Video Article

A Technique to Simultaneously Visualize Virus-Specific CD8+ T Cells and Virus-Infected Cells *In situ*

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Abstract

The numbers and locations of virus-specific CD8+ T cells relative to the numbers and locations of their infected cell targets is thought to be critical in determining outcomes that range from clearance to chronic persistent infections. We describe here a method for assessing the spatial and quantitative relationships between immune effector (E) virus-specific CD8+ T cells and infected targets (T) that combines in situ tetramer (IST) staining to detect virus-specific CD8+ T cells and in situ hybridization (ISH) to detect viral-RNA+ cells in the tissues where the battle between immune defenses and infection takes place. The combination of IST staining and ISH, abbreviated ISTH, enables visualization and mapping of the locations of immune effector cells and targets, and facile determination of E:T ratios. These parameters in turn can then be used to determine the relationships between spatial proximity, and the timing and magnitude of the immune response that predict outcomes in early infection.

Video Link

The video component of this article can be found at https://www.jove.com/video/1561/

Protocol

This method was used in the research reported in Li *et al., Science* 323, 1726-1729 (2009).

Combined in situ tetramer staining and in situ hybridization (ISTH) procedures

ISTH procedures can be divided into 4 steps: 1) IST staining of antigen-specific CD8+ T cells in tissues; 2) ISH to detect virus-infected-RNA +cells; 3) confocal microscopic imaging of IST-stained cells and viral-RNA+ cells; 4) image analysis, including construction of image montages and mapping of tetramer+ and virus RNA+ cells.

1. IST staining of antigen-specific CD8+ T cells in tissue sections.

1. Cut fresh tissues into 200 um thick sections using a vibratome or scalpel.
2. Chill vibratome bath with sterile PBS-H and chill to 0-2° C. Set the vibratome blade angle to a rather steep 27°. Whenever possible, keep tissues chilled on ice in order to minimize degradation. Fresh tissue is also easier to section using a vibratome when it is chilled.
3. Cut tissue with surgical scissors or scalpel into small approximately 0.5cm wide by 0.25 cm tall pieces. Put tissue pieces in a weigh boat or small dish and cover with ~40°C melted 4% PBS buffered low melt agarose. Make sure tissue is in contact with the bottom of the dish. Push tissue pieces down if they float. Solidify on ice. This usually takes 3-5 minutes.
4. Coat vibratome tissue block with Loctite glue. Cut agarose around tissue with a scalpel, and then using a forceps, carefully transfer the fragile agarose embedded tissue to a vibratome block coated in glue. Do not move the piece of tissue once it is set on the tissue block. Let dry approximately 10 minutes on ice.
5. Mount tissue block in the vibratome and cut the tissue into 200um thick sections using a dead slow forward speed and relatively high amplitude. Speed and amplitude settings vary with each vibratome. If a vibratome is not available, or tissue is not amenable to vibratome sectioning, cut tissues into thin strips using a scalpel.
6. Transfer sections with a paintbrush into a tissue chamber set in the well of a 24-well tissue culture plate containing 1 ml of chilled PBS-H. Put up to four tissue sections into each tissue chamber. Label the lid of the tissue culture plate with experimental sample information and secure lid to the plate with a rubber band.
7. Alternatively, for tissues that don't cut well with vibratome, like gut, can cut as thin as possible strips with scalpel or razor blade. Put only one section per well for scalpel cut sections.
8. Proceed to staining immediately after finished cutting tissues. Keep sections chilled to minimize degradation at all times until fixed. Do not let sections dry out. Keep sections in chilled sterile PBS-H.

9. Incubate tissue sections over night with 0.5 mg/ml FITC conjugated tetramers. Can include mouse or non-rabbit antibodies directed at extracellular epitopes in this incubation, e.g. anti-CD8 antibodies, diluted 1:200 in blocking solution. Use 1 ml solution per well for this and all subsequent incubations, and perform this and all subsequent incubations at 4°C with plates on a rocking platform. Keep tissue chambers containing different experimental samples separated by at least one empty well to prevent cross contamination of solutions in subsequent steps.
10. After primary incubation, wash sections with chilled PBS-H twice (2X) for 20 minutes each. Do this by transferring the tissue chambers to a different 24-well tissue culture plate containing chilled PBS-H. Be careful not to drip contents from one experimental sample into another, when moving tissue chambers. For all subsequent incubations and washes similarly transfer the tissue chambers to a clean plate containing the appropriate solution. Monitor sections in tissue chambers during procedure to make sure that sections don't get stuck to the sides of the tissue chambers.
11. Fix sections with fresh PBS buffered 4% paraformaldehyde for 2 hours at room temperature. Wash with cold PBS-H 2X 5 min each.
12. Incubate with rabbit anti-FITC antibodies, e.g. Biodesign1:10,000, in blocking solution for one to three days. For co-labeling, can include non-rabbit antibodies directed at intracellular epitopes in this incubation as well. For this option, expose epitopes if needed and permeate and block the cells prior to the second incubation.
13. If using antibodies directed at intracellular epitopes that require antigen retrieval from formaldehyde fixation, expose epitopes by boiling 3 times in 0.01M Urea, 10 seconds each time, in a microwave oven. Wait 2 minutes between each heating. Be very careful as boiling solution can force the sections out of the wells. If this happens, use a paint brush to push sections from the sides of the tissue chamber or from the lid of the plate back into the bottom of the appropriate tissue chamber. If antibodies do not require antigen retrieval omit this step.
14. If using antibodies directed at intracellular epitopes, prior to the second incubation, permeabolize and block cells in tissue sections with PBS-H containing 0.3% triton X-100, and 2% normal goat serum for one hour. Then perform remaining antibody incubations in PBS-H containing 0.3% triton X-100 and 2% normal goat serum.
15. After second incubation, wash sections in PBS-H for 20 minutes to several hours. Repeat twice for a total of three washes. Perform a final incubation with appropriate fluorescently labeled antibodies. For example, goat anti-rabbit-Cy3 and goat anti-mouse-Alexa 488 diluted 1:1000 each in blocking solution. Incubate for one to three days. Keep sections protected from light by wrapping the plates in tin foil during this incubation and thereafter, as light quenches fluorophores.
16. Wash sections in PBS-H for 20 minutes to several hours. Repeat twice for a total of three washes.
17. For IST combined with ISH post-fix sections post-fix in 4% paraformaldehyde for 1 hour to secure tetramers and antibodies in place, then rinse sections with PBS-H again and mount.
18. Use a paintbrush to transfer sections to a microscope slide. Coat each section with glycerol/gelatin containing 4mg/ml n-propyl gallate or other mounting media containing a fluorophore preservative and cover with a coverslip. Store slides in light protected slide container at -20°. Rinse tissue culture plates and remove labels on lids with alcohol. Can reuse plates.
19. Analyze stained tissue sections with a confocal microscope. Then can proceed to ISH.

2. Detect virus-infected-RNA+ cells by in situ hybridization.

This in situ hybridization protocol was modified from our previously published protocols[4,5] as follows:

1. Re-cut 5-8-micron-thick-sectionss from thick in situ tetramer stained sections.
    1. 200-micron-thick-sections are heated at 70°C for 5 min to detach the section from the slide.
    2. The section is then embedded in OCT on dry ice.
    3. 8-micron sections are cut with a cryostat from the thick sections and adhered to silanized slides.
    4. Slides can be stored at -80°C in a sealed box for subsequent in situ hybridization.

2. In situ Hybridization
    1. Rehydrate tissue sections by immersing slides for 3 min each in 90% ethanol and DEPC-treated water.
    2. Permeabilize tissue sections by heating in 10mM citrate buffer (pH 6.0) at 98°C in a water bath for 15 min.
    3. Cool tissue sections at room temperature for 20-30 min.
    4. Wash twice in DEPC-treated water for 3 min.
    5. Acetylate tissue section by immersing slides in 0.25% acetic anhydride in 0.1 M TEA (triethanolamine, pH8.0) for 10 min.
    6. Transfer slides into 0.1 M TEA (pH8.0) for 5 min.
    7. Hybridize sections to ^{35}S-labeled virus-specific (such as HIV-1, SIV) antisense probe or sense (negative control for HIV-1, SIV), or ^{35}S-labeled LCMV riboprobes (pooled sense and antisense).
    8. Wash sections twice with 2xSSC for 5 min after overnight hybridization at 42°C.
    9. Wash sections in STE (0.5 M NaCL, 1mM EDTA, 20 mM Tris-HCL, pH 7.5) for 5 min.
    10. Digest sections with RNases A and T1 in STE at 37°C for 30 min
    11. Wash sections in 2x SSC plus 50% formamide for 5 min.
    12. Wash sections in 1x SSC 10 min.
    13. Wash sections in 0.5 x SSC 15 min.
    14. Dehydrate sections in 70, 80 and 100% ethanol containing 0.3 M ammonium acetate, each 5 min.
    15. Coat sections with nuclear track emulsion
    16. Dry in a dark room for 1 hour.
    17. Expose sections at 4°C for 11 day (SIV) and 3 days (LCMV).
    18. Develop sections in D19 (Kodak, Cat# 146 4593) for 3 min, wash in dH2O 30 seconds, fix in Rapid Fixer (Kodak, Cat# 146 4106) for 4 min, wash in dH2O 5 min x 3 times.
    19. Dehydrate in 70%, 80% and absolute ethanol, 5 min each.
    20. Mount slides in fluorescent compatible Permount medium.

3. Acquire confocal microscope images.

1. Capture ISTH images with a Bio-Rad MRC 1000 Confocal Microscope, or any other confocal microscope with Epi2-Blue Reflection device.
2. Use Epi1-605 for the red tetramer fluorochrome, Epi2-522 DF32 for the green CD8 fluorochrome, and the Epi2-Blue Reflection for silver grains (ISH signal in the developed radioautographs) at 20x (for SIV) or 60x (for LCMV)
3. Sequentially collect Z-serial images at 1-micron intervals in the three channels of each field.
4. Acquire images from each section from left to right, and top to bottom, and name each image according to its row and column in an Excel File.
5. Capture each image with a ~20% overlap with its neighboring images to avoid gaps in the reconstructed montage image.

4. Image analyses: montage image reconstruction, cell centroid calculation and Spatial analysis.

1. Project Z-serial images into a single image for each channel with Confocal Assistant (Version 4.02.).
2. Automatically merge the projected image from three channels into one RGB image, using a Photoshop 7.0 Action procedure.
3. Generate a montage image by stitching merged images together in layers in Photoshop 7.0.
4. Associate individual silver grains and tetramer stain with cells, and assign the X,Y coordinates of the centers (centroids) of the SIV RNA+ and tetramer+ cells, using MetaMorph (version 7.1.3.) or Photoshop (available from the author)
5. Export centroid data into Excel files as numeric numbers for each cell. E:T ratios are determined from the Excel files from the total numbers of tetramer+ and viral RNA+ cells in the section.

Spatial relationships between red tetramer+ and blue SIV RNA+ cells are captured by copying and pasting their respective plots from the Excel files into a Photoshop document. After decreasing the opacity of a layer to align and scale the grids so that they coincide, color-selecting and copying and pasting into a new layer the positions of the blue RNA+ cells, and then discarding the layer used to align the grids, the positions of the tetramer+ and viral RNA+ cells will be revealed in the flattened image.

5. Additional Notes

If cutting infectious tissue:

Work in a BSL2.5 lab. Take special precautions with razor blades. Put the razor into the vibratome blade mount with a forceps. If you can't put the blade in with a forceps, then put it in prior to adding tissue to the bath, and don't replace the blade. When finished cutting, remove the blade with a forceps. Spray blade with 70% EtOH or DMQ prior to removal. As always, dispose of blades in a sharps container. Change outer gloves or rinse in disinfectant after each contact with tissue or contaminated PBS in tank. When finished, if working with infectious material, add DMQ disinfectant to PBS in tank and let sit a couple of minutes prior to removing. Dispose of decontaminated PBS down the drain. Then rinse the tank with water to remove salt and disinfectant. Clean workspace and utensils with DMQ. Rinse utensils with water.

To Make tetramers from biotinylated MHC monomers:

Obtain biotinylated MHC class I molecules such as HLA-A *0201/B_2m/peptide molecules produced with either Gag (SLYNTVATL), Pol (ILKEPVHGV), or irrelevant MART peptide (ELAGIGILTV) peptides from Beckman Coulter. Biotinylated HLA-A *0301/B_2m/peptide molecules produced with either Gag (KIRLRPGGK) or Nef (QVPLRPMTYK) at the NIAID tetramer facility. Tetramers are generated by adding 6 aliquots of FITC labeled ExtraAvidin (Sigma) to biotinylated Mamu A*01/B_2m/peptide monomers over the course of 8 hours to a final molar ratio of 4.5:1. The rationale behind adding the Extravidin slowly is that during the early time points there is an overabundance of monomer which assures that all of the Extravidin added gets all four biotin sites bound. Later in the reaction, this process is less efficient because there is less monomer left and more of a chance that ExtrAvidin will not have all 4 sites bound. If added all of the ExtrAvidin at once the reaction would be less efficient and more Extravidin molecules would have only 2 or 3 monomers attached.

Discussion

Since virus RNA is used as a marker for virus-infected cells in this report, all the reagents before hybridization should be RNAase free. The in situ hybridization procedure described here has been modified to preserve the fluorescent signal from in situ tetramer staining. Since the radioautographic signal from virus-infected-cells detected by in situ hybridization is at a depth of about one-cell-diameter, the thick sections used for in situ tetramer staining have to be recut into 5-8-micron-thick sections.

This novel technique of combining in situ tetramers and in situ hybridization (ISTH) described here enables simultaneous visualization, mapping and analysis of the spatial relationship of virus-specific CD8+ T cells and virus-infected target cells. This method can be applied to evaluate CD8+ T cell based vaccine and in other non-virus pathogen and host interactions. The image analysis method described here can also be adapted to study the spatial relationships of any two-cell populations interacting in situ, e.g. we recently used the centroid mapping procedure in studying of HIV-1 sexual transmission in the nonhuman primate model of simian immune deficiency virus infection of rhesus macaques[6]

Disclosures

The authors have nothing to disclose.

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