

Tech Note

Studying Unbiased Spatial Proteomes with Microscop[®]: Sample Preparation and Experimental Design

Introduction

The Microscop[®] is a powerful platform for spatial proteomic explorations. It integrates microscopy, optics, mechatronics, photochemistry, and advanced image processing—leveraging modular traditional algorithms or deep learning—to enable high-content *in situ* photolabeling. The system photochemically labels proteins at user-defined regions of interest (ROIs) under a microscope (Figure 1). Photolabeled proteins are then extracted and analyzed by mass spectrometry (MS) to identify proteins localized to those ROIs. Proper experimental design and sample preparation are critical for the success of MS data analysis. Here we provide guidelines for these aspects in spatial proteomics discovery studies with the Microscop[®].

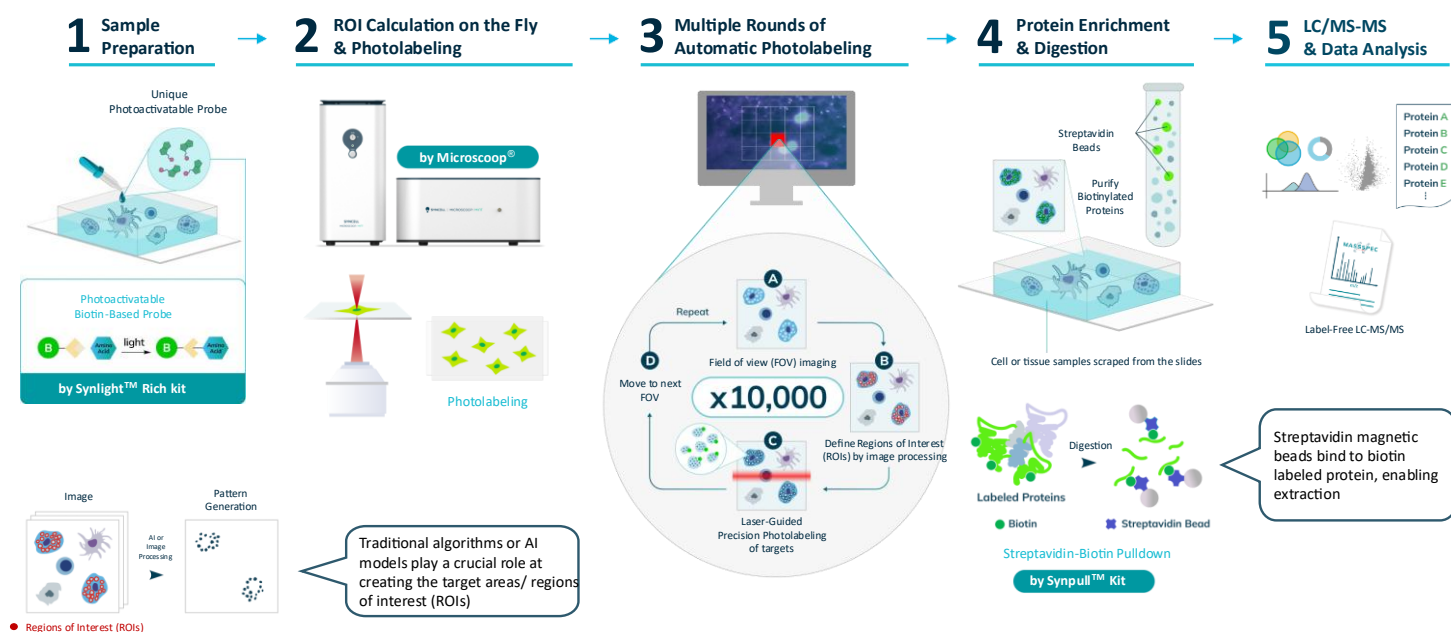


Figure 1. Overview of Microscop[®]-mass spectrometry (MS) workflow

Sample Preparation

The Microscop[®] to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) workflow is compatible with a broad range of sample types, including fixed cultured cells, formalin-fixed paraffin-embedded (FFPE) tissues, and cryopreserved tissues (cryosections). The guidelines for preparing different types of samples are shown in Table 1 and described below.

Table 1. Guidelines for sample preparation

	Cell slide	FFPE section	Cryosection
Sample condition	<ul style="list-style-type: none">• Monolayer• 70-80% confluence	Paraffin-embedded tissues	OCT-embedded tissues
Fixation	<ul style="list-style-type: none">• 2-4% PFA• Cold methanol	<ul style="list-style-type: none">• 4% PFA• 10% Formalin	
Section thickness	N/A	5-10 μm	10-20 μm
Slide material	High-performance chambered coverslips, such as μ -Slide (ibidi), chambered cover glass (Cellvis).	Standard microscope slides, such as Superfrost [®] Plus microscope slides and poly-L-lysine coated slides.	

Fixed cells. Cultured cells are typically prepared as a monolayer of 70-80% confluency. Cells should be seeded onto high-performance chambered coverslips, such as μ -Slide (Cat No. 82107, ibidi, Germany), chambered cover glass (Cat No. C1-1.5H-N, Cellvis, USA), or other chambered coverslips of comparable quality. One-well chambered coverslips are used most often in experiments. The cells can be fixed with 2-4% paraformaldehyde (PFA) solution or ice-cold methanol, permeabilized (0.5% Triton X-100 for PFA-fixed cells; unnecessary for methanol-fixed cells) and blocked according to the Synligh-Rich[™] kit (Cat No. SYN-RI0106) instructions, before proceeding with a standard immunofluorescence staining protocol.

FFPE tissues. Tissue samples should be sectioned at a thickness of 5-10 μm and placed onto standard microscope slides, such as Superfrost[®] Plus, poly-L-lysine coated or other high-quality slides for immunostaining. Each slide can hold multiple sections, depending on their size, as long as they do not overlap. Importantly, do not seal the slides with mounting medium after staining, as the sections must remain accessible for subsequent photolabeling and protein retrieval as indicated in the Synligh-Rich[™] kit instructions.

Frozen tissues (cryosections). Fixed frozen tissues are typically embedded in optimal cutting temperature (OCT) compound and cut into 10-20 μm thick sections. Similar to the FFPE tissue procedure, frozen tissue sections should be placed onto standard or high-quality microscope slides without the use of mounting media. The slides can be stored at -80 °C for up to 2-3 months prior to immunostaining. If longer-term storage is desired, it is preferable to keep the tissue in its OCT-embedded block form. Fresh cryo-sectioning and staining

are strongly recommended for better protein preservation. Minimize the number of freeze-thaw cycles, which may damage tissue structure and impact immunostaining quality.

Considerations for ROI Visualization

Microscoop® uses fluorescent images to define ROIs and guide precise photolabeling. Therefore, the fluorescence signal quality of ROI is critical for image processing to generate accurate binary mask patterns and avoid inclusion of false targets. Ideally, the ROI signal should have high intensity, clear morphology with no blurry edge under the epi-fluorescence microscope of Microscoop using the 40X objective. To achieve this, high-quality primary antibodies with low non-specific binding are recommended for the immunostaining. For secondary antibody selection, antibodies conjugated to Alexa Fluor™ 647, Alexa Fluor™ 568, Alexa Fluor™ 488 or other fluorescent dyes with similar excitation/emission wavelength ranges are compatible with Microscoop® photolabeling. In contrast, fluorophores excited at 405 nm or shorter wavelengths are not suitable and should be avoided due to the risk of off-target labeling that can be caused by the high-energy illumination. Expression of fluorescent protein tags or staining with fluorescent dyes (e.g. MitoTracker, DRAQ5, etc.) can also be used to label ROIs, provided their fluorescent properties meet the same wavelength and clarity requirements recommended for immunostaining. If the signal intensity from fluorescent protein tags is insufficient, antibody staining against the tags can be applied to enhance signal quality.

Input Protein Quantity

Once cells or tissue sections have been photolabeled, the samples are ready for downstream processing. This includes protein extraction, labeled-protein enrichment, on-bead digestion and peptide desalting, using the Synpull™ kit. The input protein quantity in the enrichment step is a critical factor affecting the outcome of LC-MS analysis. To achieve optimal results, we recommend using 200-400 µg total protein input at the enrichment step for a single MS sample, with a minimum requirement of 100 µg—based on data from Thermo Scientific Orbitrap Fusion Lumos mass spectrometer; these values may vary depending on the instrument used.

Experimental Design

Control Samples

Depending on the type of proteomics study, discovery or comparative, the control group samples will vary. For discovery proteomic studies, unlabeled samples are typically used as the control group. These samples should be prepared in parallel with the experimental group. Unlabeled (UL) control samples are prepared identically to the photolabeled (PL) experimental samples, including the addition of the photolabeling reagent. However, only the PL samples are exposed to the laser, resulting in photobiotinylation of the PL sample. Once the labeling reagent is added (refer to the Synlight-Rich™ kit protocol), the UL control sample should be kept in a dark environment until the photolabeling of the PL sample is complete.

For comparative proteomic studies, the control group typically consists of samples receiving a placebo treatment or with the same genetic background, such as solvent-treated cells, wild-type animals, or healthy individuals. The control sample should undergo the same ROI photolabeling step as the experimental group. In addition, to account for background and non-specific signals, paired UL control samples should always be prepared in parallel for each group (both the experimental and the primary control groups) as in the discovery studies.

Sample Number Determination

The total number of samples (cell slides or tissue sections) required for a Microscoop-LC-MS/MS project is estimated by (1) the average number of mask pixels per field of view (FOV), (2) the number of FOVs per chamber slide or section, (3) the number of desired biological replicates, and (4) the number of samples needed for preliminary photolabeling pre-tests. At least 2 slides or sections are typically required for the pre-test experiments to establish the mask pattern, obtain the average ROI pixel count per FOV, and assess the number of FOVs per slide or section.

Pixel count refers to the number of pixels within the area of ROI defined by the binary mask pattern for photolabeling. The pixel count number in an FOV can be measured in the Autoscoop software's Pattern Generation module after a satisfactory image processing procedure is established to mark the ROI. It is directly correlated with the relative amount of proteins that can be biotinylated and impacts on the quality of subsequent mass spectrometry (MS) data. To ensure sufficient protein yield for MS analysis, we recommend a total pixel count exceeding 5×10^7 pixels (minimum 1×10^7 pixels) per MS sample for cell samples, and over 1×10^7 pixels (minimum 5×10^6 pixels) for tissue samples. These recommended pixel counts are based on high-resolution imaging using the 40X objective of Microscoop®. Pooling multiple cell chambers or tissue sections may be required to obtain the desired total pixel counts for downstream applications.

Discovery Proteomics Study

Table 2. An experimental design example for discovery proteomics study with cultured cells.

	Experimental group	Control group
Condition	Photolabeled (PL)	Unlabeled (UL)
Cell line	RPE-1	
Cell seeding number	2 x 10 ⁵ cells/slide	
Target (ROI)	Cilia	N/A
Pixels per FOV*	2,000	N/A
FOVs per slide**	10,500	N/A
Pixels per slide	2 x 10 ⁷	N/A
Number of slides	3	3
Total pixel count***	6.3 x 10 ⁷	N/A
Total protein****	200-400 µg (minimum 100 µg required) per MS sample. Ensure equal input quantities are used for the pulldown for both groups within each biological replicate.	
* FOV, field of view. ** 1-well chambered coverslip. *** For one MS sample. **** The values are based on data from the Thermo Scientific Orbitrap Fusion Lumos mass spectrometer, and they may vary depending on the instrument used.		

- 6.3 x 10⁷ (estimated total pixel count) = 2,000 (pixels/ FOV) x 10,500 (FOVs/ slide) x 3 (slides)
- 20 (total number of slides) = (3 (PL) + 3 (UL)) x 3 (biological repeats) + 2 (pre-tests)

Table 2 shows an experimental design example for a discovery proteomics study on cilia in cultured RPE-1 cells. In this setup, the experimental group is photolabeled cells and the control group is unlabeled cells. Initial pretests determined the average pixel number per FOV and number of FOVs per slide. The estimated total pixel count per MS sample is calculated as follows:

If there are **2,000** pixels per FOV and **10,500** FOVs per slide, as a standard 1-well chambered slide generally offers about 70 x 150 FOVs across its growth area, pooling **3** slides would generate **6.3x10⁷** labeled pixels—sufficient for one MS sample. For the full experiment, a minimum of 20 slides are required based on the following calculation:

$$(3 \text{ [PL group]} + 3 \text{ [UL group]}) \times 3 \text{ (biological replicates)} + 2 \text{ (pretest)} = 20 \text{ (total number of slides)}$$

Comparative Proteomic Study

Table 3. An experimental design example for comparative proteomic study on the tissue specimens.

Condition	Experimental groups		Control groups	
	Photolabeled (PL)	Unlabeled (UL)	Photolabeled (PL)	Unlabeled (UL)
Genotype	Gene A knockout (KO)		Wild-type (WT)	
Specimen	Mouse brain, FFPE			
Section thickness	10 μ m			
Target (ROI)	Microglia			
Pixels per FOV*	5,000	N/A	5,000	N/A
FOVs per section	400	N/A	400	N/A
Pixels per section	2×10^6	N/A	2×10^6	N/A
Number of slides	6	6	6	6
Total pixel count**	1.2×10^7	N/A	1.2×10^7	N/A
Total protein***	200-400 μ g (minimum 100 μ g required) per MS sample. Ensure equal input quantities are used for the pulldown for all groups within each biological replicate.			
* FOV, field of view. ** For one MS sample. *** The values are based on data from the Thermo Scientific Orbitrap Fusion Lumos mass spectrometer, and they may vary depending on the instrument used.				

- 1.2×10^7 (estimated total pixel count) = 5,000 (pixels/ FOV) x 400 (FOVs/ section) x 6 (sections)
- 38 (total number of sections/genotype) = (6 [PL] + 6 [UL] (sections)) x 3 (biological repeats) + 2 (pre-tests)

Table 3 Illustrates the experimental design for a comparative proteomics study aimed at distinguishing microglial proteomes between wild-type (WT) and Gene A knockout (KO) mice. In this study, the experimental group is the KO mouse sample, while the control group is the WT mouse sample. Both groups are subjected to ROI photolabeling and have their respective UL controls. Using the average pixel count per FOV and number of usable FOVs per section obtained from a pre-test, the estimated total pixel count per MS sample is calculated as follows:

If there are **5,000** pixels per FOV and **400** FOVs per section, pooling **6** sections would generate **1.2×10^7** pixels—sufficient for one MS sample.

For the full experiment, including 3 replicates, a minimum of 38 sections *per genotype* are required based on the following calculation:

(6 [PL group] + 6 [UL control group]) x 3 (from different mice for 3 biological replicates) + 2 (pretest) = 38
(total number of sections per genotype).

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