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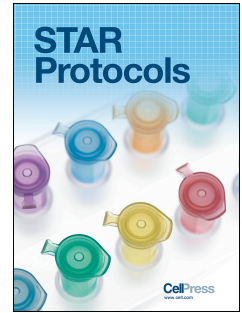
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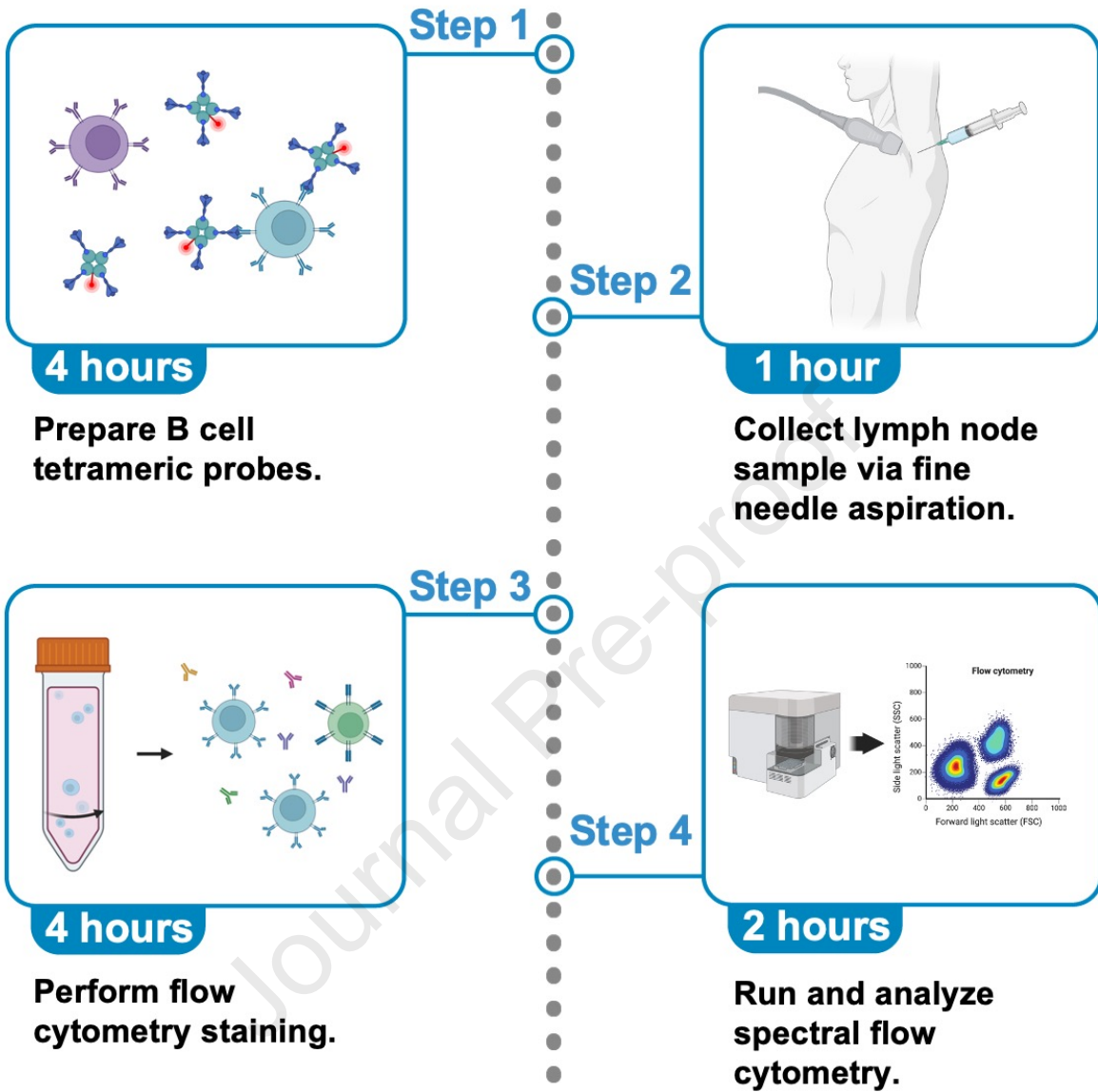
Step 1

4 hours

Prepare B cell
tetrameric probes.

Step 2

1 hour

Collect lymph node
sample via fine
needle aspiration.

Step 3

4 hours

Perform flow
cytometry staining.

Step 4

2 hours

Run and analyze
spectral flow
cytometry.

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Protocol to assess human germinal center responses to SARS-CoV-2 vaccination using a combined fine needle aspiration and spectral flow cytometry approach

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Summary

SARS-CoV-2 mRNA vaccines drive the generation of affinity-matured B cell responses through germinal center (GC) reactions in vaccine draining lymph nodes. Herein, we describe a procedure for the acquisition of human lymph node samples via an ultrasound-guided fine needle aspiration based-approach. Additionally, we outline a suggested approach for the analysis of CD4 T helper cell subsets as well as antigen-specific GC B cells, memory B cells, and plasmablasts by high-parameter, spectral flow cytometry.

For complete details on the use and execution of this protocol, please refer to [1].

Graphical abstract

Before you begin

This protocol describes both the collection and analysis of lymph node biopsy samples obtained via fine needle aspiration (FNA). The FNA procedure needs to be approved by the Institutional Review Board and performed by a trained, board-certified radiologist. Having the same radiologist/s performing all FNAs for a particular study can help to reduce the variability in FNA sample collection and cell recovery. Ideally, multiple FNAs will be scheduled to be collected on the same day, by the same radiologist. If this is not feasible, to limit sample-to-sample variability, it is important to ensure that all radiologists receive the same training and follow a standard operating protocol, such as the one outlined here.

Additionally, we have used tetrameric probes for the labeling of antigen-specific germinal center (GC) B cells, memory B cells, and plasmablasts. These probes must be prepared and titrated in advance, as described in detail below. We have found that the tetrameric probes described in this protocol are stable when stored at 4°C for up to six months. Thus, probes may be made days to months in advance. Antigen-specific T cell analysis is not discussed here but has been described by others [2].

Institutional permissions

Patient samples should be collected in accordance with the Institutional Review Board rules, including appropriate recruitment and patient consent. Our FNA protocol was approved by the Institutional Review Board at the University of Pennsylvania (IRB# 844882).

Preparation of probes for labeling SARS-CoV-2-specific B cells

We use tetramerized-recombinant protein probes for the identification of antigen specific GC B cells, memory B cells, and plasmablasts. We have found that using tetrameric probes, as opposed to monomeric probes, increases the quality of signal detection. Additionally, we typically use a dual probe approach, along with a decoy (irrelevant protein) probe, to ensure the specificity of the binding cells.

Specifically, for this protocol we used a recombinant biotinylated SARS-CoV-2 spike protein from R&D Systems. Additionally, we used a recombinant SARS-CoV-2 receptor binding domain (RBD) protein and a recombinant A/Puerto Rico/8/1934 (PR8, H1N1) influenza virus hemagglutinin (PR8-HA) protein,

which were produced as described in [3–5]. The recombinant RBD and PR8-HA proteins were biotinylated using the EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit following the manufacturer's instructions, with minor modifications, as described below.

CRITICAL: B Cell tetrameric probes should be made with non-tandem dyes (i.e. PE, APC, etc). As is discussed later, the use of tandem dyes (i.e. APC-Cy7, PerCP-Cy5.5, etc.) for the probes can make flow cytometry compensation challenging.

Timing: 4 hours

1. Biotinylation of recombinant RBD and PR8-HA:
   a. Start with 20-200 micrograms (μg) of protein dissolved in 200-700 microliters (μL) of PBS. If protein is outside of this range, dilute it down to the appropriate range.
   b. Calculate the millimoles of Sulfo-NHS-LC Biotin to add to the reaction for a 50-fold molar excess.

$$V(protein) \; x \; c(protein) \; x \; \frac{1}{MW(protein)} \; x \; 50 = n(biotin)$$

   V = volume (milliliters, mL)

   c = concentration (milligram (mg)/mL)

   MW = molecular weight (millimols (mmol)/mg)

   n = mmol

   c. Remove the No-Weigh™ Sulfo-NHS-LC-Biotin tube (included with the EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit) containing 1 mg of biotin from the freezer.
      i. Add 200 μL of molecular biology grade water to the tube.
      ii. Mix by pipetting up and down.
      iii. This creates a 9mM Sulfo-NHS-LC-Biotin solution.
   d. Calculate the microliters of 9mM Sulfo-NHS-LC-Biotin to add to the reaction.

$$n(biotin) \; x \; 557 \; mg \; x \; \frac{200 \; \mu L}{1.0 \; mg} = \; V(biotin)$$

   557 mg = molecular weight of Sulfo-NHS-LC-Biotin

   200 μL = volume of solvent used in step 1c to make a 9mM Sulfo-NHS-LC-Biotin solution

V = volume (μL)

MW = molecular weight (mmol/mg)

n = mmol

e. Add the appropriate volume of the biotin solution to the protein solution (step 1d).

f. Incubate the reaction at on ice (or at 4°C) for 2 hours.

g. After the incubation, prepare the Zeba™ Spin Desalting Columns (included with the EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit) by breaking off the bottom plug and placing in a 15 mL conical tube.

h. Spin at 1000 x g for 2 minutes and discard the flow through.

i. Mark directly on the side of the column where the resin is slanted upward.

 i. Place column back in the 15 mL conical tube.

 ii. Place column in the centrifuge with the mark facing outwards for all remaining spins.

j. Prepare BupH Phosphate Buffered Saline (PBS) by dissolving the provided packet (included with the EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit) in 500 mL of deionized water.

k. Equilibrate the column by adding 1 mL of PBS.

 i. Spin at 1000 x g for 2 minutes.

 ii. Discard the flow-through.

 iii. Repeat step 1k 2X.

l. Place column in a clean 15 mL conical tube.

 i. Apply the protein sample directly to the resin bed.

 ii. If the total volume is less than 400 μL, add 100 μL of molecular biology grade water on top of the resin after the sample is fully absorbed.

m. Spin column at 1000 x g for 2 minutes. Collect the flow-through which contains the biotinylated protein.

Pause point: Biotinylated protein can be stored at 4°C before tetramerization. Theoretically, the protein should be stable for prolonged storage at 4°C, but we have not tested the storage for longer than 24 hours.

2. Once the protein is biotinylated, follow the steps outlined below (steps 2-7) for tetramerizing RBD and PR8-HA proteins, similarly to what was reported in [6]. First, calculate the mmols of protein. We typically assume no protein loss and negligible weight from the biotin.

$$V(protein) \; x \; c(protein) \; x \; \frac{1}{MW(protein)} = n(protein)$$

V = volume (mL)

c = concentration (mg/mL)

MW = molecular weight (mmol/mg)

n = mmols

3. Calculate the molarity of the fluorochrome-labeled streptavidin (SA:fluorochrome) that will be conjugated to the biotinylated protein.

$$c(SA\!:\!fluorochrome) \; x \; \frac{1}{MW(SA\!:\!fluorochrome)} = M(SA\!:\!fluorochrome)$$

c = concentration (mg/mL)

MW = molecular weight (mmol/mg)

M = molarity (mmol/mL)

4. Determine the volumes of biotinylated protein and streptavidin-labeled fluorochrome that will be needed for a 6:1 molar ratio (6 mol biotinylated protein to 1 mol SA:fluorochrome).

$$\frac{n(protein)}{6} = n(SA\!:\!fluorochrome)$$

$$n(SA\!:\!fluorochrome) \; x \; \frac{1}{M(SA\!:\!fluorochrome)} = V(SA\!:\!fluorochrome)$$

V = volume (mL)

n = mmols

M = molarity (mmol/mL)

5. Once the volume of SA:fluorochrome needed is determined, sequentially add 1/10 of the required volume of SA:fluorochrome to the biotinylated protein and mix gently while on ice (or at 4°C).

6. Repeat step 5 every 10 minutes for a total of 10 additions (this will result in the total volume calculated in step 4 being added sequentially over 10 additions).

Note: The biotinylated-spike tetramers used for these studies was made as described in [7]. Biotinylated-spike protein was mixed with SA:fluorochrome at a 10:1 mass ratio, during a single addition, and incubated at on ice (or at 4°C) for at least 2 hours before use.

Note: Please note that for the conjugation of recombinant proteins to phycoerythrin (PE), this method may not provide optimal results. PE is substantially larger than most other fluorochromes (~240kDa), and when conjugating to smaller proteins, the volume of PE required to reach a 6:1 molar ratio may dilute out your protein more than is desirable. We recommend titrating to find the optimal amount of streptavidin-PE to conjugate your protein of interest.

Note: We have stored the RBD and PR8-HA tetramers described here at 4°C for up to six months without appreciable loss in staining quality. However, some proteins (e.g., SARS-CoV-2 full length spike) may be less stable over time and/or at higher temperatures. Due to this, the shelf-life for all tetramer formulations should be assessed individually.

Titration of tetrameric probes for labeling SARS-CoV-2-specific B cells

Titration of tetrameric probes to label antigen-specific B cells should be performed every time that a new probe is made. For the titration, peripheral blood mononuclear cells (PBMC) from COVID-19 convalescent or SARS-CoV-2 vaccinated individuals, ideally within 3-4 months post infection or vaccination, can be used. For negative controls, use pre-pandemic PBMCs if available.

In this protocol, tetrameric probes are titrated alongside a small antibody panel to follow the titration of probes directly on B cells. If comparing a new probe batch to a pre-existing probe, also include a stained sample with the pre-existing probe at the appropriate concentration.

Timing: 4-6 hours

7. Prepare FACS Buffer as described in the Materials and equipment section.

8. Prepare the eBioscience™ Fixable Viability Dye eFluor™ 780 by diluting it 1:2000 in FACS Buffer (for a final dilution of 1:4000).

9. Prepare the mix of antibodies to stain B cells (B Cell Mix) in a volume of 50 µL per sample/well in the viability dye prepared in step 8 to create a 2X B Cell Mix.

B Cell Mix

Marker	Concentration (mg/mL; 2X)	Dilution (2X)
CD4 BUV563	0.00012	1:100
CD8α PerCP-eFluor710	0.0005	1:100
CD19 PE-Cy5.5	0.00025	1:100

10. Separately, prepare a 2-fold serial dilution of the probes (Probe Titration Solution) to be tested in FACS Buffer.
    a. For a 2-fold serial dilution, start by adding the highest desired probe concentration to 100 µL of FACS Buffer.
    b. Next, resuspend and transfer 50uL of the probe mix to 50 µL of FACS Buffer. Repeat step as many times as necessary to reach the desired concentrations.
    c. Recommended final dilutions (1X) are: 0, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600.
11. Seed cells at approximately 1x10^6 cells per well. Ensure you plate has enough positive and negative wells for all titrations and control samples.
12. Spin cells at 400 x g for 2 minutes at 4°C.
13. Aspirate supernatant and add 50 µL of the B Cell Mix directly to each well.
14. Immediately add 50 µL of the 2X Probe Titration Solution to the appropriate wells and pipette to mix.
15. Incubate for 1 hour at 4°C.
16. Add 100 µL of FACS Buffer and spin at 400 x g for 2 minutes at 4°C.
17. Aspirate supernatant and add 200 µL of a 1% paraformaldehyde solution (in FACS Buffer).
18. Resuspend and incubate for 30 minutes at 4°C
19. Spin cells at 400 x g for 2 minutes at 4°C and discard supernatant.
20. Resuspend cells in 200 µL of FACS Buffer and proceed to flow cytometry acquisition.

Note: For analysis follow the gating strategy for CD19$^+$ B cells (**Figure 1**). Within the B cell population, gate on the probe-positive cells as outlined in **Figure 2**. Compare the staining of probes in the positive samples to the negative samples (pre-pandemic sample, if available). Ascertain the optimal concentration for each probe, determined by the highest signal to noise ratio (calculated as the ratio between mean fluorescence intensity of the probe positive and probe negative B cells). Ensure that the

negative control samples do not exhibit irrelevant probe binding to B cells at the desired concentration. Compare staining to a pre-validated batch of probe if applicable.

Note: If the probes have a high level of irrelevant binding in the negative controls, the probes should be remade with a new batch of protein. If persistent difficulty occurs with minimal probe staining or high background staining across batches, an alternative source of protein should be considered.

Preparation for FNA collection

FNAs are collected into ice cold Culture Media and kept on ice or at 4°C until processing.

Timing: 1-5 minutes

21. Prepare Culture Media as described in the Materials and equipment section.

22. Add 20 mL of Culture Media into a 50 mL conical tube.

23. Store the tube on ice (or at 4°C) and keep cold throughout the entire FNA procedure.

Key resources table

Materials and equipment

Culture Media

Component	Final Concentration	Volume
FBS	10%	50 mL
Glutamax	1X (Equivalent to 2 mM L-alanyl-L-glutamine dipeptide)	5 mL
Penicillin-Streptomycin	1X (Equivalent to 100 units/mL of penicillin and 100 µg/mL of streptomycin)	5 mL
RPMI	1X	440 mL

Store at 4°C for up to 1 month.

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FACS Buffer

Component	Final Concentration	Volume
FBS	2%	10 mL
EDTA	5mM	5 mL
PBS	1X	485 mL

Store at 4°C for up to 1 month.

Freezing Media

Component	Final Concentration	Volume
FBS	90%	900 μL
DMSO	10%	100 μL

Make fresh and store at 4°C until use.

1% Paraformaldehyde Solution

Component	Final Concentration	Volume
16% Paraformaldehyde	1%	3.125 mL
FACS Buffer	1X	46.875 mL

Store at 4°C for up to 1 month.

Step-by-step method details

Fine Needle Aspiration

In this step of the protocol, ultrasound is used to identify superficial lateral axillary lymph nodes in donors. The lymph node to biopsy is selected based on accessibility, larger size/cortical thickness, and relative distance to the surface of the skin. If no lateral lymph node is accessible, a central lymph node may be biopsied instead, however, posterior and/or apical lymph nodes are never sampled. Once a suitable lymph node is identified, ultrasound guidance is used to insert the tip of the needle into the lymph node and gently aspirate lymphoid cells from the cortex. After aspiration, the specimen is

expelled from the syringe into ice cold media to collect the cells. FNAs are performed similar to those reported in [8], with a few minor differences highlighted below.

Timing: 30 - 60 minutes

24. Prior to the FNA procedure, prepare the collection syringes by attaching a 25-gauge needle to a 10 mL syringe and withdraw the plunger to pull 1 mL of air into the syringe.

Note: This helps to eject the specimen. A separate sterile needle/syringe is prepared for each pass (5 total).

Note: In the original protocol [8], a 22-, 25-, or 27-gauge needle along with a 3 mL or 5 mL syringe were all successfully used. We opt to consistently use a 25-gauge needle with a 10 mL syringe.

25. Use an ultrasound instrument (Philips EPIQ ELITE or PHILIPS IU222) to visualize superficial axillary draining lymph nodes. Select the most accessible and best visualized lymph node for biopsy.
26. Clean the skin around the lymph node with 4% chlorhexidine gluconate solution, or another antiseptic solution. Anesthetize this area by injecting 2-6 mL of 0.9% buffered lidocaine solution.
27. Under ultrasound guidance, place the tip of the 25-gauge needle into the cortex of the lymph node and move back and forth for 10-20 seconds to collect cells.
    a. Eject the sample is the into 10 mL of ice-cold Culture Media.
    b. This step is considered as a single "pass". See **Methods Video S1**.

Note: Negative pressure (light suction) is usually applied to the syringe in step 26 to increase the cell yield and is recommended in the original protocol [8]. Suction should be released prior to removing the needle from the lymph node to avoid aspirating subcutaneous fat.

28. Rinse the needle with 1-2 mL of media to collect residual cells before disposal.
29. Perform a total of five passes (as defined in step 27).

Note: In the original protocol [8], four lymph nodes, with a total of four passes each, were probed per donor. In our recent study, we opted for a single lymph node per donor with a total of five passes [1]. In some donors, it can be difficult to visualize multiple lymph nodes that are accessible for FNA, thus a single lymph node per donor tends to be more feasible for collection.

Flow Cytometry

After the FNA is performed, cells are immediately processed and stained with fluorescent antibodies to identify B cell and T cell populations by spectral flow cytometry.

Timing: 4-6 hours

30. Prepare single-color compensation controls.

    a. In a V-bottom 96-well plate, set up an unstained control and a compensation control for the viability dye by adding 1 x10^6 cells per well into two separate wells.

Note: Since FNA cell recovery can be limiting, fresh PBMC or other human cells (e.g. tonsillar cells) can be used for these compensation controls.

    b. Bring the volume of the well to 200 μL with FACS Buffer, then spin the plate at 400 x g for 2 minutes at 4°C.

    c. Aspirate the supernatant and resuspend the cells with 100 μL of FACS Buffer (unstained control) or eBioscience™ Fixable Viability Dye eFluor™ 780 diluted 1:2000 in FACS Buffer (viability die control). Keep the plate at 4°C (or on ice) until step 30g.

    d. For the other single-color compensation controls, add 15 μL per well of UltraComp eBeads™ Compensation Beads to the plate. One well is needed for each fluorochrome included in the panel.

    e. The total volume of each well is brought to 100 μL by adding 85 μL of FACS Buffer.

    f. Add 1 μL (or more) of each individual antibody to the appropriate wells.

    g. Incubate all controls at 4°C for 30 minutes.

CRITICAL: It is critical that, for each fluorochrome, the single-color compensation controls are as bright, or brighter, than the stained sample. For this reason, the concentration of the antibody used to stain the samples (see Stain Mix 1 and Stain Mix 2 tables for the suggested concentrations), is an appropriate starting point for staining the compensation beads. From there, the concentration may need to be adjusted depending on signal brightness (use lower concentration if the signal is too bright, or higher concentration if the signal is too dim).

Note: The fluorescently labelled tetrameric B cell probes do not bind to these compensation beads. To set up compensation controls for the tetrameric probes, we instead stain compensation beads with an antibody conjugated to the same fluorochrome as the one conjugated to the probes (i.e. we use an anti-CD4-PE antibody as the single-color compensation control for the RBD-PE tetrameric probe). Importantly, if possible, tandem dyes should be avoided for making tetrameric probes as tandem dyes have a much higher variability from lot-to-lot (due to the dissolution of the tandem fluorochromes) and cannot be accurately compensated in this manner.

 h. After incubation add 100 μL of FACS Buffer to each well and spin the plate at 400 x g for 2 minutes at 4°C.

 i. Aspirate supernatant and wash the single-color compensation controls one additional time by adding 200 μL of FACS Buffer to each well and spin as above.

 j. Resuspend the single-color compensation controls in 100-200 μL of FACS Buffer and use to set the compensation matrix on the spectral flow cytometer.

Note: Single color compensation controls can be prepared during any incubation steps.

Note: Spectral flow cytometers, such as the Cytek Aurora, tend to be quite stable over time. For this reason, we have had success with acquiring single-color compensation controls once and reusing the same controls across multiple weeks to set the compensation matrix.

CRITCAL: If adopting the above strategy and staining fresh FNA samples on different days, the same selected frozen human sample should be used as an internal control for every new FNA sample acquisition. This internal control is used to validate the accuracy of the compensation matrix over time

and ensure the consistency of the gating strategy between batches. A tonsil sample is preferred for this purpose, as it contains the cell populations of interest. However, PBMCs could also be used if human tonsils are not available, keeping in mind that certain markers (e.g. BCL6) are not be expressed, or expressed at low levels in cell populations from blood.

31. After obtaining the FNA sample, spin at 300 x g for 10 minutes at 4°C.

32. Remove supernatant by aspiration, leaving 100-200 μL of residual volume. If red blood cells appear abundant in the pellet, proceed to step 33. Otherwise, proceed to step 36.

33. To lyse red blood cells, resuspend cells in 2 mL of ACK Lysis Buffer and incubate on ice (or at 4°C) for 3-5 minutes.

Note: Longer incubation times in ACK Lysis Buffer can decrease cell viability. We tend to keep this step as short as possible by ending the incubation as soon as the solution clears (becomes transparent).

34. Stop the lysis reaction by adding 20 mL of ice-cold PBS and spin cells at 300 x g for 10 minutes at 4°C.

35. Remove supernatant by aspiration.

36. Resuspend cell pellet in 500 μL of Culture Media. Count cells on a hemacytometer. Cells should be kept at 4°C (or on ice) throughout processing, unless otherwise denoted.

37. Prepare staining mixes. It is recommended to prepare a two-sample excess of these mixes.

Note: The concentrations listed below are 2X concentrations, as Stain Mix 1 and Stain Mix 2 are ultimately combined 1:1 (see step 42).

a. Stain Mix 1: Prepare by mixing the appropriate volume of each antibody in FACS Buffer as described in the table below. 50 μL of mix is required per sample.

Stain Mix 1

Marker	Concentration (mg/mL; 2X)	Dilution (2X)
CXCR5 BUV395	0.005	1:40
CCR4 PE-CF594	0.01	1:20
CCR6 BV480	0.003	1:33
CXCR3 BV510	0.005	1:20

b. Stain Mix 2:

i. First prepare the eBioscience™ Fixable Viability Dye eFluor™ 780 by diluting it 1:1000 in FACS Buffer (for a final dilution of 1:4000).

ii. Then combine diluted viability dye 1:1 with Brilliant Stain Buffer to achieve a total volume of 50 μL of mix per sample (mix 25 μL of diluted viability dye with 25 μL of Brilliant Stain Buffer per sample).

iii. Add the appropriate volume of each antibody/tetramer as described in the table below to obtain a 2x dilution.

Stain Mix 2

Marker	Concentration (mg/mL; 2X)	Dilution (2X)
IgM BUV496	0.002	1:100
CD4 BUV563	0.00012	1:100
CD11c BUV661	0.004	1:50
IgD BUV737	0.001	1:100
CD45RA BUV805	0.002	1:100
CD138 BV605	0.004	1:25
ICOS BV650	0.004	1:25
CD20 BV750	0.002	1:100
CD38 BV785	0.003	1:50
CD8α PerCP-eFluor710	0.0005	1:100

CD27 PE-Cy5	0.002	1:25
CD19 PE-Cy5.5	0.00025	1:100
CD11b Alexa Fluor 700	0.004	1:50
PD-1 PE-Cy7	0.004	1:25
Full S BV421	varies	Based on titration
RBD PE	varies	Based on titration
PR8-HA Alexa Fluor 488	varies	Based on titration

38. Add the appropriate number/volume of cells to a V-bottom 96-well plate. 0.5-1x10^6 cells per staining are recommended, but lower numbers can be stained according to the FNA yield.

Note: If larger numbers of cells have been collected, they can be cryopreserved as described in the FNA Cryopreservation section.

39. Spin cells at 400 x g for 2 minutes at 4°C.

40. Aspirate supernatant and resuspend cells in 50 μL of Stain Mix 1.

41. Incubate plate for 10 minutes at 37°C.

CRITICAL: Chemokine staining with Stain Mix 1 must be performed at 37°C. Staining for chemokine receptors at 4°C yields poor separation of positive and negative signals, while staining at 37°C markedly improves separation.

42. After incubation, add 50 μL of Stain Mix 2 directly to each well (without washing) and gently resuspend by pipetting.

43. Incubate for 1 hour at 4°C. During this incubation, prepare the solutions for the fixation/permeabilization step (see step 45a).

44. After the incubation is complete, add 100 μL of ice-cold FACS Buffer to each sample and spin cells at 400 x g for 2 minutes at 4°C.

45. Fix and permeabilize the cells using the FoxP3 / Transcription Factor Staining Buffer Set:

   a. During the incubation in step 43, prepare 200 µL per sample of the Fixation/Permeabilization Solution by diluting the 4X Fixation/Permeabilization Concentrate 1:3 in the Fixation/Permeabilization Diluent (for 200 µL total: mix 50 µL Concentrate with 150 µL Diluent).

   b. Aspirate supernatant and resuspend in 200 µL of Fixation/Permeabilization Solution per well.

   c. Incubate for 1 hour at 4°C. During this time, complete steps 46 and 47.

46. Prepare a 1X Permeabilization Buffer by diluting the Permeabilization Buffer (10X) in milliQ water (for 1 mL total: mix 100 µL of 10X Buffer in 900 µL of water).

47. Prepare 100 µL of BCL6 staining mix per sample by diluting the BCL6 antibody 1:50 in 1X Permeabilization Buffer.

48. After incubation is complete, spin cells at 400 x g for 2 minutes at 4°C. Then, aspirate supernatant and resuspend samples in 100 µL of BCL6 staining mix.

49. Incubate for 1 hour at 4°C.

50. After incubation, add 100 µL of 1X Permeabilization Buffer to each sample and spin cells at 400 x g for 2 minutes at 4°C.

51. Aspirate supernatant and resuspend in 200 µL of FACS Buffer and proceed to flow cytometry sample acquisition.

CRITICAL: To ensure an adequate number of events for analysis, the entire sample volume should be acquired on the flow cytometer.

Note: Because of the intracellular BCL6 staining, samples are best acquired as soon as possible on the flow cytometer. In extreme circumstances they can be left up to 16 hours before acquiring, but this will decrease the intensity of BCL6 signal.

FNA Cryopreservation (Optional)

If enough cells are recovered from the FNA procedure, excess sample can be cryopreserved for future use. In this section, we describe basic how to cryopreserve these samples for long term storage.

Timing: 24 hours

52. If freezing cells, gently resuspend the desired number of cells in the appropriate volume (2-10x10^6 cells/mL) of Freezing Media and rapidly transfer to a cryogenic vial.

53. Immediately transfer vials to a pre-chilled isopropanol freezing container, such as a Nalgene® Mr.Frosty, and place in a -80°C freezer.

54. The following day (approximately 24 hours following step 53), transfer the vials into cryoboxes and place in vapor phase liquid nitrogen (below -135°C) for long-term storage.

Note: Performing the flow analysis on frozen samples, instead of fresh, is possible if enough cells are recovered from the FNA procedure to allow for cryopreservation. FNA cell recovery will depend on the time point of collection post-vaccination and on whether the subjects undergoing the FNA collection are immunocompetent or immunocompromised. In our published study [1], we opted to perform spectral flow cytometry analysis of fresh FNA samples because of the variability in cell yield at certain time points for immunocompromised individuals. It is also worth mentioning that while the concurrent analysis of multiple cryopreserved samples might reduce variability introduced by performing the staining on different days and the cytometer set up, cryopreservation itself can affect the quality of detection for certain markers. For instance, the signal intensity of some chemokine receptors might be reduced after cryopreservation.

Expected outcomes

The yield of FNA samples collected 14 days (+/- 2) after the 1st dose (V2) and 8 days (+/-2) after the 2nd dose (V3) of SARS-CoV-2 mRNA vaccines is variable and can range from 0.3 x 10^6 – 40 x 10^6 cells in immunocompetent donors. In our study [1], we found that GC B cells and Tfh cells were present at high frequencies at V2, and that these frequencies typically increased at V3. The same finding held true for antigen-specific GC B cells, antigen-specific memory B cells, and both total and antigen-specific plasmablasts. Below is a table highlighting the expected ranges of cell frequencies at V2 and V3:

Cell Type	V2 (% of lymphocytes)	V3 (% of lymphocytes)
GC Tfh cells	0.04 – 1.58	0.34 – 3.08
GC B cells	0.01 – 4.03	0.26 – 8.22

S+ RBD- GC B cells	0.00 – 0.87	0.03 – 1.87
S+ RBD+ GC B cells	0.00 – 0.19	0.00 – 1.17
S+ RBD- MBCs	0.00 – 0.04	0.01 – 0.05
S+ RBD+ MBCs	0.00 – 0.01	0.00 – 0.03
Plasmablasts	0.01 – 0.11	0.05 – 1.00
S+RBD- Plasmablasts	0.00 – 0.01	0.00 – 0.03
S+ RBD+ Plasmablasts	0.00 – 0.00	0.00 – 0.01

Table 1. Expected frequencies of specific cell types from FNAs. Expected frequencies of the denoted cell types as measured from immunocompetent donors at V2 and V3 as detailed in [1].

Quantification and Statistical Analysis

FlowJo v10 is used for the analysis of flow cytometry files. Compensation should be run and applied on your spectral flow cytometer. Before beginning analysis, the accuracy of the compensation matrix should be validated.

Gating strategies for GC B cells, memory B cells and plasmablasts (**Figures 1-2**), GC Tfh cells (**Figure 3**), and functional polarization of CD4 T cells (**Figure 4**), are outlined below.

Limitations of the study

This procedure outlines a fine needle aspiration-based method to access cells from vaccine draining lymph nodes in humans. This approach, however, is limited in that it does not preserve any information about the location of recovered cells or the architecture of the lymph node. The cells recovered using this method are in a single-cell suspension, and therefore most suited to analysis applications such as flow cytometry. If spatial information about the draining lymph nodes is required for a particular study, then supplementary methods will need to be developed.

Additionally, while FNAs are usually successful, there have been occasions where the FNA procedure does not result in sufficient viable cell recovery for staining. Due to this, we plan for about a 10% failure rate in FNAs.

Troubleshooting

Problem 1:

Low cell yield and/or low viability of FNA biopsy.

Potential solutions:

1. If it is difficult to identify a lymph node (step 25), it may be helpful to switch between high frequency linear transducers (L15-7io or L12-4) and lower frequency small footprint transducers (C8-5), as well as optimizing other scanning parameters such as focal zone.
2. While performing the biopsy (step 27), it is important to ensure that the tip of the needle remains in the cortex and not in the adjacent fat or in the lymph node hilum and to stay within the lymph node an adequate amount of time (at least 10 seconds).
3. Selecting a lymph node with a thicker cortex in step 25 allows for a greater needle excursion and lowers the likelihood of inadvertently coming out of the lymph node cortex.
4. Ensure media tube is kept at 4°C or on ice at all times during the procedure through processing until after the fixation/permeabilization (step 45) is complete. This approach will help to limit cell death.
5. If initial cell pellet after centrifugation in step 31 is small, consider decreasing the time of ACK lysis to preserve viability.

Problem 2:

Little or no cells binding probe staining

Potential solution:

In steps 7-20, validate tetrameric probe staining using a sample with known positive, probe-binding cells. PBMCs from individuals recently vaccinated with a SARS-CoV-2 mRNA vaccine or COVID-19 convalescent subjects will suffice for validation purposes if human lymph node biopsies are not readily available. If no cells from the positive control sample bind the probes, follow the steps outlined in this protocol to remake tetramers. Biotinylated RBD can be purchased from R&D Systems to eliminate a step in the process of making tetramers for an additional cost.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michela Locci (michela.locci@pennmedicine.upenn.edu).

Materials availability

All unique reagents generated in this study will be available from the lead contact upon reasonable request.

Data and code availability

All raw data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Additional Supplemental Items are available from Mendeley Data at

https://dx.doi.org/10.17632/4kh76pv4sj.1.

Author contributions

M.L and K.L. adapted the protocol. K.L. and E.B. developed the FNA analysis strategy. E.B. and M.L. wrote the manuscript with help from K.L., H.S., and L.J. M.K. performed all clinical coordination. M.L supervised the generation of this protocol.

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Declaration of interests

The authors have no interest to declare.

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Figure legends

Figure 1. GC B cell, memory B cell, and plasmablast gating strategy. For the B cell analysis, we first gate on singlets and then on lymphocytes. We next separate out live $CD8^-$ cells, and further gate on the $CD19^+CD4^-$ population. Class-switched B cells (IgM^-IgD^-) are further selected to define: memory B cells ($CD38^-CD27^+$), GC B cells ($CD38^+CD27^{lo/int}BCL6^+$), and plasmablasts ($CD38^+CD20^{lo/-}CD27^+BCL6^-$). Memory B cells, which are $BCL6^-$, are overlaid in the plot showing CD38 versus BCL6 to guide the GC B cell gating (lower right panel). GC B cells, which are $BCL6^+$, are overlaid in the plot showing CD38 versus BCL6 to guide the plasmablast gating (lower left panel).

Note: Memory B cells ($CD38^-CD27^+$) can also be analyzed in the IgM^+ population, as some memory B cells are not class-switched.

Note: Plasmablasts can also be defined as $BLIMP1^+$ cells as opposed to $BCL6^-$ cells. Since BLIMP1 and BCL6 antagonize each other, we have found that the definition of plasmablasts as $CD38^+CD20^-CD27^+BCL6^-$ largely overlaps with the population of $CD38^+CD20^-CD27^+BLIMP1^+$ cells.

Figure 2. Antigen-specific B cell gating strategy. Following the gating strategy from **Figure 1**, we can determine the antigen-specificity of the B cell subsets by using a combination of tetrameric probes. (**A**) First, an irrelevant tetrameric probe (PR8-HA in this example) is used to gate out B cells potentially binding tetramers in an unspecific manner (i.e., streptavidin binders). (**B**) We then use a combination of SARS-CoV-2 full spike and RBD tetrameric probes to stratify antigen-specific GC B cells, memory B cells, and plasmablasts into spike-specific B cells binding the spike within or outside the RBD region.

Figure 3. Tfh cell gating strategy. For Tfh cell analysis, we first gate on singlets and then on lymphocytes. We next separate out live $CD8^-$ cells, and further gate on the $CD19^-CD4^+$ population. Antigen-experienced CD4 T cells, defined as $CD45RA^-$ cells, are selected for further analysis. GC Tfh cells are then defined as $PD-1^{hi}CXCR5^{hi}$ cells.

Figure 4. Evaluation of CD4 T cell functional polarization. The initial analysis of live $CD8^-CD19^-CD4^+$ cells is shown in **Figure 3**. The live $CD8^-CD19^-CD4^+$ cell population is further subdivided into naïve CD4 T cells ($CD45RA^+$), serving as a negative control to define chemokine receptor gating, antigen-experienced

non-Tfh CD4 T cells (CD45RA⁻CXCR5⁻) or Tfh cells (CXCR5^{hi}PD-1^{hi}, gating shown in **Figure 3**). These 3 cell populations are further subdivided into (**A**) Th1 (CXCR3^{+}) polarized cells and (**B**) Th2 (CXCR3^{-}CCR4^{+}CCR6^{-}) or Th17 (CXCR3^{-}CCR4^{+}CCR6^{+}) polarized cells.

Methods Video S1. Cine of an ultrasound guided FNA pass, related to step 27.

A

GC B Cells	Memory B Cells	Plasmablasts

B
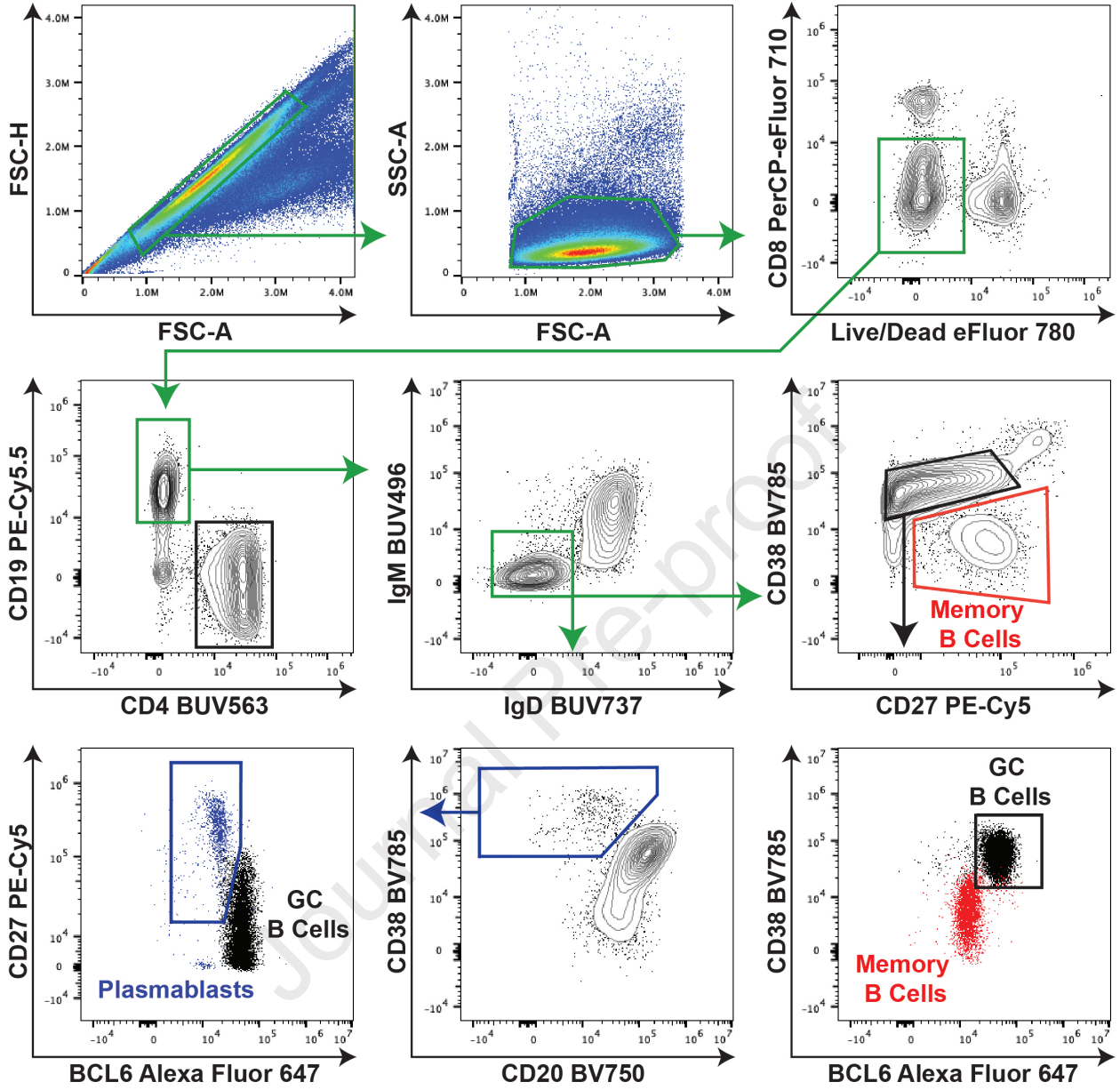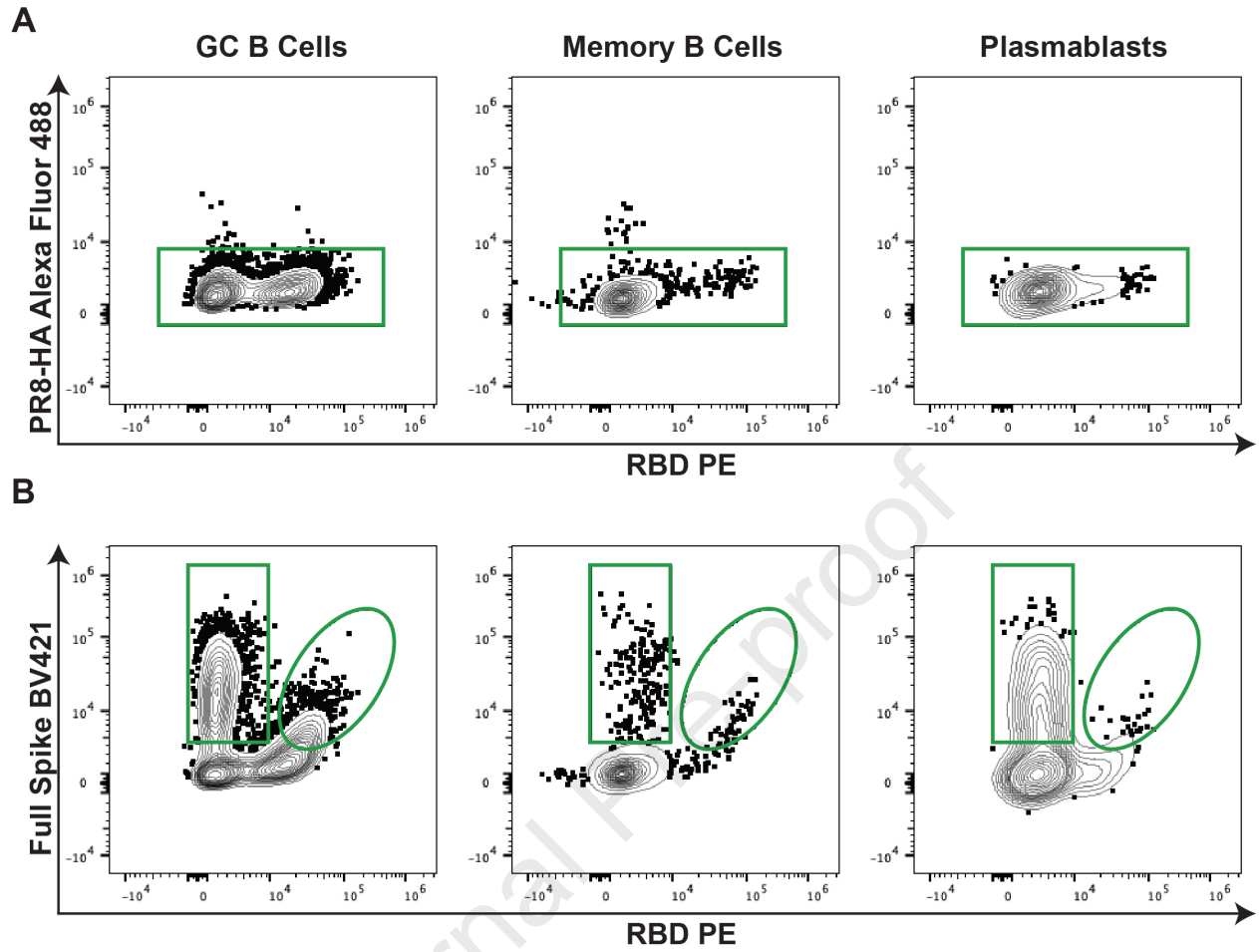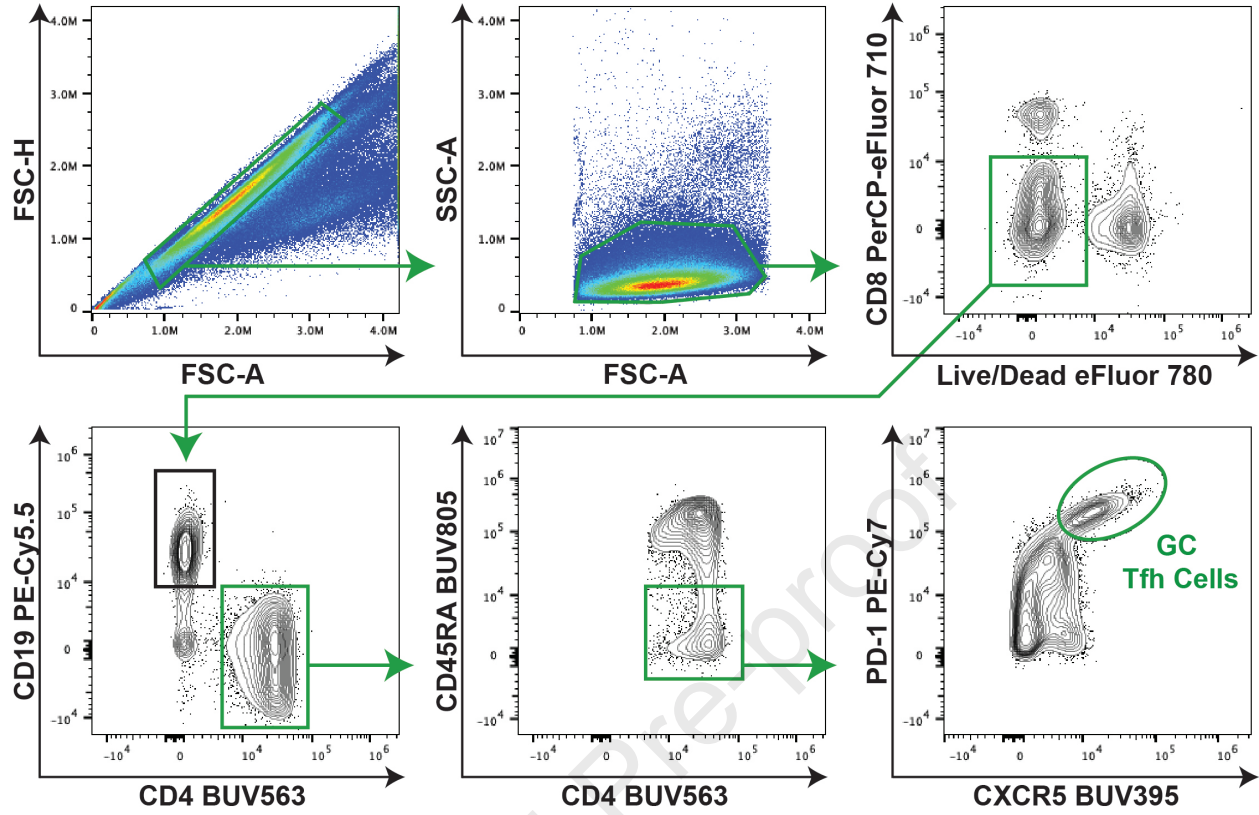
A

B

Highlights

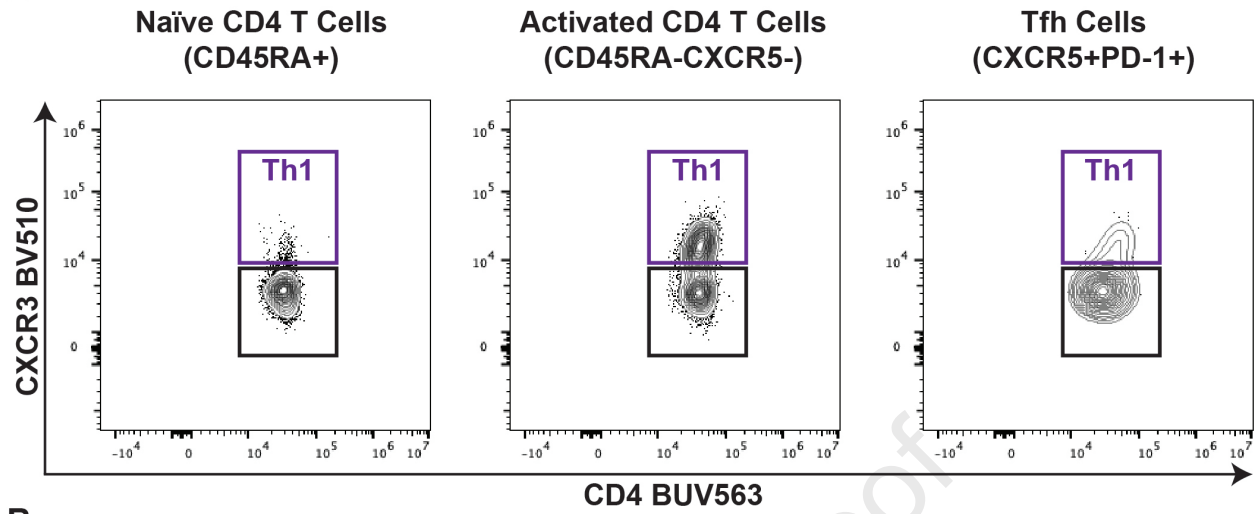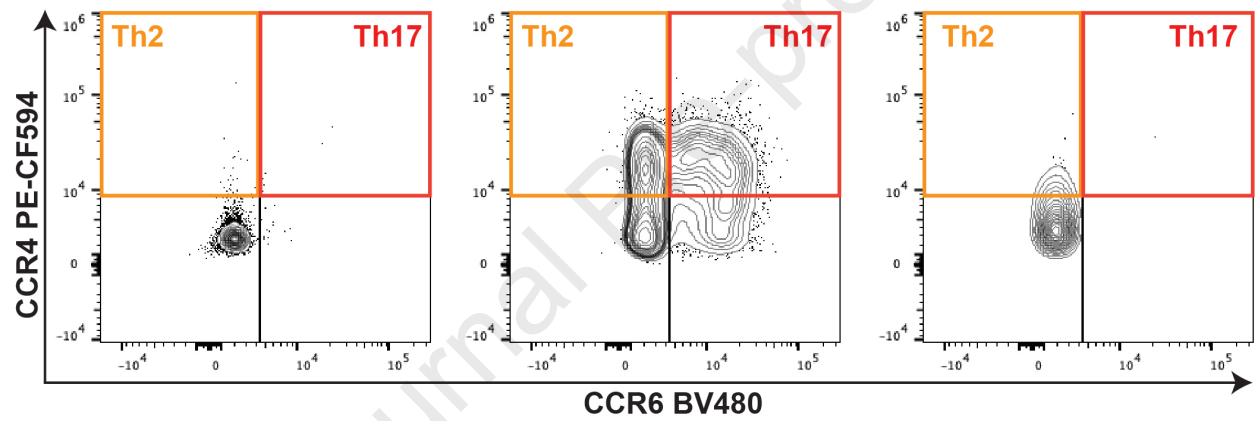- Step-by-step procedure for SARS-CoV-2-specific B cell tetrameric probe generation.
- Detailed procedure for collecting fine needle aspirate (FNA) samples from lymph nodes.
- Long-term storage of FNA samples.
- 23-color panel for analysis of human germinal center responses by spectral flow cytometry.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-human CXCR5 BUV395 (Final dilution 1:80)	BD Biosciences	Cat# 740266; RRID: AB_2740008
anti-human CCR4 PE-CF594 (Final dilution 1:40)	BD Biosciences	Cat# 565391; RRID: AB_2739215
anti-human CCR6 BV480 (Final dilution 1:66)	BD Biosciences	Cat# 566130; RRID: AB_2739529
anti-human CXCR3 BV510 (Final dilution 1:40)	Biolegend	Cat# 353726; RRID: AB_2563642
anti-human IgM BUV496 (Final dilution 1:200)	BD Biosciences	Cat# 750366; RRID: AB_2874541
anti-human CD4 BUV563 (Final dilution 1:200)	BD Biosciences	Cat# 612912; RRID: AB_2739451
anti-human CD11c BUV661 (Final dilution 1:100)	BD Biosciences	Cat# 612967, RRID: AB_2870241
anti-human IgD BUV737 (Final dilution 1:200)	BD Biosciences	Cat# 612798; RRID: AB_2738894
anti-human CD45RA BUV805 (Final dilution 1:200)	BD Biosciences	Cat# 742020; RRID: AB_2871317
anti-human CD138 BV605 (Final dilution 1:50)	Biolegend	Cat# 356520; RRID: AB_2562862
anti-human ICOS BV650 (Final dilution 1:50)	Biolegend	Cat# 313550; RRID: AB_2749929
anti-human CD20 BV750 (Final dilution 1:200)	BD Biosciences	Cat# 747062; RRID: AB_2871819
anti-human CD38 BV785 (Final dilution 1:100)	Biolegend	Cat# 303530; RRID: AB_2565893
anti-human CD8α PerCP-eFluor710 (Final dilution 1:200)	Invitrogen	Cat# 46-0086-42; RRID: AB_2848331
anti-human CD27 PE-Cy5 (Final dilution 1:50)	Invitrogen	Cat# 15-0279-42; RRID: AB_10717249
anti-human CD19 PE-Cy5.5 (Final dilution 1:200)	Invitrogen	Cat# 35-0198-42; RRID: AB_11218903
anti-human CD11b Alexa Fluor 700 (Final dilution 1:100)	Biolegend	Cat# 301356; RRID: AB_2750075
anti-human PD-1 PE-Cy7 (Final dilution 1:50)	Biolegend	Cat# 329918; RRID: AB_2159324
anti-human/mouse BCL6 Alexa Fluor 647 (Final dilution 1:50)	BD Biosciences	Cat# 561525; RRID: AB_10898007
Bacterial and virus strains		
Biological samples		
Human FNA	Perelman Center for Advanced Medicine	N/A
Human PBMCs	Perelman Center for Advanced Medicine	N/A
Human pediatric tonsils	Children's Hospital of Philadelphia	N/A

Chemicals, peptides, and recombinant proteins		
Fixable viability dye eFluor 780	Invitrogen	Cat# 65086518
Recombinant RBD	Norbert Pardi	N/A
Recombinant Spike-Biotin	R&D Systems	Cat# BT10549-050
Recombinant PR8 HA	Norbert Pardi	N/A
Streptavidin BV421	Biolegend	Cat# 405225
Streptavidin Alexa Fluor 488	Biolegend	Cat# 405235
Streptavidin Alexa Fluor 647	Biolegend	Cat# 405237
EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit	Thermo Fisher Scientific	Cat# 21935
EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format (Included in EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit)	Thermo Fisher Scientific	Cat# A39257
Zeba™ Spin Desalting Columns (Included in EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit)	Thermo Fisher Scientific	Cat# 89889
BupH Phosphate Buffered Saline (Included in EZ-Link™ Micro Sulfo-NHS-LC-Biotinylation Kit)	Thermo Fisher Scientific	Cat# 28372
FoxP3 / Transcription Factor Staining Buffer Set	ThermoFisher Scientific	Cat# 00-5523-00
ACK Lysis Buffer	Gibco	Cat# A1049201
DMSO	Sigma	Cat# D2650
FBS	Corning	Cat# 35-010-CV
EDTA	Invitrogen	Cat# 15575-038
PBS	Corning	Cat# 21-031-CV
Glutamax	Gibco	Cat# 35050-061
Penicillin-Streptomycin	Gibco	Cat# 15140-122
RPMI	Gibco	Cat# 22400-089
Brilliant Stain Buffer	BD BioSciences	Cat# 563794
0.9% buffered lidocaine	Hospital of the University of Pennsylvania	N/A
4% Chlorohexadine Gluconate	SC Johnson	Cat# 134439
16% Paraformaldehyde	Electron Microscopy Sciences	Cat# 15710
Molecular Biology Grade Water	Corning	Cat# 46-000-Cl
Critical commercial assays		
Deposited data		
Experimental models: Cell lines		

Experimental models: Organisms/strains		
Oligonucleotides		
Recombinant DNA		
Software and algorithms		
FlowJo v10	FlowJo LLC	RRID:SCR_008520
Other		
UltraComp eBeads™ Compensation Beads	ThermoFisher	Cat# 01-2222-42
Spectral Cytometer	Cytek	Aurora Spectral Analyzer (5 lasers, 64 detectors)
Ultrasound Machine	Phillips	EPIQ ELITE or IU222
Ultrasound Probes	Phillips	L15-7io or C8-5