Technical Note

Ex Vivo Imaging Quick Start Guide

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I. Near-infrared Dye Labeled Targeting Agents and Optical Imaging

You probably know that fluorescently labeled targeting agents and optical imaging are effective for monitoring disease progression and evaluating treatment efficacy in animals. Near-infrared (NIR) labeled targeting agents are valuable for non-invasive, longitudinal study of disease (including tumorigenesis and metastasis). A properly developed NIR-labeled targeting agent can even enable preclinical study of therapeutic effects, pharmacokinetics, and pharmacodynamics drug-target interactions.

But what does "properly developed" mean? How do you even get started if the possibilities and complexities of developing a probe are completely new to you?

The goal of this guide, and the other three guides in this series, is to help you with the basic questions you need to answer when confronted with the task of developing an optical probe. Each guide tackles a specific segment of the process, and resources are linked at every step for further exploration.

To get started, here are the main phases of the probe development process and the guides that introduce each phase.

Probe Design and *In Vitro* Validation (licor.com/probe_design_guide)

In Vivo Validation of Optical Probes (licor.com/in_vivo_guide)

Ex Vivo Validation of Optical Probes (licor.com/ex_vivo_guide)

> **More Info:** If you should encounter any questions during the probe development process, the LI-COR Custom Services team is available to help. Contact us (licor.com/customservices) with your questions or if you would like help under the hood taking your early phase project to a clinical trial.

II. Introduction to *Ex Vivo* Imaging

Once the specificity, clearance, and biodistribution of an optical probe have been determined non-invasively, the next questions that need to be addressed include:

- Are there specific organs of retention?
- Are signals uniform or mottled within an organ?

NIR-labeled targeting agents provide the unique ability to see deeper into tissue to answer these questions. NIR fluorescent signal will remain constant for an extended period, enabling you to image properly preserved organs and tissues later, at your convenience.

Pearl Trilogy Organ Imaging

Imaging organs and tissues with the Pearl Trilogy is simple and intuitive. The Pearl Trilogy Organ Tray Base and Disposable Organ Trays (licor.com/organ_tray) are designed specifically for excised organ and whole tissue evaluations after administration of labeled targeting agents.

Illumination on the Pearl Trilogy imaging bed is even and consistent, ensuring that multiple organs can be imaged together under uniform light. You can then make a rapid visual assessment of multiple organs from an individual animal or for a specific organ from multiple animals.

After imaging, the Small Animal Image analysis key for Image Studio Software provides a fast, intuitive way to analyze images.

> **More Info:** Read the *Pearl Imager Precision In Organ Analysis Guide* (licor.com/organ_analysis) for more information.

Odyssey CLx Imager Organ Section and Tissue Section Imaging

Macroscopic analysis of tissue and organ sections on the Odyssey CLx Imager gives you quantitative localization and biodistribution data. On the CLx Imager, you can screen up to 30 tissue section slides in one scan at 21 µm resolution.

III. Tissue Section Imaging and Microscopy

Tissue section imaging and microscopy are effective for granular identification of targeting agent locations within an organ. With appropriate microscopy equipment, you can assess NIR-labeled targeting agent location (e.g. membrane bound, intracellular, or interstitial).

However, most microscopes are not configured for NIR fluorescence detection. If you're ready to try IRDye® Infrared Dyes on an epifluorescence microscope, this list of microscope requirements will help you get started (licor.com/microscope_requirements).

The following procedure outlines the basic steps required to prepare tissue sections for imaging on the Odyssey CLx or for examination under a microscope.

> **More Info:** You can also send your samples to LI-COR Custom Services for imaging with the Odyssey CLx or the Olympus IX81 microscope (licor.com/customservices).

IV. Fluorescence-based Tissue Analysis Procedure

Some testing and optimization will be necessary to fine-tune this general protocol for your specific experiment. Research the current literature from antibody vendors to quickly develop the best procedure (references are provided at the end of this procedure).

Note:

- **Some post-processing steps, ie steps performed after the tissue section has been cut, can deplete NIR fluorescent signal from an in vivo administered optical probe in the tissue** (especially the paraffin removal and epitope retrieval steps). If you plan to use immunofluorescent procedures after cutting a tissue section, be sure to document the tissue section signal before post-processing.
- **If you plan to use antibodies, carefully select and validate primary and secondary antibodies.** Try to find antibodies that have been validated for immunohistochemistry. Antibodies that have been validated for use in Western blot applications need to also be evaluated in tissue to ensure the antibody specificity within the tissue.

1. Prepare tissue
2. Preserve tissue
3. Cut tissue sections
4. Remove paraffin or optimal cooling temperature compound (OCT)
5. Retrieve epitope, if necessary
6. Permeabilize
7. Block
8. Mount
9. Image

V. Tissue Preparation

Begin by fixing and dehydrating the sample.

Fixation

The goal of fixation is to preserve the tissue's cellular structure as closely as possible to its native state. If fixation is done correctly, the sample can be used throughout the rest of the analysis without losing the protein of interest.

Fixation may damage or mask antigenic sites, potentially compromising the intensity of your immunostaining. You may need to test several fixatives to determine which fixative adequately preserves both effective antibody binding and structural integrity of the sample.

Fixatives Options

There are two common types of chemical fixatives:

- **Cross-linking fixatives**: These fixatives act by creating covalent bonds between proteins in the tissue. The most common fixative in histology is formaldehyde gas dissolved in water (37% w/v), but other fixatives can be used.
- **Precipitating fixatives**: Alcohols are commonly used to fix frozen sections and smears.

A variety of factors affect fixation. Some fixatives, if present in tissue, can diminish intended dye/fluorophore signal. This problem can occur when a labeled biomarker is used *in vivo* prior to tissue excision.

Dehydration

You must dehydrate tissues prior to paraffin embedding, because paraffin is immiscible with water. Dehydration is accomplished by displacing water in the tissue through a series of alcohol baths containing increasing concentrations of alcohol.

> **Important:** Never allow tissues to air dry.

VI. Preserve Tissue

You have many options for tissue preservation. The most common methods are described below. See the *Tissue Preservation Tech Note* (licor.com/tissue_section_guide) for more detail about each of the following methods.

Paraffin-embedded

Wax preserves tissue morphology and can be used to preserve tissues for years at room temperature. The most commonly used waxes are commercial paraffin waxes. Tissue

processors will likely have instrument specific procedures for paraffin embedding.

Frozen Section

The Frozen Tissue section method allows you to preserve enzyme and antigen function in your sample. The tissue section must be kept thin so it will freeze quickly before ice crystals form, which can cause artifacts in the sample. Properly preserved specimens can be kept for about a year at -80°C.

Optimal cutting temperature compounds, made of water-soluble glycols and resins, are often used as a convenient specimen matrix for cryostat sectioning of tissues at temperatures of -10°C and below. Tissues are usually sectioned into 2cm cubed pieces.

Free Floating Section

Free-floating immunochemistry is performed with sections floating in solution, usually in a large well plate. Sections are not mounted on slides until after the immunochemistry process has been completed.

Free-floating immunohistochemistry is ideal for fixed tissues. This method can be used on thicker sections, making it practical for seeing distribution of staining through the entire section.

> **More Info:** This example paper shows the use of free floating tissue sections (licor.com/brain_sections).

VII. Cut Tissue Sections

For microscopy, sections are generally cut to a thickness of 4-10 µm. Thicker sections may facilitate visualization of lower-abundance protein targets when imaging tissues on the Odyssey CLx. The following tools are commonly used to cut tissue sections.

- **Microtome**: Tool used for cutting thin slices of tissue for microscopy.
- **Cryostat**: Tool used to cut frozen sections.

VIII. Remove Paraffin or Optimal Cooling Temperature Compound

If you have used OCT to preserve your tissue, you do not necessarily need to remove it before imaging. Consult the manufacturer's instructions for more information. See the *Tissue Section Guide* (licor.com/tissue_section_guide) for more information.

IX. Retrieve Epitope

Fixation can alter protein biochemistry, causing the epitope of interest to be masked from the primary antibody. The epitope can be masked by cross-linking of amino acids within the epitope or cross-linking with unrelated peptides at or near the epitope. The process of epitope/antigen retrieval refers to unmasking the epitope to restore epitope/antibody binding. The epitope can be unmasked in several ways:

- Acid, basic, neutral treatment
- Heat
- Microwaves

X. Permeabilize

Permeabilization helps get antibodies into fixed tissue samples. The extent of permeabilization required depends on the target. If you are detecting a cell surface protein, little, if any, permeabilization will be required. If you do permeabilize, be careful not to remove the entire membrane. Permeabilization, like fixation, can affect the morphology of cells, which will affect the antigen of interest.

Commonly used detergents include NP-40, Triton X-100, Tween-20, and Saponin.

> **Important:** Optimize your procedure for each target of interest.

XI. Block

The staining procedure can be improved by blocking the non-specific binding of your antibody to the tissue section. BSA, milk, or serum can be used for this.

> **Important:** Be careful not to block with BSA from the same species as the species in which the primary antibody was raised!

The blocking procedure should be optimized prior to examining valuable tissue samples.

XII. Mount

When mounting sample on a slide, it is important to use low fluorescence mounting media. Low fluorescence mounting media are available from several vendors.

> **Important:** Some mounting media have fluorescent properties and are visible in either the 700 nm or 800 nm channel.

Test your mounting medium before using it on valuable samples!

XIII. Image Tissue Section

Finally, tissues can be imaged using microscopy techniques or whole tissue sections can be imaged on the Odyssey CLx Imager.

XIV. Imaging Tissue Sections Resources

- "Immunofluorescence General Protocol." Cell Signaling Technology. Web. 21 July 2017.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if
- "Antibody Validation for Immunofluorescence." Cell Signaling Technologies.cellsignal.com/common/content/content.jsp?id=ourApproach-validation-if
- "Protocol for the Preparation and Fluorescent IHC Staining of Frozen Tissue Sections." R&D Systems. Web. 21 July 2017. rndsystems.com/resources/protocols/protocol-preparation-and-fluorescent-ihc-staining-frozen-tissue-sections
- "Double immunofluorescence – simultaneous protocol." Abcam. Web. 21 July 2017abcam.com/ps/pdf/protocols/double%20immunofluorescence%20-simultaneous%20protocol.pdf
- "IHC staining protocol for paraffin, frozen and free floating sections." Abcam. Web. 21 July 2017. abcam.com/protocols/immunostaining-paraffin-frozen-free-floating-protocol
- "Free-Floating Versus Slide-Mounted Sections for Immunohistochemistry." Bitesize Bio. Web. 21 July 2017. bitesizebio.com/26549/free-floating-versus-slide-mounted-sections-ihc

XV. From Probe Development to Scale-up and Manufacturing

Do you have questions about developing your optical probe? From compound synthesis and dye conjugation, to quantitative assay development, to manufacturing under current Good Manufacturing Practices (cGMP), the LI-COR Custom Services team is equipped to expand your capabilities and support your projects through to completion. With over 40 years of experience as a leading innovator in NIR fluorescence technology, LI-COR is ready to help accelerate your research.

LI-COR is an ISO 9001:2015 certified company.

Global Headquarters: 4647 Superior Street • Lincoln, NE 68504
Toll free: 800-645-4267 • Fax: +1-402-467-0819 • biosales@licor.com

LI-COR GmbH, Germany, Serving Europe, Africa, and the Middle East.
LI-COR Biosciences GmbH • Siemensstraße 25A • 61352 Bad Homburg • Germany
Phone: +49 (0) 6172 17 17 771 • Fax: +49 (0) 6172 17 17 799 • bio-eu@licor.com

LI-COR Ltd., United Kingdom, Serving Denmark, Finland, Iceland, Ireland, Norway, Sweden, and UK
LI-COR Biosciences UK Ltd. • St. John's Innovation Centre • Cowley Road • Cambridge • CB4 0WS
United Kingdom • Phone: +44 (0) 1223 422104 • Fax: +44 (0) 1223 422105 • bio-eu@licor.com

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