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DOI: https://doi.org/10.1007/978-1-4939-8709-2_5

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-184006>

Book Section

Accepted Version

Originally published at:

Siliņa, Karīna; Burkhardt, Chiara; Casanova, Ruben; Solterman, Alex; van den Broek, Maries (2018). A Quantitative Pathology Approach to Analyze the Development of Human Cancer-Associated Tertiary Lymphoid Structures. In: Dieu-Nosjean, Marie-Caroline. Tertiary Lymphoid Structures : Methods and Protocols. Switzerland: Springer, 71-86.

DOI: https://doi.org/10.1007/978-1-4939-8709-2_5

A quantitative pathology approach to analyze the development of human cancer-associated tertiary lymphoid structures

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Running Head: Analysis of TLS by quantitative pathology

Funding: This work was supported by the Swiss National Science Foundation, the Sciex Foundation, the Science Foundation for Oncology, the Cancer League Zurich, the University Research Priority Program “Translational Cancer Research”, and the Swiss Cancer League.

Abstract

Tertiary lymphoid structures (TLS) develop in the human tumor microenvironment and correlate with prolonged survival in most cancer types. We recently demonstrated that TLS development follows sequential maturation stages and culminates in the generation of a germinal center (GC) reaction. This maturation process is crucial for the prognostic relevance of TLS in lung and colorectal cancer patients.

The mechanisms underlying TLS development in various inflammatory conditions or their functional relevance in tumor immunity is not fully understood. Investigating which cell types and soluble mediators orchestrate lymphoid neogenesis in human tissues requires a method that allows simultaneous detection of multiple markers.

Here we describe a quantitative pathology approach to identify and quantify different TLS maturation stages in combination with other parameters. This approach consists of 7-color immunofluorescence protocol using tyramide signal amplification combined with multispectral microscopy and quantitative data acquisition from histological images.

Key Words

Germinal center, tertiary lymphoid structure, lymphoid organ neogenesis, cancer, quantitative pathology, multispectral imaging, multispectral microscopy, Vectra, tyramide signal amplification, immunofluorescence.

1. Introduction

Lymphoid neogenesis resulting in the development of tertiary lymphoid structures (TLS) occurs under chronic inflammatory conditions and involves different cell types including perivascular stromal cells, B cells, high endothelial venules (HEVs), T cells,

macrophages and dendritic cells (DCs) (reviewed in (Jones et al. 2016; Silina et al. 2014)). We recently demonstrated that TLS development in the microenvironment of lung and colorectal cancer follows sequential maturation stages ((Posch et al. 2017; Silina et al. 2017)). The process begins with the accumulation of lymphocytes in response to chemokine cues. Next, follicular dendritic cells (FDCs) differentiate to generate TLS that resemble primary follicles of the secondary lymphoid organs. Finally, a germinal center (GC) reaction develops resulting in TLS that resemble secondary follicles. The presence of TLS in human tumor microenvironment is often associated with improved patient outcome (Dieu-Nosjean et al. 2016; Sautes-Fridman et al. 2016; Silina et al. 2014). We showed that the generation of GCs was crucial for the prognostic power of TLS in untreated lung and colorectal cancer patients (Silina et al. 2017). This suggests that TLS promote anti-tumor immunity. However, despite the frequent observation of GC-containing TLS in various human tissues, the molecular and cellular mechanisms that initiate TLS development and drive TLS maturation in different inflammatory contexts is incompletely understood.

The dissection of the interplay between different cell types and signaling molecules leading to TLS formation within the histological context requires the detection of multiple parameters on tissue sections. We focus here on immunofluorescence because it enables analysis of more markers simultaneously than bright field imaging. Several intrinsic limitations must be considered when using immunofluorescent staining of multiple parameters on the same tissue section. First, the signal of tissue immunofluorescence has lower intensity when compared to flow cytometry, which often precludes the use of directly labeled primary antibodies. In such cases, signal amplification using labeled secondary antibodies is necessary. Second, the limited number of species in which most primary antibodies are produced thus restricts the

number of markers one can analyze within the same tissue section. This obstacle can, however, be overcome by using tyramide signal amplification (TSA): Dye-conjugated tyramide molecules are activated by horseradish peroxidase (HRP), which initiates a radical chain reaction leading to the covalent binding of dye-tyramide residues to the antibody-bound protein (Faget and Hnasko 2015). Because of this permanent labeling, the detection antibodies can be removed by heating without losing the fluorescent signal of the target antigen. A second antigen can now be detected using primary antibodies raised in the same species as for the first antigen, followed by HRP-conjugated secondary antibody and a tyramide conjugated to a different dye. This detection/removal process can be repeated several times. TSA considerably increases the detection sensitivity of tissue immunostaining (Faget and Hnasko 2015) thus allowing the detection of molecules that are present in low concentrations such as secreted cytokines.

Multispectral imaging (MSI) is a recent technological advancement that allows increasing the number of fluorescent colors that can be simultaneously detected by a conventional fluorescence microscope (Mansfield 2014). MSI also provides the possibility to separate tissue autofluorescence from the specific fluorophore signals. This involves the use of a multispectral camera for the acquisition of spectral profiles of each individual fluorophore as well as the spectral profile of tissue autofluorescence using unstained tissue. Further, using a dedicated image-processing software, the spectral profiles present in each pixel can be assigned to specific fluorophores and/or autofluorescence through a process called spectral unmixing. Thus, the main factor limiting the number of parameters that can be detected simultaneously is the spectral overlap of fluorophores, which cannot be properly unmixed if their emission spectra overlap too much. The development of fluorophores

with more narrow emission spectra in combination with the multispectral imaging and TSA currently allow for robust 7-color immunofluorescence protocols. This number may still increase by development of new dyes.

Here we describe a quantitative pathology approach for the analysis of TLS in different maturation stages and in combination with other relevant TLS-associated cell types including HEVs and conventional DCs. This method can be further adapted to other tissues or scientific questions. To distinguish the different TLS maturation stages, a minimal combination of CD20-, CD21- and CD23-expression needs to be analyzed. The remaining three markers can be used to assess chemokine expression, vasculature, tissue-specific niche cells or other parameters.

The method we developed and describe here consists of (i) immunostaining of formalin-fixed paraffin-embedded (FFPE) human tissues for the detection of 6 markers plus a nuclear staining, (ii) multispectral imaging using the Vectra 3.0™ automated microscopy system (PerkinElmer), and (iii) image data analysis using Inform™ (PerkinElmer) and RStudio software. We provide details on tissue section preparation, staining procedure using tyramide-conjugated Opal™ dyes (PerkinElmer), multispectral image acquisition and analysis of quantitative imaging data using tissue and cell segmentation algorithms of Inform™ software (PerkinElmer).

2. Materials

2.1. Preparation of FFPE tissue sections

1. Freshly cut FFPE tissue sections (2-5 µm) on routine pathology glass sections (see **Note 1**) including sections from secondary lymphoid organs.
2. 55°C incubator.

3. Pressure cooker (routine kitchen cooker, 5 liters) and stove for antigen-retrieval by autoclaving (see **Note 2**).
4. Metal forceps, at least 20-cm long.
5. Pre-treatment and antigen-retrieval buffer: 1x Trilogy™ solution (CellMarque) (see **Note 3**).
6. Heat-resistant section trays (glass jars with glass section holders or plastic 50-mL tubes, depending on the number of sections being processed simultaneously) for antigen retrieval.
7. Regular section trays for washing steps.
8. Washing buffer (WB): 1x phosphate buffered saline (PBS) containing 0.1% (v/v) Triton X-100.
9. Rocking platform.
10. PAP pen, for example ImmEdge Hydrophobic Barrier PAP Pen (Vector Laboratories) (see **Note 4**).
11. 3% H₂O₂ (v/v) in double distilled water (ddW).
12. Wet chamber (a tray with a lid containing a support for holding sections and water).

2.2. Immunostaining of human FFPE tissues

1. Blocking buffer (BB): 4% bovine serum albumin (w/v), 4% normal donkey serum (v/v) in WB.
2. 37°C incubator.
3. Antibody-dilution buffer (ADB): 1% bovine serum albumin (w/v), 1% normal donkey serum (v/v) in WB.
4. Primary antibodies (**Table 1**): rabbit anti-human CD3 (clone SP7), mouse anti-human CD20 (clone L26), rat anti-mouse/human PNAd (clone MECA-

- 79), rat anti-mouse/human DC-LAMP (clone 1010E1.01), rabbit anti-human CD23 (clone SP23), mouse anti-human CD21 (clone 2G9) (see **Note 5**).
5. HRP-conjugated secondary antibodies (**Table 1**): donkey anti-rabbit IgG (H+L), donkey anti-mouse IgG (H+L), donkey anti-rat IgG (H+L), goat anti-rat IgM (μ chain-specific) with minimal cross-reactivity to immunoglobulins from other species (see **Note 6**).
 6. Tyramide-fluorophore conjugates: Opal™ 7-color Manual IHC Kit (PerkinElmer) (**Table 2**). This kit also contains the Amplification Diluent buffer necessary for generating the working dilutions of the tyramide-conjugated dyes. The kit contains an optimized combination of 6 modified fluorescent dyes with narrow emission spectra and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). The use of such dyes is crucial for multispectral image processing to enable unmixing of the spectra from dyes that are detected with the same emission filter.
 7. 95°C water bath (see **Note 7**).
 8. Heat-resistant section trays (glass jars with glass section holders or plastic 50 mL tubes, depending on the number of sections being processed simultaneously) for antibody removal.
 9. Regular section trays for washing steps.
 10. Antibody removal buffer (ARB): 10 mM citric acid, pH 6.0.
 11. WB, rocking platform and wet chamber as in Section 2.1.
 12. Antifade mounting medium without DAPI like ProLong™ Diamond (ThermoFischer) (see **Note 8**).
 13. Glass coverslips.

2.3. Multispectral imaging

1. Vectra 3.0™ multispectral imaging system (PerkinElmer).
2. Mounted tissue sections:
 - a. A fully unstained tissue section that has been subjected to the same processing protocol as the stained sections (antigen-retrieval, blocking, washing, heating) but omitting all fluorescent dyes. This section is necessary to determine the spectral profile of autofluorescence of the tissue of interest (see **Note 9**).
 - b. Tissue sections that have been stained with only one fluorescent dye, including a separate section for the DAPI staining (see **Note 10**). These sections are necessary to determine the spectral profile of each dye used in the multiplex protocol.
 - c. Sections stained with the 7-color protocol.

2.4 Image and data processing

1. Phenochart™ software (PerkinElmer).
2. Inform™ software (PerkinElmer).
3. RStudio software (see **Note 11**).

3. Methods

3.1. Immunostaining

The protocol described here has been optimized for the manual processing of up to 20 sections in parallel with the least possible number of heating steps for antibody removal to reduce the risk of tissue and epitope damage. If not indicated otherwise, all steps are performed at room temperature (RT). During all incubation steps the sections should be protected from light (i.e. washing containers and wet chambers covered with aluminum foil). Apart from the sections of interest, 10 additional

sections must be included (see above under Step 2.3.2): One section that is not stained with any of the fluorophores, 7 sections that are stained with each individual fluorophore only, 1 negative control section where all primary antibodies are omitted, and 1 positive control section (any of the secondary lymphoid organs). The single-color and unstained sections need to be included only once if the same combination of fluorescent dyes is used and the same tissue is analyzed. The single-stained sections are necessary for the generation of the spectral profile for each dye and the unstained section is necessary to establish the autofluorescence profile of the tissue of interest. These spectral profiles are used for the unmixing of fluorophore signals in the multiplex-stained tissue sections using the Inform™ software. Of note, positive and negative control sections need to be included in every experiment.

3.1.1. Section pre-processing

1. Incubate FFPE tissue sections at 55°C for 2-16 h.
2. Prepare two equal heat-resistant glass trays containing 1x Trilogy™ solution.
Place sections in a glass section holder and transfer it into one of the trays so that all tissues are fully immersed in the buffer without filling the tray to the brim. Leave the second tray with an equal amount of Trilogy™ solution without sections.
3. Fill the pressure cooker with 2 liters of ddW, place a heat-resistant platform (for example an autoclavable tip box) to prevent submersion of the section trays inside the pressure cooker.
4. Cover both trays with loose lids and place them in the pressure cooker on a stove. Close the lid of the pressure cooker, turn the stove on and wait until the pressure has reached its maximum, which is indicated by the fully lifted

pressure nob on the pressure cooker's lid.

5. Autoclave the sections for 15 minutes, then remove the cooker from the heat and release the pressure by keeping the pressure nob lifted using long forceps or other heat-resistant tool (see **Note 12**).
6. Immediately after opening the pressure cooker, carefully transfer the sections to the second tray containing the hot Trilogy™ solution. Do not shake any of the trays or the sections to avoid boiling retardation, which will damage the tissue.
7. Incubate sections in the second tray for 5 minutes to remove remaining paraffin residues.
8. Transfer the sections to another tray containing ddW and place it on a rocking platform for 5 minutes (see **Note 13**).
9. One by one, dry the glass area surrounding the tissue with a paper napkin and encircle the tissue with the PAP pen. Do not allow the tissue to dry at any time throughout the duration of the protocol.
10. Place sections in a wet chamber with tissue facing upwards, apply 3% H₂O₂ solution and incubate for 15 minutes to block endogenous peroxidases. Depending on the size of the tissue section, 100-300 µL/section is sufficient.
11. Decant the H₂O₂ solution and wash the sections in WB for 15 minutes on a rocking platform.
12. Continue with immunostaining or leave the sections in the WB at 4° C until further use (see **Note 14**).

3.1.2. Immunostaining

1. Place sections in a wet chamber with tissue facing upwards, apply BB and incubate for 15 minutes at 37°C. Depending on the size of the tissue section,

100-300 μL /section is sufficient.

2. Decant BB, place sections back in the wet chamber, apply the first primary antibody mix in ADB containing mouse anti-human CD21, rabbit anti-human CD23 and rat anti-mouse PNAd antibodies, and incubate at 4°C overnight. Depending on the size of the tissue section, 50-150 μL /section is sufficient (see **Notes 5** and **15**).
3. Decant the primary antibody mixture and wash in WB for 3x5 minutes on a rocking platform.
4. Place sections in a wet chamber with tissue facing upwards, apply the first HRP-conjugated secondary antibody (anti-mouse IgG in ADB) and incubate for 1 h (see **Note 6**). Depending on the size of the tissue section, 50-150 μL /section is sufficient.
5. Decant the secondary antibody mix and wash in WB for 3x5 minutes on a rocking platform.
6. Place sections in a wet chamber with tissue facing upwards, apply the first tyramide-fluorophore conjugate Opal-520 diluted in the Amplification Diluent buffer, and incubate for 10 minutes to detect the CD21 antigen (see **Note 16**).
7. Decant the tyramide-dye mix and wash in WB for 5 minutes on a rocking platform.
8. Place sections in a wet chamber with tissue facing upwards, apply 3% H_2O_2 , and incubate for 15 minutes to block the activity of the first HRP-conjugated secondary antibody (see **Note 17**).
9. Decant the H_2O_2 solution and wash the sections in WB for 15 minutes on a rocking platform.
10. Place sections in a wet chamber with tissue facing upwards, apply the second

- HRP-conjugated secondary antibody (donkey anti-rabbit IgG in ADB), and incubate for 1 h (see **Note 6**). Depending on the size of the tissue section, 50-150 μL /section is sufficient.
11. Repeat steps 5 to 9, but use the tyramide-fluorophore conjugate Opal-570 to detect CD23 antigen.
 12. Place sections in a wet chamber with tissue facing upwards, apply the third HRP-conjugated secondary antibody (goat anti-rat IgM in ADB), and incubate for 1 h (see **Note 6**). Depending on the size of the tissue section, 50-150 μL /section is sufficient.
 13. Repeat steps 5 to 7, but use the tyramide-fluorophore conjugate Opal-620 to detect PNAd antigen.
 14. Place sections in a heat-resistant tray containing ARB such that tissues are covered and the tray is not filled to the brim.
 15. Place the tray in a 95°C water bath for 10 minutes to remove the bound antibodies.
 16. Wash the sections in WB for 10 minutes on a rocking platform.
 17. Place sections in a wet chamber with tissue facing upwards, apply BB, and incubate for 15 minutes at 37°C. Depending on the size of the tissue section, 100-300 μL /section is sufficient.
 18. Decant BB, place sections back in the wet chamber, apply the second primary antibody mix in ADB containing mouse anti-human CD20, rabbit anti-human CD3 and rat anti-mouse DC-LAMP antibodies, and incubate overnight at 4°C (see **Note 5**). Depending on the size of the tissue section, 50-150 μL /section is sufficient.
 19. Repeat steps 3 to 13, but use donkey anti-mouse IgG, donkey anti-rabbit IgG

and donkey anti-rat IgG antibodies with tyramide-fluorophore conjugates Opal-650, Opal-690 and Opal-540 to detect CD20, CD3 and DC-LAMP antigens, respectively.

20. Place sections in a wet chamber with tissue facing upwards, apply the DAPI solution (2 drops/1 mL PBS), and incubate for 5 minutes. Depending on the size of the tissue section, 100-300 µL/section is sufficient.
21. Decant the DAPI solution and wash sections in WB for 5 minutes on a rocking platform.
22. One by one, mount the coverslips using an antifade mounting medium that is suitable for the detection of fluorophores emitting also in the far-red spectrum (see **Note 8**).
23. Store sections at 4°C until imaging as well as for long-term storage.

3.2. Multispectral imaging

The process described below corresponds to the standard operating procedure of the Vectra 3.0™ multispectral imaging system with automated section loading. This microscope is equipped with a LED white and UV light source, 1 bright field (position 1) and 5 fluorescence excitation/emission filters (positions 2-6) and the Nuance™ multispectral camera (PerkinElmer). The excitation/emission data and the respective detection filters for each fluorophore/maker are depicted in **Table 2**.

3.2.1. Establishment of imaging protocol

We have highlighted buttons and tabs in **grey bold italics**, and choosable options in *italics* in the description of how to use of the dedicated software for clarity.

1. Using the **Edit Protocol** tab of Vectra™ software, indicate the use of 10x objective for the whole section overview scan and 20x objective for the

multispectral high-power field (HPF) acquisition. Determine the optimal exposure times for each of the 5 fluorescent channels for each of the objectives using sections that have been stained with only a single fluorophore. Exposure times between 50 and 100 ms are recommended. Set **DAPI** as the reference signal for finding the focal plane and choose the **Saturation Protection** option. Save the scanning protocol in the respective study folder.

2. Using the single color-stained sections and the determined exposure times, find an optimal region of interest with the highest marker expression and acquire a multispectral HPF snapshot for each individual fluorophore. Save these snapshots under the respective fluorophore name.
3. Using the unstained section and the determined exposure times, acquire a multispectral HPF snapshot of a tissue area containing blood vessels, fibers or other autofluorescent structures. Save this snapshot under the Autofluorescence name.

3.2.2. Section scanning

1. Using the **Scan Sections** tab of Vectra™ software, generate the task list containing the section names with the respective positions in the section loading cassette, indicate the name of the generated scanning protocol and choose the **Prescan** task, which will generate whole section overview scans.
2. Perform whole section scans of all sections of interest stained with the 7-color protocol.
3. Open whole section scan images using Phenochart™ software and select the regions of interest for multispectral HPF acquisition for all sections of interest, optimally containing one TLS per HPF.

4. In the **Scan Sections** tab of Vectra™ software, modify the task list by selecting the **Acquire MSI Fields** task, which will automatically find the position of the selected HPFs from the position annotations generated by Phenochart™ software for each section.
5. Perform multispectral HPF acquisition.

3.3. Image analysis

3.3.1. Spectral Unmixing

1. Generate the spectral library of the used fluorophores by choosing the **Build Library** tab in Inform™ software. Open the multispectral HPF snapshots of each individual fluorophore, extract and save the respective spectral profiles (**Figure 1**, top left).
2. Open 3-5 multispectral HPF images from each section of interest and the Autofluorescence image in Inform software™. To perform spectral unmixing, select the 7 fluorophores from the generated spectral library. Using the **Autofluorescence Selection** tool draw a line over the regions with most autofluorescent signal in the Autofluorescence image. By clicking on the **Prepare Sections** button, the software will perform unmixing of all indicated fluorophore and autofluorescence profiles, which can now be viewed as individual channels or in any combination assigning colors as necessary (**Figure 1**, top right). **Figure 2A** depicts the detection of each individual marker and the marker combination in human tonsil. **Figure 2B, left** depicts a GC-containing TLS in a human lung tumor section Next, save the established image view settings as an Inform™ project file.

3.3.2. Tissue and cell segmentation

1. **Configure** the Inform™ project by activating the **Trainable Tissue**

Segmentation, *Cell Segmentation* and *Phenotyping* options. Because FDCs and HEVs are cells with highly irregular shape, we recommend using tissue segmentation rather than cell segmentation for quantification of these cell types. The cell segmentation and phenotyping algorithms can be reliably trained to recognize T cells, DCs and B cells.

2. In the *Tissue Segmentation* tab, generate tissue segmentation categories named Germinal Centre, Follicular Dendritic Cells, B Cell Cluster, High Endothelial Venule, Other, Empty. Next, mark training areas corresponding to each of the generated tissue categories: (i) germinal centers that are positive for CD20, CD21 and CD23, (ii) areas with follicular dendritic cells that positive for CD21 and CD20 but not CD23, (iii) areas containing dense B cell aggregates that are positive for CD20 but not CD21 and CD23, (iv) areas containing PNAd signal, (v) all other areas containing tissue, (vi) empty spaces (**Figure 1**, bottom left). Train tissue segmentation algorithm using pattern scale *Large* and segmentation resolution *Fine* until the training indicator stops moving. Check the quality of the algorithm by segmenting all images in the project and retrain, as required. **Figure 2B, middle** depicts an example of a segmentation map of human lung tissue containing TLS from a lung cancer patient.
3. Proceed with the *Cell Segmentation* tab and train the cell segmentation algorithm based on *Nuclear Counterstain DAPI*. Next, segment the cytoplasm as a 4-pixel band surrounding the nucleus (**Figure 1**, bottom left).
4. Proceed with the *Phenotyping* tab and train the cell phenotyping algorithm. First, generate phenotype categories to be detected: T cell, mature Dendritic Cell, B cell, Other. Next, assign at least 30 cells corresponding to each

category and train the algorithm. Test the performance of the algorithm by segmenting all images in the project and retrain, as required (**Figure 1**, bottom left). **Figure 2B, right** depicts an example of detected cell phenotypes in lung tissue containing TLS from a lung cancer patient.

3.3.3. Image data processing

1. **Export** the *Composite* images, *RGB images with Tissue segmentation map* and *with Phenotype map* and the *Tissue segmentation* and *Cell segmentation Tables* (**Figure 1**, bottom right).
2. In the **Review and Merge** tab of Inform™ software, select the folder containing the exported data. This opens a review window where exported images can be verified and images with mistakes in segmentation or artifacts can be excluded from the final data file. After reviewing all images, click on the **Merge** button to generate a merged text file containing data from all approved images. This also creates a second file containing data from the rejected images (**Figure 1**, bottom right).
3. Using RStudio, create an excel file by arranging the data from the merged text file according to tissue categories for each patient, and generate a summary data table containing the information about the size of analyzed tissue categories and the number of each cell phenotype in the different tissue categories for each patient (**Figure 1**, bottom right). The data can now be subjected to various statistical analyses. Specific R scripts are available upon request.

4. Notes

1. Cut FFPE tissue sections can be stored at 4°C for up to 3 months without

apparent decrease in signal for the described antigens.

2. A microwave, which allows for the regulation of power intensity, can be used instead of a pressure cooker (see the Opal™ 7-color Manual IHC Kit description for the requirements of the microwave).
3. We have compared the Trilogy™ solution pre-treatment with the conventional pre-treatment protocol (xylene deparaffinization, graded ethanol rehydration and antigen retrieval by autoclaving in 10 mM citric acid pH 6.0, or Tris-EDTA buffer pH 9.0) and found that the immunostaining signal intensity was superior for all antibodies using the Trilogy™ solution.
4. We have tested several PAP pens and found that the ImmEdge pen from Vector Laboratories stays on the sections after multiple washing and heating rounds without the necessity to redraw the border, which saves a considerable amount of time especially when processing many sections in parallel.
5. The optimal dilution of the primary antibodies should be determined for your experimental setting and antibody manufacturer. As a rule of thumb, the use of TSA provides strong immunostaining signals using 5-10 times lower primary antibody concentrations than in conventional immunostaining protocols using fluorophore-labeled secondary antibodies.
6. It is important to use secondary antibodies with the least possible cross-reactivity due to the high sensitivity of the tyramide signal amplification approach. The dilutions will depend on the manufacturer.
7. A microwave (see **Note 2**) can be used instead of the water bath.
8. Not all antifade mountants are suitable for the detection of far-red dyes. Make sure to use one that is. In our experience, Prolong™ Diamond mounting medium provides reliable detection of all above described fluorophores for up

to 3 months.

9. Use a separate washing tray for the unstained section to avoid any fluorescent dye transfer especially DAPI from the stained sections.
10. Use separate washing trays for the single Opal™ color-stained sections to avoid any fluorescent dye transfer especially DAPI from other stained sections. This is crucial for the identification of the pure spectral profiles for each dye, which is necessary for the spectral unmixing step by the Inform™ software.
11. Matlab, Excel or other suitable software can be used instead of RStudio.
12. Protecting your hand with a towel keep the pressure nob lifted. Take care not to release the pressure too fast as this might cause boiling of the solution inside the section trays causing tissue damage.
13. Using ddW instead of WB for this step makes it easier to dry the glass surface surrounding the tissue in order to draw the barrier.
14. Sections can be kept in the WB at 4° C for up to 3 days.
15. The incubation time for all antibodies except anti-PNAd could be reduced to 2h at RT.
16. The manufacturer recommends diluting the Opal™ tyramide-dyes 1:50. In our experience, 1:100 dilution is detected equally well, with the exception of Opal-690. Because Opal-650 and Opal-960 are detected through the same microscope filter (see **Table 2**), it is crucial to establish such antibody and tyramide-dye dilutions that are detected with the same exposure time. A recommended exposure time for all filters is 50-100 ms. In our experience, Opal-650 needs to be diluted 1:150 and Opal-690 1:50 to achieve the same exposure time and to obtain signals that can be reliably unmixed by Inform™

software.

17. Here we have combined three primary antibodies raised in different species in the same incubation step. This allows to use TSA by sequential incubation of HRP-conjugated secondary antibodies (with minimal cross-reactivity) in combination with H₂O₂ treatment after each tyramide reaction. This quenches the HRP of the previous antibody before applying the next antibody followed by the next tyramide-dye conjugate. This way no heat-induced antibody removal is necessary, which decreases the chance of tissue and epitope destruction and considerably shortens the duration of the manual protocol. Antibody removal by heating is necessary only after all three secondary antibodies have been used in order to proceed with the next round of primary antibodies. If, however, most primary antibodies were raised in the same species, heat-induced antibody removal is necessary after each tyramide reaction. In this case, it is important to determine, which epitopes are most sensitive to multiple heating rounds in order to establish the optimal staining order.

Table 1. Immunostaining reagents for the analysis of TLS in human tissues

Maker	Antibody clone	Antibody dilution	Producer, catalogue number	HRP-conjugated secondary antibody *
CD21	2G9	1:5000	Leica, NCL-L-CD21-2G9	Donkey anti-mouse IgG
DC-LAMP	1010E1.01	1:1000	Dendritics	Donkey anti-rat IgG
CD23	SP23	1:1000	Abcam, ab16702	Donkey anti-rabbit IgG
PNAD	MECA-79	1:500	Biolegend, 120801	Goat anti-rat IgM

CD20	L26	1:5000	Dako	Donkey anti-mouse IgG
CD3	SP7	1:1000	ThermoFisher	Donkey anti-rabbit IgG

*All HRP-conjugated secondary antibodies were purchased from Jacksons ImmunoResearch. The optimal working dilutions were 1:500 for anti-mouse IgG, anti-rat IgM and IgG, and 1:1000 for anti-rabbit IgG.

Table 2. The detection of each fluorescently stained marker by Vectra 3.0™

Maker	Fluorophore	Excitation (nm)	Emission (nm)	Detection filter
Nucleus	DAPI	358	461	2
CD21	Opal-520	494	525	3
DC-LAMP	Opal-540	523	536	4
CD23	Opal-570	550	570	4, 5
PNAD	Opal-620	588	616	5
CD20	Opal-650	627	650	6
CD3	Opal-690	676	694	6

5. References

- Dieu-Nosjean MC, Giraldo NA, Kaplon H, Germain C, Fridman WH, Sautes-Fridman C (2016) Tertiary lymphoid structures, drivers of the anti-tumor responses in human cancers *Immunological reviews* 271:260-275 doi:10.1111/imr.12405
- Faget L, Hnasko TS (2015) Tyramide Signal Amplification for Immunofluorescent Enhancement Methods in molecular biology 1318:161-172 doi:10.1007/978-1-4939-2742-5_16
- Jones GW, Hill DG, Jones SA (2016) Understanding Immune Cells in Tertiary Lymphoid Organ Development: It Is All Starting to Come Together *Frontiers in immunology* 7:401 doi:10.3389/fimmu.2016.00401
- Mansfield JR (2014) Multispectral imaging: a review of its technical aspects and applications in anatomic pathology *Veterinary pathology* 51:185-210 doi:10.1177/0300985813506918

- Posch F et al. (2017) Maturation of tertiary lymphoid structures predicts the risk for recurrence in stage II and III colorectal cancer *Oncoimmunology* Accepted
- Sautes-Fridman C, Lawand M, Giraldo NA, Kaplon H, Germain C, Fridman WH, Dieu-Nosjean MC (2016) Tertiary Lymphoid Structures in Cancers: Prognostic Value, Regulation, and Manipulation for Therapeutic Intervention *Frontiers in immunology* 7:407 doi:10.3389/fimmu.2016.00407
- Silina K, Rulle U, Kalnina Z, Line A (2014) Manipulation of tumour-infiltrating B cells and tertiary lymphoid structures: a novel anti-cancer treatment avenue? *Cancer immunology, immunotherapy* : CII 63:643-662 doi:10.1007/s00262-014-1544-9
- Silina K et al. (2017) Germinal centers determine the prognostic relevance of tertiary lymphoid structures and are impaired by corticosteroids in lung squamous cell carcinoma *Cancer research* doi:10.1158/0008-5472.CAN-17-1987

Figure Legends

Figure 1. Multispectral image analysis workflow.

Schematic representation of the main 4 steps of multispectral image analysis using the Inform™ software. Representative images of lung cancer tissues undergoing each processing step are shown.

Figure 2. MSI of 7-color immunostained human tissues for the analysis of TLS

Tissue sections were immunostained to detect B cells (CD20), T cells (CD3), mature dendritic cells (DC-LAMP), high endothelial venules (PNAd), follicular dendritic cells (CD21) and germinal centers (CD23) in combination with the nuclear stain DAPI using the Opal™ 7-color Manual IHC Kit (PerkinElmer). Multispectral images were acquired with the automated microscopy system Vectra 3.0™ (PerkinElmer). Autofluorescence profile was determined in unstained human lung tumor tissue and each individual marker signal was unmixed using Inform™ software (PerkinElmer). **A** Human tonsil tissue was used as a positive control, each individual marker staining is displayed as a pseudo-bright field image and the composite image depicting the tissue expression of all analyzed markers. **B** A representative image of a GC-positive TLS in human lung tissue from a lung cancer patient with autofluorescent

erythrocytes (left), image overlaid with tissue segmentation map (middle) and cell phenotype map (right).

1. Build spectral library

2. Prepare MSI images

3. Train algorithms

Select trainable tissue, cell and phenotype segmentation

4. Process image data

1. Batch Export unmixed composite images, RGB images with tissue and phenotype segmentation maps and tissue and phenotype segmentation data tables with parameters of interest for all acquired MSI fields.
2. Review exported image segmentation quality and Merge data table for all appropriately segmented images.
3. Arrange the exported data for statistical analysis using R, Matlab or Excel software

A

B