mode to test the CellProfiler settings
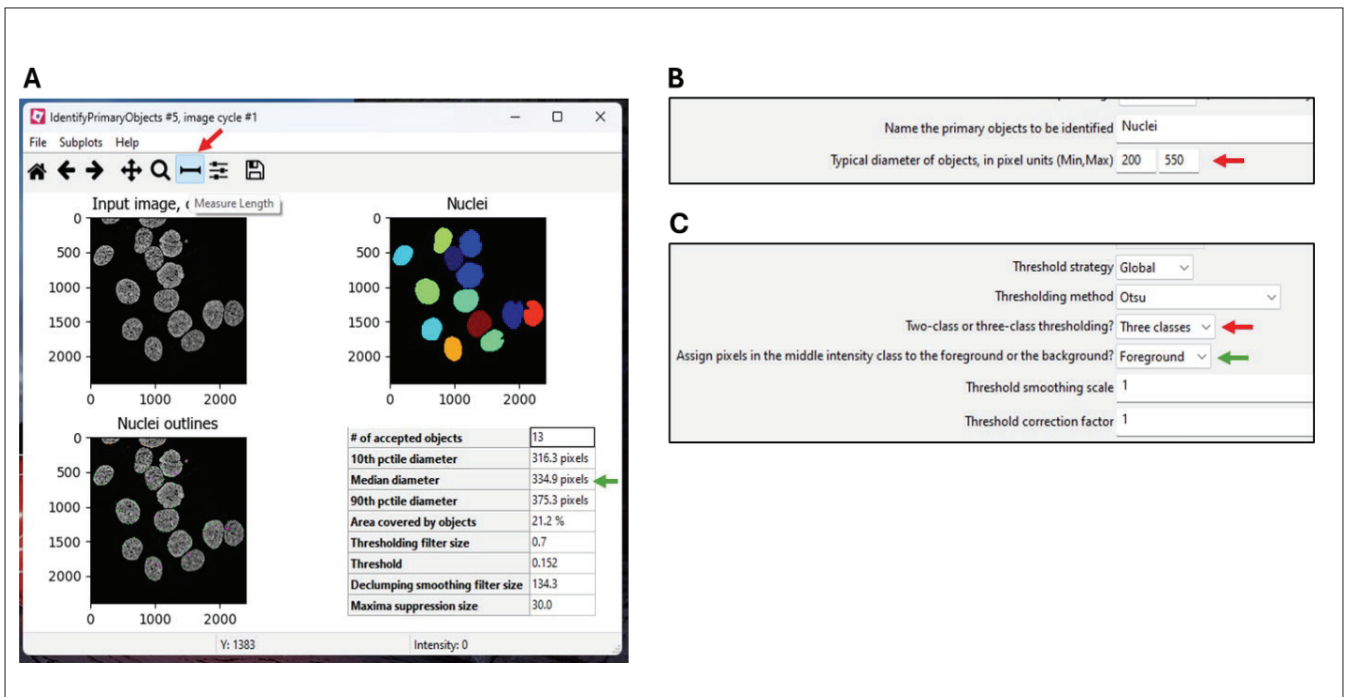
Figure 13. Identify nuclei in the images.

Figure 14. Outline of cell borders

Figure 15. Enhance the RCP objects in the images.

4. Identify Primary Objects

This module is performed for both the ATTO565 and ATTO647 channels. It aims to identify and segment all the RCPs in the image. At this stage, keep the default settings and move on to the next modules. Adjustments will not be made in this module until sizes of the RCPs have been measured in 'MeasureObjectSizeShape' and displayed in 'DisplayHistogram' modules.

### 5. Measure Object Size Shape

Use the default settings and move on to the next module.

6. Display Histogram

CellProfiler will generate an output window showing a size distribution of the RCPs in the image (*Figure 16A*). Use this histogram to visualize the size of the smallest and largest RCPs in the image. To get a closer look of the scale on the x-axis, one can use the magnification glass symbol (red arrow in *Figure 16B*) and select a region in the graph that should be enlarged (green arrow in *Figure 16B*). Use the enlarged images (*Figure 16C* and *D*) to determine the minimum and maximum RCPs sizes in the image. In the example presented here, this was empirically decided at 3 and 40 pixels, respectively (see red arrows in *Figure 16C* and *D*).

7. Now go back to the 'Enhance Or Supress Features' modules

and insert the maximum object size in the 'Feature size' field (red arrow *Figure 15A*). Note that when the input is changed in an earlier module, CellProfiler will automatically revert back to that module and one needs to click 'step' again (red arrow in *Figure 12C*) to progress to the next module.

8. In the 'Identify Primary Objects' modules,

in the 'Typical diameter of objects' insert the 'Min' and 'Max' values obtained in the 'DisplayHistogram' module (red arrow in *Figure 17A*). Click 'Step' and an output window will open showing the segmented RCPs that have been outlined by green borders (*Figure 17C*).

Use the magnification glass symbol (red arrow in *Figure 17C*) and select a region in the image that should be enlarged (boxed region in *Figure 17C*). In the enlarged image (*Figure 17D*), ensure that all the RCPs in the field have been selected, indicated by the presence of a green border around each object. If some RCPs are not selected, the 'Min' and 'Max' diameter threshold may have to be adjusted again. Alternatively, RCPs with low fluorescent intensities may have been discarded. To include weaker RCPs, the 'Manual threshold' may have to be lowered (red arrow in *Figure 17B*).
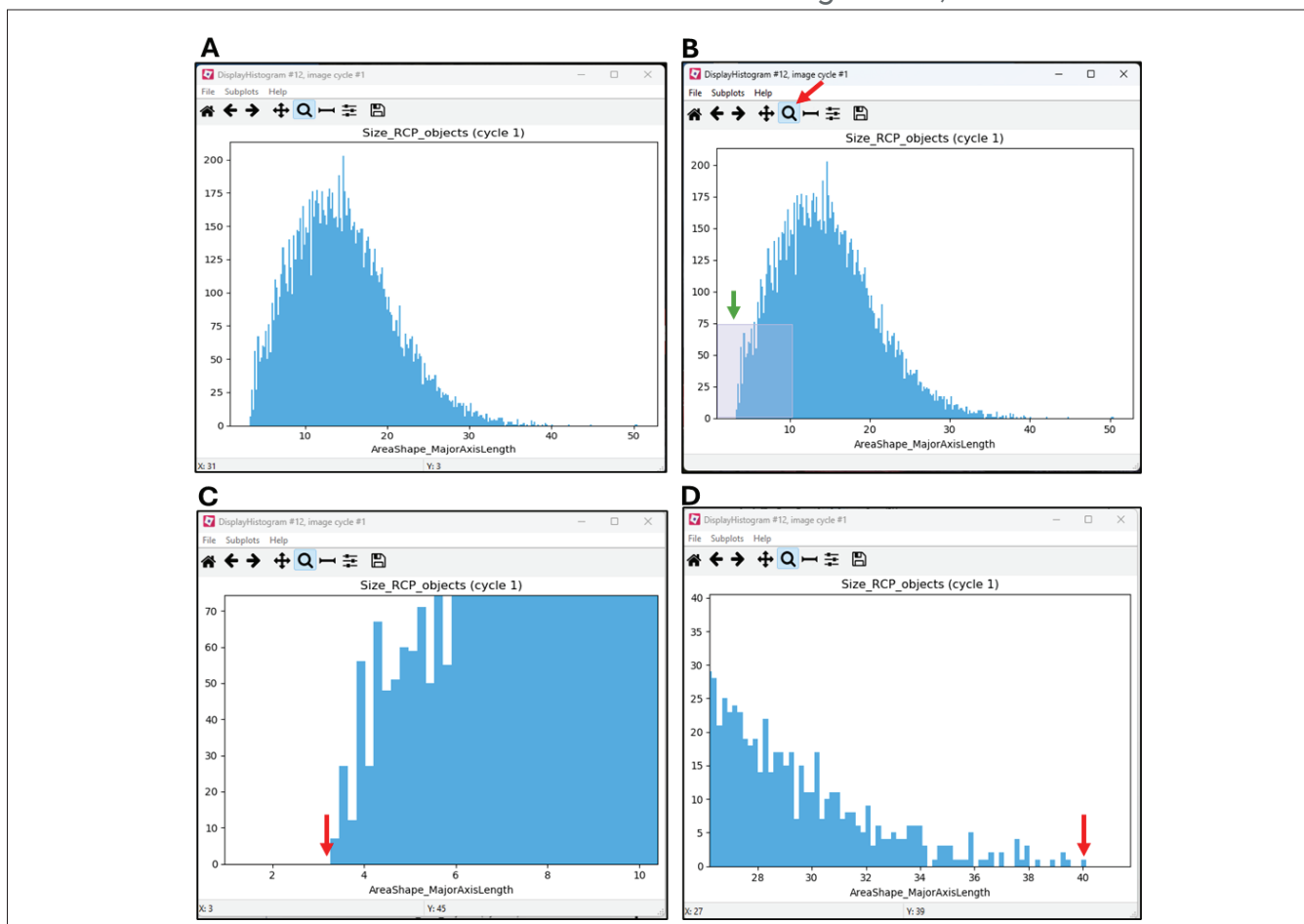
Figure 16. Size distribution of the RCPs
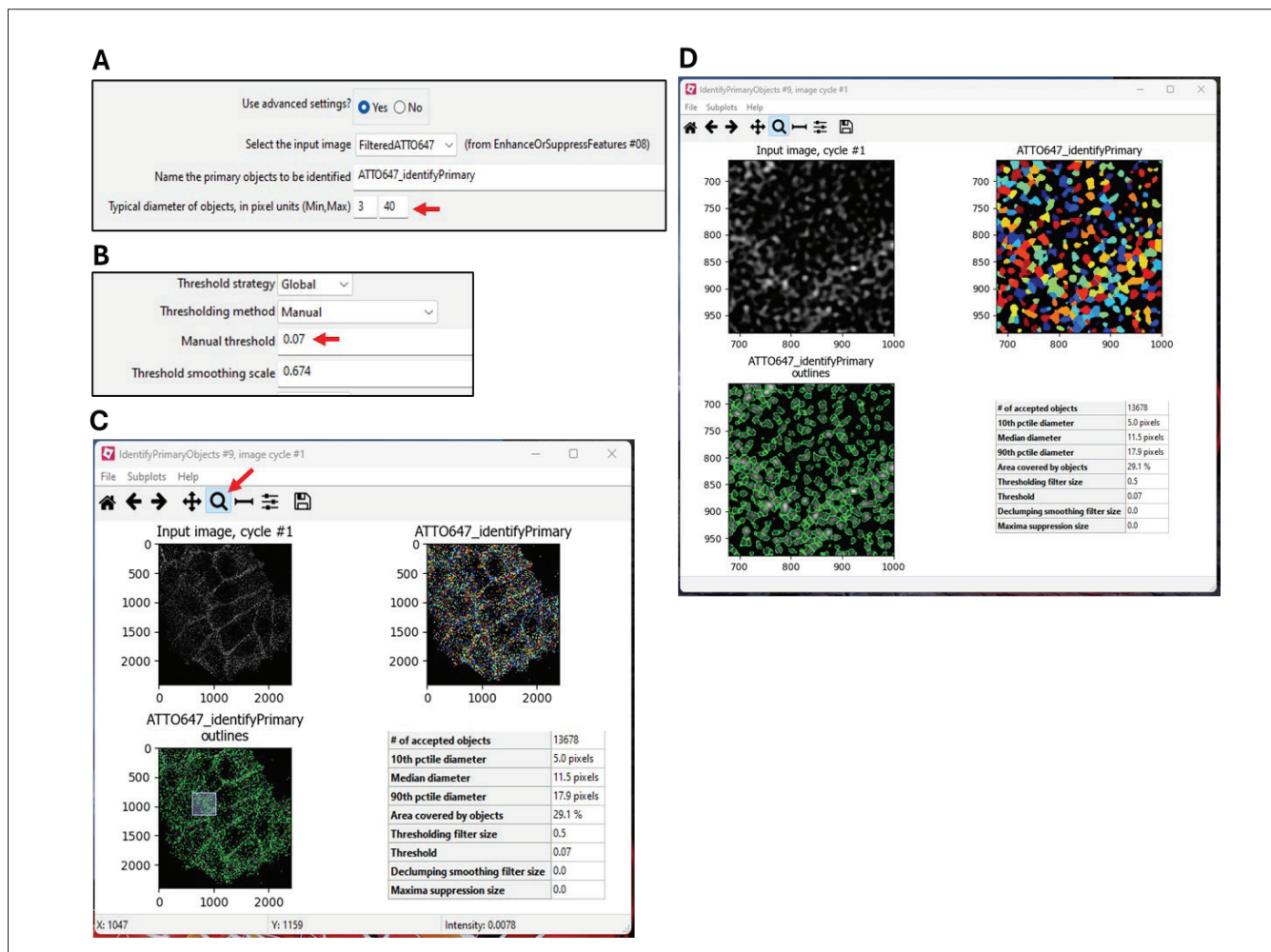
Figure 17. Segmentation of RCPs in the 'IdentifyPrimaryObjects' module.

9. Expand Or Shrink Objects

This module is performed for both the ATTO565 and ATTO647 channels. It aims to shrink all the RCPs into single pixels so that they can be expanded by a defined number of pixels (see next module), effectively reducing the area of each RCP where fluorescence intensity will be measured.

This procedure improves RCP segmentation and therefore lowers the risk of generating false positive protein interaction results, i.e. two closely located, but separate RCPs being falsely classified as overlapping (in the 'ClassifyObjects' module).

Use the default settings and proceed directly to the 'IdentifySecondaryObjects' module.

10. Identify Secondary Objects

This module expands the shrunken RCPs (from the 'ExpandOrShrinkObjects' module) by a defined number of pixels. Start by using the default settings (pixel expansion of 4) (*Figure 18B*). An output window will open (*Figure 18A*), showing the original RCPs but with and overlay of magenta-coloured circles, which represent the expanded area starting

from the single pixel objects generated in the 'ExpandOrShrinkObjects' module.

Use the magnification glass symbol and enlarge a selected region in the image. Aim to generate magenta circles that are covering as much of your original RCPs as possible, but without the circles touching each other. If, after expanding with a given number, the circles are touching (as illustrated in *Figure 18D*), reduce the expansion number until the circles are no longer touching (as illustrated in *Figure 18C*). Compare *Figure 18C* and *18D* where an expansion by 4 or 6 pixels has been made, respectively. One can observe higher degree of overlap between the magenta circles in *Figure 18D* compared to *18C*.

11. Combine Objects

This module combines the expanded ATTO565 and ATTO647 RCPs into one image to give all RCPs 'TotaRCPs'. Use the default settings and move on to the next module.

12. Mask Objects

This module will select the RCPs that are located within the cell borders (identified in the second module), naming them 'TotalRCPsInCell'. Use the default settings and move on to the next module.

NB 25. As discussed for module 1 and 2, if the number of nuclei and cells in the image cannot be determined, this module will not be used. See instructions in section 7.3.3 for how to proceed in those cases.

13. Measure Object Intensity

This module will measure the intensity for the 'TotalRCPsInCell' in the ATTO565 and ATTO647 channels, respectively. Use the default settings and move on to the next module.

14. Display Density Plot

The 'TotalRCPsInCell' are plotted based on their integrated intensities in the ATTO565 and ATTO647 channels. Keep the default settings and click 'step' to generate an output window displaying a density plot with the ATTO565 and the ATTO647 intensities on the x and y-axis, respectively (*Figure 19*). The density plot displays the distribution for the RCPs. High density areas, representing RCPs of similar fluorescent intensities in the two fluorescent channels, are indicated using a 'coolwarm' color map.

15. Classify Objects

This module uses the density plot to perform classification and filtering of RCPs as either 'ATTO565_RCPs', 'ATTO647_RCPs', 'overlapping RCPs' or 'background_RCPs'.

In the example presented herein (*Figure 19*), MolBoolean was performed for E-cadherin (ATTO565 channel) and β-catenin (ATTO647 channel) proteins. The RCPs generated from free E-cadherin and β-catenin proteins display intensities in only one of the fluorescent channels and are therefore lining the x or y-axis on the density plot, respectively. On the other hand, RCPs generated from interacting E-cadherin/β-catenin proteins have intensity in both fluorescent channels and will appear relatively high on both the x and y-axis of the density plot.

Add intensity cutoff values in the 'Enter the cutoff value' (red arrows in *Figure19C*) for the ATTO565 and ATTO647 channels. The cutoff values can be estimated based on the density plot. Enlarge the area near the lower part of the density plot. Draw an imaginary vertical line at the value on the x-axis where the main bulk of RCPs start to appear in the ATTO565 channel (*Figure 19B*).

Perform the same procedure for ATTO647 on the y-axis. In the example presented here, the cutoff values on both the x and y-axis have been rounded up to nearest whole number (*Figure 19C*).

Any RCPs with intensity above cutoff value on x-axis but below the cutoff value on the y-axis will be classified as ATTO565_RCPs (free E-cadherin protein) and vice versa for ATTO647_RCPs (free β-catenin protein). Any RCPs above both cutoff values will be classified as Overlapping_RCPs (interacting E-cadherin/β-catenin proteins).
*Figure 19D* shows an imaginary illustration of the RCPs after dividing them into different groups based on the cutoff values. The output window generated from this module will plot the number of RCPs in the image according to the cutoff values and classification bin names (*Figure 19E*).

NB 26. The cutoff values are set by manual interpretation. It is up to the user to decide the stringency for the classification of single versus overlapping fluorescent RCPs, which will ultimately affect the determined number of free versus interacting proteins in the output data.

- The remaining modules of the pipeline relate to the data output and do not need to be tested in Test mode. Move to 'Exit Test Mode', section 7.3.2.3.

7.3.2.3. Exit Test Mode

- Click 'Exit Test Mode' at the left bottom corner of the window (see red arrow in *Figure 20A*).
- Deactivate the 'MeasureObjectSizeShape' and DisplayHistogram' modules by removing the green tick (red arrows *Figure 20B*). These modules were only used as help when setting the RCP size thresholds and should not be part of the image analysis.
- Deactivate the 'eye' symbol from all the modules (red arrows in *Figure 20C*).
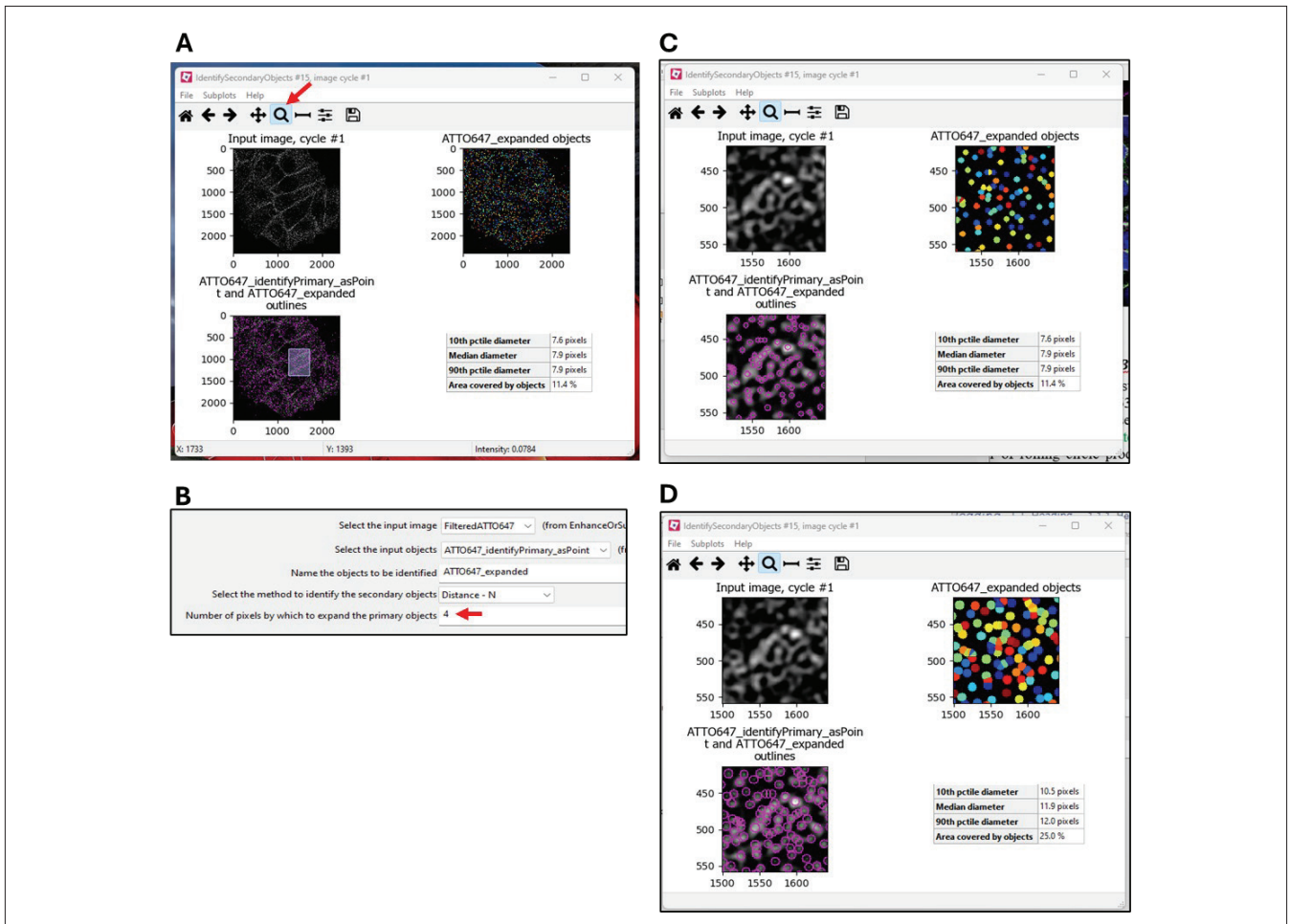
Figure 18. Expansion of shrunk RCPs by a defined number of pixels.
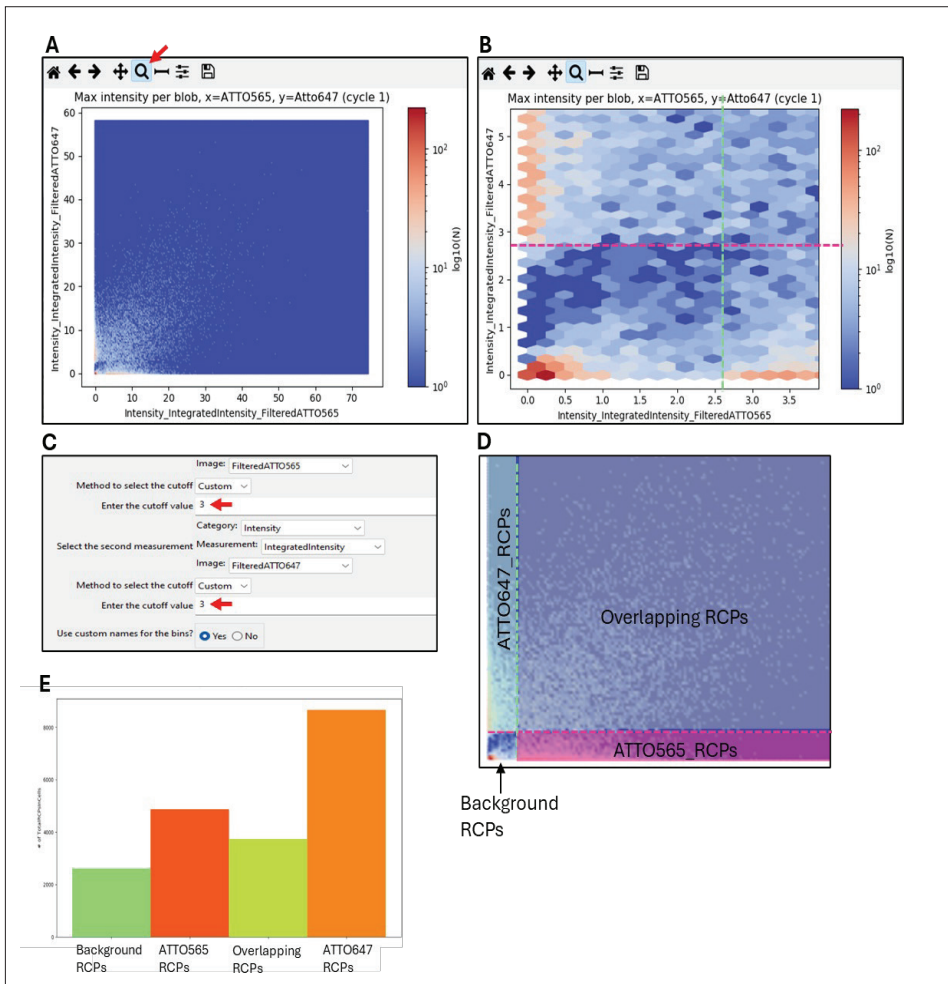
Figure 19.
Choosing cutoff values to classify RCPs based on fluorescent intensity in ATTO565 and ATTO647 channels.

Figure 20. Steps involved when exiting the test mode.

7.3.2.4 Generation of image analysis data

- In the 'SaveImages' and 'ExportToSpreadsheet' modules (red arrows in *Figure 21A*), in their respective sections for the 'Output file location'- 'Sub-folder' (red arrow in *Figure 21B*), choose a location on the computer where output data should be saved.
- In the 'ExportToSpreadsheet' module, name the output files with a 'Filename prefix' (red arrow in *Figure 21C*).
- Start image analysis by clicking the 'Analyze Images' at the left bottom corner of the window (red arrow in *Figure 21D*).
- The time it takes to analyze images depends on the number of image sets (1 set consists of three tiffs; ch00, ch01 and ch02). To process three image sets takes roughly 5 minutes.

7.3.2.5 Data output and processing

- For each image set, CellProfiler™ will generate an output image file ('_classified_RCPs.tiff'). It shows the distribution of the RCPs in the cells, classified according to the cutoff values set in the 'ClassifyObjects' module (see *Figure 22A*). An additional image is also generated, showing the same classified RCPs with nuclei outlines ('_OrigOverlay.tiff') (image not shown here).

CellProfiler™ will also generate two CSV files: one is named 'Experiment.csv' that lists the modules that were used during the CellProfiler run. The other file, 'image.csv', contains output data on the number of RCPs that were quantified in the different classification groups.

The following steps describe how to process the data located in the 'image.csv' file.

- The 'image.csv' file is comma delimited. To convert the text format to columns, start by highlighting the first column in the file (green arrow in *Figure 23A*).
- In the 'Data' menu, select the 'Text to Columns' option. In the window that appears, select the 'Delimited' option and click 'Next' (see red arrows in *Figure 23A*).
- In the following window, make sure that 'Comma' and 'Tab' is selected (red arrows in *Figure 23B*) and then click 'Finish' (green arrow in *Figure 23B*).
- The data is now sorted into columns. If needed, convert all the decimal separators from dot to comma. Save the file on the computer as an Excel Workbook file.
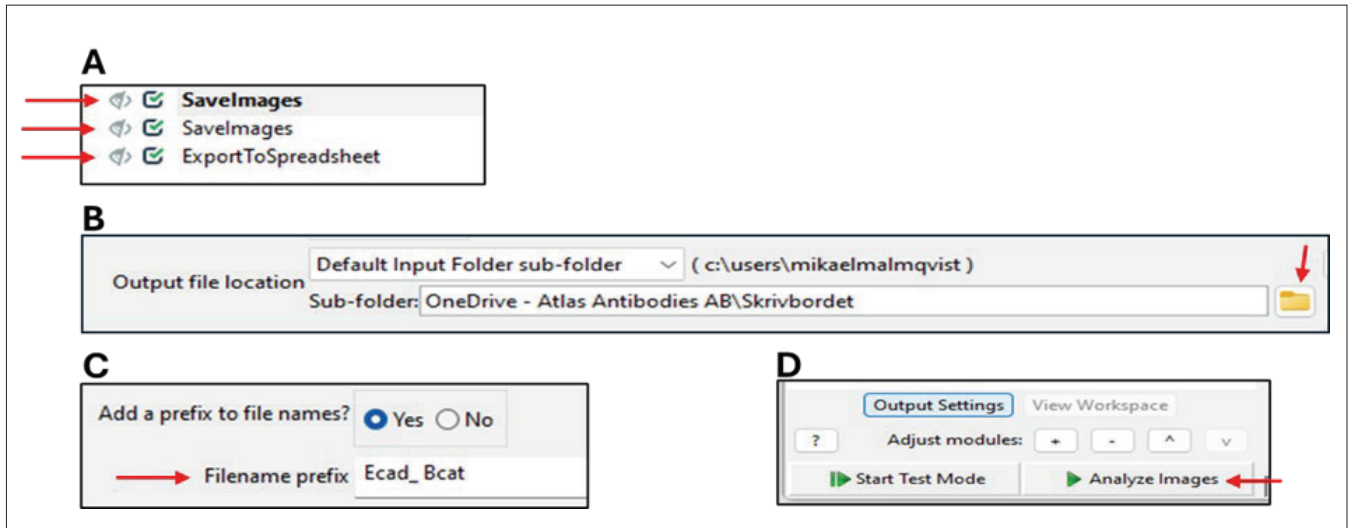
Figure 21. Steps involved when starting the image analysis.
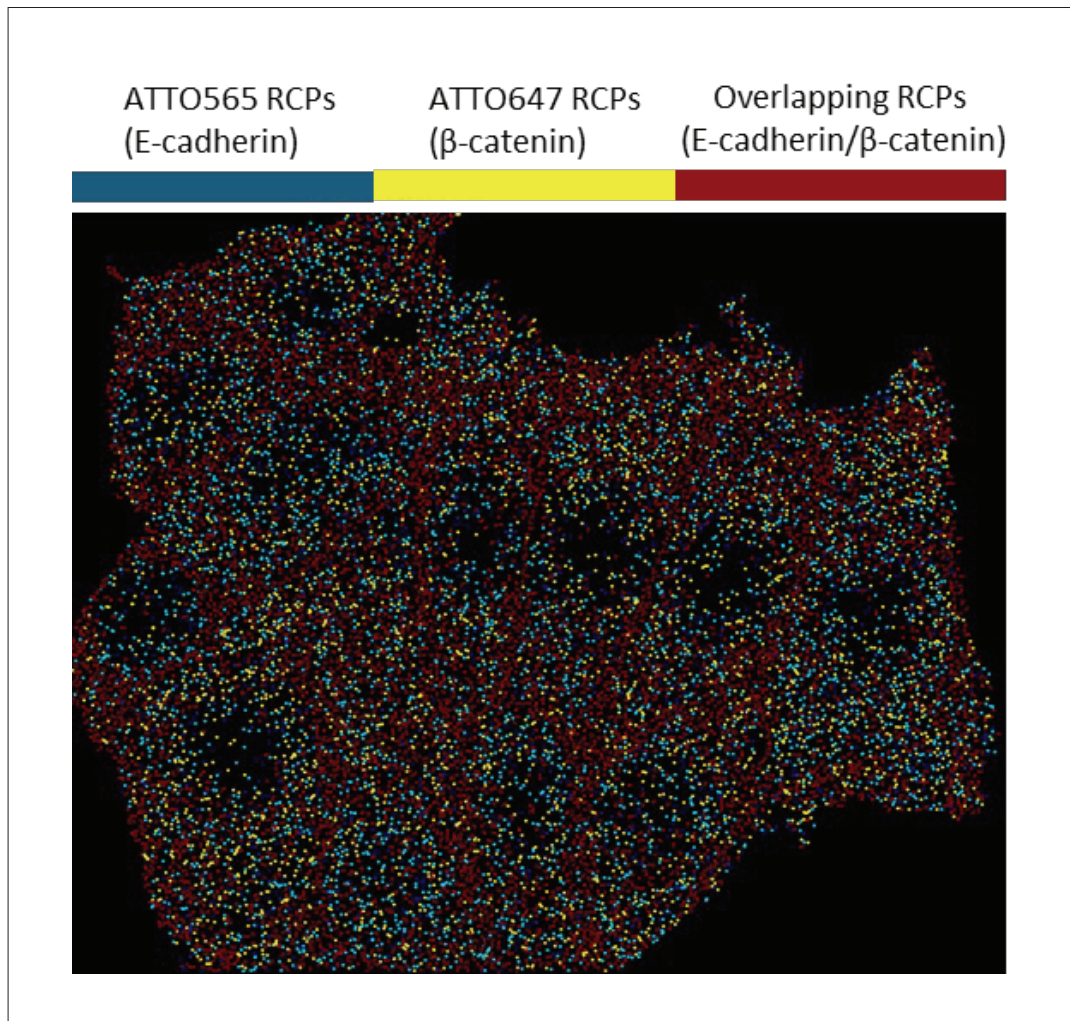
Figure 22. Distribution of RCPs in cells, separated in three different classification groups.
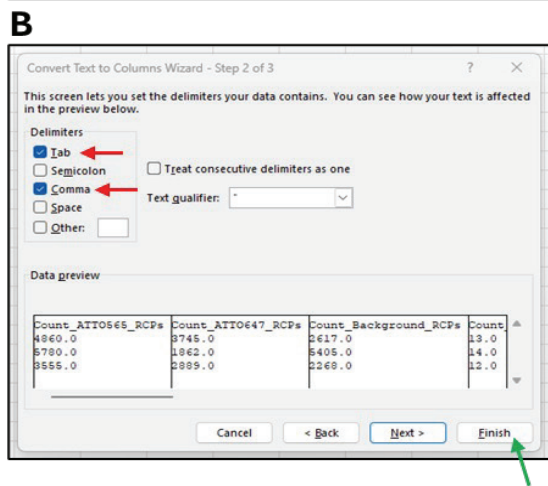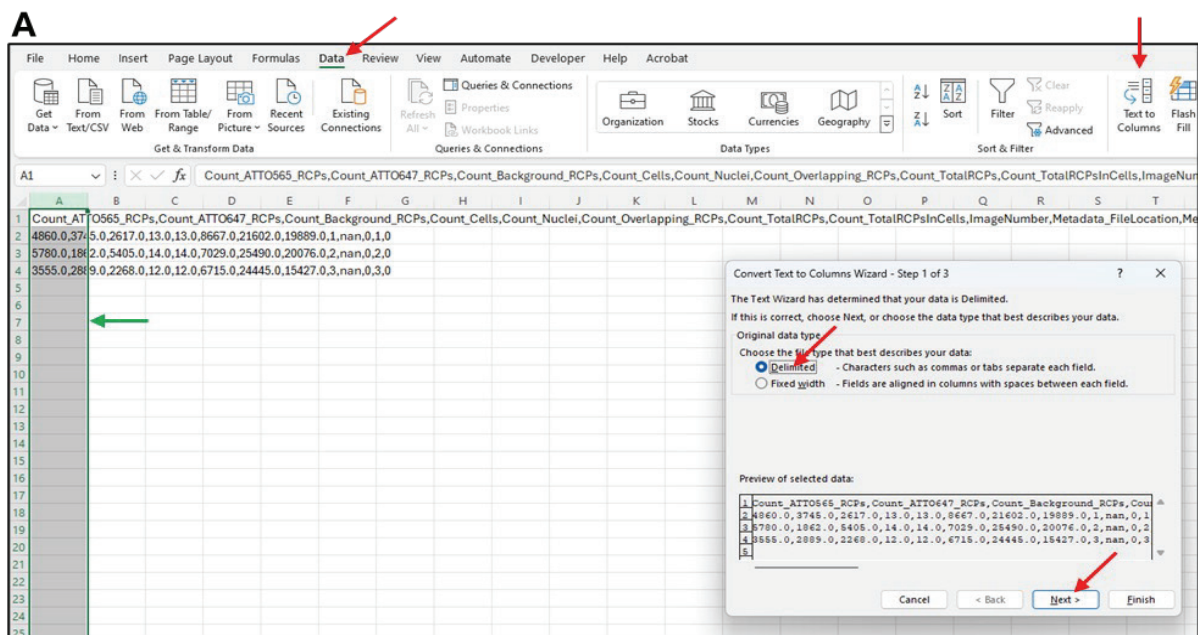
Figure 23. Converting csv file text format to columns

The output columns contain information on the number of counted RCPs in cells for each classification group. Each row represents a different image set.

The columns listed in Table 1 are as follows:
- Count_ATTO565_RCPs; free E-cadherin proteins
- Count_ATTO647_RCPs; free β-catenin proteins
- Count_Overlapping_RCPs; interacting E-cadherin/β-catenin proteins
- Count_Background_RCPs; RCPs with intensities below cutoff values in both the ATTO565 and ATTO647 channels.

- Count_TotalRCPsInCells; The sum of all RCPs in cells (ATTO565_RCPs+ATTO647_RCPs+Overlapping_RCPs+Background_RCPs)
- Count_TotalRCPs; The sum of all RCPs detected in the image set (including the RCPs located outside of the cell borders).
- Count_Nuclei; identified in the first 'IdentifyPrimaryObject' module.
- Count_Cells, identified in the first 'IdentifySecondaryObject' module.
- Image number and MetaData number.
- Count_Cells, identified in the first 'IdentifySecondaryObject' module.
- Image number and MetaData number.

Table 1. Output data with number of RCPs in cells for each classification group

Count_ATTO565_RCPs	Count_ATTO647_RCPs	Count_Background_RCPs	Count_Cells	Count_Nuclei	Count_Overlapping_RCPs	Count_Total RCPs	Count_TotalRCPsInCells	Image Number	Metadata_Number
4786	3658	2517	13	13	8662	21303	19623	1	1
5786	1857	5396	14	14	7036	25493	20075	2	2
3525	2855	2198	12	12	6653	24103	15231	3	3

Table 2. Organizing output data for presentation

Image	Count_Total RCPs	Count_Total RCPsInCells	Count_TotalRCPsIn Cells_background subtracted	Count_Background _RCPs	Count_ATTO565 _RCPs	Count_ATTO647 _RCPs	Count_Overlapping _RCPs	Count_ Nuclei
1	21303	19623	17106	2517	4786	3658	8662	13
2	25493	20075	14679	5396	5786	1857	7036	14
3	24103	15231	13033	2198	3525	2855	6653	12

7.3.2.6 Data presentation

Present the data from Table 1 as preferred. This section outlines two different approaches to present the data.

Before starting, subtract the 'Count_background_RCPs' from the 'Count_TotalRCPsInCells'. This gives the 'Count_TotalRCPsInCells_background subtracted' (see Table 2).

NB 27 Compared to Table 1, in Table 2 the order of columns has been changed and a couple of unused columns have been removed.

Normalization to number of cells (nuclei)
- Separately divide the RCP count for the different classification groups in Table 2 ('Count_TotalRCPsInCells_background subtracted', 'Count_ATTO565_RCPs', 'Count_ATTO647_RCPs' and 'Count_Overlapping_RCPs') with the count of nuclei in the same image set.
- The table in **Figure 24** shows the RCP data when normalized to nuclei count.

Normalization to total number of RCPs in cells
- Separately divide the RCP count for the different classification groups in Table 2 ('Count_ATTO565_RCPs', 'Count_ATTO647_RCPs' and 'Count_Overlapping_RCPs') with the total count of RCPs in cells of the same image set ('Count_TotalRCPsInCells_background subtracted').
- Convert the fold values to percentages by multiplying each number with 100.
- The table in **Figure 25** shows the RCP data when normalized to total RCPs per image. Also see Appendix, section 8.1.1 for example MolBoolean results in cells (E-cadherin and β-catenin MolBoolean results in MCF7 cells).

	Image	Total	Overlapping	ATTO565	ATTO647
	1	1316	666	368	281
	2	1049	503	413	133
	3	1086	554	294	238
Average		1150	574	358	217
STDEV		145	84	60	76
SEM		84	48	35	44
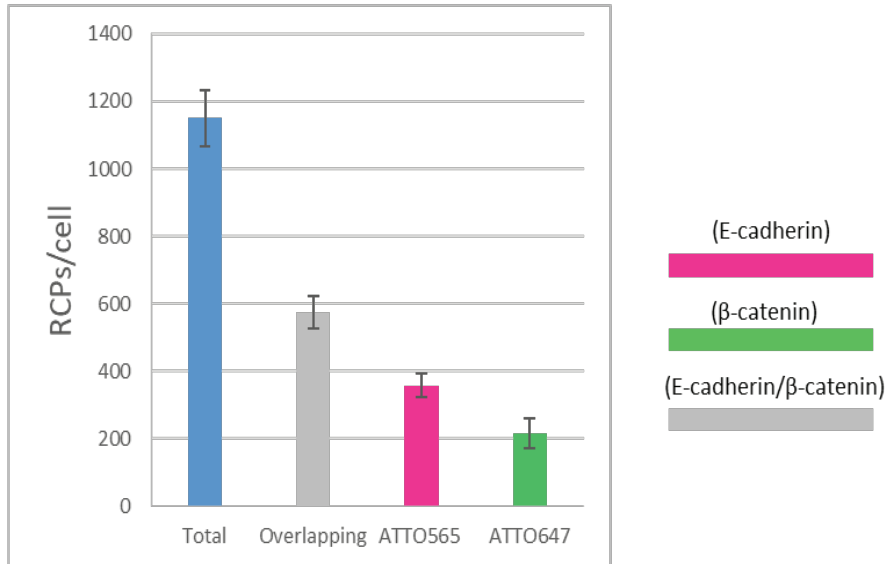
Figure 24. RCPs in cells normalized to nuclei count in the image. The graph represents averages from the images, error bars are standard error of mean (SEM).

	Image	Overlapping	ATTO565	ATTO647
	1	0,51	0,28	0,21
Fold	2	0,48	0,39	0,13
	3	0,51	0,27	0,22
	Image	**Overlapping**	**ATTO565**	**ATTO647**
	1	51	28	21
Percentage	2	48	39	13
	3	51	27	22
Average		50	31	19
STDEV		1,7	6,9	5,2
SEM		1	4	3
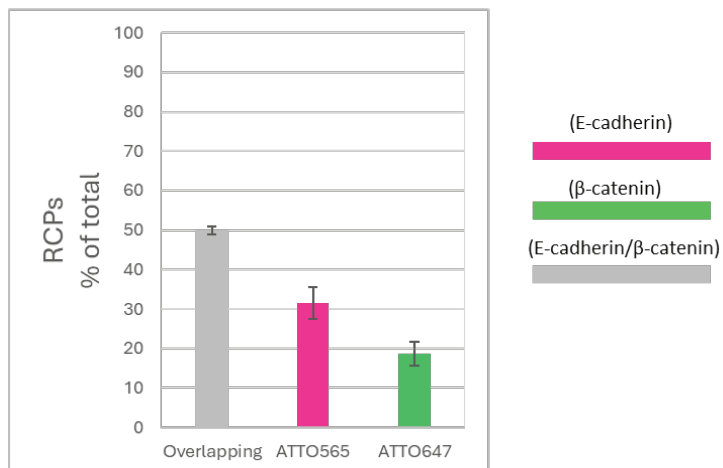
Figure 25. RCPs normalized to total RCPs in cells in the image. The graph represents averages from three images, error bars are standard error of mean (SEM).

7.3.3 Performing Image analysis without nuclei and cell segmentation (tissue)

CellProfiler may not always be able to determine the number of nuclei and cell borders in the image sets. For example, this could be difficult in tissue images where the nuclei shape and staining intensity are often less uniform.

The image in *Figure 26* illustrates such an example, where in human small intestine the nuclei appear in different shapes and intensities. When trying to segment the nuclei in the 'IdentifyPrimaryObjects' module, CellProfiler™ struggles to identify nuclei, with no '#of accepted objects'.

In these instances, it will not be possible to count RCPs within cell borders. Instead, one will have to count RCPs throughout the entire image. The number of counted RCPs for each classification group can still be normalized as a percentage of total RCPs (as in section 7.3.2.6, option 2), the only difference is that the results will represent total RCPs per image set rather than total RCPs in cells.

When in Test mode (section 7.3.2.2), if CellProfiler™ struggles to segment and identify the nuclei in the 'IdentifyPrimaryObjects' module (see *Figure 26*). Follow the steps described below to perform image analysis without nuclei and cell segmentation:

- In the 'IdentifySecondaryObjects' module, where cell borders are identified, use the default settings and move on to the next module. Ignore the results in this module, it will not be used in the output data.
- Go through all the modules as explained in section 7.3.2.2 except a few small alterations:

1. Unclick the 'Mask Objects', 'OverlayOutlines' and 'SaveImages' (the second one) modules (red arrows in *Figure 27A*). This will result in error messages (indicated by cross symbols) in the subsequent modules (green arrows in *Figure 27A*). How to correct this, see instructions below.

2. In the 'MeasureObjectIntensity' module, deselect 'TotalRCPInCells' (green arrow in *Figure 27B*). Instead, select 'TotalRCPs' (green arrow in *Figure 27C*). These changes should remove the error message from the module.

3. In the 'DisplayDensityPlot' module, change from 'TotalRCPInCells' to 'TotalRCPs' (red arrow in *Figure 28A*). These changes should remove the error message from the module.

4. In the 'ClassifyObjects' module, change from 'TotalRCPInCells' to 'TotalRCPs' (red arrow in *Figure 28B*). These changes should remove the error message from the module.

5. In the 'FilterObjects' module, change from 'TotalRCPInCells' to 'TotalRCPs' (red arrow in *Figure 28C*). Repeat this for all the 'FilterObjects' modules (four in total). These changes should remove the error message from these modules.

6. In the 'ExportToSpreadsheet' module, click the 'Press button to select measurements' option (red arrow in *Figure 29A*). In the opened window, unclick the 'Cells' and 'Nuclei' option under image>Count (red arrow in *Figure 29B*).

Data output, processing and presentation:
- Exit the test mode as explained in section 7.3.2.3 and proceed to generate output data as explained in section 7.3.2.4.
- For data processing, follow the instructions in section 7.3.2.5. However, there are a few alterations to the output data compared to section 7.3.2.5:

1. The 'OrigOverlay.tiff' will not be generated.

2. The data output columns in the 'Image.csv' file will not include 'Count_TotalRCPsInCells', 'Count_Nuclei' or 'Count_Cells'. For a list of columns generated in the 'Image.csv' file, see Table 3. Subtract the 'Count_background_RCPs' from the 'Count_TotalRCPs', giving the 'Count_TotalRCPs_background subtracted' (see Table 4).

3. The output data for respective classification group will not represent the RCP count in cells. Instead, it represents the RCP count per image set.

- The results can be presented as explained in section 7.3.2.6, option 2 ('Normalization to total number of RCPs). The table in *Figure 30* shows the RCP data when normalized to total RCPs per image set in tissue. Also see Appendix, section 8.1.2 for example of MolBoolean results in tissue (E-cadherin and β-catenin MolBoolean results in human small intestine).
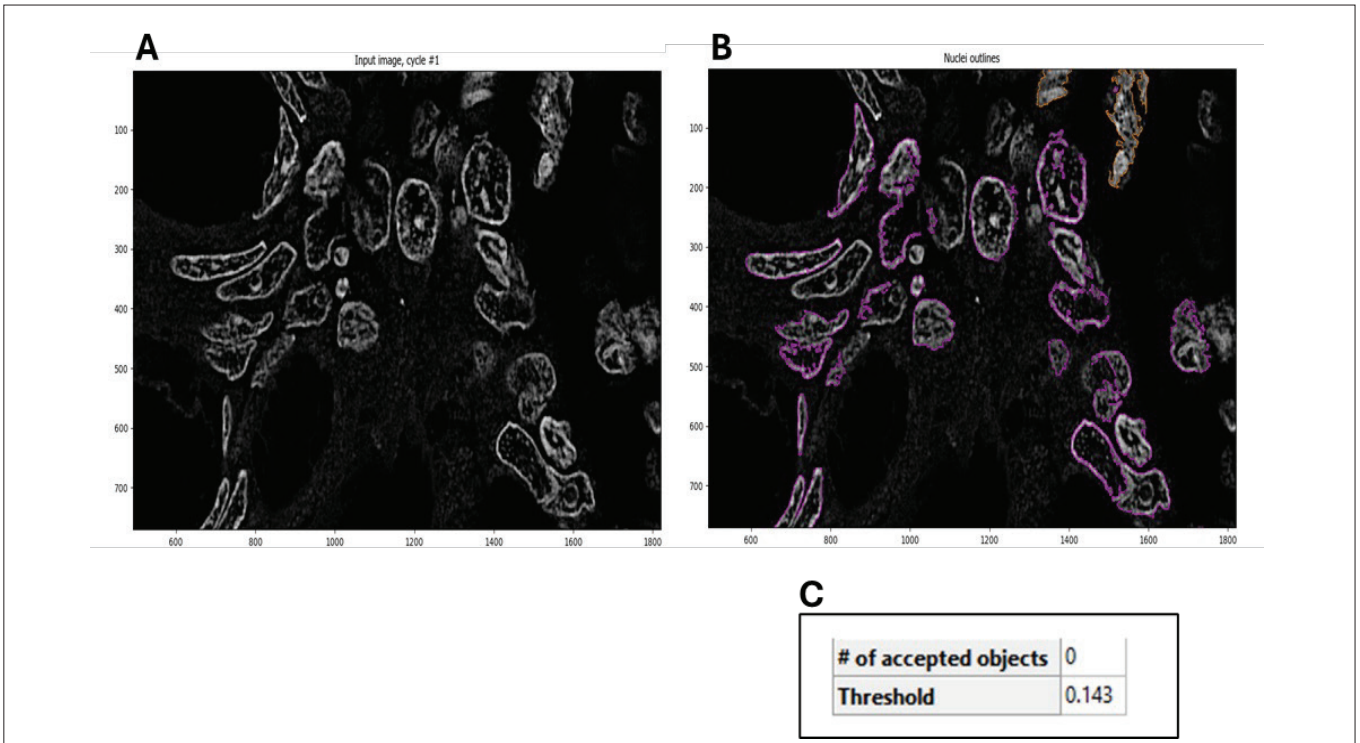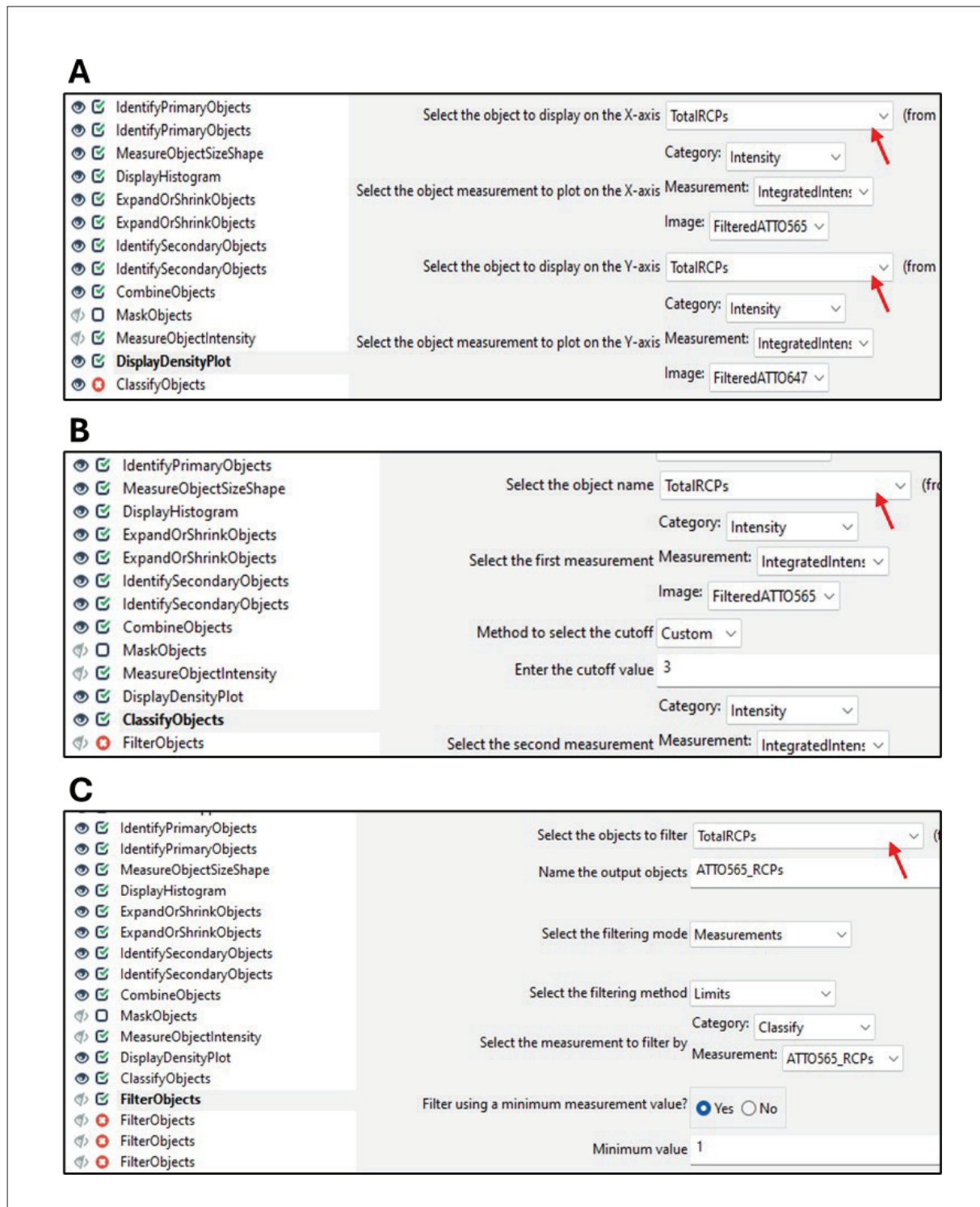
Figure 26. Nuclei cannot be segmented in human small intestine tissue.

Figure 27. Switching from 'TotalRCPInCells' to 'TotalRCPs' in modules

Figure 28. Switching from 'TotalRCPInCells' to 'TotalRCPs' in modules, continued.

**Figure 29.** Deselect 'Cells' and 'Nuclei' in the 'ExportToSpreadsheet' module

Table 3. Output data for respective classification group with RCP count per image set

Count_ATTO565 _RCPs	Count_ATTO647 _RCPs	Count_Background _RCPs	Count_Overlapping _RCPs	Count_TotalRCPs	Image Number	Metadata_ Number
1824	1514	854	3201	7393	1	1
1458	1490	838	2972	6758	2	2
1534	1297	662	2859	6352	3	3

Table 4. Organizing output data for presentation

Image	Count_Total RCPs	Count_TotalRCPs_ background subtracted	Count_Background _RCPs	Count_ATTO565 _RCPs	Count_ATTO647 _RCPs	Count_Overl apping_RCP s
1	7393	6539	854	1824	1514	3201
2	6758	5920	838	1458	1490	2972
3	6352	5690	662	1534	1297	2859

	Image	Overlapping	ATTO565	ATTO647
Fold	1	0,49	0,28	0,23
	2	0,50	0,25	0,25
	3	0,50	0,27	0,23
	Image	Overlapping	ATTO565	ATTO647
Percentage	1	49	28	23
	2	50	25	25
	3	50	27	23
Average		50	26	24
STDEV		0,7	1,7	1,3
SEM		0	1	1
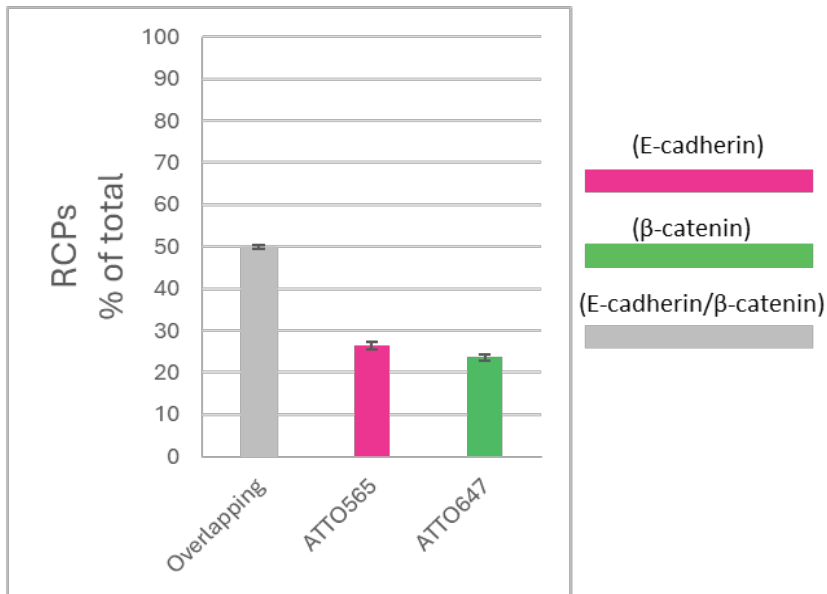
Figure 30.
RCPs normalized to total RCPs in the image.
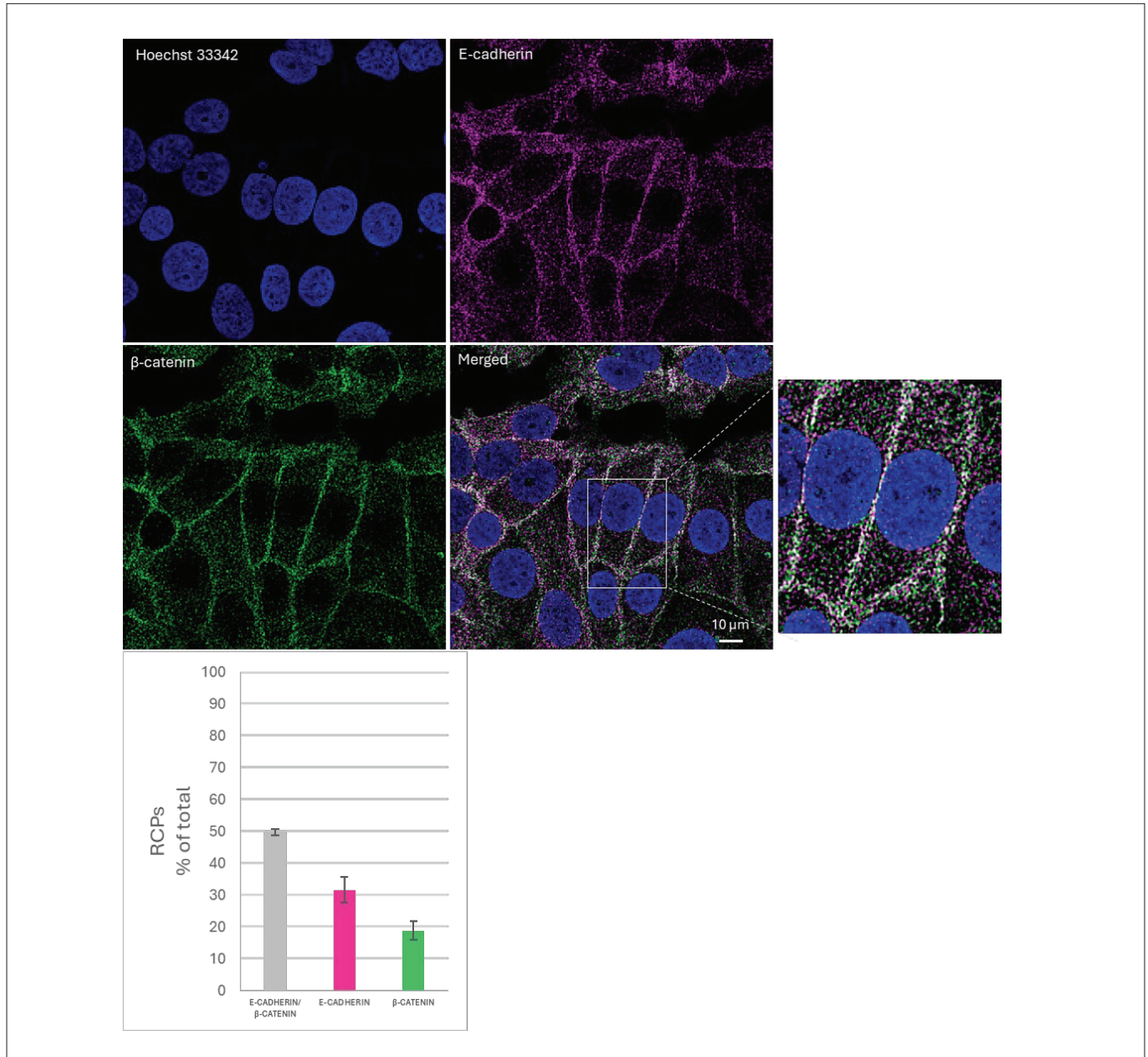The graph represents averages from three images, error bars are standard error of mean (SEM).

8.1   MolBoolean example images

The following sections show example data from MolBoolean analysis in cells and human tissue.

8.1.1   E-cadherin and β-catenin MolBoolean results in MCF7 cells

Figure 31.
E-cadherin and β-catenin MolBoolean results in MCF7 cells
Images were acquired using a PL APO 63x (1.2 NA) water immersion objective on a Leica TCS SP8x confocal microscope, using lasers and detectors matching the ATTO565 (E-cadherin, Cat. AMAb90862) and ATTO647 (β-catenin, Cat. HPA029159) fluorophores. Cell Profiler was used to segment the number of RCPs in each fluorescent channel and the fractions of overlapping (interacting proteins) versus single (free proteins) fluorescently labelled RCPs were classified based on density scatter plots. The graphs represent count of RCPs in each group normalized to total RCP count in cells per image (presented as a percentage). Error bars are standard error of the mean, three separate image sets.

8.1.2 E-cadherin and β-catenin MolBoolean results in tissue
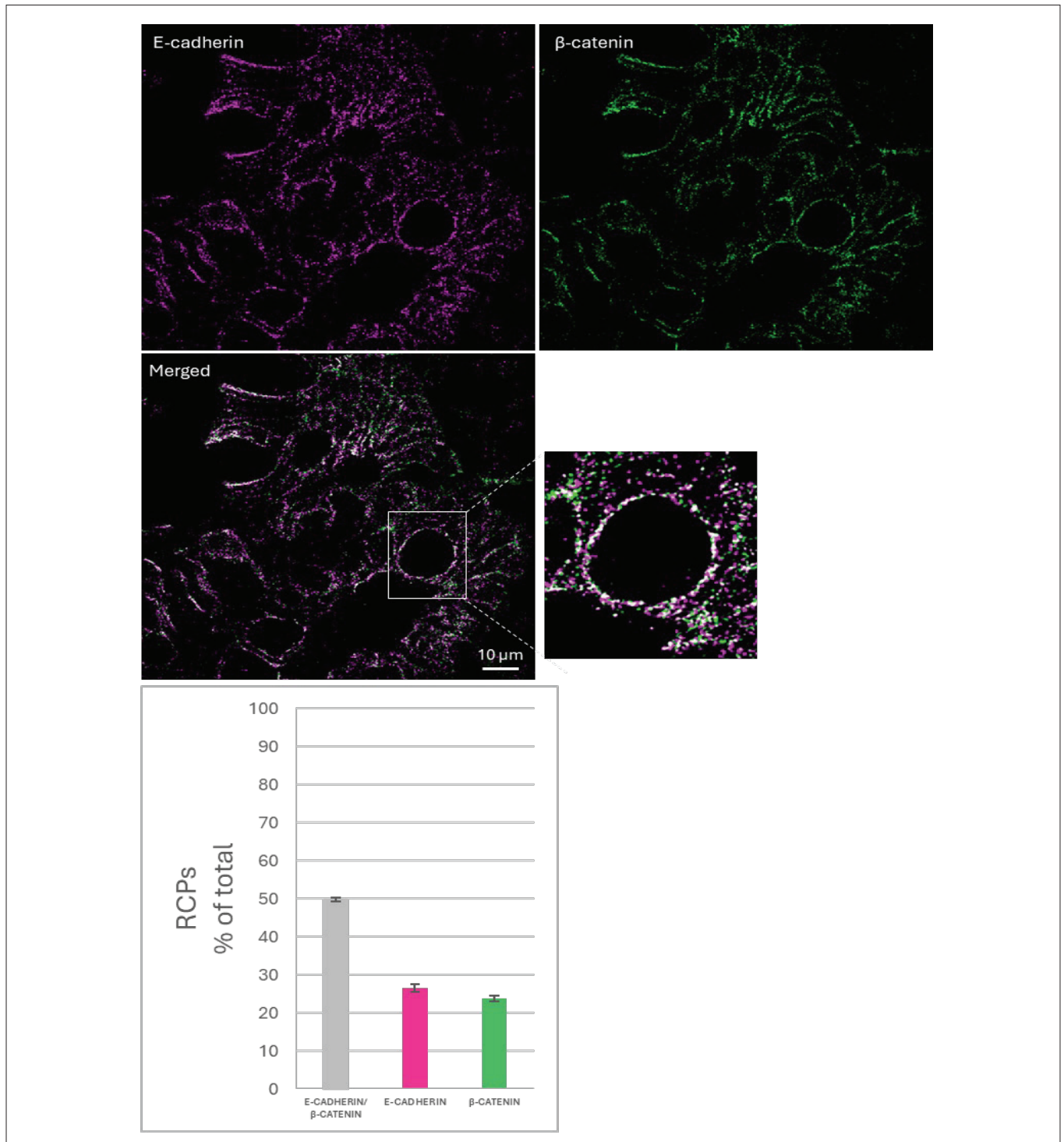
Figure 32.
E-cadherin and β-catenin MolBoolean results in human small intestine.
Images were acquired using a PL APO 63x (1.2 NA) water immersion objective on a Leica TCS SP8x confocal microscope, using lasers and detectors matching the ATTO565 (E-cadherin, Cat. AMAb90862) and ATTO647 (β-catenin, Cat. HPA029159) fluorophores. Cell Profiler was used to segment the number of RCPs in each fluorescent channel and the fractions of overlapping (interacting proteins) versus single (free proteins) fluorescently labelled RCPs were classified based on density scatter plots. The graphs represent count of RCPs in each group normalized to total RCP count per image (presented as a percentage). Error bars are standard error of the mean, three separate image sets. Installing

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