Video Article

In Situ MHC-tetramer Staining and Quantitative Analysis to Determine the Location, Abundance, and Phenotype of Antigen-specific CD8 T Cells in Tissues

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Abstract

T cells are critical to many immunological processes, including detecting and eliminating virus-infected cells, preventing autoimmunity, assisting in B-cell and plasma-cell production of antibodies, and detecting and eliminating cancer cells. The development of MHC-tetramer staining of antigen-specific T cells analyzed by flow cytometry has revolutionized our ability to study and understand the immunobiology of T cells. While extremely useful for determining the quantity and phenotype of antigen-specific T cells, flow cytometry cannot determine the spatial localization of antigen-specific T cells to other cells and structures in tissues, and current disaggregation techniques to extract the T cells needed for flow cytometry have limited effectiveness in non-lymphoid tissues. *In situ* MHC-tetramer staining (IST) is a technique to visualize T cells that are specific for antigens of interest in tissues. In combination with immunohistochemistry (IHC), IST can determine the abundance, location, and phenotype of antigen-specific CD8 and CD4 T cells in tissues. Here, we describe a protocol to stain and enumerate antigen-specific CD8 T cells, with specific phenotypes located within specific tissue compartments. These procedures are the same that we used in our recent publication by Li *et al.*, entitled "Simian Immunodeficiency Virus-Producing Cells in Follicles Are Partially Suppressed by $CD8^+$ Cells *In Vivo*." The methods described are broadly applicable because they can be used to localize, phenotype, and quantify essentially any antigen-specific CD8 T cell for which MHC tetramers are available, in any tissue.

Video Link

The video component of this article can be found at https://www.jove.com/video/56130/

Introduction

T cells are critical to many immunological processes, including detecting and eliminating virus-infected cells, preventing autoimmunity, assisting in B-cell and plasma-cell production of antibodies, and detecting and eliminating cancer cells. The development of peptide/MHC class I tetramer staining of antigen-specific CD8 T cells[1] and the more recent development of MHC class II tetramer staining of CD4 T cells[2] by flow cytometry revolutionized our ability to study and understand the immunobiology of T cells. While extremely useful for determining the quantity and phenotype of antigen-specific T cells, flow cytometry does not allow for the detection of the spatial localization of antigen-specific T cells to other cells and structures in tissues, and current disaggregation techniques to extract the T cells needed for flow cytometry have limited effectiveness in non-lymphoid tissues[3].

We and others have developed methods using peptide-loaded MHC class I and class II tetramer or multimer reagents to stain antigen-specific CD8 and CD4 T cells in tissues[4,5,6,7,8,9,10,11,12,13]. These IST methods allow for the determination of the location, abundance, and phenotype of antigen-specific CD8 and CD4 T cells in tissues and provide a means to detect of these cells relative to other cells and structures in the tissues. Our group has extensively used MHC-I tetramer staining to study human immunodeficiency virus (HIV)- and simian immunodeficiency virus (SIV)-specific CD8 T cells in lymphoid, genital, and rectal tissues to gain an understanding of HIV and SIV immunopathogenesis and to identify correlates of successful vaccination strategies[14,15,16,17]. In addition, we also developed a technique that combines IST with *in situ* hybridization (ISH) to localize and quantify virus-specific CD8 T cells and virus-infected cells in tissues and to determine the *in vivo* effector-to-target levels[18,19].

Here, we describe a protocol using peptide-loaded MHC-I tetramers to stain antigen-specific CD8 T cells in fresh tissue sections, to counterstain tissues using IHC, and to quantify cells with specific phenotypes in specific tissue compartments. These procedures are the same as were used in our recent publication by Li *et al.*, in which we determined the location, abundance, and phenotype of SIV-specific T cells in lymphoid tissue during chronic SIV infection in macaques[20].

For this procedure, fresh tissues are sectioned and incubated overnight with peptide-loaded MHC-I tetramers conjugated to fluorescein thiocyanate molecules (FITC). They are then fixed in paraformaldehyde. After fixing the tissue, the signal from the MHC tetramers is amplified using rabbit anti-FITC antibodies and incubated with fluorescently tagged anti-rabbit IgG antibodies, which further amplify the signal from the

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bound tetramers. IHC is used in conjunction with IST to characterize antigen-specific T cells and surrounding cells. Antibodies that recognize epitopes on the surface of cells or in the extracellular space are included in the primary incubation with the tetramers. Antibodies that recognize intracellular epitopes require permeation of the cell wall prior to staining. The stained tissue sections are imaged using a confocal microscope and analyzed using confocal software. Labeled cells are quantified using confocal microscopy software or ImageJ. The described protocol can be used to stain essentially any antigen-specific CD8 T cell in any tissue for which MHC-I tetramers are available.

Protocol

1. Day 1: Fresh Tissue Sectioning and Primary Incubation

1. Use a scalpel to cut fresh tissue into small (approximately 0.5 cm wide by 0.5 cm tall) pieces. Separately glue each tissue to a plunger and embed them with 3 - 5 mL of 4% low-melt agarose in PBS. Label the plunger with the tissue information using a sticker. Put it in a chilled holder in an ice bucket to solidify.
2. Turn on the microtome and set the thickness of the sections to 200 µm. Install a razor blade on the microtome and insert the plunger mounted with tissue in the microtome bath.
3. Prepare phosphate-buffered saline with heparin (PBS-H) by adding 100 µg/mL or 18.7 U/mL heparin to PBS to preserve the RNA and to permit potential ISH applications downstream. Fill the microtome bath, covering the embedded tissue, with 100 - 120 mL of chilled, sterile PBS-H. Add PBS-H ice cubes to the bath to maintain the temperature at 0 - 2 °C. Start the microtome and cut the tissue into 200 µm sections.
   Note: It is important to keep the tissues chilled on ice to minimize cellular activity within the tissues and because fresh tissue is easier to section when it is chilled. PBS alone can be used if there are no plans for downstream ISH.
4. Alternatively, for tissues that do not cut well with a microtome (*e.g.,* gut and lung), use a scalpel or razor blade to cut the tissue into thin strips as close as possible to 200 µm.
5. Label the lid of 24-well tissue culture plates with the experimental sample information and place the tissue chambers in the corresponding wells. Use a paintbrush to transfer the sections to a tissue chamber set in the well of a 24-well tissue culture plate containing 1 mL of chilled PBS-H.
   NOTE: Reusable tissue chambers should be made prior to initiating staining. Tissue chambers can be made using a 14 mL polypropylene round-bottom snap-cap tube and wire mesh. Use a sharp razor blade to cut the bottom of a 14 mL polypropylene round-bottom snap-cap tube off. Cut the wire mesh in a circle to fit the hole at the bottom of the tube. Heat the wire mesh circle using a Bunsen burner until it is red-hot. Set the wire mesh circle down very quickly and push the tube onto the mesh. Check that the wire mesh is securely attached to the bottom of the tube and then carefully cut off the top of the tube at the 3 mL mark using a sharp razor blade. Put up to 3 tissue sections into each tissue chamber, or up to 1 cm^2 of tissue per well. Keep at least one empty well between wells with different antibody combinations to avoid cross-contamination.
6. Proceed to the primary tetramer and antibody staining immediately after finishing the transfer of all cut sections into the tissues chambers. Keep the sections submerged and chilled in 1 mL of PBS-H at all times.
7. Incubate the tissue sections overnight with 0.5 µg/mL FITC-conjugated, peptide-loaded MHC-I tetramers diluted in PBS-H with 2% normal goat serum (NGS). Include mouse or other non-rabbit antibodies directed at extracellular epitopes of interest in this incubation (*e.g.,* rat anti-CD8 antibodies diluted 1:500 in PBS-H with 2% NGS). Place 1 mL of diluted antibodies in each well.
   NOTE: Care should be taken when selecting CD8 antibodies, as some can enhance and some can inhibit MHC tetramers binding to T-cell receptors[4,21]. The rat anti-human CD8 antibody described here is unstable and sometimes results in somewhat weak staining. It is used here for triple labeling because it is the only non-rabbit and non-mouse CD8 antibody tested that stained rhesus macaque CD8 T cells.
8. Use 1 mL of solution per well for the primary antibody and all subsequent incubations, and perform this and all subsequent incubations at 4 °C, with the plates on a rocking platform.
   NOTE: Tissues should float freely in the chamber.

2. Day 2: Fixation and Secondary Incubation

1. After the primary incubation, wash the sections twice with 1 mL of chilled PBS-H for 20 min each wash. Do this by transferring the tissue chambers to a different 24-well tissue culture plate containing 1 mL of chilled PBS-H in the corresponding wells.
   NOTE: Be careful not to drip contents from one experimental sample into another when moving between tissue chambers. For all subsequent incubations and washes, similarly transfer the tissue chamber to a clean plate containing the appropriate solution. Be sure to monitor the sections in the tissue chambers during the procedure to make sure that the sections do not get stuck to the sides of the tissue chambers. If they do, push them back into the solution.
2. Fix the sections with 1 mL of fresh PBS-buffered 4% paraformaldehyde for 2 h at room temperature (do not over-fix). Wash with cold PBS-H twice for 5 min.
   Caution: Paraformaldehyde is toxic; wear appropriate personal protective equipment.
   NOTE: If antigen retrieval and permeabilization is needed to detect intracellular epitopes, antigens can be retrieved by boiling the sections in 0.01 mol urea after the paraformaldehyde fixation.
3. Transfer the sections into 24-well culture plates containing 0.01 mol urea and place these plates in a microwave oven. Boil the sections three times for about 10 s each, for a total of 30 s.
   NOTE: Be very careful, as boiling the solution can force the sections out of the wells. If this happens, use a paintbrush to push the sections from the sides of the tissue chamber or from the lid of the plate back into the bottom of the appropriate tissue chamber.
4. Prior to the secondary antibody incubation, permeabilize and block the tissue sections by incubating them in blocking solution containing PBS-H, 0.3% detergent (PBS-H-T), and 2% NGS on a rocker for 1 h at 4 °C. Perform subsequent antibody incubations with PBS-H-T/2% NGS.
5. For the secondary incubation, transfer the sections in the tissue chambers to wells containing rabbit anti-FITC antibody diluted 1:10,000 in PBS-H-T/2% NGS. Incubate overnight.

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6. Perform counterstaining using mouse anti-CD20 antibody diluted 1:200 in PBS-H-T/2% NGS. For this option, retrieve the epitopes if needed, permeate the cells, and block prior to this incubation, as described above.

3. Day 3: Tertiary Incubation

1. After the second incubation, wash the sections three times in PBS-H at 4 °C for at least 20 min.
2. Perform a final incubation with the appropriate fluorescently labeled antibodies (*e.g.,* goat anti-rabbit conjugated greenish yellow, goat anti-rat conjugated far red dye, and goat anti-mouse conjugated green dye antibodies diluted 1:5,000, 1:5,000, and 1:2,000, respectively, in PBS-H-T/2% NGS). Incubate overnight.
   NOTE: At this point, the incubation can be extended for up to three days if needed. Keep the sections protected from light by wrapping the plates in tin foil during this incubation step and thereafter, as light quenches fluorophores.

4. Day 4: Mounting the Sections

1. Wash the sections three times in PBS-H for at least 20 min.
   NOTE: If planning for downstream ISH[19], fix the sections in 4% paraformaldehyde for 1 h to secure the tetramers and antibodies in place and then wash the sections twice with PBS-H for 5 min each.
   Caution: Paraformaldehyde is toxic; wear appropriate personal protective equipment.
2. Use a paintbrush to transfer the sections to a microscope slide. Be careful not to poke the tissue too much. Coat each section with glycerol/gelatin containing 4 mg/mL n-propyl gallate or another mounting medium containing a fluorophore preservative. Cover with a coverslip.
3. Store the slides in a light-protected slide container at -20 °C. Rinse the tissue culture plates and remove the labels on the lids using alcohol.
   NOTE: The plates can be reused.

5. Acquisition of Confocal Microscope Images

1. Capture high-resolution images with a confocal microscope, using the appropriate lasers and filters for each fluorophore (**Figure 1A** and **B**).
   NOTE: In this example, a confocal microscope (see the **Table of Materials**) was used. Images were collected using the 561 nm laser at 20% power for the greenish yellow-labeled antigen-specific T cells, the 488-nm laser at 10% power for green-labeled CD20-expressing B cells, and the 640-nm laser at 15% power for far red-labelled CD8 T cells. The 20X objective and a numerical aperture of 0.8 were used.
2. Sequentially collect z-series at 3 µm (or other) intervals in the three channels in multiple 800 x 800 pixel fields. Construct a montage of the collected fields (**Figure 1C-E**). Name each montage image based on the information of the corresponding slide and save for analysis.
3. Perform quantitative image analysis using the respective confocal microscope analysis and quantification software or using ImageJ.

6. Quantitative Image Analysis

NOTE: Quantitative image analysis can be accomplished using confocal microscope analysis and quantification software or by using ImageJ software. Here, ImageJ was used as an example.

1. Open a confocal montage by dragging it to the ImageJ window (**Figure 2A**).
   NOTE: ImageJ can directly open montages collected by many different confocal microscopes. If the montage cannot be directly opened by ImageJ, export the selected z-scan as a TIFF file to open it.
2. Duplicate the selected z-scan for analysis ("Image"-> "Duplicate") (**Figure 2B**).
3. Split the different channels ("Image"-> "Color"-> "Split Channels") (**Figure 2C**).
4. Draw the ROI for quantitative analysis in the corresponding channel to be objective and add it to the ROI manager by pressing "T" on the keyboard. Measure the area.
   NOTE: The ROI manager of ImageJ shows the area in μm^2 (**Figure 2D**).
5. Adjust the fluorescence brightness and contrast of the channel to be analyzed ("Image" -> "Adjust" -> "Brightness/Contrast") (**Figure 2E**).
6. Flatten the ROI on the image ("ROI manager" -> "Flatten") (**Figure 2F**).
7. Quantify the positive cells in the image using the "Multi-point" tool (**Figure 2G**).

Representative Results

Figure 1 shows how to collect confocal images using a confocal microscope. **Figure 2** demonstrates quantitative image analysis using ImageJ. **Figures 3** and **4** show representative images of lymph node tissues from an SIV infected rhesus macaque stained with MHC tetramers, CD8 antibodies, and CD20 antibodies, and serve to demonstrate the specificity of the MHC-tetramer staining. **Figure 3** compares MHC class I tetramers loaded with a peptide from SIV compared to sections from the same tissue stained with MHC class I tetramers loaded with an irrelevant peptide. **Figure 4** shows that cells stained with the MHC/SIV-peptide tetramer are co-stained with CD8 antibodies, but not CD20 antibodies, which stain B cells. **Figure 5** shows an example of a montage created from multiple confocal z-series fields used for quantification of tetramer stained cells with specific phenotypes in different anatomical compartments on the lymph node. The enlargement shows an area of the lymph node with a B cell follicle delineated by CD20 staining, and surrounding T cell zone, in which MHC-tetramer-stained cells can be detected, some of which co-express Ki67 which is a marker of T cell activation and proliferation.This staining combination allows the determination of the phenotype of SIV-specific CD8 T cells inside and outside of B cell follicles, in relationship to SIV-specific CD8 T cells and to other Ki67 expressing cells, and B cells.

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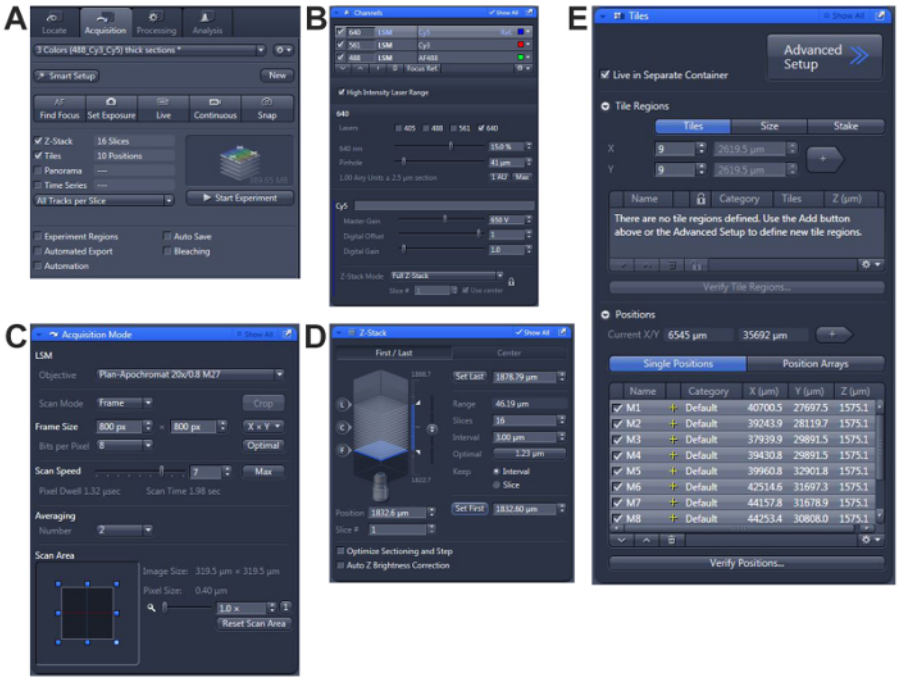

Figure 1: Representative Screenshots Showing How to Collect Confocal Images Using a Confocal Microscope. (**A**) Acquisition mode used to collect confocal images. (**B**) Channels used for image collection. (**C**) 20X objective and 800 x 800 pixel fields were used in image collection. (**D**) A sequential z-stack was collected at 3 µm intervals. (**E**) Tiles were adopted to delineate and collect images in multiple fields. Please click here to view a larger version of this figure.

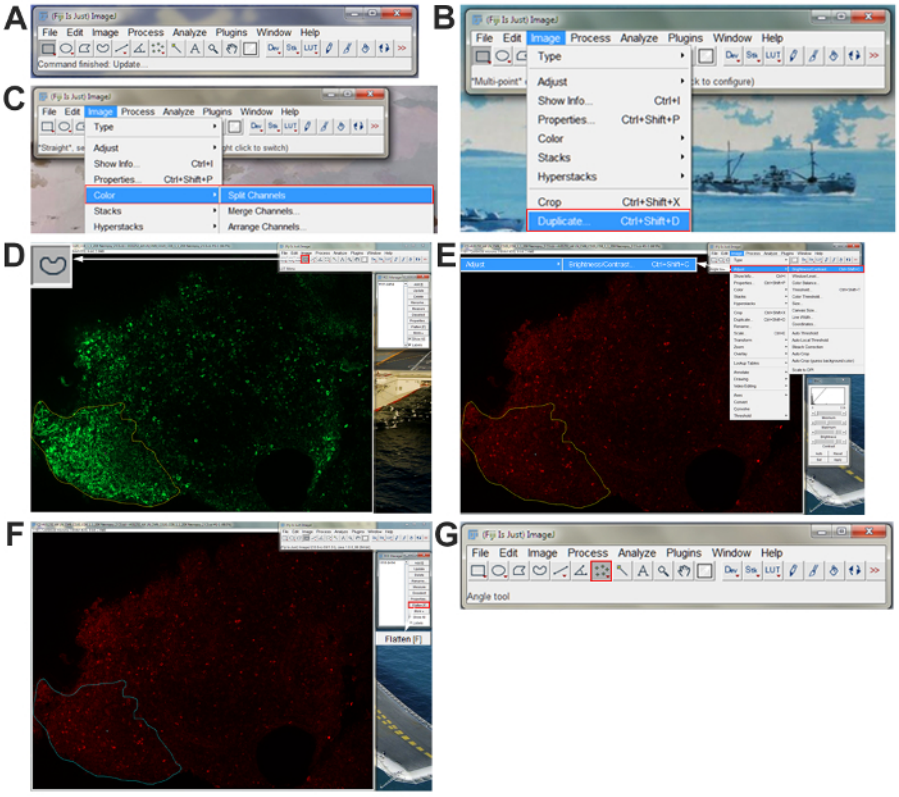

Figure 2: Representative Screenshots Demonstrating Quantitative Image Analysis Using ImageJ. (**A**) Open a confocal montage by dragging it to the ImageJ window. (**B**) Duplicate the selected z-scan for analysis. (**C**) Split the different channels. (**D**) Draw the ROI for quantitative analysis in the corresponding channel to be objective and add it to the ROI manager by pressing "T." (**E**) Adjust the fluorescence brightness and contrast of the channel to be analyzed. (**F**) Flatten the ROI on the image. (**G**) Count the positive cells in the image. Please click here to view a larger version of this figure.

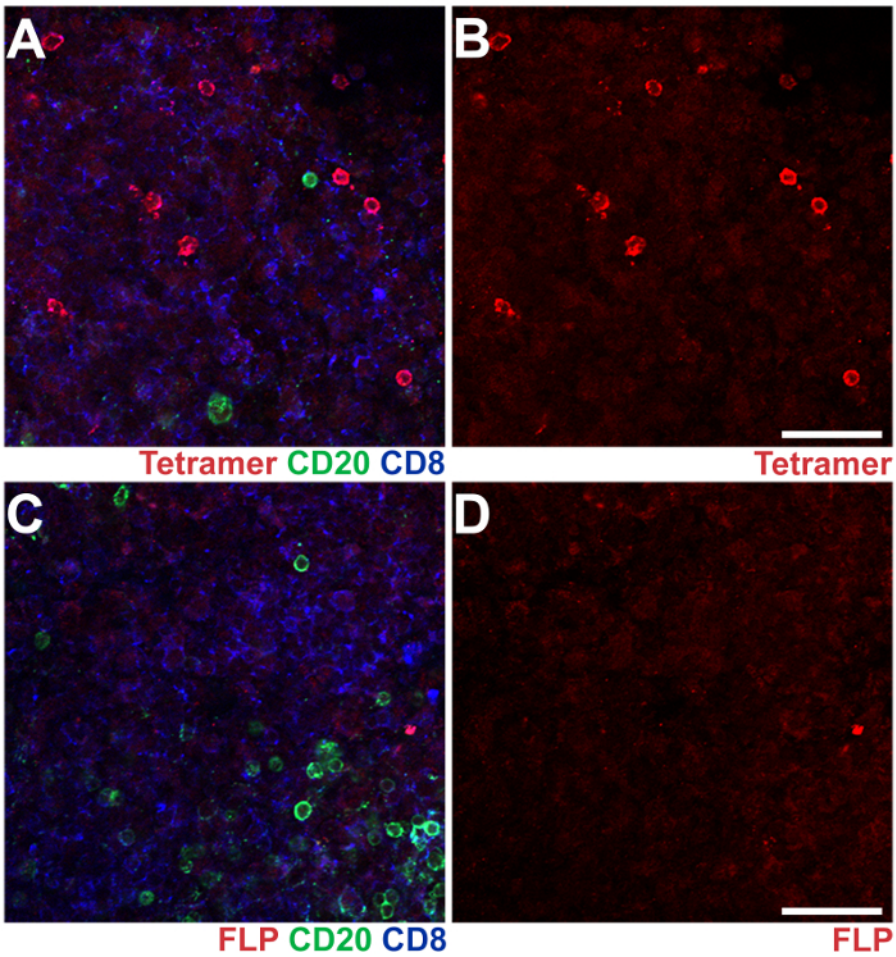

Figure 3: IST Combined with IHC in Axillary Lymph Node Sections from an SIV-infected Rhesus Macaque. Mamu-A*02 tetramers loaded with SIV Nef YY9 peptides were used to stain antigen-specific CD8 T cells, and similar tetramers loaded with an irrelevant FLP peptide from the hepatitis B virus were used as a negative control (red). Mouse anti-CD20 antibodies were used to stain $CD20^+$ B cells (green), and rat anti-CD8 antibodies were used to stain $CD8^+$ T cells (blue). (**A**) A representative image from an axillary lymph node section stained with YY9 tetramers, CD20, and CD8 antibodies. (**B**) The same image as in panel A showing the YY9 tetramer stain alone. (**C**) Representative image of a section from the same axillary lymph node, stained with FLP tetramers and CD20 and CD8 antibodies. (**D**) The same image as in panel C with the FLP tetramer stain alone. Scale bars = 100 µm. Please click here to view a larger version of this figure.

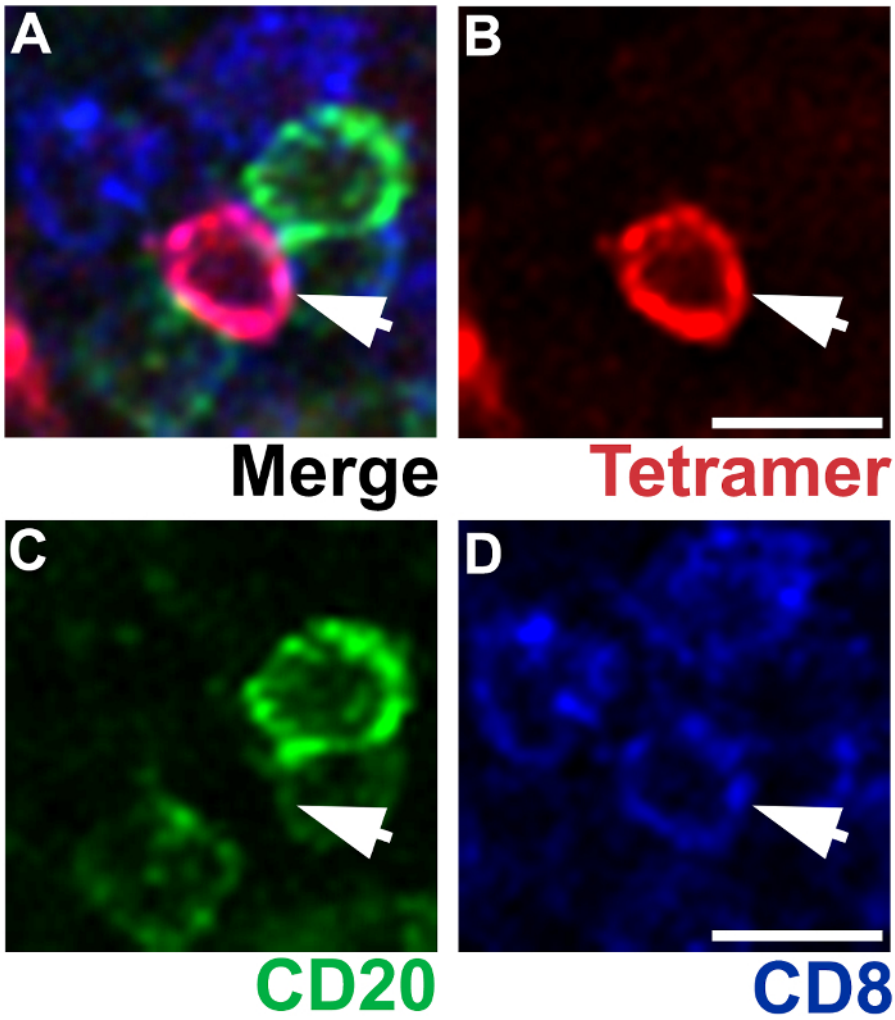

Figure 4: IST Combined with IHC Showing the Specificity of MHC-tetramer Staining. Representative axillary lymph node section stained with Mamu-A*02 tetramers loaded with Nef YY9 peptides to label SIV-specific CD8 T cells (red), mouse anti-CD20 antibodies to label B cells (green), and rat anti-CD8 antibodies to label CD8 T cells (blue) (**A**). An SIV-specific CD8 T cell is tetramer+ (**B**), $CD20^-$ (**C**), and $CD8^+$ (**D**). Scale bars = 10 µm. Please click here to view a larger version of this figure.

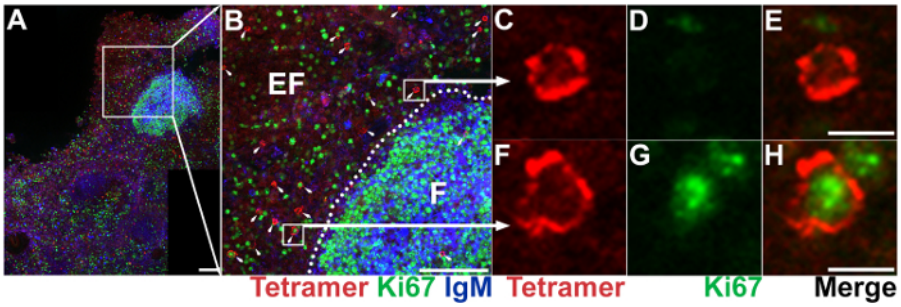

Figure 5: IST Combined with IHC to Show the Location, Abundance, and Phenotype of Antigen-specific CD8 T Cells. (**A**) Representative axillary lymph node section stained with Mamu-A*02 tetramers loaded with Nef YY9 peptides to label SIV-specific CD8 T cells (red), Ki67 antibodies to label proliferating cells (green), and IgM antibodies to label B cells (blue). Scale bar = 100 µm. (**B**) Enlargement of a selected region in panel A. IgM staining defines the follicular area, which is marked as "F;" the extrafollicular area is marked as "EF." $Tetramer^+$ cells are indicated with arrows. Scale bar = 100 µm. Representative $tetramer^+$ $Ki67^-$ cell (**C**, **D**, **E**) and $tetramer^+$ $Ki67^+$ cell (**F**, **G**, **H**). Scale bar = 10 µm. Please click here to view a larger version of this figure.

Discussion

IST combined with IHC provides an essential tool for detecting, characterizing, and quantifying antigen-specific CD8 T cells in native environments with the context of other cells and tissue structures. Here, we described detailed procedures for IST combined with IHC, followed by quantitative image analysis, to determine the location, abundance, and phenotype of antigen-specific CD8 T cells in lymph nodes from rhesus

macaques. Similar staining can be applied to human, mouse, or other species tissues for which MHC-I tetramers are available. In addition, peptide MHC Class II tetramer or dextramer staining can be performed using relatively similar methodologies to label antigen-specific CD4 T cells in tissues[4,5,6,7,8,9,10,11,12,13]. IST can also be combined with ISH to determine, for example, *in vivo* effector-to-target cell levels[18,19]. In the future, it will be interesting to carry IST/IHC further by combining IST/IHC with advanced *in situ* RNA and DNA detection methodologies. Recent advancements in *in situ* hybridization assays include the development of RNAscope and DNAscope[24]. These techniques allow for the detection of target RNA and DNA in tissues. It will be exciting to combine these methodologies with IST and IHC to simultaneously detect virus-specific-CD8 T cells, viral RNA, viral DNA, and antibody-labeled antigens of interest.

While we originally described IST methods with fresh tissues, tissues that were fixed for a short duration, and frozen tissues[4], in recent years, we have exclusively used fresh tissue sections, as they consistently produce the highest-quality stains and allow for the examination of cells in thick tissue sections. As an alternative to the procedures presented here, one can apply tetramers to tissues, incubate overnight, fix, embed, and cryopreserve in freezing medium *(e.g.,* OCT), produce frozen thin sections, and perform IHC later[22]. Similarly, we routinely stain a subset of tissue sections with tetramers alone and then freeze the sections in OCT to allow for additional counterstaining combinations in the future. In addition, Qdot 655-conjugated peptide-MHC multimers can be used to directly visualize antigen-specific T cells in cryopreserved tissue sections[13].

We have described here indirect tetramer staining. Direct staining using APC- or PE-conjugated tetramers has also been shown to work[4,22]. In this case, the concentration of MHC tetramer required is higher than that used in indirect tetramer staining. In our hands, a concentration of 20 µg/mL of APC-labeled tetramer was effective at detecting antigen-specific cells. However, the staining intensity was much lower than that obtained with indirect labeling, which includes amplification with anti-FITC antibodies.

We found that the use of a compression-based microtome (see the **Table of Materials**) for fresh tissue cutting eased the process of cutting fresh tissue sections as compared to using a vibrating microtome[23]. However, in instances where a compression-based microtome is not available, a vibrating microtome or scalpel can be used for fresh tissue sectioning.

A major limitation of this technique is the use of fresh tissues. Using fresh tissues is much more difficult than fixed or frozen tissues because they require immediate attention and processing. We have successfully shipped fresh tissues overnight on ice in tissue culture medium or PBS-H. However, there have been occasional issues with shipping; for example, snow storms have delayed the shipment of tissues for 48 h or more. In these instances, we found that fresh tissues sectioned and those stained 48 h post-extraction generally show specific staining, with signs of some tissue degradation; tissues stained 72 h post-extraction are too degraded for staining. We also found that the shipment of fresh tissues with ice blocks that are too cold or too close to the tissues can freeze the tissues during shipping; this freezing generally destroys the tissue for staining. Thus, it is extremely important to ship fresh tissues chilled, but not frozen, and to initiate IST staining within 24 h. Fresh tissue processing also requires a great deal of student and staff time, as tissues from multiple animals or study participants cannot be collected and stained together on a later date. Despite these difficulties, we find that fresh tissue sections are the best choice for beautiful, specific IST/IHC staining.

Another limitation of the IST/IHC method described here is the indirect staining approach. Due to limitations on the number of distinct species of animals available to generate secondary antibody combinations, we are limited by indirect antibody staining methods to only three or four fluorescent antibody staining combinations at a time. This limits the amount of information that can be collected on one tissue slab. Direct IHC staining overcomes this limitation and can expand the capabilities, detecting eight or more antibody-tagged antigens simultaneously, albeit with each antibody producing a much weaker fluorescent signal compared to indirect methods. Thus, indirect IHC might be used as an alternative to indirect IHC for counterstaining IST-stained tissues, allowing for the detection of increased numbers of cellular antigens when combined with the IST detection of antigen-specific CD8 T cells.

In some instances, substantial autofluorescence and/or non-specific tetramer or antibody binding may occur with IST/ISH. Because of this, good positive and negative controls are necessary to discern specific stains from background and autofluorescent staining. Good negative controls for MHC I tetramers include negative control tissues (*e.g.,* non-infected tissues; tissues stained with MHC I tetramers loaded with irrelevant peptides or irrelevant MHC tetramers; or, in a pinch, tissues stained with no tetramers but with amplifying antibodies).

In summary, MHC I IST combined with IHC is a valuable tool to determine the location, abundance, and phenotype of antigen-specific CD8 T cells in tissues. This methodology allows for the detection of antigen-specific CD8 T cells in native environments, with the relative localization to other cell types and tissue structures maintained. This method is broadly applicable because it can be used to localize, phenotype, and quantify essentially any antigen-specific CD8 T cell for which MHC tetramers are available, in any tissue.

Disclosures

The authors have nothing to disclose.

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