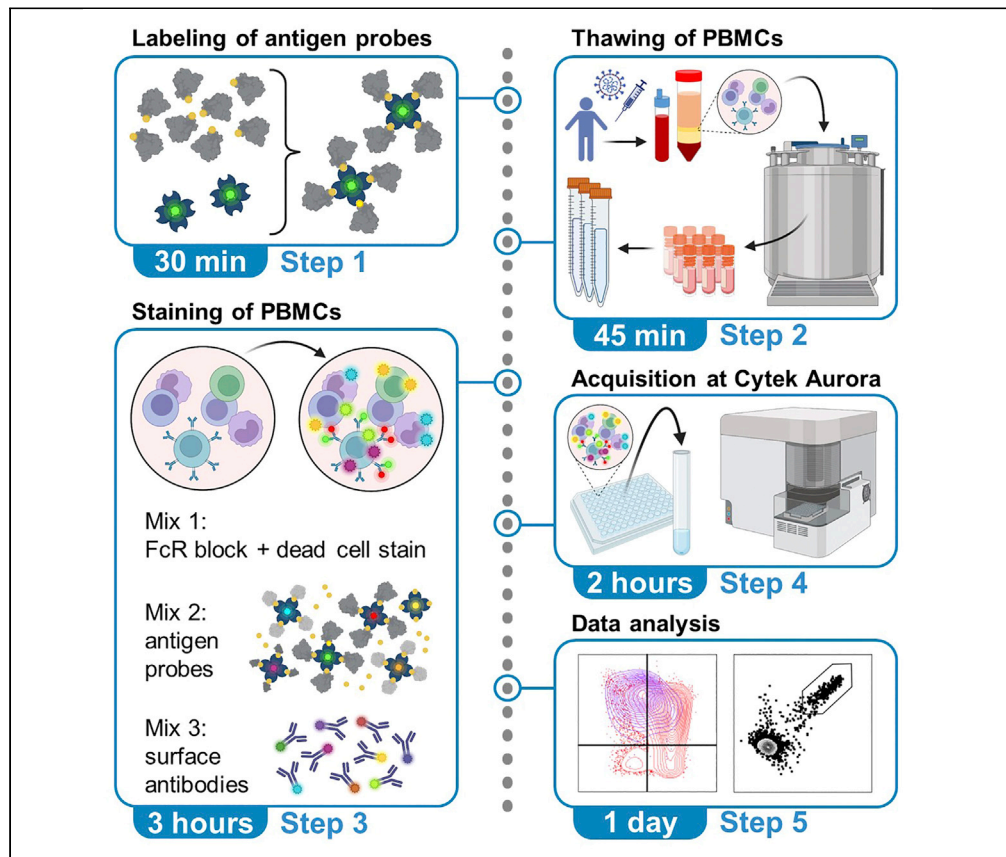


## Protocol

# Flow cytometric protocol to characterize human memory B cells directed against SARS-CoV-2 spike protein antigens



Memory B cells (MBCs), part of the immune response elicited by infection or vaccination, can persist in lymphoid organs and peripheral blood and are capable of rapid reactivation upon secondary antigen exposure. Here, we describe a flow cytometric assay to identify antigen-specific MBCs from peripheral blood mononuclear cells and characterize their isotypes and activation status. We detail steps to use fluorescently labeled antigen probes derived from the SARS-CoV-2 spike protein. These can be adapted to detect MBCs against other antigens.

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

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### Highlights

Characterization of memory B cell isotypes and activation status

Simultaneous identification of three antigen specificities: spike S1, S2, RBD

Protocol can easily be adjusted to other protein-based antigens

Detailed information on staining, acquisition (Cytex Aurora), and gating strategy

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## Protocol

## Flow cytometric protocol to characterize human memory B cells directed against SARS-CoV-2 spike protein antigens

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## SUMMARY

Memory B cells (MBCs), part of the immune response elicited by infection or vaccination, can persist in lymphoid organs and peripheral blood and are capable of rapid reactivation upon secondary antigen exposure. Here, we describe a flow cytometric assay to identify antigen-specific MBCs from peripheral blood mononuclear cells and characterize their isotypes and activation status. We detail steps to use fluorescently labeled antigen probes derived from the SARS-CoV-2 spike protein. These can be adapted to detect MBCs against other antigens.

For complete details on the use and execution of this protocol, please refer to Weskamm et al. (2022).<sup>1</sup>

## BEFORE YOU BEGIN

Here we describe a multiparametric flow cytometric assay to identify antigen-specific memory B cells (MBCs) from human peripheral blood mononuclear cells (PBMCs). Besides antigen specificity, MBCs are further characterized to identify their antibody isotype and activation status. We provide here detailed information about the staining procedure, a basic workflow for acquisition of samples on a spectral flow cytometer (Cytek Aurora) and a proposed gating strategy.

The protocol below describes the specific steps for detection of MBCs directed against SARS-CoV-2. Several fluorophores are used to differentiate between 3 different antigen specificities: the spike protein subunits S1 and S2, as well the receptor binding domain (RBD). We here used proteins from the SARS-CoV-2 Wuhan-Hu-1 strain, but the protocol could easily be adapted to other variants of SARS-CoV-2. The examples provided in this protocol are based on the staining of cryopreserved PBMCs from healthy donors, collected before and at different time points after vaccination against SARS-CoV-2. Notably, the protocol could also be used to analyze spike-specific B cells in convalescent individuals or be adapted to stain B cells with specificity towards other antigens or pathogens. The protocol has also been established for staining of MERS-CoV spike-specific B cells in MERS vaccinees.<sup>1</sup>



### Institutional permissions

The cryopreserved PBMCs used in this study were isolated from EDTA blood obtained from healthy donors. Blood donations were approved by the Competent National Authority (Paul-Ehrlich-Institut, Langen, Germany) and the ethic committee of the Medical Association Hamburg, Germany (reference numbers: 2020-10376-BO-ff, PV4780), and conducted at the University Medical Centre Hamburg-Eppendorf, Germany. All participants provided written informed consent prior to enrollment in the study.

### Preparation of reagents and buffers

⌚ Timing: 30 min

1. Prepare staining buffer.
  - a. Mix 500 mL 1× PBS with 10 mL heat-inactivated Fetal Bovine Serum (FBS) and 2 mL 0.5 M EDTA (see also [materials and equipment](#) section).
2. Reconstitute biotinylated SARS-CoV-2 spike antigens.
  - a. Reconstitute lyophilized proteins with 80 µL of sterile water to obtain a stock concentration of 0.25 mg/mL.
  - b. Use immediately or store in aliquots at –20°C to –80°C for up to three months.
  - c. Avoid repeated freeze and thaw cycles.
3. Aliquot Bio-200 biotin solution for long-term storage (several years) at –20°C or –80°C.
  - a. At 4°C Bio-200 biotin solution is stable for up to 2 months.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
PerCP anti-human CD3 Antibody (final dilution 1:54)	Biolegend	Cat#344813; RRID: AB_10641841
PerCP anti-human CD14 Antibody (final dilution 1:54)	Biolegend	Cat#301847; RRID: AB_2564058
Alexa Fluor® 700 anti-human CD56 (NCAM) Antibody (final dilution 1:54)	Biolegend	Cat#318316; RRID: AB_604104
Brilliant Violet 650™ anti-human CD20 Antibody (final dilution 1:54)	Biolegend	Cat#302335; RRID: AB_11218609
Brilliant Violet 785™ anti-human IgD Antibody (final dilution 1:54)	Biolegend	Cat#348241; RRID: AB_2629808
Brilliant Violet 570™ anti-human IgM Antibody (final dilution 1:54)	Biolegend	Cat#314517; RRID: AB_10913816
BD OptiBuild™ BUV805 Mouse Anti-Human IgG (final dilution 1:54)	BD	Cat#742041; RRID: AB_2871333
BUV563 Mouse Anti-Human CD19 (final dilution 1:54)	BD	Cat#612917; RRID: AB_2870202
BD Horizon™ BUV737 Mouse Anti-Human CD21 (final dilution 1:54)	BD	Cat#612788; RRID: AB_2870115
BD Horizon™ BB515 Mouse Anti-Human CD27 (final dilution 1:54)	BD	Cat#564643; RRID: AB_2744354
IgA Antibody anti-human, APC-Vio 770 (final dilution 1:54)	Miltenyi Biotec	Cat#130-113-999; RRID: AB_2733153
Biotinylated detection mAbs (MT78/145) (final dilution 1:100)	Mabtech	Cat#3850-6-250; PRID: AB_10666158
<b>Biological samples</b>		
Cryopreserved PBMCs from healthy volunteers vaccinated against SARS-CoV-2	University Medical Center Hamburg-Eppendorf, Germany	N/A

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 (2019-nCoV) Spike S1-His Recombinant Protein, Biotinylated	Sino Biological	40591-V08H-B
SARS-CoV-2 (2019-nCoV) Spike S2 ECD-His Recombinant Protein, Biotinylated	Sino Biological	40590-V08B-B
SARS-CoV Spike/RBD Protein (RBD, His Tag), Biotinylated	Sino Biological	40150-V08B2-B
FcR Blocking Reagent human	Miltenyi Biotec	Cat#130-059-901
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Thermo Fisher	Cat#L23105
PE-Cy5.5 Streptavidin	Thermo Fisher	Cat#SA1018
AF647 Streptavidin	Biologend	Cat#405237
PE-Cy7 Streptavidin	Biologend	Cat#405206
PE Streptavidin	Biologend	Cat#405203
BV711 Streptavidin	Biologend	Cat#405241
BV421 Streptavidin	Biologend	Cat#405225
BIO200 - BIO-200 biotin solution	Avidity	Cat#BIO200
Brilliant Stain Buffer	BD	566349
UltraComp eBeads™ Compensation Beads	Thermo Fisher	01-2222-41
SpectroFlo® QC Beads	Cytek Biosciences	SKU B7-10001
Software and algorithms		
SpectroFlo® v3.0.1	Cytek Biosciences	<a href="https://cytekbio.com/">https://cytekbio.com/</a>
FlowJo v10.8.0	FlowJo, LLC	<a href="https://www.flowjo.com/">https://www.flowjo.com/</a>

## MATERIALS AND EQUIPMENT

### Staining buffer

Reagent	Final concentration	Amount
DPBS	N/A	500 mL
Heat-inactivated FBS	2% (v/v)	10 mL
EDTA (0.5 M)	2 mM	2 mL
<b>Total</b>	<b>N/A</b>	<b>50 mL</b>

Store at 4°C up to two months.

### Optical configuration of the 5 laser Cytek Aurora (Cytek Biosciences, Fremont, California)

Laser	Excitation wavelength	Channels for detection
Ultraviolet	355 nm	UV1-UV16
Violet	405 nm	V1-V16
Blue	488 nm	B1-B14
Yellow Green	561 nm	YG1-YG10
Red	640 nm	R1-R8

## STEP-BY-STEP METHOD DETAILS

### Preparation of antigen probes

⌚ Timing: 30 min preparation + 60 min incubation

In this step, antigen probes for detection of SARS-CoV-2 spike-specific B cells are prepared by multimerization of biotinylated antigens with fluorescently labeled streptavidin (SA).

1. Dilute SA conjugates to a concentration of 50 ng/μL in staining buffer.

**Table 1. Preparation of antigen probes**

	Concentration [mg/mL]	Amount [ng/sample]	Volume [μL/sample]
<b>SA-AF647/SARS-CoV-2-S1-biotin</b>			
SA-AF647 (52 kDa)	0.05	15	0.3
SARS-CoV-2-S1-biotin (76.5 kDa)	0.25	90	0.36
Staining buffer	N/A	N/A	0.34
<b>SA-PE-Cy7/SARS-CoV-2-S1-biotin</b>			
SA-PE-Cy7 (52 kDa)	0.05	15	0.3
SARS-CoV-2-S1-biotin (76.5 kDa)	0.25	90	0.36
Staining buffer	N/A	N/A	0.34
<b>SA-PE/SARS-CoV-2-S2-biotin</b>			
SA-PE (52 kDa)	0.05	15	0.3
SARS-CoV-2-S2-biotin (59.37 kDa)	0.25	75	0.3
Staining buffer	N/A	N/A	0.3
<b>SA-BV711/SARS-CoV-2-S2-biotin</b>			
			0.9
SA-BV711 (52 kDa)	0.05	15	0.3
SARS-CoV-2-S2-biotin (59.37 kDa)	0.25	75	0.3
Staining buffer	N/A	N/A	0.3
<b>SA-BV421/SARS-CoV-2-RBD-biotin</b>			
SA-BV421 (52 kDa)	0.05	15	0.3
SARS-CoV-2-RBD-biotin (26.5 kDa)	0.25	33	0.13
Staining buffer	N/A	N/A	0.17

- Mix SA conjugates (15 ng/ sample) with the respective biotinylated antigens at a molar ratio of 1:4 and add staining buffer according to [Table 1](#). Mass ratios were calculated as 1:6 for SA/SARS-CoV-2-S1, 1:5 for SA/SARS-CoV-2-S2 and 1:2.2 for SA/SARS-CoV-2-RBD.
- Incubate for 60 min at 4°C and use the incubation time to thaw cryopreserved PBMCs.

**Note:** As antigen-specific B cells are very rare, bright fluorophores are used for labeling of antigen probes. To exclude B cells binding to the respective fluorophores, each antigen is separately multimerized with SA labeled with two different fluorophores. Only double positive cells are classified as spike-specific. Note that only one fluorophore is used for the RBD, as it is part of the S1 subunit and can be plotted against one of the S1 antigen probes.

**Alternatives:** In this step, the antigens can be exchanged to adjust the protocol to detect B cells specific to other variants of the spike protein or other antigens of SARS-CoV-2, as well as antigens of other pathogens. For adjustment to other SARS CoV-2 variants, the amounts of S1, S2, and RBD can be adopted from [Table 1](#), as they have the same molecular weight. For different proteins, the mass ratio to be used for labeling with fluorophore-conjugated SA has to be calculated based on a molar ratio of 1:4 (SA: protein), as described above. If there is only one antigen of interest, the other antigen probes/ fluorophores can be excluded from the staining protocol.

### Thawing of cryopreserved PBMC

⌚ **Timing:** 45 min, depending on number of samples

In this step, cryopreserved human PBMCs are thawed and counted for subsequent staining.

- Prepare a 15 mL conical tube with 9 mL of ice-cold staining buffer for each sample and cool down centrifuge to 4°C.

5. Thaw cells.
  - a. Place cryotube with frozen PBMCs in a 37°C water bath until partially thawed.
  - b. Rapidly transfer vial into sterile working bench and decant cells into 15 mL conical tube containing staining buffer.
  - c. Use 1 mL of fresh staining buffer to collect remaining cells from cryotube and transfer them into the same 15 mL conical tube.

△ **CRITICAL:** Rapidly conduct steps 5a–c. Subsequently place samples on ice until continuation with step 6 to reduce toxicity of DMSO contained in the freezing medium.

6. Wash and count PBMCs.
  - a. Centrifuge samples for 8 min at 600 g and 4°C.
  - b. Discard the supernatant and resuspend cells using a 200 µL pipette.
  - c. Fill tube up to 10 mL with ice-cold staining buffer and resuspend directly before counting.
  - d. Mix 20 µL of cell suspension with 20 µL of Trypan Blue, transfer 10 µL into counting slide and count cells manually or using an automated cell counter.
  - e. Centrifuge samples for 8 min at 600 g and 4°C.
  - f. Discard the supernatant and resuspend cells in a maximum volume of 200 µL of staining buffer.
7. Transfer 5 to 10 million PBMCs to a 96 well V-bottom plate for staining.

**Note:** This protocol was established for staining of 5–10 million PBMCs per sample, but can also be used for staining lower or higher amounts of cells. Note that lower cell numbers may reduce the sensitivity of the assay due to the low frequency of antigen-specific B cells within PBMCs.

### Staining of PBMCs for flow cytometric analysis

⌚ **Timing:** 3 h

This section describes the procedure of staining PBMCs for flow cytometric analysis and consists of three staining steps and subsequent fixation. At first, the PBMCs are stained with an amine-reactive dye for detection of dead cells, in combination with an Fc receptor (FcR) blocking reagent to prevent non-specific binding of antibodies via their Fc region. The subsequent staining of antigen-specific cells is performed using the antigen probes prepared in steps 1–3. Additionally, SA labeled with PE/Cy5.5 is added as a decoy probe to distinguish B cells specific to the spike antigens from those binding to SA. Brilliant Stain Buffer containing free d-biotin is used to minimize potential cross-reactivity between the antigen probes. In a third staining step, different surface markers are stained using fluorophore-conjugated antibodies, followed by fixation with the cross-linking agent paraformaldehyde (PFA).

8. Prepare staining mixes 1 to 3 according to [Table 2](#) and store them at 2°C–8°C until needed.

△ **CRITICAL:** For staining mix 2, free biotin has to be added prior to the sequential addition of SA-labeled antigen probes. This way, any possible free binding sites of SA resulting from the individual labeling reactions are occupied by the free biotin before adding the next antigen probe.

**Note:** Volumes are indicated for staining of a single sample and can be scaled up depending on sample number. Some excess volume should be included into staining mixes.

9. Perform blocking of Fc receptors and staining of dead cells.
  - a. Centrifuge samples in 96 well plate for 3 min at 500 g and 4°C.
  - b. Decant supernatant.

**Table 2. Preparation of staining mixes 1–3**

Reagent	Volume [ $\mu\text{L}/\text{sample}$ ]
<b>Mix 1: Dead cell staining and FcR blocking</b>	<b>105</b>
Staining buffer	100
LIVE/DEAD™ Fixable Blue Dead Cell Stain	0.1
FcR Blocking Reagent	5
<b>Mix 2: Antigen-specific staining</b>	<b>55</b>
Brilliant Stain Buffer	50
BIO-200 biotin solution	0.5
Streptavidin-PE-Cy5.5, diluted 1:10 in staining buffer	0.2
SA-AF647/SARS-CoV-2-S1-biotin	1
SA-PE-Cy7/SARS-CoV-2-S1-biotin	1
SA-PE/SARS-CoV-2-S2-biotin	0.9
SA-BV711/SARS-CoV-2-S2-biotin	0.9
SA-BV421/SARS-CoV-2-RBD-biotin	0.6
<b>Mix 3: Antibody surface staining</b>	<b>135</b>
Staining buffer	50
Brilliant Stain Buffer	50
BUV563 $\alpha$ -CD19	2.5
BUV737 $\alpha$ -CD21	2.5
BUV805 $\alpha$ -IgG	2.5
BV480 $\alpha$ -CD71	2.5
BV570 $\alpha$ -IgM	2.5
BV650 $\alpha$ -CD20	2.5
BV785 $\alpha$ -IgD	2.5
BB515 $\alpha$ -CD27	2.5
PerCP $\alpha$ -CD3	2.5
PerCP $\alpha$ -CD14	2.5
AF700 $\alpha$ -CD56	2.5
APC-Vio770 $\alpha$ -IgA	2.5
APC/Fire810 $\alpha$ -CD38	2.5

- c. Resuspend cells in 105  $\mu\text{L}$  per well of staining mix 1 as prepared in step 8.
- d. Incubate for 15 min at 4°C.
10. Wash cells.
  - a. Add 100  $\mu\text{L}$  of staining buffer and centrifuge samples for 3 min at 500 g and 4°C.
  - b. Decant supernatant.
  - c. Resuspend cells in 200  $\mu\text{L}$  staining buffer and centrifuge for 3 min at 500 g and 4°C.
  - d. Decant supernatant.
11. Perform staining of antigen-specific B cells.
  - a. Resuspend cells in 55  $\mu\text{L}$  of staining mix 2 as prepared in step 8.
  - b. Incubate for 60 min at 4°C.
12. Wash cells as described in steps 10a–d.
13. Stain surface markers.
  - a. Resuspend cells in 135  $\mu\text{L}$  of mix 3 as prepared in step 8.
  - b. Incubate for 30 min at 4°C.
14. Wash cells as described in steps 10a–d.
15. Fix cells using 4% paraformaldehyde (PFA).
  - a. Resuspend cells in 100  $\mu\text{L}$  of 4% PFA.
  - b. Incubate for 15 min at 20°C–25°C.
16. Wash cells as described in steps 10a–d.
17. Resuspend cells in 100  $\mu\text{L}$  of staining buffer and store at 4°C until acquisition.

**△ CRITICAL:** For incubation steps and storage, samples and staining mixes should be placed in the dark, as the fluorophores are sensitive to light exposure.

**Table 3. Panel overview and preparation of single stained controls**

Peak emission channel	Fluorophore	Antibody/ Antigen	Sample type used for reference control	Reagent for single cell staining	Volume [ $\mu$ L]
UV	fixable BLUE	dead cell stain	killed PBMCs	fixable BLUE	0.1
	BUV563	$\alpha$ -CD19	PBMCs	BUV563/ $\alpha$ -CD19	2.5
	BUV737	$\alpha$ -CD21	PBMCs	BUV737/ $\alpha$ -CD21	2.5
	BUV805	$\alpha$ -IgG	PBMCs	BUV805/ $\alpha$ -IgG	2.5
Violet	BV421	SARS-CoV-2-RBD	beads	biotin/ $\alpha$ -IgG + SA-BV421	1 + 1
	BV480	$\alpha$ -CD71	beads	BV480/ $\alpha$ -CD71	1
	BV570	$\alpha$ -IgM	PBMCs	BV570/ $\alpha$ -IgM	2.5
	BV650	$\alpha$ -CD20	PBMCs	BV650/ $\alpha$ -CD20	2.5
	BV711	SARS-CoV-2-S2	beads	biotin/ $\alpha$ -IgG + SA-BV711	1 + 1
	BV785	$\alpha$ -IgD	PBMCs	BV785/ $\alpha$ -IgD	2.5
Blue	BB515	$\alpha$ -CD27	PBMCs	BB515/ $\alpha$ -CD27	2.5
	PerCP	$\alpha$ -CD3	PBMCs	PerCP/ $\alpha$ -CD3	2.5
		$\alpha$ -CD14	beads	PerCP/ $\alpha$ -CD14	1
Yellow/Green	PE	SARS-CoV-2-S2	beads	biotin/ $\alpha$ -IgG + SA-PE	1 + 1
	PE-Cy5.5	Streptavidin	beads	biotin/ $\alpha$ -IgG + SA-PE-Cy5.5	1 + 1
	PE-Cy7	SARS-CoV-2-S1	beads	biotin/ $\alpha$ -IgG + SA-PE-Cy7	1 + 1
	AF647	SARS-CoV-2-S1	beads	biotin/ $\alpha$ -IgG + SA-AF647	1 + 1
Red	AF700	$\alpha$ -CD56	beads	AF700/ $\alpha$ -CD56	1
	APC-Vio770	$\alpha$ -IgA	beads	APC-Vio770/ $\alpha$ -IgA	1
	APC-Fire810	$\alpha$ -CD38	PBMCs	APC-Fire810/ $\alpha$ -CD38	2.5

▮▮ **Pause point:** Samples can be acquired directly or stored at 4°C to be measured the next day.

### Single stained controls for Cytex Aurora

⌚ **Timing:** 90 min (or in parallel with regular staining protocol)

This section describes the preparation of single stained reference controls, which have to be prepared for each fluorophore included in the assay. Reference controls are needed for unmixing of raw data files, as they define the signature of each fluorophore across the full emission spectrum. If possible, reference controls should be prepared using the same cell type and staining protocol as for the regular staining procedure. However, it might be more feasible to use compensation beads (e.g., UltraComp eBeads, Thermo Fisher) for antibodies directed against rare antigens, to obtain sufficient events in the fluorophore-positive population. To provide reference controls for the fluorophores used in the antigen probes, compensation beads can be coated with any biotinylated antibody of host species mouse, rat or hamster, and subsequently labeled with the respective fluorophore-conjugated SA. Staining of reference controls for dead cell stains can be performed on killed PBMCs (e.g., by heat shocking, as described below).

**Note:** Reference controls only have to be prepared and acquired once and can then be saved and re-used for all subsequent measurements.

18. Kill PBMCs for dead cell stain reference control.
  - a. Transfer approximately 2 million PBMCs into a 1.5 mL tube and incubate in the water bath at 65°C for 10 min.
19. Distribute PBMCs and compensation beads in 96 well plate, as needed for reference controls (see Table 3). Include two additional wells for unstained PBMCs and beads, respectively.
  - a. Killed PBMCs: add approximately 2 million cells per well.



- b. PBMCs: add approximately 2 million cells per well.
- c. Beads: add one drop of beads per well.
- 20. Wash samples.
  - a. Fill up wells to approximately 200  $\mu$ L with staining buffer.
  - b. Centrifuge samples for 3 min at 500 g and 4°C.
  - c. Discard supernatant.
- 21. Perform single stain of dead cells.
  - a. Dilute LIVE/DEAD™ Fixable Blue Dead Cell Stain 1:1000 in staining buffer.
    - i. Add 100  $\mu$ L of diluted fixable BLUE to killed PBMCs.
    - ii. Add 100  $\mu$ L of staining buffer to all other samples.
  - b. Resuspend samples and incubate for 15 min at 4°C.
- 22. Wash samples.
  - a. Add 100  $\mu$ L of staining buffer and centrifuge samples for 3 min at 500 g and 4°C.
  - b. Decant supernatant.
  - c. Resuspend samples in 200  $\mu$ L staining buffer and centrifuge for 3 min at 500 g and 4°C.
  - d. Decant supernatant.
- 23. Perform single stains on PBMCs and beads according to [Table 3](#).
  - a. Resuspend all samples in 100  $\mu$ L of staining buffer.
    - i. PBMCs: add 2.5  $\mu$ L of the respective antibody.
    - ii. Beads: add 1  $\mu$ L of the respective antibody or 1  $\mu$ L of biotinylated anti-IgG antibody plus 1  $\mu$ L of fluorescently labeled SA.
  - b. Incubate for 30 min at 4°C.

**Alternatives:** Instead of biotinylated anti-IgG antibody, a biotinylated antibody directed against any antigen can be used, as the compensation beads capture any mouse, rat, or hamster antibody.

- 24. Wash samples as described in steps 22a–d.
- 25. Fix cells using 4% paraformaldehyde (PFA).
  - a. Resuspend cells in 100  $\mu$ L of 4% PFA.
  - b. Resuspend beads in 100  $\mu$ L of staining buffer.
  - c. Incubate for 15 min at 20°C–25°C.
- 26. Wash samples as described in steps 22a–d.
- 27. Resuspend samples in 100  $\mu$ L of staining buffer and store at 4°C in the dark until acquisition.

**Note:** Fixation with PFA is only required for cellular samples, and steps 25 to 26 may be skipped for the wells containing compensation beads. However, it may be more feasible to stain cells and beads in the same plate and to resuspend beads in staining buffer during fixation of cellular samples, as described above.

### Acquisition at Cytex Aurora

⌚ **Timing:** 2–5 h, depending on number of samples

Warm-up of lasers and running of Quality Control (QC): 60 min.

Setting up experiment and reference controls: 30 min.

Acquisition: approximately 5 min per sample.

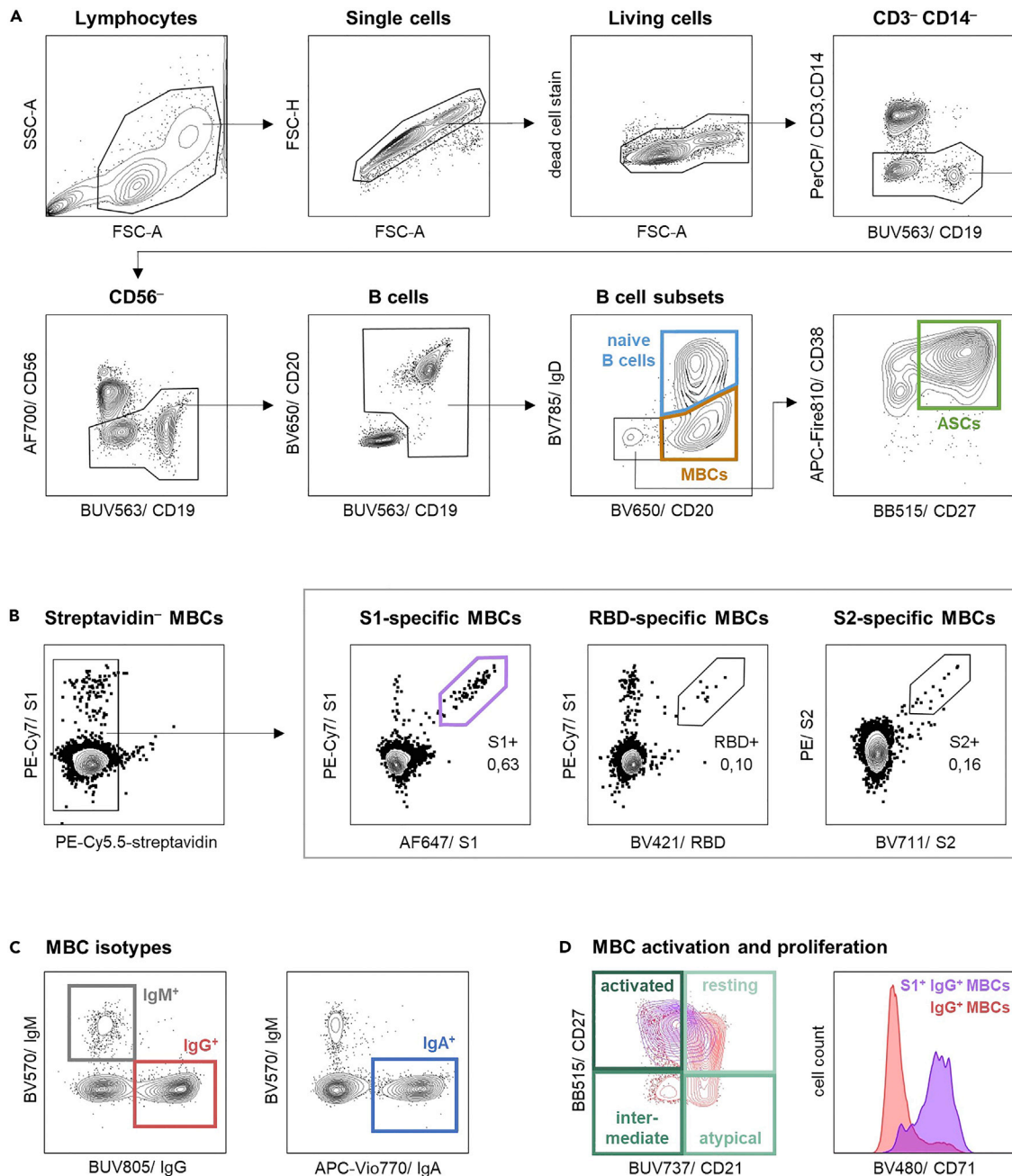
This section describes the process of starting up the Cytex Aurora, setting up a new experiment and acquiring reference controls as well as samples. Parts of this section are adopted from the Cytex® Aurora Quick Reference Guide (available from <https://cytekbio.com/pages/user-guides>).

28. Start-up Cytex Aurora.
  - a. Start SpectroFlo software and switch on Cytex Aurora.
  - b. Perform "Clean Flow Cell" and run ddH<sub>2</sub>O for 30 min.
  - c. After at least 45 min of laser warm-up, run QC with SpectroFlo® QC Beads.
29. Set up experiment.
  - a. Create a new experiment.
  - b. Select fluorescent tags used in your experiment (see [Table 3](#) for complete panel).
  - c. Add groups.
    - i. Create a reference group and select control type (Cells or Beads) for each single-stained control.
    - ii. Create groups for your samples. Add tubes and label them with sample IDs (tubes and labels can be edited at any time during the experiment).
  - d. Enter labels for markers associated with each fluorescent tag.
  - e. Define acquisition settings. For groups containing samples (not reference controls), adjust stopping criteria to high thresholds to make sure that the whole sample is acquired, as an example:
    - i. Events to Record: 1,000,000.
    - ii. Stopping Volume (μL): 3,000.
    - iii. Stopping Time (sec): 10,000.
  - f. Save and open experiment.
30. Acquire reference controls.
  - a. Select the Default Raw Worksheet (Raw) for the reference controls.
  - b. Adjust SSC and FSC gains to be on scale for cells or beads, respectively.
  - c. Record unstained and single stained controls of the reference group.
  - d. Select live unmixing.
31. Acquire samples.
  - a. Select the Default Unmixed Worksheet for the samples.
  - b. Create plots resembling the basic gating strategy to be able to monitor cell populations while recording.
  - c. Adjust SSC and FSC gains for the lymphocyte population to be on scale.
  - d. Transfer each sample from the 96 well plate to a 5 mL flow cytometry tube. Pass through a cell strainer cap to ensure single cell suspension and wash out with 50–100 μL of staining buffer. The volume of buffer can be adjusted to change the event rate.
  - e. Vortex each tube prior to acquisition to resuspend cells.
  - f. Acquire samples.
32. Export unmixed fcs files.

### EXPECTED OUTCOMES

For identification and quantification of cell populations, the fcs data files can be analyzed using FlowJo or comparable software packages. An exemplary analysis using the FlowJo software is provided in [Figures 1](#) and [2](#), based on samples from four healthy donors obtained before and at different time points after vaccination against SARS-CoV-2. Donor characteristics and type of vaccinations are indicated in [Table 4](#).

Our proposed gating strategy for identification of B cell subsets within the PBMC population is shown in [Figure 1A](#). Lymphocytes are first identified based on their size and granularity using the forward scatter (FSC) and sideward scatter (SSC) signals, followed by exclusion of doublets and dead cells. Subsequently, T cells, monocytes and NK cells are excluded based on their expression of CD3, CD14 and CD56, respectively, and B cells are identified by their expression of CD19. MBCs are then gated as IgD<sup>-</sup>CD20<sup>+</sup> cells as opposed to IgD<sup>+</sup> naïve B cells and IgD<sup>-</sup>CD20<sup>-</sup> antibody-secreting cells (ASCs). The majority of the ASC population is short-lived and only transiently present after vaccination or infection, whereas MBCs can persist for years or even decades after immunization.<sup>2–4</sup>



**Figure 1. Gating strategy for identification of B cell subsets and characterization of memory B cells (MBCs)**

(A) Identification of B cell subsets from PBMCs. Lymphocytes are first identified based on their size and granularity as indicated by the forward scatter (FSC) and sideward scatter (SSC) signals. Subsequently, doublets and dead cells are excluded from the analysis, as well as T cells, monocytes and NK cells, based on expression of CD3, CD14 and CD56, respectively. B cells are identified by expression of CD19 and/or CD20 and subsequently further divided into B cell subsets. Memory B cells (MBCs) are identified as IgD<sup>-</sup>CD20<sup>+</sup> cells as opposed to IgD<sup>+</sup> naïve B cells and IgD<sup>-</sup>CD20<sup>-</sup> antibody-secreting cells.

(B) Identification of antigen-specific MBCs. After exclusion of streptavidin-binding MBCs, antigen-specific cells are identified as double positive for the respective antigen probes (S1, RBD, S2).

(C) Identification of isotypes IgM, IgG and IgA within the MBC population.

(D) MBC activation and proliferation status, depicted as overlaid contour plots and histograms of total IgG<sup>+</sup> MBCs (red) and S1-specific IgG<sup>+</sup> MBCs (purple). Resting, intermediate, atypical, and activated MBCs are identified by expression of CD21 and CD27 (left panel). CD71 serves as a marker of proliferation activity (right panel). Representative contour plots are shown for donor 1, day 19 after the second SARS-CoV-2 vaccination (A and B) and donor 2, day 8 after the third vaccination (C and D).

The MBC compartment can be further characterized based on antibody isotypes and activation status, as well as specificity towards SARS-CoV-2. Spike-specific cells directed against the S1 and S2 subunits and the RBD can be identified as shown in [Figure 1B](#). After exclusion of streptavidin-binding MBCs, antigen-specific cells are identified as double positive for the respective antigen probes (S1, S2, RBD). This way, MBCs specifically binding to the respective fluorophores can be excluded from the analysis. Furthermore, unswitched MBCs (IgM<sup>+</sup>) can be distinguished from class-switched MBCs of the isotypes IgG and IgA ([Figure 1C](#)). CD21 and CD27 can be used to assess the distribution of resting (CD21<sup>+</sup>CD27<sup>+</sup>), intermediate (CD21<sup>-</sup>CD27<sup>-</sup>), activated (CD21<sup>-</sup>CD27<sup>+</sup>) and atypical (CD21<sup>+</sup>CD27<sup>-</sup>) MBCs, and CD71 serves as an additional proliferation marker ([Figure 1D](#)). Activated/proliferating MBCs resemble plasma cell precursors<sup>5</sup> and are expected to be induced in response to vaccination or infection.

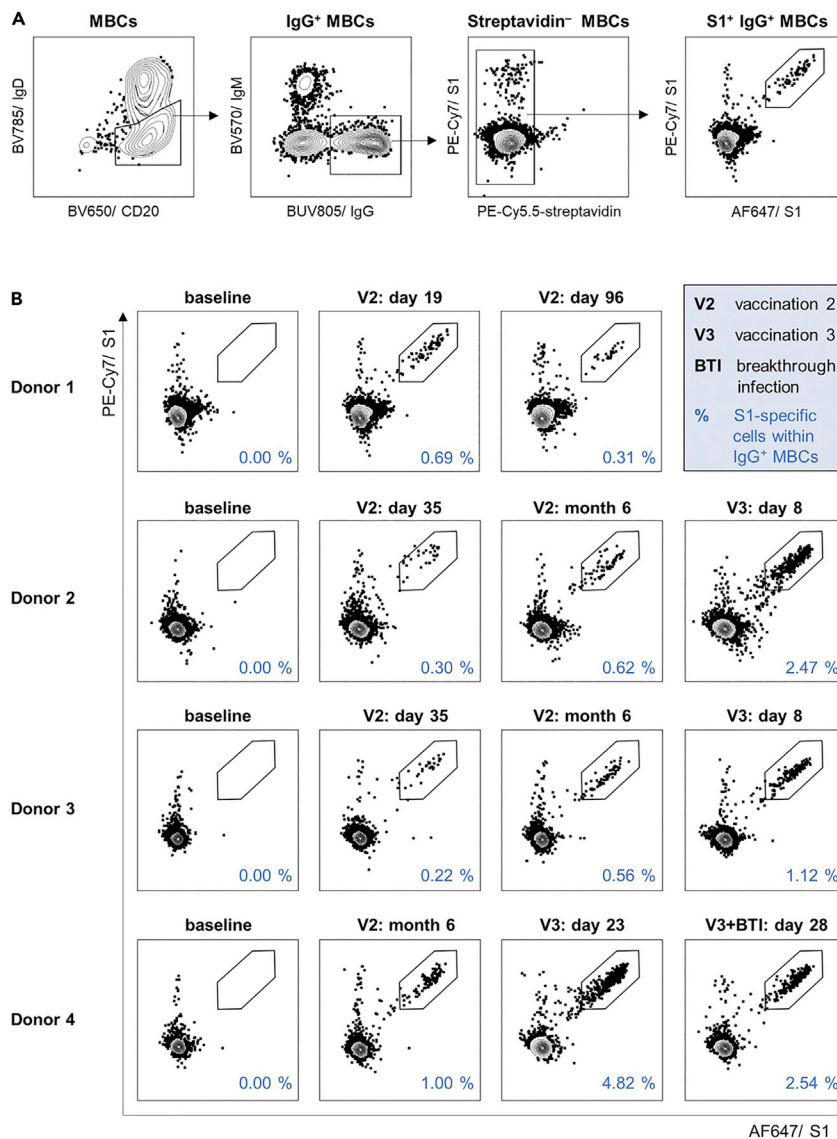
The gating strategies shown in [Figures 1B–1D](#) can be conducted in different orders and combinations, depending on the objective of the analysis. The gating hierarchy shown in [Figure 1B](#) followed by [Figure 1C](#) can be used to analyze the relative contribution of each isotype to the total spike-specific B cell population. This may be useful for understanding differences in isotype distribution between infection and vaccination. Reversing the order, i.e., applying gates shown in [Figure 1C](#) before gates shown in [Figure 1B](#), can be more feasible to monitor the frequency of spike-specific B cells within each MBC isotype over time. The activation status of MBCs can differ between the spike-specific cell population and the total MBC compartment, as illustrated for the IgG isotype in the exemplary plots ([Figure 1D](#)). However, it might also be interesting to characterize the activation status of spike-specific cells at different time points (e.g., before and after booster vaccination or infection) or in different settings (e.g., vaccination versus infection).

Generally, antigen-specific MBCs are expected to be rare or non-existent in naïve individuals and to be induced upon vaccination or infection. They can persist in the human body for up to years or decades, with their frequency strongly depending on the time point after vaccination or infection, the type and schedule of vaccines administered, as well as the individual preconditions.<sup>2,6</sup> In [Figure 2](#), we illustrate the frequencies of S1-specific MBCs in the IgG<sup>+</sup> MBC population, as detected at different time points before and after SARS-CoV-2 vaccination in four healthy donors (see also [Table 4](#)). In all four individuals, S1-specific IgG<sup>+</sup> MBCs are not detectable at baseline, but emerge following vaccination. The frequencies of S1-specific cells range between 0.22%–1.00% of IgG<sup>+</sup> MBCs after two vaccinations (depending on donor and time point) and further increase to 1.12%–4.82% following a third vaccination. For donor 4, S1-specific cells are additionally characterized following a breakthrough infection experienced 15 weeks after the third vaccination, showing a frequency of 2.54% in the IgG<sup>+</sup> MBC population.

The exemplary plots shown in [Figure 2](#) aim to illustrate possible outcomes of the protocol described above. However, they do not suffice to comprehensively describe MBC dynamics induced by SARS-CoV-2 vaccination and infection, which have been addressed in numerous studies by different research groups. For example, Dan et al.<sup>7</sup> analyzed frequencies of IgM<sup>+</sup>, IgG<sup>+</sup> and IgA<sup>+</sup> MBCs for up to 8 months after SARS-CoV-2 infection. Goel et al.<sup>8</sup> describe differences in the MBC population induced by mRNA vaccination in naïve and SARS-CoV-2 recovered individuals, providing frequencies and isotype distribution. A comprehensive analysis of MBCs induced by different SARS-CoV-2 vaccines has been conducted by Zhang et al.<sup>9</sup> and the impact of booster vaccinations and breakthrough infections on the MBC compartment is addressed in a study by Buckner et al.<sup>10</sup>

## LIMITATIONS

Peripheral blood is an important resource for the study of immune responses, as it can be obtained easily from study participants. However, the frequency of antigen-specific B cells in peripheral blood is very low,<sup>11</sup> leading to the amount of PBMCs being a limiting factor for this analysis. We recommend to use at least 5 million PBMCs for each sample, as lower numbers would reduce the sensitivity



**Figure 2. Frequencies of S1-specific IgG<sup>+</sup> MBCs detected at different time points after vaccination**

(A) Gating Strategy for identification of S1-specific IgG<sup>+</sup> MBCs from the MBC population. Representative contour plots are shown for donor 1.

(B) Frequencies of S1-specific IgG<sup>+</sup> MBCs. Exemplary plots are shown for donors 1–4 at baseline and at different time points after the second and third vaccination (V2, V3) as well as after breakthrough infection (BTI). Percentages indicate the frequency of S1-specific cells within the IgG<sup>+</sup> MBC population.

of the assay. To gain further insights into the compartments of MBCs and especially long-lived plasma cells, an additional analysis of lymphoid tissue and bone marrow would be of interest, as only a small portion of antigen-experienced B cells is trafficking through peripheral blood.<sup>4</sup>

Despite performance of QC prior to each experiment, slight changes in the laser intensities might occur between measurements conducted on different days. If possible, samples and the related controls (e.g., baseline and later time points of the same study participant) should be measured the same day to reduce batch effects. Clearly separated negative and positive populations are not as sensitive to batch effects, but especially data from continuously expressed markers have to be interpreted carefully if measured on different days.

**Table 4. Healthy donors: Baseline characteristics and SARS-CoV-2 vaccinations**

Baseline characteristics			SARS-CoV-2 vaccinations		
Donor	Sex	Age	V1	V2	V3
1	female	43 years	ChAd	mRNA	N/A
2	female	29 years	mRNA	mRNA	mRNA
3	female	29 years	mRNA	mRNA	mRNA
4	male	33 years	mRNA	mRNA	mRNA

V1, V2, V3: Vaccination 1, 2, 3; ChAd: Vaxzevria (Astra Zeneca); mRNA: Comirnaty (Biontech/ Pfizer) or Spikevax (Moderna Biotech); N/A: not applicable.

## TROUBLESHOOTING

### Problem 1

This protocol requires 5 to 10 million PBMCs per staining. However, this amount might not always be available when working with clinical samples (steps 4–7/[expected outcomes](#)).

### Potential solution

The staining can in principle also be performed on lower numbers of PBMCs, using the same concentration of antibodies and antigen probes. The identification of B cell populations (naïve B cells, ASCs, MBCs) as well as MBC subsets (isotypes, activation state) can be performed as described above. Using lower numbers of PBMCs mostly affects the ability to detect antigen-specific MBCs. Due to their low frequency in PBMCs, their detection may be difficult and less reproducible when working with low numbers of PBMCs. This has to be kept in mind, especially when analyzing samples with very low antigen-specific MBC counts expected (e.g., late follow-up time points after infection or vaccination). However, analysis of antigen-specific cells is possible with lower numbers of PBMCs, when analyzing samples with relatively high frequencies of MBCs expected (e.g., a few weeks after booster vaccination or breakthrough infection).

### Problem 2

Low numbers of cells are detected during acquisition of samples at the Cytex Aurora, despite staining of 5–10 million PBMCs (step 31).

### Potential solution

Low detectable cell numbers may be caused by the loss of cells during the staining procedure. During staining and washing steps, make sure to decant the supernatant directly after centrifugation of cells (steps 9–16). Otherwise, the cell pellet might dissolve and parts of it may be discarded with the supernatant. Cell loss during the washing steps may also be reduced by increasing the duration of the centrifugation steps. Furthermore, cells may stick to the wells of the V-bottom plate and can be lost upon transfer to the flow cytometry tube. Make sure to thoroughly resuspend the cells prior to transfer and collect residual cells from the wells with additional staining buffer (step 31d).

### Problem 3

High frequencies of dead cells are detected during acquisition, resulting in a smaller population of living lymphocytes for further analysis (step 31).

### Potential solution

High numbers of dead cells may result either from bad sample quality of frozen PBMCs, or from inappropriate handling of cells during the thawing and staining procedure. As freezing media containing DMSO are toxic to PBMCs upon thawing, make sure to work fast when thawing PBMCs and only thaw up to 4 vials at a time (step 5). When counting the PBMCs, make sure to check their viability prior to proceeding to the next step and keep the samples on ice during the whole staining procedure. If desired, the portion of dead cells can be reduced by resting of PBMCs for

16–24 h at 37°C, 5% CO<sub>2</sub> and a concentration of  $4 \times 10^6$  PBMCs per mL in RPMI medium complemented with 10% FBS (before proceeding to step 7). However, this does not improve the absolute cell count of living cells.

#### **Problem 4**

No or very few antigen-specific B cells are detected in samples collected following vaccination or infection (step 31/[expected outcomes](#)).

#### **Potential solution**

The frequency of antigen-specific B cells might be very low (see [Figure 2](#) for examples), depending on the time point of blood drawing after infection or vaccination, as well as the kind of vaccine administered and the individual characteristics of the donor. Thus, if frequencies of IgG<sup>+</sup> MBCs are undetectable or lower than expected, it may be difficult to conclude if the results are valid. It may therefore be useful to include a positive control into each measurement, i.e., a sample that is known to have a relatively high frequency of antigen-specific MBCs. For time points where antigen-specific MBCs are expected to be very rare, it might be more efficient to stain more than 10 million PBMCs, adjusting the volume of staining reagents, or to enrich B cells from PBMCs prior to the flow cytometric staining, e.g., via magnetic activated cell sorting (MACS).<sup>12,13</sup>

#### **Problem 5**

We designed our antibody panel for acquisition on a 5 laser Cytex Aurora. However, this flow cytometer may not always be available ([materials and equipment/Table 3](#)).

#### **Potential solution**

The staining protocol can be adjusted for acquisition on different flow cytometers with less lasers available or different configurations of detection channels. To reduce the number of fluorophores, different markers can be left out and/or changed to other fluorophores, depending on the objective of the analysis (step 8). For example, the antigen-specific staining can be reduced to one antigen, resulting in three fluorophores less. For some studies, it might be sufficient to analyze MBCs of the IgG isotype and remove the antibodies for detection of IgM and IgA from the panel. If changing the fluorophores used for certain antibodies or antigen probes, make sure to use bright fluorophores for the detection of markers that are expressed at low levels or cell populations of low frequency, such as antigen-specific MBCs. Note that the antibody dilutions indicated in the [key resources table](#) refer to the staining mix using the whole antibody panel described in this protocol. If leaving out some antibodies, we recommend to stick with the volumes indicated in [Table 2](#), which will result in slightly increased final antibody concentrations due to reduced total volume of the staining mix.

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Leonie M. Weskamm ([m.weskamm@uke.de](mailto:m.weskamm@uke.de)).

### **Materials availability**

This study did not generate new unique reagents.

### **Data and code availability**

This study did not generate or analyze datasets or code.

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### AUTHOR CONTRIBUTIONS

Conceptualization, L.M.W., C.D.; methodology, L.M.W.; investigation, L.M.W.; writing – original draft, L.M.W.; writing – review & editing, L.M.W., C.D.; funding acquisition, C.D., M.M.A.; resources, M.M.A.; supervision, C.D., M.M.A.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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