Abstract

This protocol synthesizes antibody-DNA conjugates using MIST-Linker as adaptors, enabling site-specific and gentle labeling of antibodies with DNA oligonucleotides. The protocol includes preparing, assembling, and purifying antibody-DNA conjugates and their application in protein in-situ detection techniques. This approach allows for rapid and flexible antibody labeling and is compatible with spatial proteomics at the single-cell level.

Assembly of Antibody-DNA Conjugates

1 Assembly of antibody-DNA conjugates (30 min)

1.1 Mix 1 μL of 0.5 mg/mL antibody with 3 μL of 0.67 mg/mL MIST-Linker thoroughly and incubate at room temperature for 15 minutes.
1.2 Add 1 μL of 100 μM biotin-DNA-647 solution to the mixture from step 1.1 and incubate at room temperature for 15 minutes.

2 Purification of antibody-DNA conjugates (5 min)

After the assembly reaction is complete, dilute the mixture to 200 μL using 1% bovine serum albumin (BSA) solution. Filter the diluted mixture using a 0.5 mL of 100 kDa molecular weight cutoff filter at 8,000 rpm for 5 minutes to obtain purified antibody-DNA conjugates.

Note: The antibody-DNA conjugate has been validated to remain stable at 4°C temporarily and should be used within one week.

Immunostaining with Prepared Antibody-DNA Conjugates

3 Immunostaining of cultured cells (1 h 20 min, except cell culture)

3.1 Preparation of cell culture medium: Mix 890 mL of RPMI medium with 100 mL of fetal bovine serum (FBS) and 10 mL of penicillin-streptomycin. Mix thoroughly, aliquot as needed, and store at 4 °C until use.
3.2 Seed the cells into a 12-well plate and incubate at 37°C with 5% CO_2 in an incubator.
3.3 When cells have fully adhered and reach a confluency of approximately 30%–40%, stop the culture. Wash the cells three times with PBS 7.4, then fix them with 4% formaldehyde solution (prepared in PBS 7.4) at RT on a shaker at 30 rpm for 10 minutes. After fixation, wash the cells three times with PBS 7.4.
3.4 Permeabilize cells with 0.1% Triton X-100 at RT for 10 minutes on a shaker at 30 rpm. After permeabilization, wash the cells three times with PBS 7.4.
3.5 Block the cells using the blocking buffer, incubating at RT on a shaker at 30 rpm for 1 hour.
3.6 After removing the blocking buffer, dilute the conjugate (from Step 2) into 100 μL of the conjugate dilution buffer. Add the diluted conjugate to the wells of the 96-well plate and incubate with the cells at RT on a shaker at 30 rpm for 1 hour.
3.7 Following the incubation step, wash the cells three times with PBS 7.4. Subsequently, add an appropriate volume of PBS 7.4 to each well of the 96-well plate, and image the cells using fluorescence microscopy.

Note:

(1) Cells can be pre-treated and blocked in parallel with the preparation of the conjugate (Steps 1 and 2).
(2) the conjugate incubation time (in step 3.6) can be extended to 3 hours or longer for detection of low abundance proteins and if high sensitivity is required.
(3) It is critical to avoid cell drying at all times to minimize high background signals.
(4) Cells should not reach high confluency to avoid tight junctions between cells, thereby achieving single-cell resolution.

4 Immunostaining of tissue sections (3 h 10 min)
4.1 Bake paraffin-embedded tissue sections at 70°C for 30 minutes.
4.2 Deparaffinization: Prepare fresh solutions in 50 mL centrifuge tubes for the following washes:

Solution	Time	Repeats
100% Xylene	3 minutes	2 times
Xylene : 100% EtOH (1:1)	3 minutes	1 time
100% EtOH	3 minutes	1 time
95% EtOH	3 minutes	1 time
70% EtOH	3 minutes	1 time
50% EtOH	3 minutes	1 time
DI water	3 minutes	2 times

For each wash, fully submerge the slides in the solution contained within a 50 mL centrifuge tube. Gently move the slides within the solution during each wash step to promote thorough deparaffinization and hydration. After processing, store the sections in PBS 7.4 solution at 4°C for future use.

Notes
(1) Always perform xylene and xylene-ethanol steps inside a chemical hood.
(2) Fresh xylene and ethanol solutions are recommended for best results.
(3) Handle slides carefully to prevent tissue detachment during washes.

4.3 Permeabilize tissue sections with 0.3% Triton X-100 at RT for 10 minutes on a shaker at 30 rpm. After permeabilization, wash the cells three times with PBS 7.4.
4.4 Block the tissue sections using the blocking buffer, incubating at RT on a shaker at 30 rpm for 1 hour.
4.5 After removing the blocking buffer, dilute the conjugate into 100 µL of the conjugate dilution buffer. Add the diluted conjugate to the tissue sections and incubate with the tissues at RT on a shaker at 30 rpm for 1 hour.
4.6 Following the incubation step, wash the tissues three times with PBS 7.4. Subsequently, add an appropriate amount of mounting buffer to the sample and cover it with a coverslip for fluorescence microscopy analysis.

Note:
(1) Tissues can be pre-treated and blocked in parallel with the preparation of the conjugate (Steps 1 and 2).
(2) the conjugate incubation time (in step 4.5) can be extended to 3 hours or longer for detection of low abundance proteins and if high sensitivity is required.

(3) It is critical to avoid tissue drying at all times to minimize high background signals.

Microarray Analysis

5 Nuclear staining (10 min)

After immunostaining of cells (step 3) or tissues (step 4), stain with POPO-3 dye at 2,000X for 10 minutes at RT with gentle shaking at 30 rpm. Wash three times with PBS 7.4 and wait for clamping.

6 Clamping (10 min)

DNA hybridization is achieved using a clamp between the tissue or cells and the microarray. Upon exposure to UV light, the pc groups on the conjugate are cleaved, resulting in the in-situ release of the fluorescently labeled SA-DNA conjugate onto the array, thereby preserving the spatial information of the sample.

6.1 The coverslip carrying a MIST array is removable on a block. If removal is required, use a tweezer to gently pick up the coverslip from the edge. Do not use sharp edged tweezer. Avoid direct contact with the beads and try to grip the glass slide from the edge as shown in Figure 1A. To place the coverslip onto the PDMS pad on the block (Figure 1B), gently press down with the tweezers to ensure firm contact between the coverslip and the PDMS.

6.2 Add 50 µL of 15% PEG solution onto the array, ensuring that the PEG fully covers the entire array surface without any air bubbles.

6.3 Place the tissue (Figure 1C) or cells (Figure 1D) on a horizontal surface. Rapidly invert the arrayed block up and down, pressing the array vertically against the surface of the cells or tissue.

6.4 Maintain contact for 1 minute, then expose to UV light (365 nm, 4 W) for 5 minutes, followed by a 3-minute post-incubation. After completion, separate the array vertically from the tissue or cells. Wash the array three times with PBS and wait for scanning.

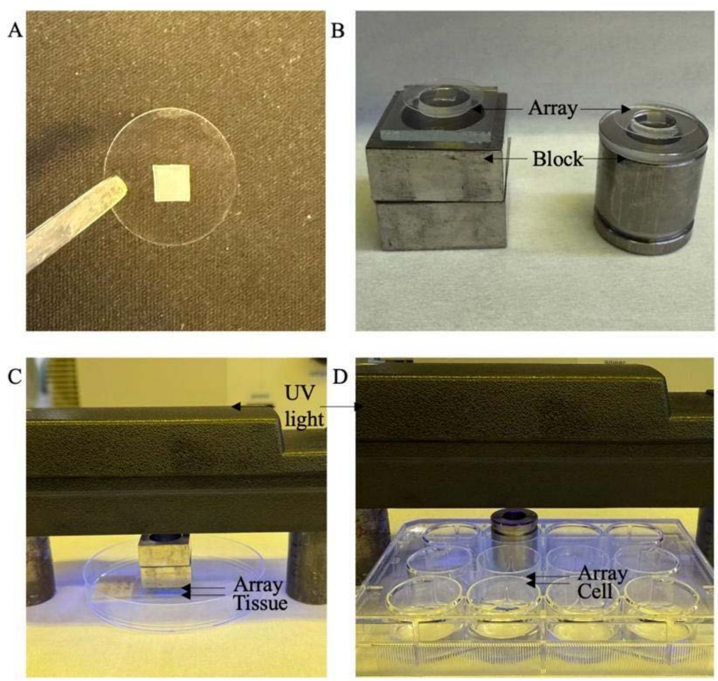

Figure 1

7 Scanning of MIST array (5 min)

Place the coverslip with the array facing down onto a clean glass slide after adding an appropriate amount of mounting buffer. Insert the slide into the scanner for imaging (20X). The scanner settings are as follows:

Channel	Intensity	Exposure time (ms)
Bright Field	80%	10
Cy3	80%	100
Cy5	80%	100

8 Scanning of tissue section (optional)

Under the same conditions as Step 7, scan the nuclear markers (stained with POPO-3) on tissue or cells for subsequent data segmentation.

9 Data analysis

Input of the images from Steps 7 and 8 into MIST-Explorer for data processing and visualization of results.

Material

Chemicals and biological reagents

Component	**Vendor**	**Part number**
MIST-Linker		
Antibody		
Biotin-DNA-Cy5		
Blocking buffer		
Conjugate diluting buffer		
Triton X-100	Merck	TX1568-1
BSA	Sigma-Aldrich	A7030
Antifade mounting medium	Vector Laboratories	
16% formaldehyde solution (w/v), methanol-free	Thermo Scientific	AB391092
RPMI medium 1640	Thermo Fisher Scientific	11875093
FBS	Thermo Fisher Scientific	A5670701
Penicillin-streptomycin	Thermo Fisher Scientific	15140122
Xylene	Sigma-Aldrich	214736-1L
EtOH	Sigma-Aldrich	459844-1L
PBS 7.4	Fisher Bioreagents	243743
POPO™-3 Iodide (534/570)	Thermo Fisher Scientific	P3584

Instrument and equipment

Microarray, homemade
Slide scanner, SLIDEVIEW VS200, Olympus
Microscopy, Nikon Eclipse Ti-U

Low Speed Orbital Shaker, Corning
Centrifuge, Eppendorf, 5425
Pipette, Eppendorf research plus
BSI Nuaire CO_2 incubator
ESCO class 2 Type A2 lab culture hood
Chemical hood
Mini Vortex Mixer, Fisher Scientific, 21073063

Consumables
Eppendorf tubes, Thermo Scientific
Centrifugal filter unit, 100 k, Sigma Aldrich Millipore
Tips
96-well cell culture plate
Microscope cover glass, fisherbrand
Plain microscope slides, fisherbrand
Block with PDMS pad, homemade

Cell lines and tissues
Human 231 cells
Mouse MC3T3-E1 cell
Ready-to-use Mouse C57 kidney paraffin sections, Zyagen, catalog number: MP-901-C57
Mouse brain tissue

Troubleshooting

Issue	Likely reasons	Solution
High background	Non-specific binding of antibody or DNA	(1) Always keep tissues/cells from drying during all steps. During cell staining, keep the lid on the 96-well plate. During tissue staining, place the tissue in a humidified dish. (2) Strictly follow every washing steps. (3) Increase blocking time. (4) Optimize conjugate concentration.
	Endogenous biotin interference	Add avidin/biotin blocking steps
Bright punctate spots in background	Conjugate aggregation	Use fresh antibody-DNA conjugates
	Insufficient washing	(1) Increase washing time (2) Use PBST buffer to wash
Weak signal	Poor conjugate quality. Some antibodies are stored at solutions containing glycerin.	Add PBS 7.4 buffer to decrease glycerin concentration (< 10%) when

		assembling antibody-DNA conjugates
	Low conjugate concentration	Increase conjugate concentration
	Wrong purification	Avoid high centrifuge speed and long time when purifying conjugates
	Incompatible mounting medium	Change antifade mounting medium types
	Photobleaching	Minimize light exposure during preparation and imaging
	The target proteins have low abundances in sample	Increase conjugate incubation time
Non-specific nuclear staining	Free DNA probes binding to nuclear	(1) Increase blocking time (2) Optimize DNA concentrations used for assembling antibody-DNA conjugates
Tissue autofluorescence interfering	Autofluorescence from tissue specific components	(1) Use additional quenching treatments (2) Use longer-wavelength dyes
	Autofluorescence from experimental reagents	(1) Removing the wax surrounding the tissue prior to the deparaffinization process (2) Ensure that hydrophobic pen markings do not touch the tissue surface
Tissue/cell detachment during protocol	Overly harsh washing	Use gentle pipetting
	Weak tissue/cell adhesion	Use chemically activated slides for better adhesion
Unclear and weak array signal	Uneven clamping	Ensure that the surfaces of the array and tissue (or cells) are completely flat and clean, without any dust or debris. Make sure there are no air bubbles in the PEG solution during clamping
	Insufficient UV photo cleavage	(1) Increase the UV time (2) Use a higher-power UV light source (3) shorten the distance between the UV light and the tissue or cells.

	Insufficient DNA hybridization	Increase the post-incubation time during post-UV clamping
Signals on the array appear diffused and lose their localization	PEG concentration is too low	Increase the PEG concentration
	During clamping, blocking on the tissue/cell surface shifts or slides	(1) When bringing the array into contact with the tissue/cells, move steadily and at a constant speed in a vertical downward direction. Avoid any shaking or lateral displacement after contact. (2) Experiments that may cause vibrations, such as operating a centrifuge or a shaker, should not be conducted on the same surface where clamping is performed.
Difficulty separating array from tissue/cells	The PEG solution is over-drying	(1) During clamping, place the entire setup inside a wet chamber or avoid strong air currents. (2) Decrease the clamping time, although it will decrease the detection sensitivity
	Unclean surroundings of the tissue or cells	Before clamping, carefully clean the area around the tissue/cell that may come into contact with the coverslip, including any adhesive, hydrophobic pen marks, and small dust particles.
High background or nonspecific signals after clamping	Washing is not enough	(1) Increase washing time. (2) Use PBST buffer to wash.
Partial beads loss after clamp	Mechanical stress	(1) Lower the strength of clamping. (2) Perform washing steps gently. (3) After the clamping step, the array should be left at room temperature for 5 minutes before proceeding with the washing steps.

	During clamping, blocking on the tissue/cell surface shifts or slides	During the contact between the array and tissue/cells, avoid any displacement or shaking. When lifting the array vertically, do so slowly and at a constant speed.