MolBoolean™

BEYOND PROXIMITY LIGATION

UNLOCK THE SECRETS OF PROTEIN-PROTEIN INTERACTIONS IN CELLS AND TISSUE

MolBoolean™ is a novel in situ proximity technology developed by Atlas Antibodies AB that enables the simultaneous detection of both free and interacting fractions for two protein targets. MolBoolean™ is applicable in fixed cells and tissue sections, making it versatile for various research settings.

ADDED CONFIDENCE TO SPATIAL PROTEIN ANALYSIS

Traditional approaches to study protein-protein interactions (PPIs) in cells and tissue could generate unreliable results due to the need of using engineered protein expression systems or lysed cell material. The introduction of in situ proximity ligation assay (in situ PLA) enabled the detection of endogenous PPIs in cells and tissue but it still lacks information on the fraction of non-interacting proteins.

The dynamic mechanisms of PPIs in cells and tissue are heavily dependent on the spatial location, but also the abundance of the respective interacting partners. Therefore, for in situ protein proximity studies, it is pivotal to not only understand the number of interaction events, but also the number of ingoing components.

Working closely together with Professor Ola Söderberg at Uppsala University, Atlas Antibodies has developed the MolBoolean™ assay, a novel in situ protein proximity technology for simultaneous detection of both interacting and non-interacting fractions of two protein targets in cells and tissue.

MolBoolean™ relies on dual target recognition with proximity probes and generates rolling circle amplification products to increase signals, meaning that even low abundant proteins can be detected. On top of that, by performing a series of proprietary molecular steps, MolBoolean™ differentiates between amplified products stemming from individual versus interacting proteins, and this is indicated by the binding of one or two separate fluorescent detection reporters (*Figure 2*).

The more comprehensive extraction of information provided by MolBoolean™ enables normalization of the interaction data to total target protein levels (*Figure 3*).

Therefore, MolBoolean™ will add more confidence to the interpretation of protein proximity data. It can be used for basic research or in drug discovery to study cell signaling PPIs in health and disease and how these may be modified by drug intervention.

WHY MOLBOOLEAN?

- Complete spatial quantitative analysis of protein-protein interactions by simultaneous detection of free and interacting proteins.
- Accurate quantification by normalization of interaction data to total target protein levels.
- Biologically relevant data without the need for engineered protein expression.
- 1000-fold increased fluorescence signal by Rolling Circle amplification, allowing detection and quantification of low abundant proteins.
- Adaptable to different research needs.
- Universal kit that can be used with the customer's choice of primary antibodies.
- Validated in both cells and tissue.

Assay	Individual Proteins	Protein Interaction
IHC/ICC	✓	✗
in situ PLA	✗	✓
MolBoolean	✓	✓

MolBoolean assay steps

MolBoolean™ Mouse/Rabbit (Cat. MolB00001) 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.

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.

- **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 2 provides a schematic representation of the MolBoolean™ assay steps.

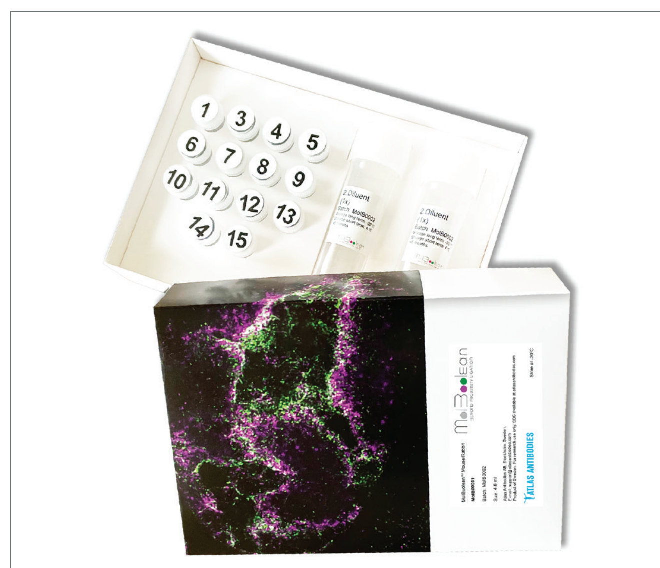

Figure 1. MolBoolean Kit
The MolBoolean™ kit contains 15 separate kit tubes: **1:**Blocker (4x); **2:**Diluent (1x); **3:**Probe A (80x); **4:**Probe B (80x); **5:**Circle (40x); **6:**Additive (40x); **7:**Buffer A (10x); **8:**Nickase enzyme (80x); **9:**Buffer B (8x); **10:**Tag oligos (40x); **11:**Ligase enzyme (80x); **12:**Polymerase enzyme (40x); **13:**Buffer C (5x); **14:**Detection oligos (40x); **15:**Buffer D (10x). The kit volume (4.8 ml) covers approximately 120 assays in cells (40 µl/assay) and 60 assays in tissue (80 µl/assay).

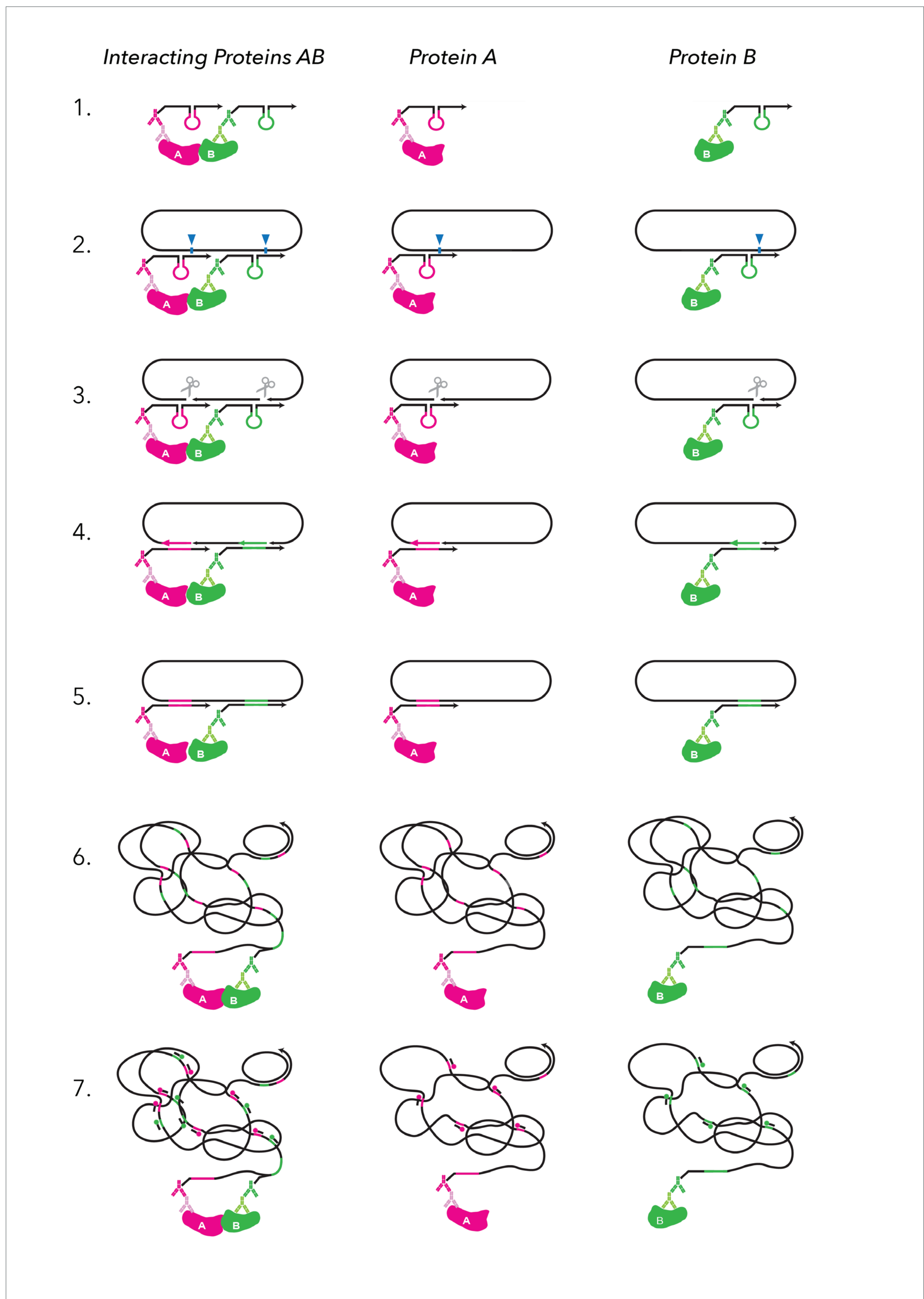

Figure 2. MolBoolean assay steps
Schematic representation of the MolBoolean™ principle for detection of interacting (AB) and free (A or B) proteins.

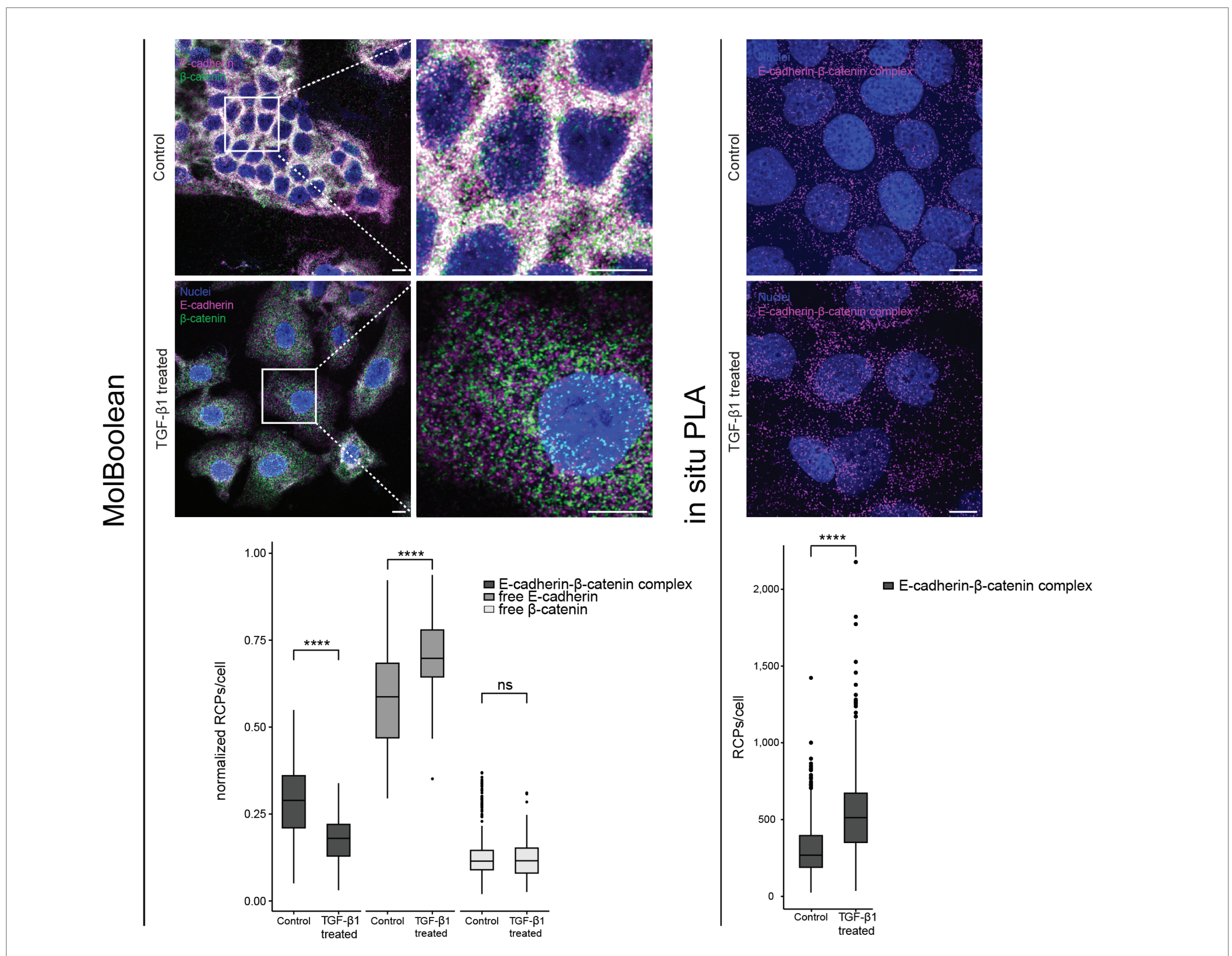

Figure 3. Protein proximity data normalization with MolBoolean to account for dynamic changes in protein complex formation in an inducible biological system.

TGF-β1 treatment in HaCaT cells disrupts the interaction between E-cadherin and β-catenin and leads to the loss of cell-cell contacts at cell junctions, resulting in cell morphological changes.
(Left) To account for the increased cell area and consequent increase in the total number of RCPs per cell that is detected in the treated condition, all the MolBoolean™ data was normalized by dividing the number of RCPs in each category (free E-cadherin, free β-catenin, or proteins in complex) with the total number of RCPs detected in cells. A significant decrease was observed in the fraction of E-cadherin–β-catenin complexes as a result of the increase in free E-cadherin proteins.
(Right) On the contrary, in situ PLA showed an approximately twofold increase in E-cadherin/β-catenin complexes for the TGF-β1treated cells compared to control. However, since there is only one category of signal detected by in situ PLA, i.e., interaction RCPs, normalization cannot be performed. Therefore, it is not possible to account for changes in target protein levels and the results may be inconclusive (*Raykova et al, 2022*).

MolBoolean™ signals are shown for E-cadherin (magenta), β-catenin (green), E-cadherin-β-catenin complex (white), and nuclei (blue). In situ PLA signals are shown in magenta and nuclei in blue.

Beyond Proximity Ligation: Overcoming in situ PLA limitations

MolBoolean™ offers several advantages over traditional methods like in situ PLA, addressing key gaps in data interpretation and providing more reliable results:

- **Normalization of Data:** With MolBoolean™, the **number of protein interactions can be normalized to the total number of target proteins**. This is crucial because target protein levels can be influenced by various factors such as cell treatments or disease states. By providing a normalized measure, MolBoolean™ allows for more accurate comparisons between samples, as demonstrated in **Figure 3**.

- **Detection of Interacting and Non-interacting Fractions:** Unlike traditional methods, MolBoolean™ can **detect both interacting and non-interacting endogenous fractions for two protein targets in cells and tissues.** The more comprehensive detection capability increases the spatial information that can be extracted from the experiment, allowing researcher to determine locations of positive or negative protein interactions.

- **Consistent Molecular Process:** The Rolling circle products (RCPs) stemming from free or interacting proteins are **generated through the same molecular process steps**. This ensures uniform signal efficiencies, reducing uncertainty during data analysis.

Overall, MolBoolean™ offers a more reliable and comprehensive solution for detecting protein interactions, addressing key gaps in data interpretation, and providing researchers with more accurate insights into molecular interactions in cells and tissues.

Furthermore, MolBoolean's versatility and precision in studying protein-protein interactions offer a multifaceted approach to therapeutic innovation.

From targeted drug development to personalized medicine and biomarker discovery, MolBoolean™ holds immense potential to advance biomedical research and translate scientific findings into tangible clinical benefits for patients.

Peer-reviewed applications of MolBoolean

Raykova et al, A method for Boolean analysis of protein interactions at a molecular level. Nat Commun. 2022.

The MolBoolean™ technology was first published in Raykova et al, (2022). This publication included several examples showcasing the sensitivity and specificity of the MolBoolean™ assay in both cells and tissue. Some of the results from MCF7 cells are shown in Figures 4 and 5, and human tissue samples in Figures 6 and 7.

Kotliar et al, Expanding the GPCR-RAMP interactome. bioRxiv [Preprint]. 2023

MolBoolean™ was used by Kotliar et al. (2023) to detect native GPCR-RAMP2 complexes in cell membranes that had previously been identified by an in vitro suspension bead array (SBA) assay, providing physiological relevance to the interaction between GPCR-RAMP2 in cells. The MolBoolean™ assay allowed the researchers to quantify selected GPCR-RAMP2 complexes in SK-N-MC cells and determine their significance in relation to total GPCR and RAMP2 levels. Image analysis results showed that several GPCR-RAMP2 complexes exhibited significant interaction, indicating the formation of these complexes in the native cell membrane environment. The validation of native GPCR-RAMP interactions using MolBoolean™ further supported their findings from the SBA assay and highlighted the role of MolBoolean™ in confirming the presence of GPCR-RAMP complexes in situ.

Rivas-Santisteban et al, Boolean analysis shows a high proportion of dopamine D_2 receptors interacting with adenosine A_{2A} receptors in striatal medium spiny neurons of mouse and non-human primate models of Parkinson's disease. Neurobiol Dis. 2023

Rivas-Santisteban et al, (2023) employed MolBoolean™ to analyze the interaction between dopamine D_2 (D_2R) and adenosine A_{2A} ($A_{2A}R$) receptors in striatal medium spiny neurons in rodent and non-human primate models of Parkinson's disease (PD). MolBoolean™ provided quantitative data on the proportion of individual receptors and receptor complexes in multiple experimental conditions such as: heterologous HEK-293 cells, primary striatal neurons, rat 6-OHDA-PD model, Macaca MPTP-lesioned PD model, highlighting the prevalence of $A_{2A}R$-D_2R heteromers in primary striatal neurons. Importantly, MolBoolean™ enabled the researchers to determine the percentage of each individual receptor that were forming $A_{2A}R$ / D_2R heterodimers, shedding more light on how $A_{2A}R$ mediate their antagonistic regulation on motor control in the brain.

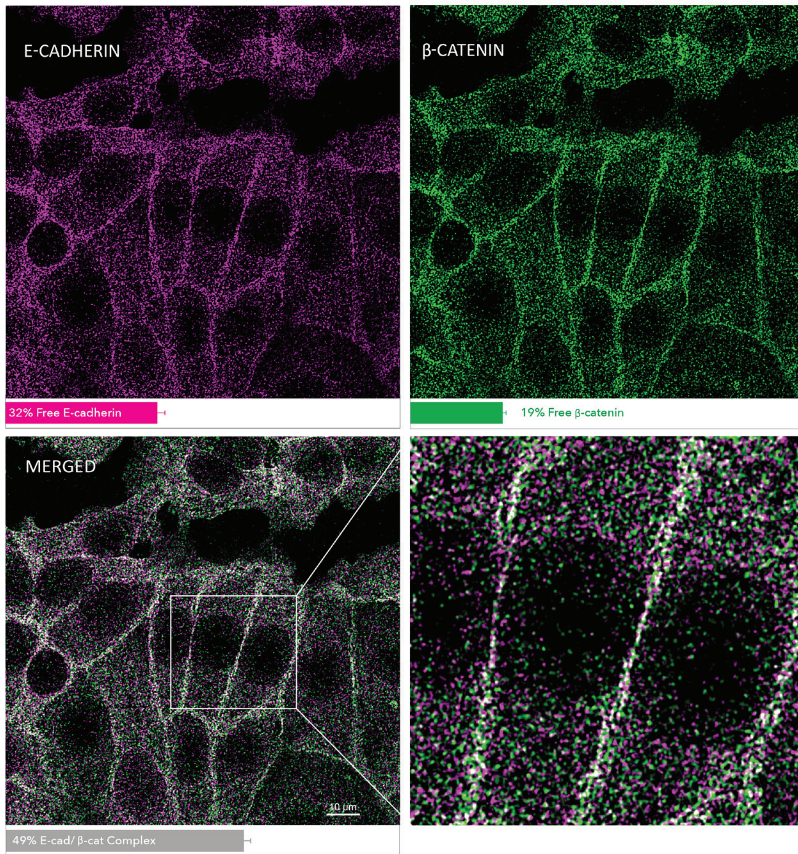

Figure 4
E-cadherin/ β-catenin MolBoolean staining in MCF7 cells.

MolBoolean™ analysis of the interaction between E-cadherin (magenta) and β-catenin (green) in MCF7 cells, using the monoclonal anti-CDH1 (Cat. AMAb90862) and the polyclonal anti-CTNNB1 (Cat. HPA029159) antibodies from Atlas Antibodies AB.

Image shows the relative quantification of free versus interacting protein fractions, indicated by the detection of rolling circle products (RCPs) in either one or two fluorescent channels: 32% free E-cadherin (magenta), 19% free β-catenin (green) and 49% E-cadherin/ β-catenin complex (white). Data is normalized to total target protein levels (total RCPs).

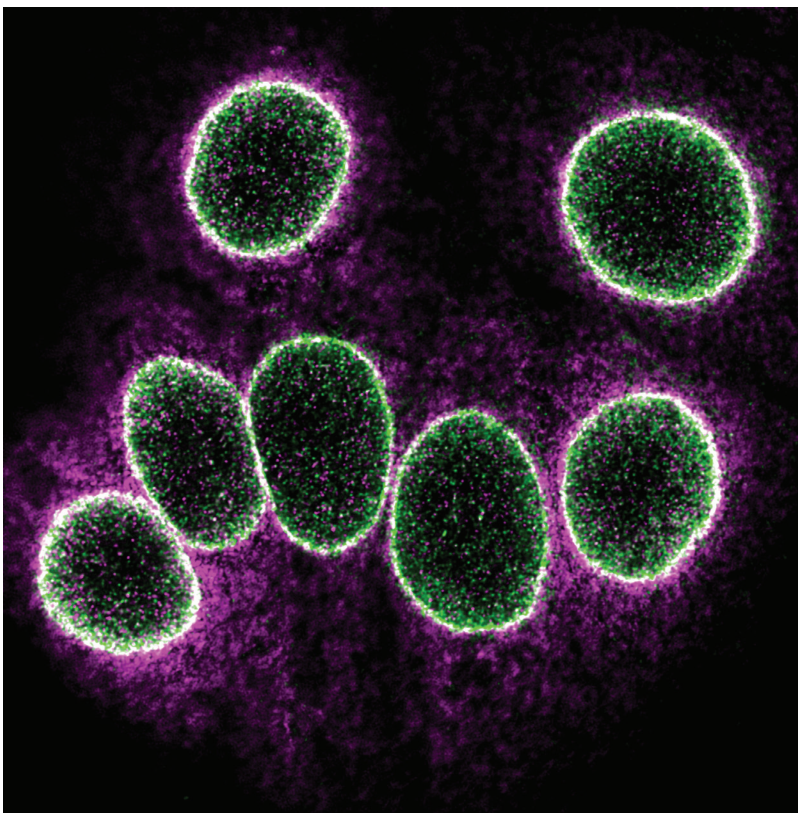

Figure 5
EMD/LMNB1 MolBoolean staining in MCF7 cells.

MolBoolean™ analysis of the interaction between EMD (magenta) and LMNB1 (green) in MCF7 cells, using the monoclonal anti-EMD (Cat. AMAb90562) and the polyclonal anti-LMNB1 (Cat. HPA050524) antibodies from Atlas Antibodies AB.

Image shows the spatial location of free versus interacting protein fractions, indicated by the detection of rolling circle products (RCPs) in either one or two fluorescent channels.

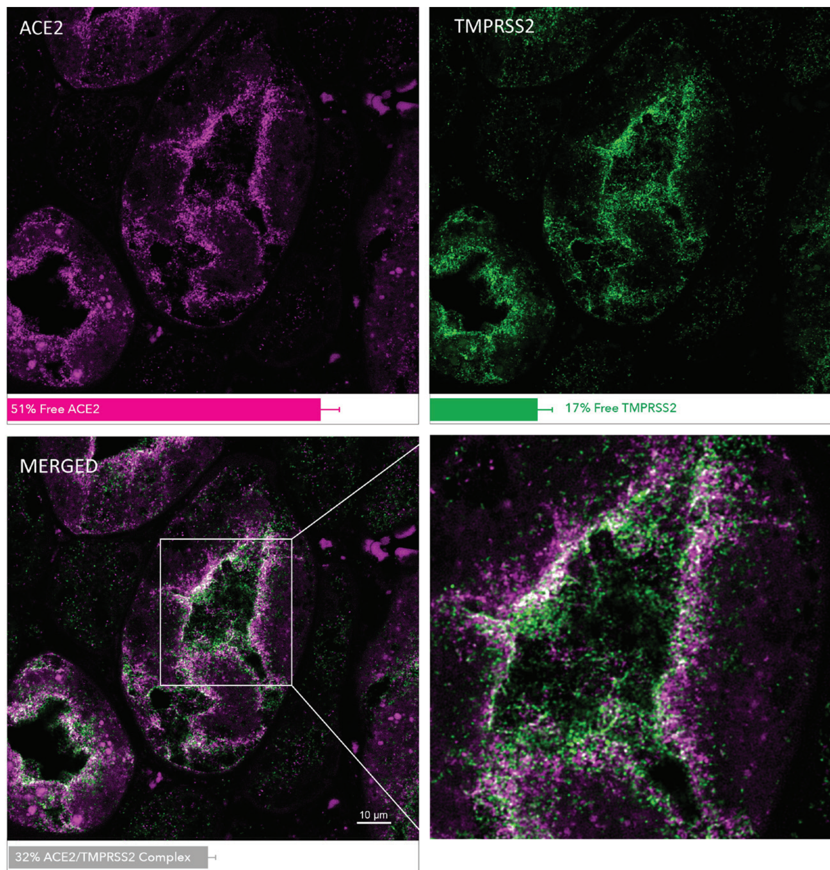

FIGURE 6
ACE2/TMPRSS2 MolBoolean staining in human kidney.

MolBoolean™ analysis of the interaction between ACE2 (magenta) and TMPRSS2 (green) in human kidney, using the monoclonal anti-ACE2 (Cat. AMAb91259) and the polyclonal anti-TMPRSS2 (Cat. HPA035787) antibodies from Atlas Antibodies AB.

Image shows the relative quantification of free versus interacting protein fractions, indicated by the detection of rolling circle products (RCPs) in either one or two fluorescent channels: 51% free ACE (magenta), 17% free TMPRSS2 (green) and 32% ACE/TMPRSS2 complex (white). Data is normalized to total target protein levels (total RCPs).

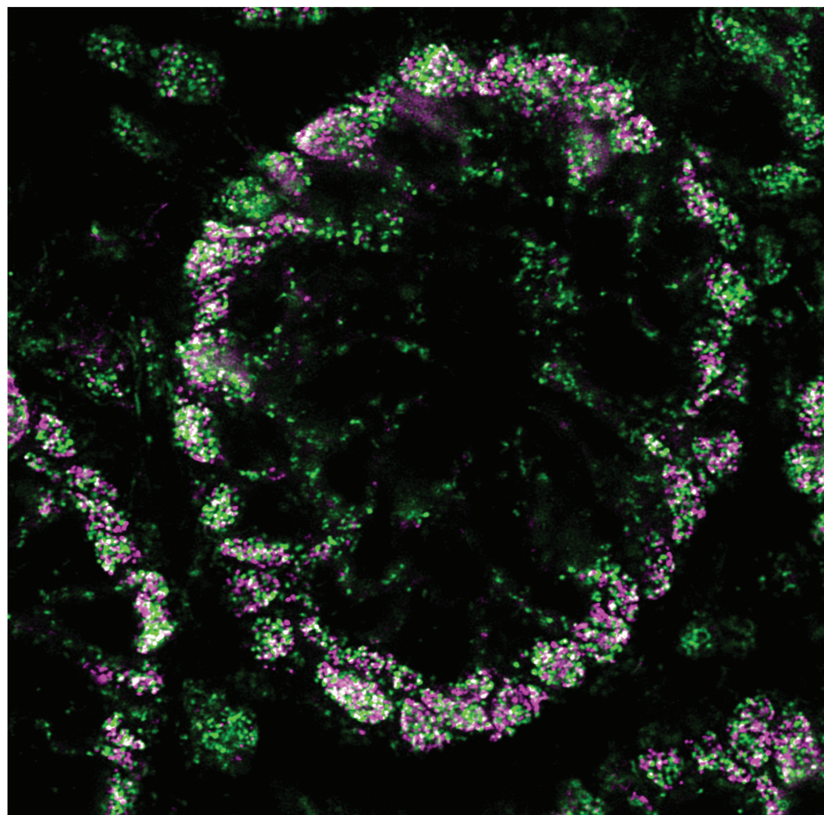

FIGURE 7
SATB2/HDAC1 MolBoolean staining in human colon.

MolBoolean™ analysis of the interaction between SATB2 (magenta) and HDAC1 (green) in human colon, using the monoclonal anti-SATB2 (Cat. AMAb90682) and the polyclonal anti-HDAC1 (Cat. HPA029693) antibodies from Atlas Antibodies AB.

Image shows the spatial location of free versus interacting protein fractions, indicated by the detection of rolling circle products (RCPs) in either one or two fluorescent channels.

Additional note

Antibody specificity and validation

The specificity of the primary antibodies used can affect the accuracy of MolBoolean™ results. It is important to validate the primary antibodies to ensure that they specifically target the proteins of interest.

At Atlas Antibodies, we take great care to validate our antibodies. We perform *Enhanced validation* as an additional layer of security in an application and context-specific manner.

Enhanced validation offers increased security of antibody specificity in a defined context. This is ensured by using the most relevant validation method for each combination of protein, sample, and application.

Enhanced validation follows the guidelines proposed by the International Working Group for Antibody Validation (IWGAV) and published in Nature Methods (Ref: Uhlen, M., Bandrowski, A., Carr, S., et al. A proposal for validation of antibodies. Nat Methods 13, 823–827; 2016).

Read more about Enhanced validation here.

References

Kotliar et al, (2023) *Expanding the GPCR-RAMP interactome. bioRxiv [Preprint]. 2023 Nov 23:2023.11.22.568247. PMID: 38045268;*

Raykova et al, (2023) *A method for Boolean analysis of protein interactions at a molecular level. Nat Commun. 2022 Aug 13;13(1):4755. Erratum in: Nat Commun. 2023 Sep 6;14(1):5450. PMID: 35963857*

Rivas-Santisteban et al, (2023) *Boolean analysis shows a high proportion of dopamine D2 receptors interacting with adenosine A2A receptors in striatal medium spiny neurons of mouse and non-human primate models of Parkinson's disease. Neurobiol Dis. 2023 Nov;188:106341. PMID: 37918757.*

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