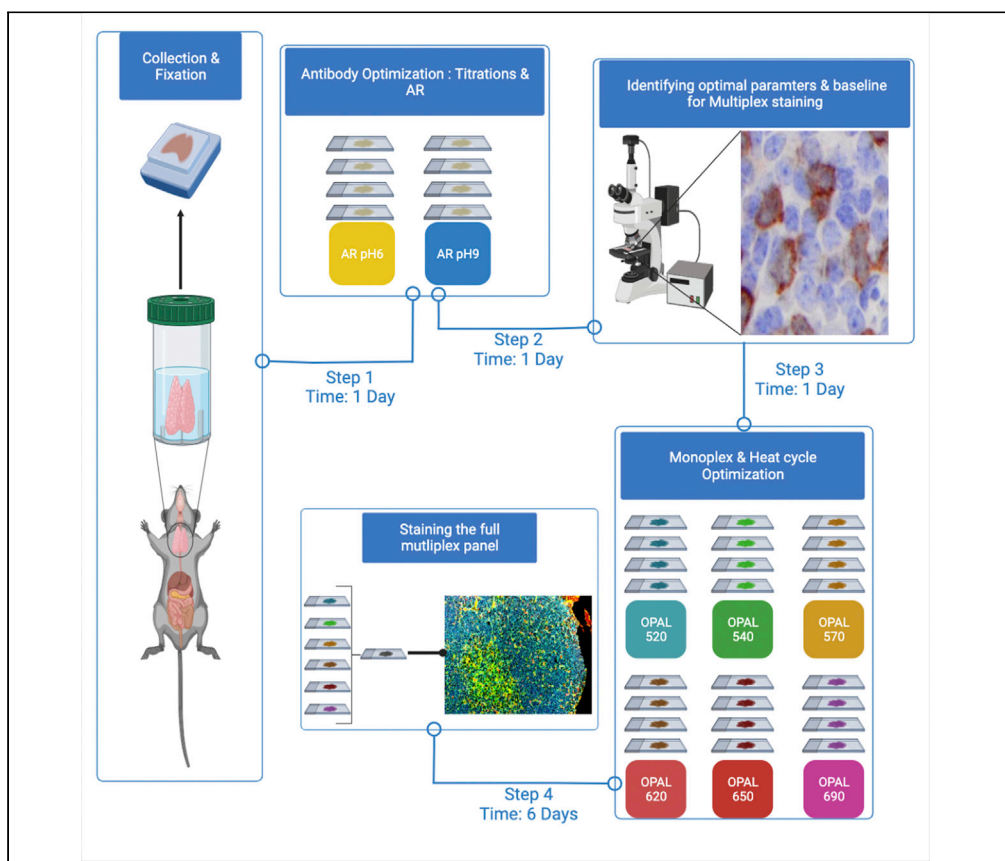


## Protocol

# Establishing a multiplex imaging panel to study T cell development in the thymus in mouse



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### Highlights

Establishing a multiplex imaging panel

Using multiplex imaging to delineate spatial characteristics of developing T cells

Integrating positional information with differentiation and signalling

Multiplexed immunohistochemistry enables analysis of cellular and signaling events in the context of an intact organ. Here, we describe protocols for applying multiplexed immunohistochemistry to the mouse thymus. In particular, we describe how to identify cells at the specific differentiation stage known as  $\beta$ -selection, and to monitor pre-TCR signaling and the cellular response at that stage.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

## Establishing a multiplex imaging panel to study T cell development in the thymus in mouse

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<https://doi.org/10.1016/j.xpro.2022.101472>

## SUMMARY

**Multiplexed immunohistochemistry enables analysis of cellular and signaling events in the context of an intact organ. Here, we describe protocols for applying multiplexed immunohistochemistry to the mouse thymus. In particular, we describe how to identify cells at the specific differentiation stage known as  $\beta$ -selection, and to monitor pre-TCR signaling and the cellular response at that stage. For complete details on the use and execution of this protocol, please refer to Allam et al. (2021).**

## BEFORE YOU BEGIN

The protocol below describes the specific steps to establish a multiplex imaging panel to investigate the immunological synapse formation at the  $\beta$ -selection checkpoint during T cell development in a mouse thymus tissue. However, the protocol can be used for other tissue types, we have also used it for immune profiling of the tumor microenvironment in colon and breast cancer tissues from human and mouse.

## Institutional permissions

Mice were euthanized under the approval of the Animal Experimentation Ethics.

Committee of the Peter MacCallum Cancer Centre before extraction of thymic tissue. Experimenters will need to acquire permissions from their relevant institutions.

## Required buffers before start

⌚ Timing: 0.5–1 h

1. Antigen retrieval buffer (AR): 1 mM EDTA pH 8.
  - a. 50 mL 0.05 M EDTA + 450 mL H<sub>2</sub>O.
  - b. Adjust pH to 9.
  - c. Store at 20°C–22°C for 2 months.
2. Antigen retrieval buffer: 10 mM Sodium Citrate pH6.
  - a. 2.94 g Citric acid, trisodium salt + 1000 mL H<sub>2</sub>O.
  - b. Adjust pH to 6.
  - c. Store at 20°C–22°C for 2 months.



3. 0.1% BSA in TBS.
  - a. 0.05 g BSA + 50 mL TBS (1 x, 50 mL).
  - b. Store at 4°C for 3 months.
4. 0.3% H<sub>2</sub>O<sub>2</sub>, 200 mL.
  - a. 2 mLs 30% H<sub>2</sub>O<sub>2</sub> + 198 mL PBS.
  - b. Make new batch or each experiment.

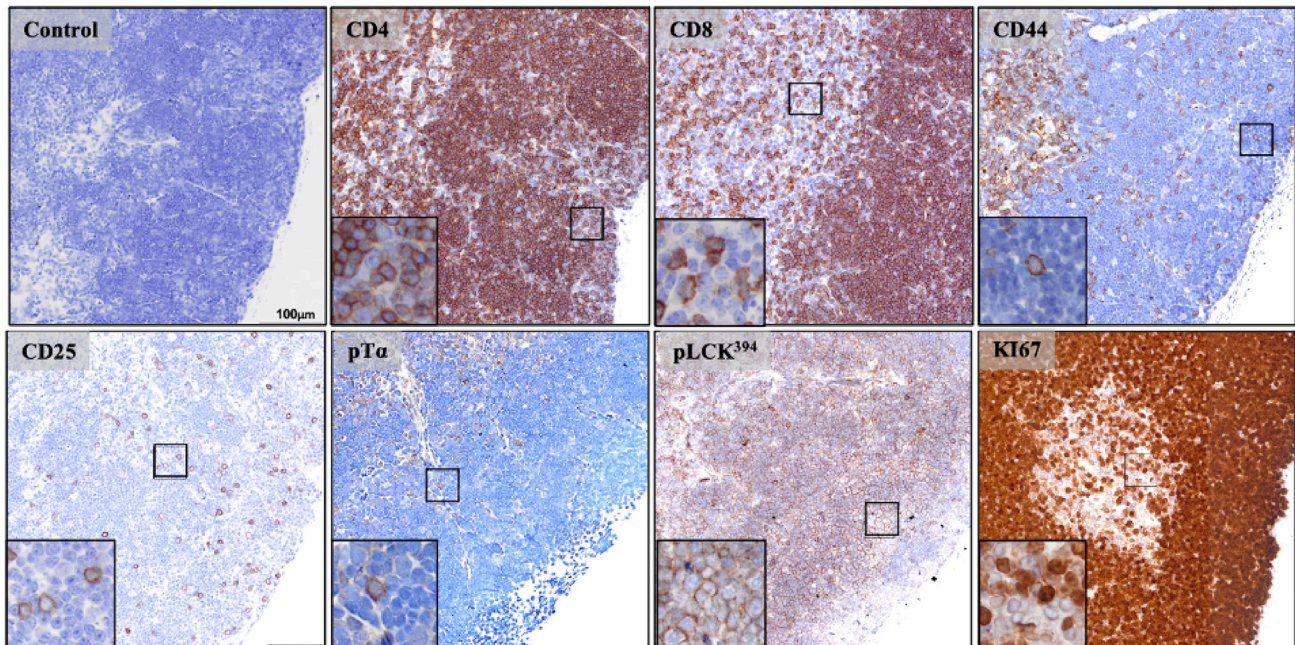
## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-CD4 (1:1000)	Thermo Fisher Scientific	CAT#14-9766-82
Anti-CD8a (1:1000)	Thermo Fisher Scientific	CAT#14-0808-80
Anti-CD44 (1:800)	BD Biosciences	CAT#553132
Anti-CD25 (1:500)	Thermo Fisher Scientific	CAT#PA546922
Anti-pTα (1:200)	Thermo Fisher Scientific	CAT#552407
Anti-Ki67 (1:800)	Thermo Fisher Scientific	CAT#14-5698-80
HRP Goat Anti-Rabbit IgG (Ready to use)	Vector Laboratories	Cat#MP-7451
HRP Horse Anti-Goat IgG (Ready to use)	Vector Laboratories	Cat#MP-7405
HRP Goat Anti-Mouse IgG (Ready to use)	Vector Laboratories	Cat#MP-7452
HRP Goat Anti-Rat IgG (Ready to use)	Vector Laboratories	Cat#MP-7404
<b>Chemicals, peptides, and recombinant proteins</b>		
Hematoxylin	Sigma-Aldrich	CAT#517-28-2
Scott's Tap Water Substitute Concentrate	Sigma-Aldrich	CAT#S5134
DAB Substrate	Sigma-Aldrich	CAT#11718096001
Ultra-Sensitive ABC Peroxidase Standard Staining Kit	Thermo Fisher Scientific	CAT#32050
<b>Biological samples</b>		
Healthy 6–8 weeks old mouse thymus tissue Genotype: C57BL/6	Peter MacCallum Cancer center	N/A
<b>Other</b>		
Vectra 3 Automated Quantitative Pathology Imaging System	PerkinElmer, Inc.	N/A
Rocker-Shaker	Fisher Biotec	CAT#MR-1 (BioSan)
TintoRetriever Pressure Cooker	Bio SB	BSB 7087
<b>Software and algorithms</b>		
inForm Advanced Image Analysis Software	Akoya Biosciences	<a href="https://www.akoyabio.com/phenoptics/software/inform-tissue-finder/">https://www.akoyabio.com/phenoptics/software/inform-tissue-finder/</a>
HALO Image Analysis Platform	Indica Labs	<a href="https://indicalab.com/halo/">https://indicalab.com/halo/</a>
Quantitative Pathology & Bioimage Analysis (QuPath)- Free Alternative software	Open Access	<a href="https://qupath.github.io/">https://qupath.github.io/</a>

## MATERIALS AND EQUIPMENT

<b>10× Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST)</b>		
Reagent	Final concentration	Amount
NaCl	n/a	175.3 gm
Tris base	n/a	121.14 gm
Tween 20 (0.1%)	n/a	10 mL
dH <sub>2</sub> O	n/a	1990 mL
Total		2000 mL

**Note:** Adjust pH to 7.4 and store at 4°C for 3 months.



**Figure 1. Chromogenic detection of antibodies used to establish multiplex panel**

Representative images of optimized chromogenic detection for antibodies needed to establish multiplex panel. Chromogenic detection was followed by counter staining to visualize nuclei (Blue). Scale bar, 100  $\mu\text{m}$ . (Images adapted from published work in (Allam et al., 2021)).

## STEP-BY-STEP METHOD DETAILS

### Chromogenic optimization

⌚ Timing: 2 days

**Note:** Antibodies of interest should be optimized using chromogenic detection before establishing a multiplex spectral library (Figure 1).

1. Fix and section tissues.
  - a. Incubate tissues in 4% Formaldehyde solution for 24 h.
  - b. Rinse tissue twice using 1  $\times$  PBS, by immersing tissue in plastic container full with PBS, then discard PBS and refill container with fresh PBS.
  - c. Put tissues in 70% Ethanol (EtOH) until embedding in paraffin blocks using standard protocols.
  - d. Dissect sections of 4  $\mu\text{m}$  thickness and place on super-frost glass (Thermofisher) slide.
2. De-wax and rehydrate slides.
  - a. De-wax slides by placing in 200 mL xylene for 10 min, then transfer the slides into another container with 200 mL of xylene for 10 min.
  - b. Place slides into 200 mL of 100% EtOH for 5 min, then transfer slides into a container with 200 mL of 100% EtOH for 5 min.
  - c. Place slides in 200 mL 70% EtOH for 5 min.
  - d. Place slides back into distilled  $\text{H}_2\text{O}$ .
3. Antigen Retrieval (AR).
 

AR can be performed using one of the following methods:

  - a. Using pressure cooker (non-electrical):
    - i. Pre-heat your AR buffer of choice (Sodium Citrate pH6 or EDTA pH9) for 1–2 min until boiling point, then place slides in the buffer and pressure cooker.

- ii. Remove your slides from pressure cooker and allow to cool down in AR buffer at approximately 20°C for 20–30 min, then wash twice using dH<sub>2</sub>O.
- b. Using water bath:
  - i. Pre-heat your AR buffer of choice (Sodium Citrate pH6 or EDTA pH9) for 1–2 min until boiling point.
  - ii. Make sure water bath is at 90°C before placing your slides inside it.
  - iii. Leave slides in a closed water bath for 20 min.
  - iv. Remove your slides from water bath and allow to cool down in AR buffer at approximately 20°C for 20–30 min, then wash twice using dH<sub>2</sub>O.

**Note:** From this stage onwards avoid allowing slides to dry at any step throughout the protocol.

**Note:** There are pros and cons for different AR buffers, for example EDTA pH9 AR buffer, offer better detection of antibodies, however, it can damage tissues. In addition, if the antibody signal to noise is strong using EDTA pH9 AR buffer can elevate background noise. On the other hand, Sodium Citrate pH6 AR buffer, is less efficient in antibody detection, however, it has minimal effect on tissue integrity. Hence, it is better to start optimizing antibody detection using Sodium Citrate pH6 AR buffer, if it does not provide a strong detection then try EDTA pH9 AR buffer.

**Note:** A number commercially available pressure cookers are pre-programmed for AR can be used as well.

4. Blocking endogenous peroxidases.
  - a. Incubate slides for 20 min in 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) buffer at approximately 20°C on a gentle rocker. Make sure the slides are in a dark container as H<sub>2</sub>O<sub>2</sub> is light sensitive.
  - b. Wash slides twice with dH<sub>2</sub>O.

**Note:** It is advisable to use 0.3% H<sub>2</sub>O<sub>2</sub> as it can damage certain receptors such as CD receptors at higher concentrations (Kim et al., 2016), however, if there is strong background after scanning slides, then repeat with higher concentration of H<sub>2</sub>O<sub>2</sub>, up to 3%.

**Note:** The step of blocking endogenous peroxidases can be performed after adding the primary antibody, this will ensure that your primary antibody detection is unaffected by the sensitivity to H<sub>2</sub>O<sub>2</sub> of the protein of interest.

5. Blocking endogenous non-specific binding with secondary antibodies.
  - a. Have your slides in dH<sub>2</sub>O before drawing with Pap-pen (Abcam) and draw around tissue carefully.
  - b. Add 5% serum of the host your secondary antibody was raised in (enough to cover the entire tissue), for example, if your secondary antibody was raised in goat, then use 5% goat serum to block endogenous non-specific binding. Incubate your slides in a humidified dark chamber for 1 h at approximately 20°C on gentle rocker.
  - c. Place slide in TBST and incubate for 10 min at approximately 20°C with gentle rocking before moving to add primary antibody.

**Note:** Avoid using Pap-pen on slides which were in TBST, as TBST sticks to glass slide and causes Pap-pen drawing to break, lift off and cover tissue, preventing their staining. If slides were in TBST wash thoroughly with dH<sub>2</sub>O before using Pap-pen.

**Note:** If you do not have blocking serum of the host the secondary antibody raised in, 2%–5% BSA can be used, however, this should be tested.

**Note:** Incubating slides in TBST allow better tissue permeabilization. In addition, TBST sticks to slides allowing primary antibody buffer to spread equally on the tissue without physically trying to spread it on tissue.

6. Adding primary antibody.
  - a. Dilute primary antibody concentrations in 2% blocking serum in 1×TBS (1×TBS in dH<sub>2</sub>O).
  - b. Start by using 4 different dilutions of the antibody of interest, 1:100, 1:500, 1:1000 and 1:2000 (antibody: buffer).
  - c. Incubate tissues with primary antibody for 1 h at approximately 20°C or 18 h at 4°C with gentle rocking.
  - d. Wash 3× with TBST for 3 min while on a shaker.

**Note:** If the signal of antibody is still strong at 1:2000 dilution, lower antibody concentrations can be tested and vice versa if the antibody signal at 1:100 dilution is weak.

7. Adding secondary antibody.
  - a. Add immPRESS secondary antibodies (Vector Laboratories) on tissues and incubate for 1 h at approximately 20°C with gentle rocking.
  - b. Wash 3× with TBST for 3 min while on a shaker.
8. Chromogenic detection.
  - a. Add DAB (3,3'-Diaminobenzidine) chromogen on tissues and incubate for 2–10 min at approximately 20°C with gentle shaking.
  - b. Wash 2× in dH<sub>2</sub>O to stop the chromogenic reaction.

**Note:** Incubation of tissue with DAB is time sensitive and variable from antibody to another, therefore, it is advisable to optimize for the best time by incubating for as short as 30 s up to 15 min. It is best to add DAB to one slide, then observe under a light microscope the development of brown stain. Once you identify the best signal to noise incubation period make a note of it, and stain the rest of your tissue with different antibody concentration using the same incubation with DAB.

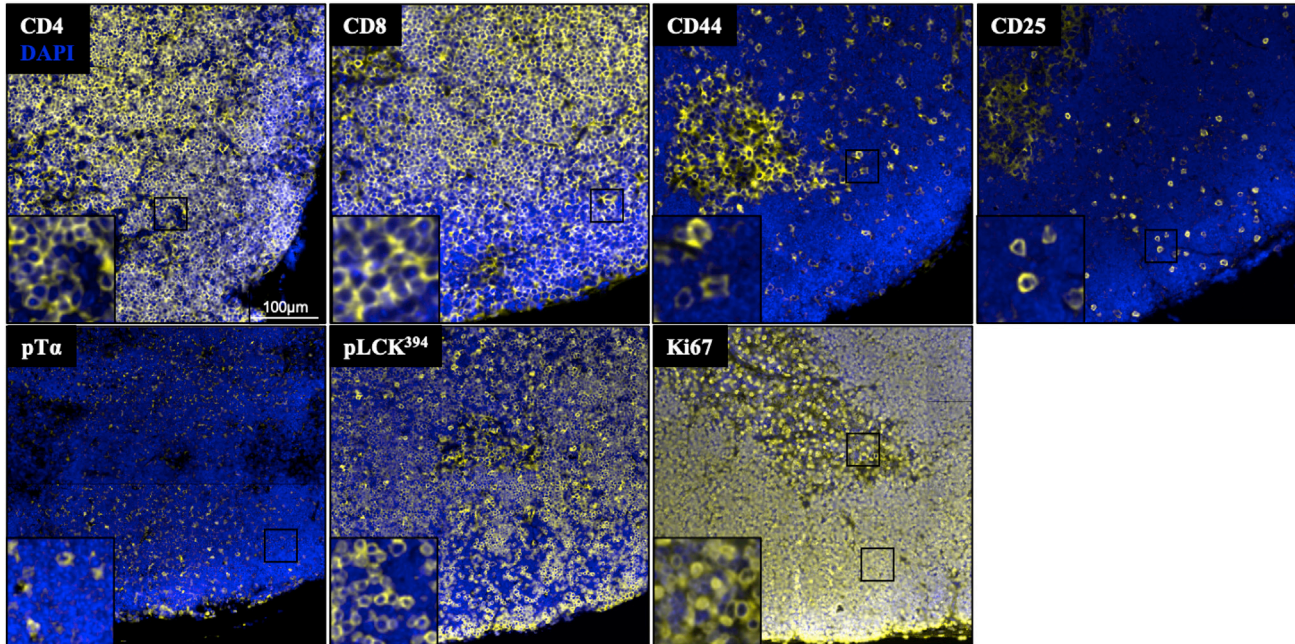
9. Nuclear counterstain.
  - a. Incubate tissues in Hemotoxylin Solution for 20–30 s.
  - b. Wash under running water for 3 min.
  - c. Incubate tissues in Scott's buffer for 20–30 s.
  - d. Wash under running water for 3 min.
10. Dehydrate and coverslip tissues.
  - a. 1 × 5 min wash in 70% alcohol.
  - b. 2 × 5 min washes in 100% EtOH.
  - c. 2 × 10 min washes in xylene.
  - d. Coverslip your slides with mounting media of choice.

### Monoplex optimization and spectral library establishment

⌚ Timing: 2 days

This section will list the major steps in optimizing antibodies for a monoplex (single antibody) staining and optimizing parameters to establish spectral library (Figure 2).

**Note:** Immunofluorescence detection using OPAL fluorophores is more sensitive than chromogenic detection, hence it will require further optimization. Chromogenic detection will provide insight into key optimization parameters for antibodies of interest, including concentration, blocking and antigen retrieval at low cost and less labor.



**Figure 2. Monoplex detection of antibodies used in establishing multiplex panel**

Images acquired using Vectra imaging system, are representative of monoplex staining of antibodies used in establishing multiplex panel. Scale bar, 100  $\mu\text{m}$ . (Images adapted from published work in (Allam et al., 2021)).

Monoplex optimization steps:

11. Fixing and section tissues (As described in step 1).
12. De-wax and rehydrate slides (As described in step 2).
13. Antigen Retrieval (AR) (As described in step 3).
14. Blocking endogenous peroxidase. (As described in step 4).
15. Blocking endogenous non-specific binding with secondary antibodies. (As described in step 5).
16. Adding primary antibody.
  - a. Dilute primary antibody concentrations in 2% blocking serum and 1 $\times$ TBS in dH<sub>2</sub>O.
  - b. Based on previously identified optimal chromogenic detection for each antibody the monoplex optimization can be planned. For example, if optimal dilution of primary antibody is 1:500 in chromogenic detection, then 1:500 should be the lowest dilution used in monoplex optimization. For the rest of the dilutions use 2 $\times$ , 3 $\times$  and 4 $\times$  of the lowest dilution (e.g., 1:1000, 1:1500 and 1:2000).
  - c. Incubate tissues with primary antibody for 1 h at approximately 20°C or 18 h at 4°C with gentle rocking.
  - d. Wash 3 $\times$  with TBST for 3 min while on a shaker.
17. Adding secondary antibody. (As described in chromogenic detection optimization).
18. OPAL fluorophore incubation.
  - a. Dilute OPAL fluorophore (Tyramide Signal Amplification (TSA)) stock into 100  $\mu\text{m}$  DMSO and pipette up and down  $\sim$ 10 times then vortex for 20 s.
  - b. Prepare OPAL fluorophore staining buffer (see Table 1 for fluorophore dilutions).
  - c. Incubate tissue with OPAL buffer (see Table 1 for incubation times).
  - d. Wash 3 $\times$  with TBST for 5 min while on a shaker.
  - e. Wash 2 $\times$  with dH<sub>2</sub>O for 2 min while on a shaker to remove any excess TBST.

*Optional:* Incubate slides DAPI (1 drop of spectral DAPI in 1.5 mL TBST) for 2–3 min, then wash 2 $\times$  for 2 min in dH<sub>2</sub>O.

**Table 1. Table shows spectral signal range of different OPAL fluorophores, their dilutions and incubation periods**

Fluorophore	Signal band	Dilution in 1 × Amplification buffer	Incubation period (mins)	Excitation	Emission
OPAL 520	FITC	1:100	8 min	494 nm	525 nm
OPAL 540	FITC/Cy3	1:100	8 min	523 nm	536 nm
OPAL 570	Cy3/Texas Red	1:150	5 min	550 nm	570 nm
OPAL 620	Cy3/Texas Red	1:150	5 min	588 nm	616 nm
OPAL 650	Cy3/Texas Red/Cy5	1:75	10 min	627 nm	650 nm
OPAL 690	Texas Red/Cy5	1:75	12 min	676 nm	694 nm

- f. Mount slides with anti-fading mounting media of choice and coverslip the slides.
- g. Incubate slides in dark chamber at approximately 20°C for 18 h.

**Note:** For spectral extraction, staining is performed for the antibody of interest without DAPI. However, it is ideal to stain the nuclei with DAPI during optimization to help identify the optimal staining parameters before moving into staining the full multiplex panel.

19. Scanning and identifying optimal dilution and OPAL fluorophore (Table 2).
  - a. Scan slides using Vectra 3 Automated Quantitative Pathology Imaging System (we will refer to as Vectra Machine).
  - b. Get your tissue in focus then use the autoexposure function to adjust exposure.
  - c. Optimal antibody dilution should have exposure between 60 ms and 120 ms.

**Note:** Optimal antibody dilution can vary between different OPAL fluorophores. Therefore, when building a multiplex panel should optimize each antibody with different OPAL fluorophores. For example, CD4 detection can be optimized to be detected using OPAL 520, 570 and 650 fluorophores, this approach allows CD4 detection in different signal bands, which in turn allows more options when building a multiplex panel.

### 20. Heat cycle optimization.

When staining a full multiplex panel, it is required to AR at the end of each cycle to remove the previous primary and secondary antibody complex. Considering that heat can damage proteins and molecules of interest, identifying how many heat cycles markers of interest can sustain before getting damaged is crucial in designating the right order of staining different markers of a full multiplex panel.

Optimize heat cycle for each antibody as following:

- a. For AR Cycle 1:
  - i. Perform the regular staining steps described above (from steps 3 to 8) with the optimal antibody dilution.
  - ii. Scan stained slides.
- b. For AR Cycle 2, 3, 4 and 5:
  - i. Perform the regular staining step described above, however, add primary and secondary antibodies only in the AR cycle being tested (e.g., for AR cycle 4 add primary and secondary antibodies only after 4 AR cycles).
  - ii. Repeat the regular staining steps described above (from steps 3 to 8) with the optimal antibody dilution.
  - iii. Scan stained slides.

**Note:** Using multiple heat cycles can help identifying best possible staining order while performing a full multiplex panel staining. For example, if the protein can be detected after AR cycle 2, but not detectable after AR cycle 3, then this protein should be stained either first or second in the staining order of the full multiplex panel.



**Table 2.** Table shows the range of excitation and emission cut on and off for each spectral channel where the OPAL fluorophores signals are detected

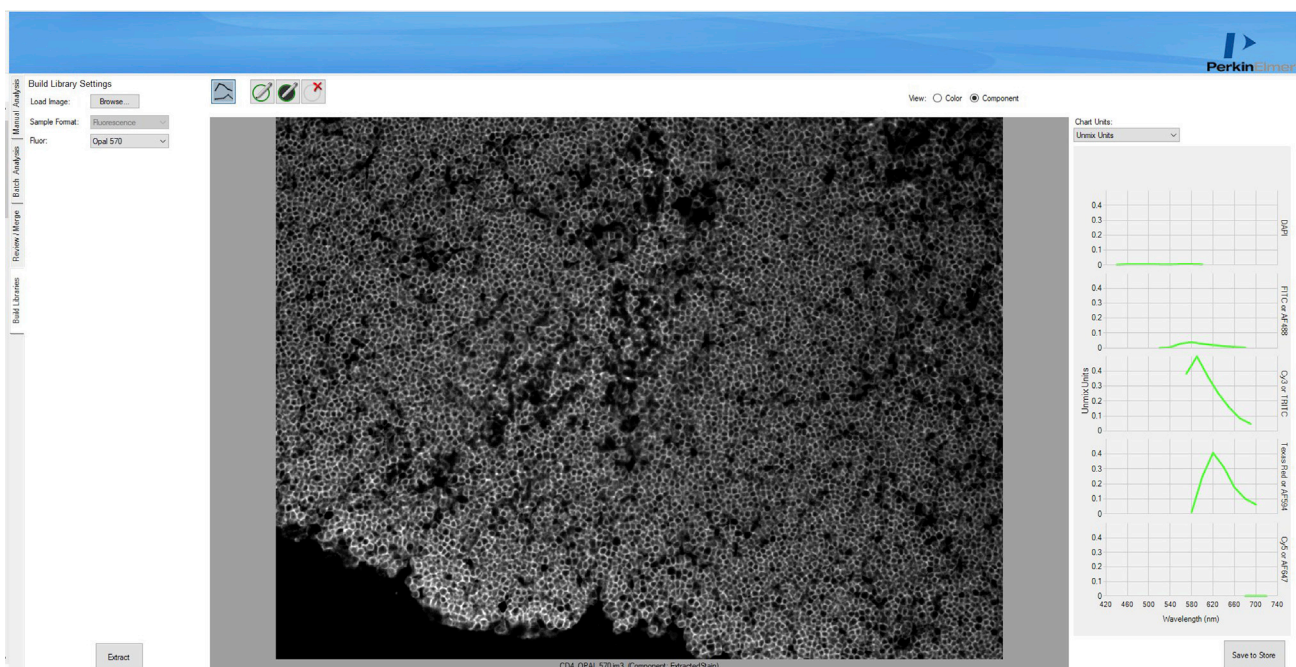
Channels	Excitation wavelength Cut on/Cut off	Emission wavelength Cut on/Cut off
DAPI	352 nm/402 nm	417 nm/1100 nm
FITC	460 nm/500 nm	510 nm/720 nm
CY3	530 nm/560 nm	570 nm/720 nm
Texas Red	540 nm/585 nm	604 nm/1100 nm
Cy5	590 nm/650 nm	663 nm/738 nm

**Note:** Multiple AR cycles can weaken the signal of the protein of interest. Therefore, proteins with low expression should be stained early in the multiplex panel and abundant proteins should be stained after, unless they can't sustain AR cycles.

## 21. Spectral library development.

- Based on the previous steps identify optimal staining settings based on antibody concentration and exposure for each OPAL fluorophore used to detect the protein of interest.
- Using the Vectra machine take a snapshot of optimized parameters for each antibody.
- Using the spectral library building function in inForm software, open the snapshot image taken using the Vectra machine and extract signal spectral range from this image (Figure 3).
- Save the extracted signal in the experiment folder.

**Note:** Optimization of the antibodies should be done on the same tissue type used to stain a full multiplex panel. For example, if multiplex panel to be performed on colon cancer tissue, then the optimization should be done using colon cancer tissue, using normal colon tissue is acceptable as well.



**Figure 3. Establishing multiplex spectral library**

The image shows spectral extraction of CD4 antibody detected using OPAL 570 fluorophore.

**Table 3.** Table shows the parameters of the multiplex panel used to identify immunological synapse formation in developing T cells in an intact thymus

Staining order	Antibody	Dilution ( $\mu$ L in TBST)	OPAL	AR/Cycle
1	CD25	1:500'	570	Cycle 1: EDTA Ph9
2	pT $\alpha$	1:200	620	Cycle 2: EDTA Ph9
3	CD4	1:1000	650	Cycle 3: EDTA Ph9
	CD8	1:1000		
	CD44	1:800		
4	pLCK	1:300	520	Cycle 4: EDTA Ph9
5	Ki67	1:800	690	Not Required
6	DAPI	1 drop in 1.5 mL TBST	–	Not Required

**Note:** It is preferable to build a spectral library for the same antibody in the same OPAL fluorophore but with different heat cycles. For example, if the antibody of interest is detectable at different AR cycles but the signal is weaker as heat cycles increase, a spectral library can be built for this antibody at each heat cycle. This will provide better signal to noise ratio when spectrally dissecting a different multiplex panels, which include the same antibody but stained after different AR cycles.

### Multiplex panel staining

In this section we will list the major steps and consideration when staining a full multiplex panel.

At this stage all antibodies should be optimized and the spectral library established. However, the following should be considered while combining all antibodies of interest in one panel.

22. Endogenous levels of expression:
  - a. Since multiple heat cycles can weaken detection signal and proteins with low expression levels usually have weaker signal than those with high expression levels. We recommend staining for proteins of interest with low expression before those with high expression levels.
23. OPAL fluorophores:
  - a. OPALs 520, 570, 620 and 650 fluorophore provide a strong signal to noise detection. Therefore, proteins with low expression should be stained in these channels.
  - b. OPAL 690 has a narrow spectral range of detection. Therefore, we recommend for best possible results using antibodies which provide strong signal to noise detection should be stained in this OPAL fluorophore.
  - c. OPAL 540 can be detected in the emission spectra of OPAL 520 and OPAL 570. Therefore, we recommend using the OPAL 540 only if a 7-color multiplex panel is being established. If using OPAL 540 is required for the panel we recommend, 1) it is best to use antibodies with the strongest signal to noise ratio, and 2) staining for proteins of interest which do not localize in the same region of those stained using OPAL 520 & OPAL 570. For example, if proteins of interest being detected using OPAL 520 & OPAL 570 are expressed on the cell surface, then OPAL 540 can be used to detect proteins expressed in the nucleus.
24. Colocalizing antibodies:
  - a. Antibodies which are expected to stain the same type of cells, should be separated spectrally by assigning two OPAL fluorophores which do not have spectral emission overlap (e.g., OPAL 520 and OPAL 620).

Using multiplex panel to identify the immunological synapse formation in developing T cells in the thymus.

Following the optimization steps described above we stained thymic sections using antibodies and parameters in a single multiplex panel as described in [Table 3](#).

## EXPECTED OUTCOMES

There are no limitations on outputs such as yield, and no limitations on the extent and scope of the analysis (other than the obvious constraints derived from the choice of markers labeled).

## LIMITATIONS

Sensitivity of proteins of interest to AR cycles can limit combining certain antibodies together, for example, if two proteins significantly lose their signals after two heat AR cycles, to the extent that the produced data would be unreliable, then both antibodies cannot be used on the same multiplex panel.

Tissues can get damaged due to multiple heat AR cycles.

Although multiplex imaging provides invaluable data, it remains a high-cost technique, especially with animal experiments and big patient sample cohorts, where there is a large number of slides to be stained.

## TROUBLESHOOTING

### Problem 1

The key staining limitation is sensitivity to repeated AR cycles (mentioned in [limitations](#) section). As an example, CD25 and pT $\alpha$  lose their fluorescence signal after two heat cycles, and CD44, CD4 and CD8 start losing their signal after 3 heat cycles.

### Potential solution

As DN3 cells transition beyond the  $\beta$ -selection checkpoint, to become DN4 cells they start expressing CD44, then transform to DP cells where they express CD4 and CD8 receptors. Considering that DN3 cells at the  $\beta$ -selection checkpoint lack the expression of CD4, CD8 and CD44, we merged CD44, CD4 and CD8 in one OPAL stain. However, to make sure of specificity of each antibody at different developmental stage we performed dual multiplex stains for CD25 and CD44 with DAPI and CD25 and CD4+CD8 with DAPI ([Figure 4](#).) This approach can be applied to any multiplex panel, given that the merged markers are optimized separately to ensure specific staining. This is done as per [multiplex panel staining](#) section, before staining the full panel.

### Problem 2

The repeated AR cycles can significantly damage tissues, especially if tissues were fixed for less than 24 h.

### Potential solution

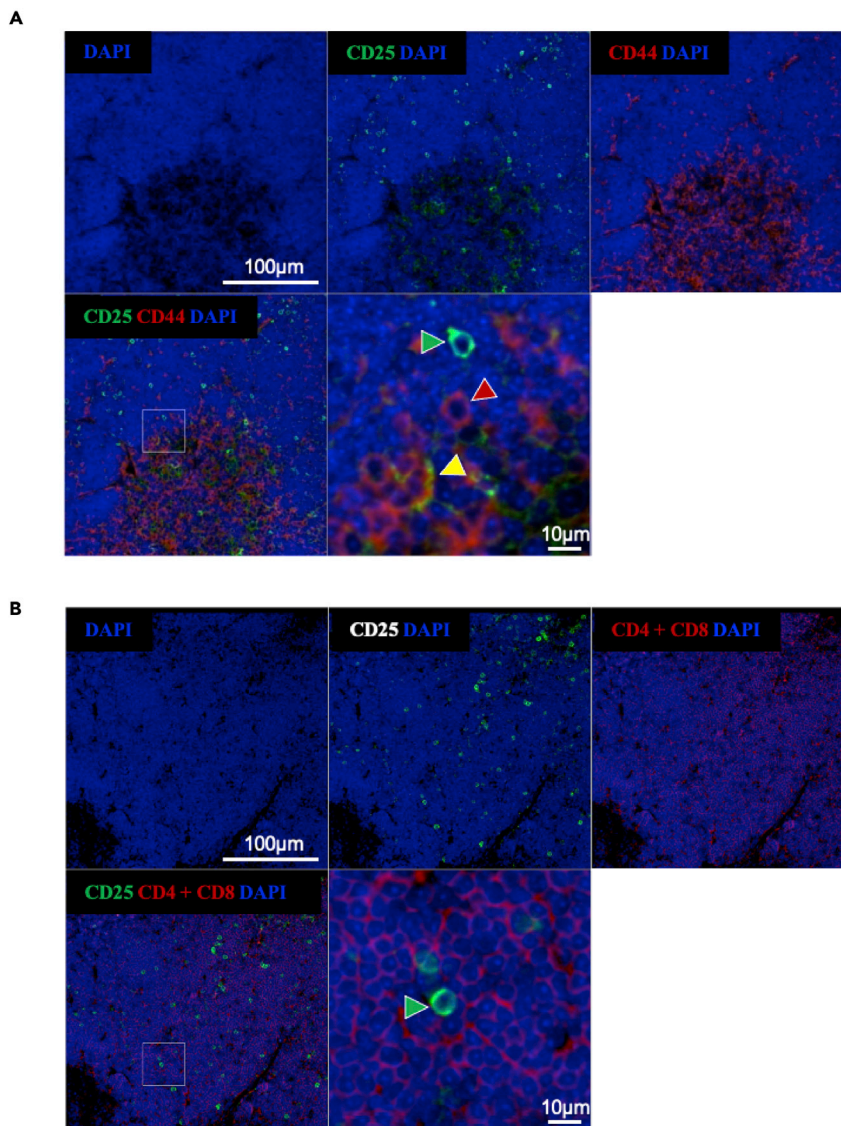
Tissues should be fixed for at least 24 h (As mentioned in step 1 in chromogenic detection section). However, if the repeated AR cycles still damage the tissues, then the slides should be incubated in a 60°C oven for 45 min prior to the de-waxing and rehydration steps. In addition, de-waxing and rehydration incubations time-periods should be lowered as following:

De-wax slides by placing in 200 mL xylene for 5 min, then transfer the slides into another container with 200 mL of xylene for 5 min.

Place slides into 200 mL of 100% EtOH for 3 min, then transfer slides into a container with 200 mL of 100% EtOH for 3 min.

Place slides in 200 mL 70% EtOH for 3 min.

Place slides back into distilled H<sub>2</sub>O.



**Figure 4. Confirming specificity of CD44 and CD4+CD8 before co-staining using OPAL 650**

(A) Representative images of dual multiplex staining of CD25, CD44 and DAPI acquired using Vectra imaging system showing CD25<sup>+</sup> CD44<sup>-</sup> cells (green arrow head, zoomed image), CD25<sup>-</sup> CD44<sup>+</sup> cells (red arrow head, zoomed image) and CD25<sup>+</sup> CD44<sup>+</sup> cells (yellow arrow head, zoomed image).

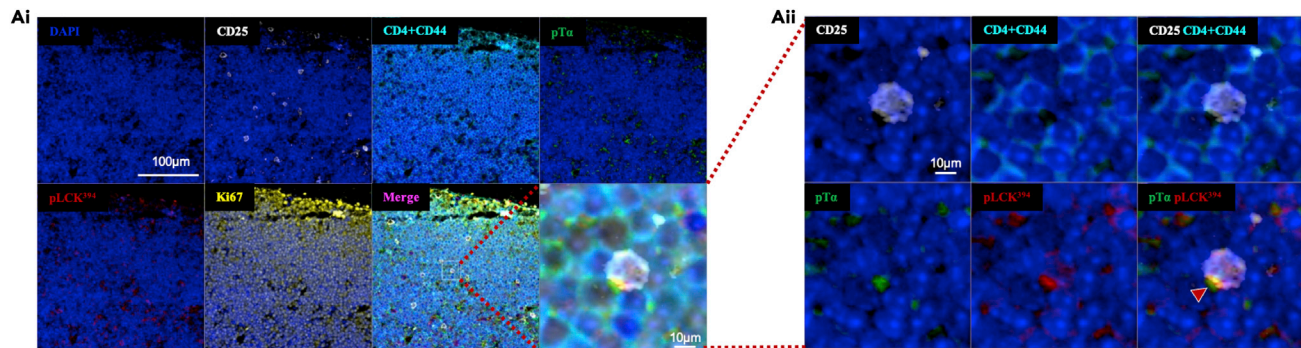
(B) Representative images of dual multiplex staining of CD25, CD44 and DAPI acquired using Vectra imaging system showing CD25<sup>+</sup> CD44<sup>-</sup> cells (green arrow head, zoomed image). Scale Bar; A&B) 100 μm; A&B) zoomed images 10 μm.

### Problem 3

In some instances, a full multiplex panel can have three essential antibodies, each of which can maintain signal, but are weaker if applied in the second or third AR cycle. Also, in this case merging antibodies as per [problem 1](#) solution, is not an option, as this panel will require the antibodies to be separated.

### Potential solution

In such situation, it is ideal to stain the antibody which provides the least signal intensity first; for the second and third antibodies, instead of using HRP-conjugated secondary antibody for detection (as mentioned in step 7, in chromogenic detection section), an avidin-biotin complex (ABC) staining kit



**Figure 5. Developing T cells at the  $\beta$ -selection checkpoint establish an immunological synapse**

(Ai and Aii) Representative images acquired using Vectra imaging system show a 6 color multiplex panel (Ai) used to show that developing T cells ( $CD25^+ CD4^- CD44^- CD8^-$ ) establish an immunological synapse (Aii, bottom right, red arrow) as indicated by the co-clustering of the  $pT\alpha$  subunit with its downstream effector  $pLCK^{394}$  the immunological synapse. Scale Bar; Ai) 100  $\mu$ m; Aii) 10  $\mu$ m.

can be used (Ultra-Sensitive ABC Peroxidase Standard Staining Kit, Thermo Fisher Scientific), which can amplify the signal significantly.

#### Problem 4

In some instances, when staining two antibodies in a full multiplex panel, where one is highly abundant in tissue, the highly abundant antibody detection signal can mask the less abundant one, which can lead to false-negative results (Figure 5).

#### Potential solution

To avoid this, the protein with less expression should be stained first when staining the full multiplex panel. Also, these stains should be separated physically (Protein A in AR cycle 1 and protein B in AR cycle 3) and spectrally (Protein A detected using OPAL 520 fluorophore and protein B detected using OPAL 620 fluorophore). This should be optimized during monoplex optimization.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sarah Russell ([sarah.russell@petermac.org](mailto:sarah.russell@petermac.org)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate/analyze datasets or code.

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## AUTHOR CONTRIBUTIONS

A.H.A. contributed to conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing, reviewing, and editing; S.R. contributed to conceptualization, formal analysis, funding acquisition, methodology, project administration, supervision, visualization, and writing, review, and editing of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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