Immunofluorescence staining for flow cytometry

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Introduction - Antibodies

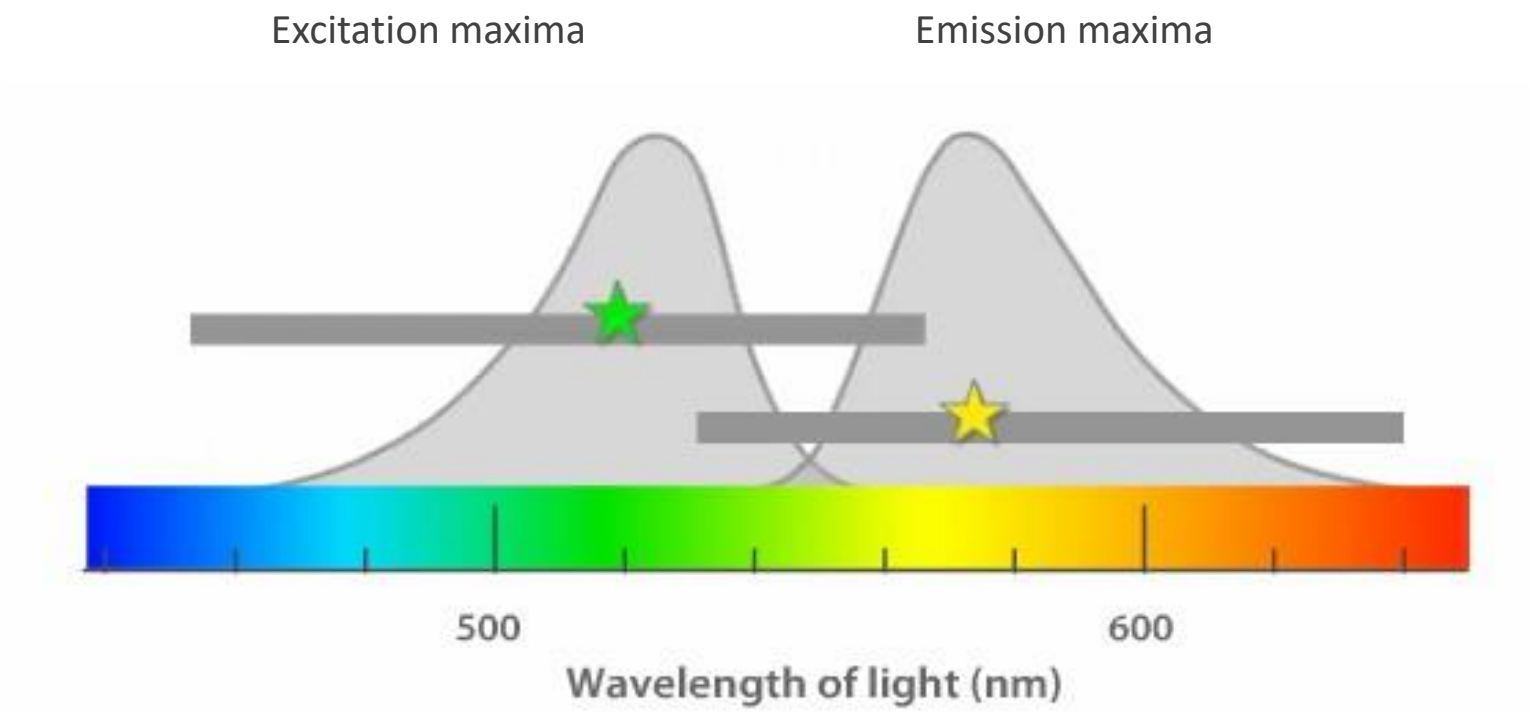
- Antibodies are produced by the immune system (B-cells). Each antibody has the unique property of highly specific binding to a particular molecule (antigens)
- Preparations of antibodies can either be polyclonal - produced by many clones of antibody producing cells but directed against the same target; or monoclonal - produced by a single clone of an antibody producing cell
- Antibodies can be used to determine if a cell expresses a particular antigen of interest
- To measure whether the antibody has bound a target on a cell you can use fluorescence. This is the technique known as immunofluorescence.
- The immunofluorescently labeled cells can be measured by flow cytometry
- Combining immunofluorescence with flow cytometry is a powerful technique that can identify different types of cells on the basis of their antigen expression, and also measure functions and other properties of the cell which are associated with the expression of particular antigens

Immunofluorescence and Flow Cytometry

- Antibodies can be tagged with fluorescent molecules (fluorochromes) and then used to label cells
- Measure cells fluorescence by flow cytometry to determine if antibody has stained the cell
- Can measure multiple antigen expression by the same cell by using differently fluorescent molecule labelled antibodies directed against different antigens
- Can measure surface, cytoplasmic, and nuclear antigens simultaneously by flow cytometry

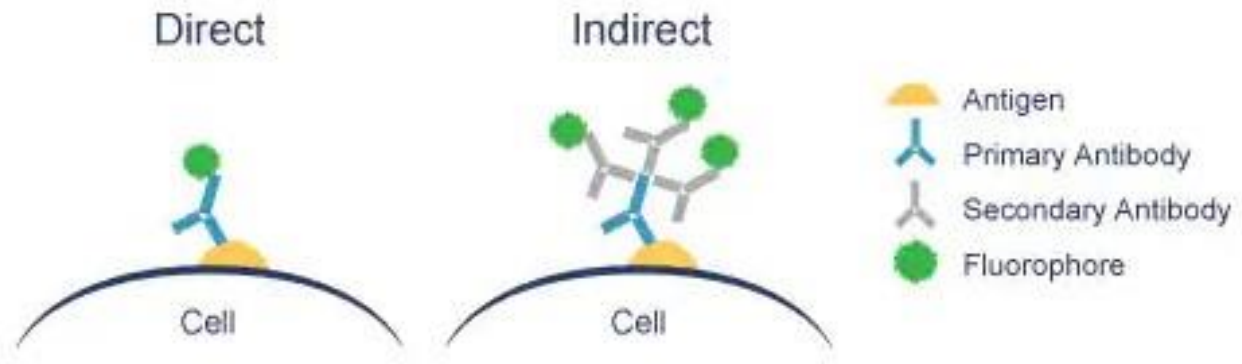
Immunofluorescence and Flow Cytometry

- Each fluorochrome has its own unique Emission-Excitation Spectrum



Immunofluorescence

The two most common ways to do immunofluorescence
-Direct Immunofluorescence
Primary antibody conjugated to fluorochrome
-Indirect Immunofluorescence
Anti- antibody (secondary) conjugated to fluorochrome



Direct Immunofluorescence - Cell Surface

1. Cells in single cell suspension.
2. Add fluorochrome conjugated antibody to cells
3. Incubate to allow antibody to bind to antigen on cells
4. Wash out unbound antibody

Run on flow cytometer

1. Cells in single cell suspension

Easy with blood, lymphoid organs, suspension cell lines.

Getting cells from solid tissues/adherent cell lines trickier…

Need enzyme digest, mesh, etc…

- Effects on cell viability/function?
- Effects on surface antigen expression?

But can be done!

1. Cells in single cell suspension

What solution should you put the cells in?

Something that keeps the cells alive and happy…

Phosphate Buffered Saline (PBS) commonly used. Also a protein buffer should be added eg 0.1-1% bovine serum albumin or calf serum. It will stop antibodies sticking to plastic, and also helps cells pellet when washing

NaAzide – 0.01% stop antibody capping and also keep solution clean

Sometimes known as FACSwash or FACS buffer

1. Cells in single cell suspension

What volume should the cells be resuspended in?

Something small (<200ul).

Determined by what you put the cell suspension in…

96 well plate wells then 25- 50ul

Tube 25-100ul

Needs to be a volume that will allow all the cells to be equally exposed to the antibody you are adding

1. Cells in single cell suspension

How many cells should you have per sample?

Enough to do the measurement you want correctly…

Minimum, $5x10^4$

Maximum, depends…

For cell lines or general lymphocyte measurements 10^6 usually enough

But what if you are trying to measure a rare cell population, eg 0.001% of cell population? If so need a lot of cells, maybe 10^7 or 10^8

2. Add fluorochrome conjugated antibody to to cells

What concentration/volume of antibody to add?

Normally when you purchase antibodies the company will give you indication what to add (eg 1:200, 5ul, etc…)

Is usually in the range of 1ug/ml final concentration

But always best to determine the best concentration for your experiment by doing antibody titration (see later)

Always ensure after addition of antibody to the cells they are well mixed so the antibody is distributed evenly among the cell suspension. Can pipette up and down but vortex or agitation is best!

3. Incubate to allow antibody to bind to antigen on cells

At what temperature?

Can be 37C, room temperature, 4C, or other temperature, but what is right temperature for your experiment?

Depends on… What antigen are you binding? Are the cells likely to clump together over time? Can the cells survive at that temperature?

Determined by the needs of the experiment…

Usually 4C or RT is ok, but you should check what have other people done

3. Incubate to allow antibody to bind to antigen on cells

How long should you leave the cells with the antibody?

Long enough to allow the antibodies to be exposed to all cells equally, and to have optimal and consistent binding

Some people like 10 minutes, some people like 1 hour. I normally like 15 minutes because it gives good labelling and also gives me time to prepare other things for the experiment. If you want to go to lunch then 1 hour should be ok

Can you incubate too long? Yes. I wouldn't leave over weekend because cells may die

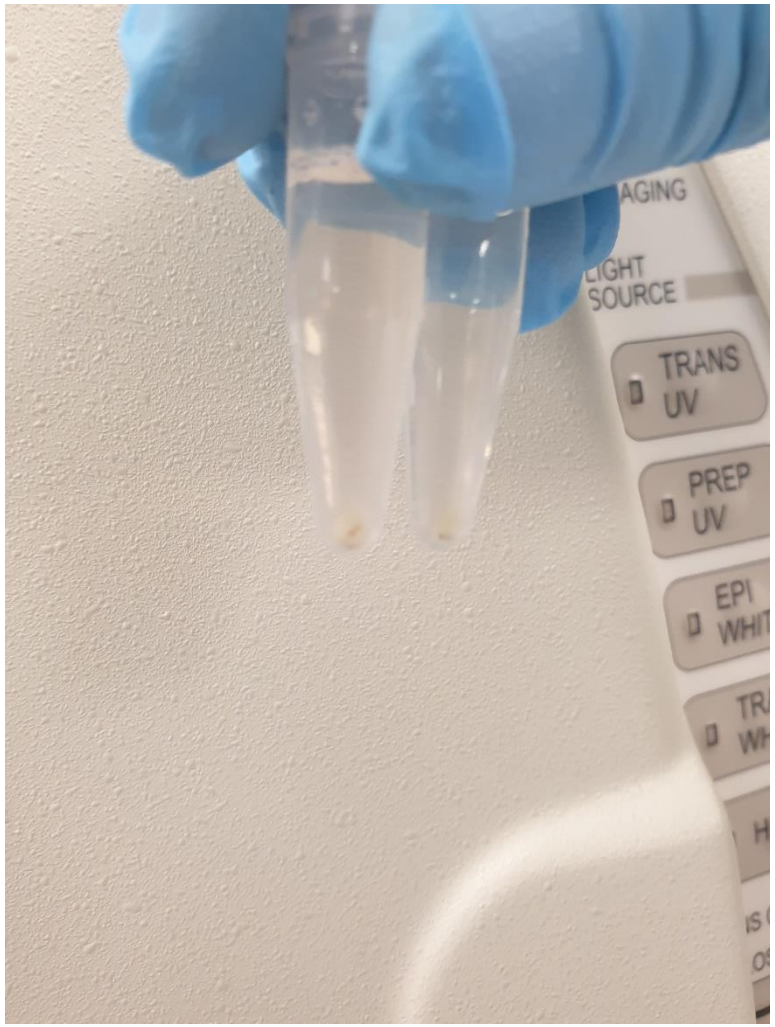
Can you incubate too short? Yes. Give long enough for antibody to bind. Also it needs to be consistent from experiment to experiment.

4. Wash out unbound antibody

Washing is a process of diluting out the unbound antibody from the cells. To do it add an extra volume of your cell buffer to the incubating cells, centrifuge the cells to pellet, and then remove the supernatant.

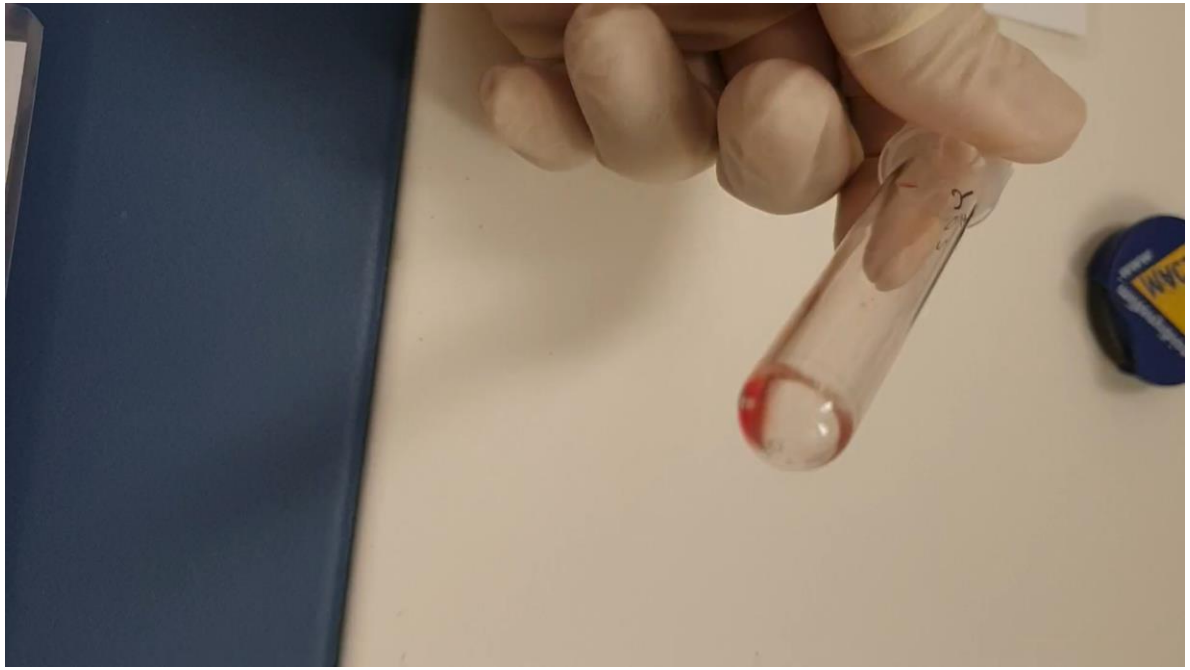
4. Wash out unbound antibody

Cell pellet after washing:



4. Wash out unbound antibody

Cell pellet after washing:



4. Wash out unbound antibody

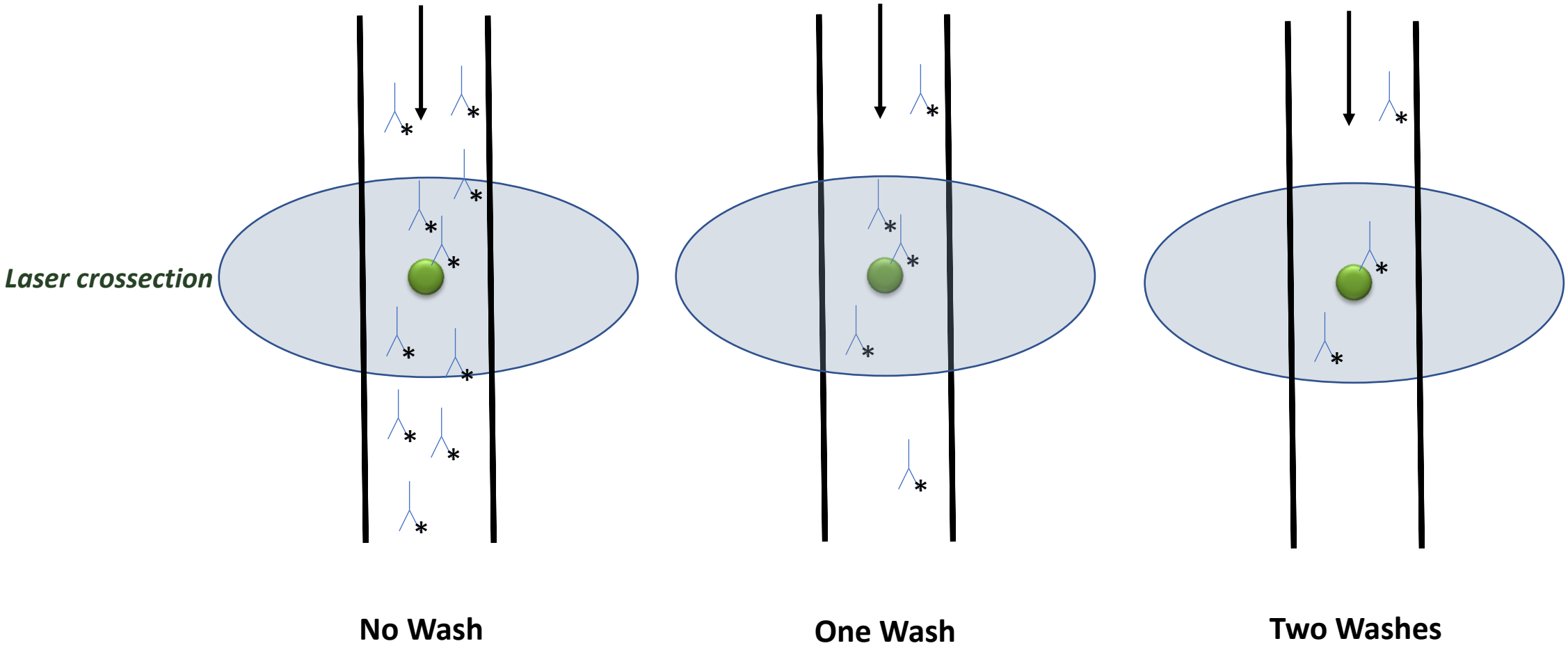
To increase the removal of the unbound antibody do more washes and/or use a larger volume of wash solution. Generally this is good as it will reduce the background staining you measure on the cells.

But, don’t wash too many times. You lose cells with each wash. You also lose time with each wash.

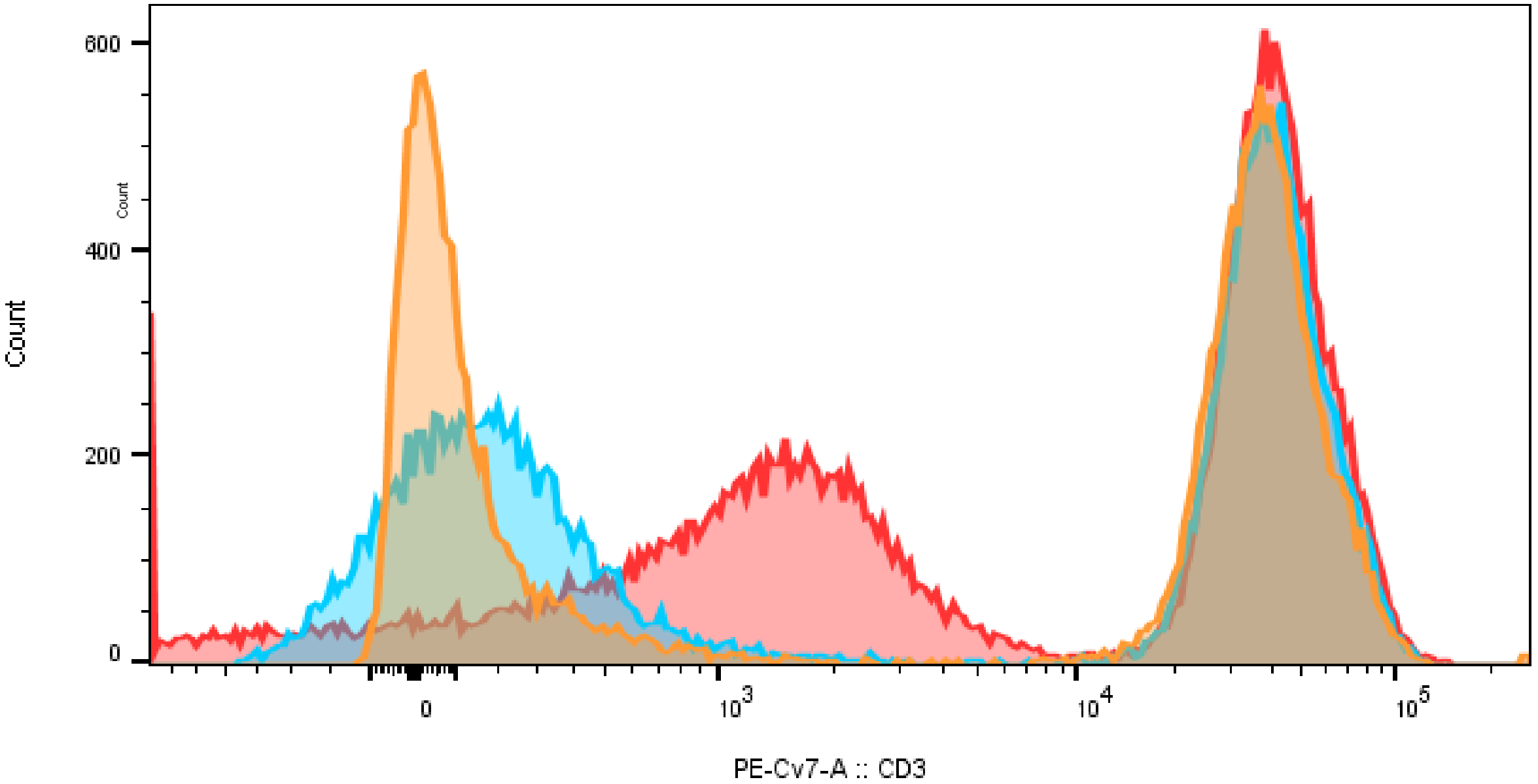
How many times to wash? If volume of wash solution is large, x50-100 of incubating volume, one wash may be enough. If wash volume is small, x5-10 of incubating volume, probably need at least 2 washes.

4. Wash out unbound antibody

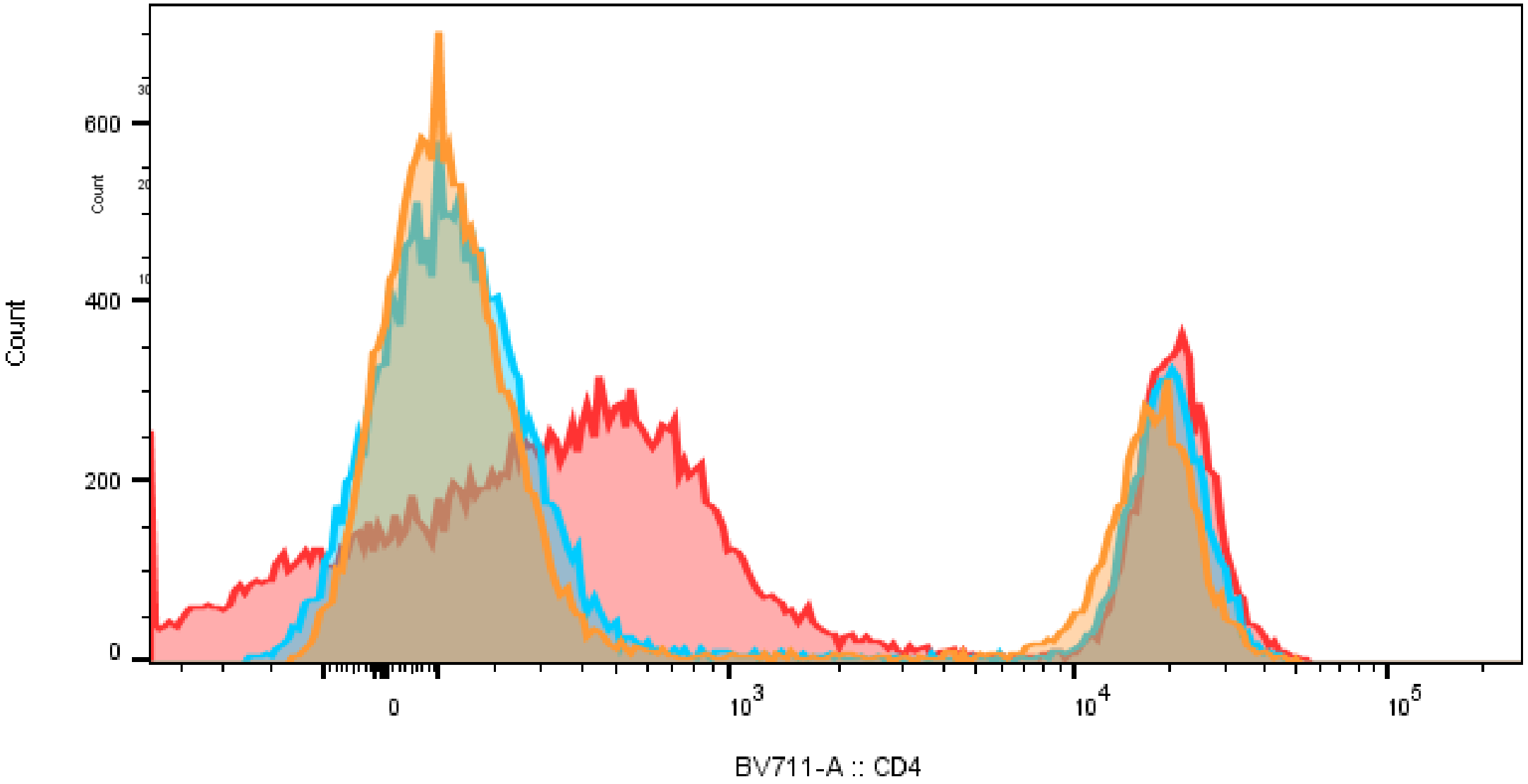
What happens if you have too much unbound antibody in your sample when you run it thru flow cytometer?



4. Wash out unbound antibody



4. Wash out unbound antibody



Antibody Titration

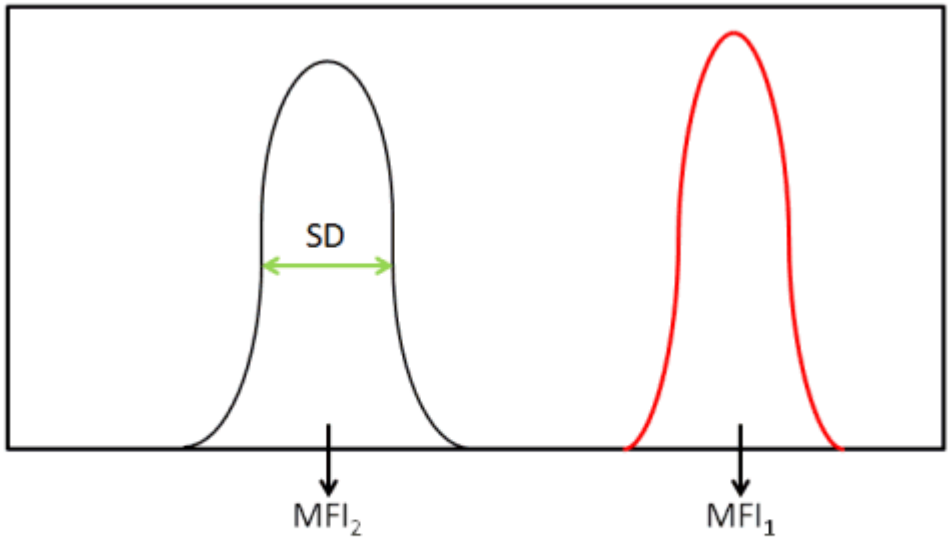
Do it to get the best staining with your antibody.

Do it to save money!

What is best staining? The one that gives the best separation between negative and positive cells

How to quantify ‘best staining’? Use the Stain Index

Antibody Titration – Stain Index

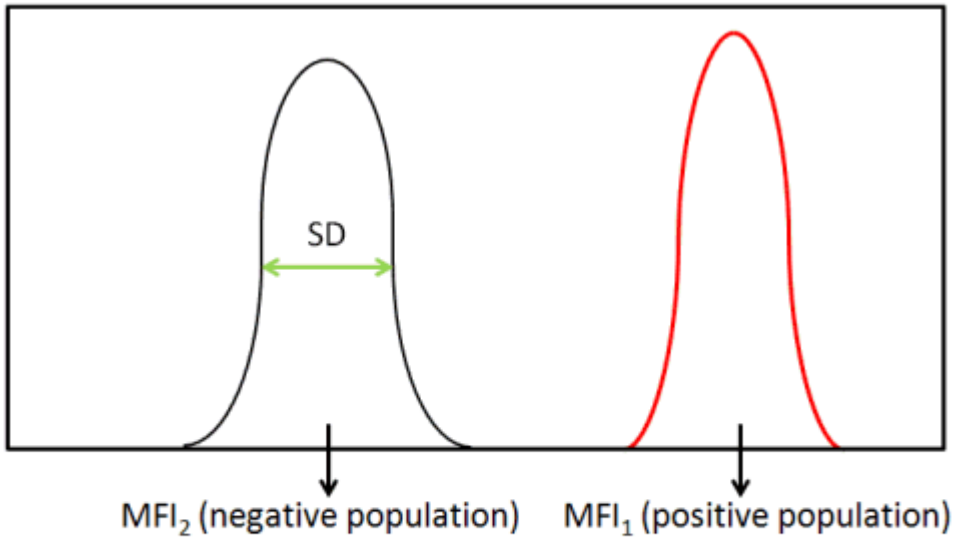


$$\text{Stain Index} = \frac{[MFI_1 - MFI_2]}{2 \times SD}$$

How strong is the positive stain relative to the negative background?

https://www.biolegend.com/en-us/blog/the-stain-index-what-is-it-and-what-does-it-tell-you

Antibody Titration – Stain Index



$$\text{Stain Index} = \frac{[MFI_1 - MFI_2]}{2 \times SD}$$

What is the background distribution relative to the positive staining?

https://www.biolegend.com/en-us/blog/the-stain-index-what-is-it-and-what-does-it-tell-you

Antibody Titration – Stain Index

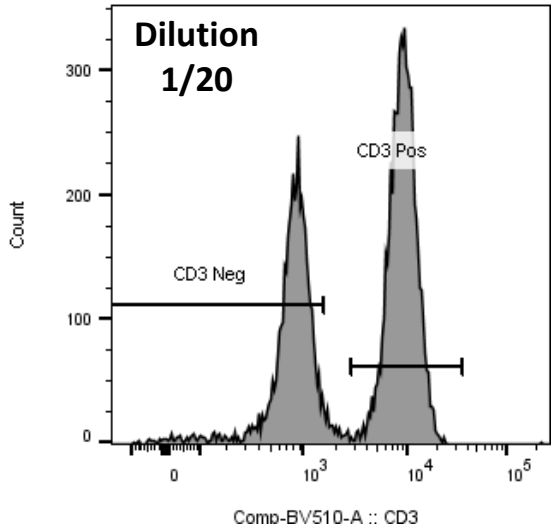
To do the antibody titration you should stain a mix of 2 cell types; one positive for your antibody and one negative for your antibody. Also, ideally the 2 cell populations should have same negative background properties (auto-fluorescence and non-specific binding) as each other

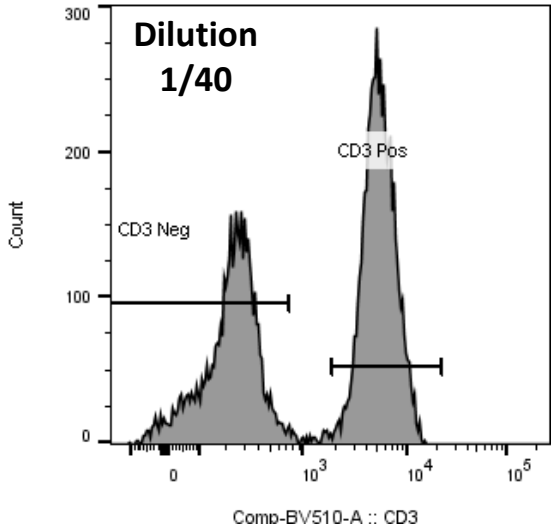
Easy with lymphocytes, sometimes harder with other cell types

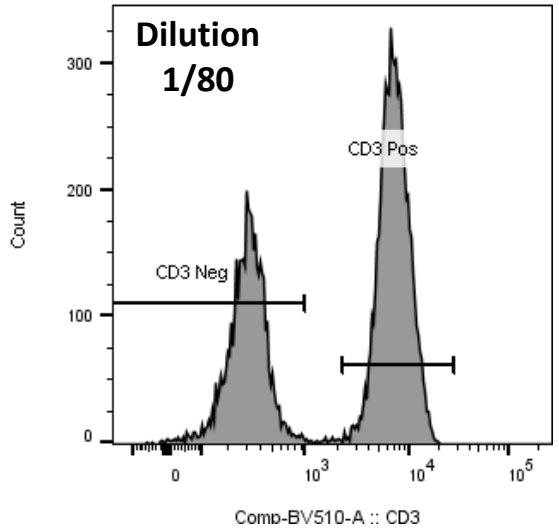
Setup samples of the same cells with a serial dilution of the antibody added to each

Eg. Start with recommended amount and then do a halving dilution up to 4 times

Antibody Titration – Calculating the Stain Index

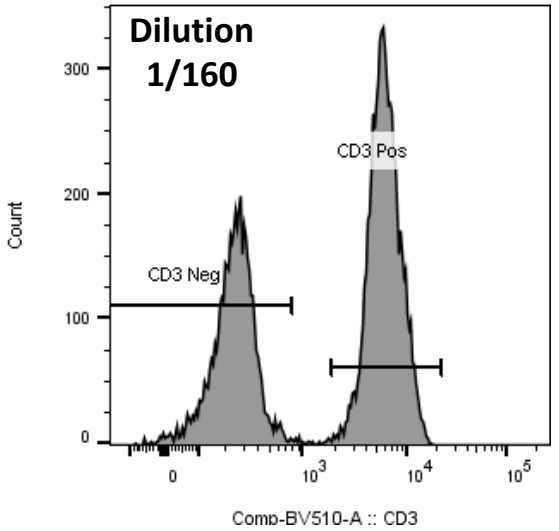


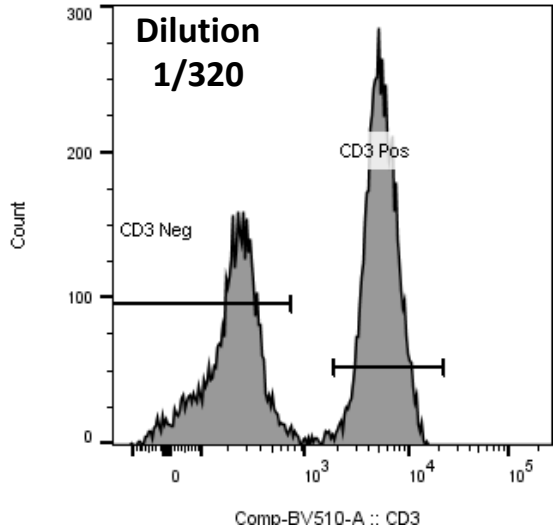




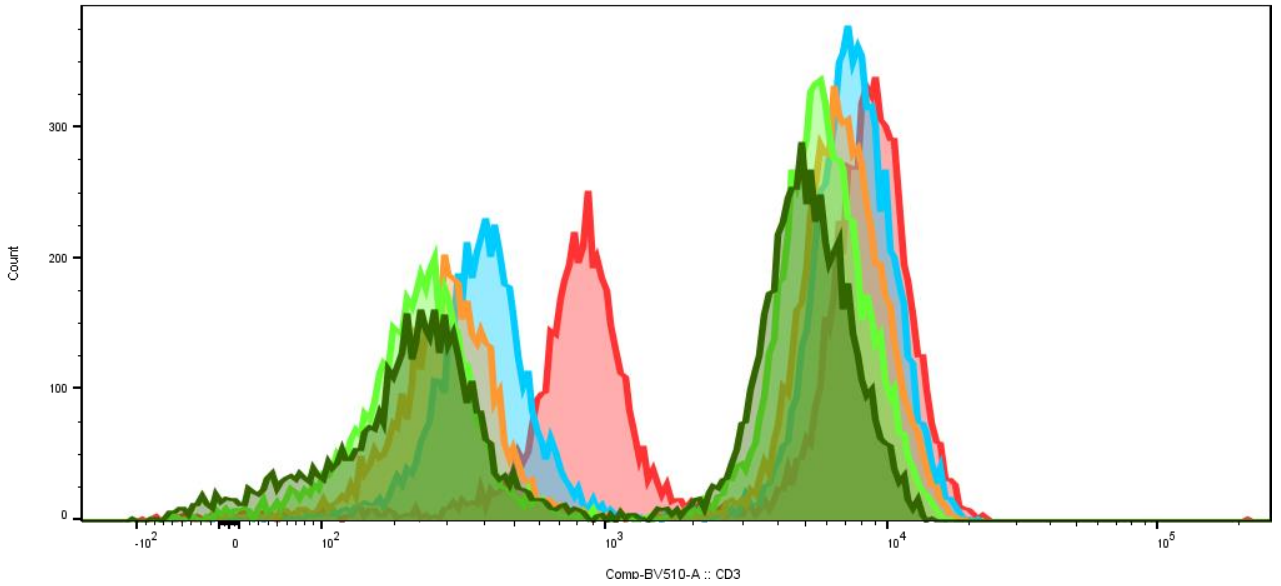
Stain Index = (MFI of Pos-MFI of Neg)/(2xSD of Neg)

	CD3 V500					Stain Index	
Dilution	Neg %	Neg Mean	Neg SD	Pos %	Pos Mean		
1/20	40.4	768	260	57.6	8719		15.29
1/40	39.2	405	128	60.1	7600		28.11
1/80	**39.1**	**316**	**111**	**60.2**	**6938**		**29.83**
1/160	40.2	252	100	59.1	5887		28.18
1/320	43.2	237	129	55.5	5187		19.19





Antibody Titration – Calculating the Stain Index



If antibody concentration too low, positive staining is too low
If antibody concentration is too high, then negative staining is too high

Indirect Immunofluorescence - Cell Surface

1. Cells in single cell suspension
2. Add primary antibody to cells
3. Incubate to allow primary antibody to bind to antigen on cells
4. Wash out unbound antibody
5. Add secondary antibody to cells
6. Incubate to allow secondary antibody to bind to antigen on cells
7. Wash out unbound antibody

Run on flow cytometer

Indirect Immunofluorescence - Cell Surface

What is the secondary antibody?

In most cases it is a polyclonal antibody directed against the animal host immunoglobulin type of the primary antibody

Eg

- Many primary antibodies are monoclonal antibodies made in mouse cells

- In this case can use a Sheep anti-mouse Ig – FITC, a polyclonal antibody reagent raised in sheep that will specifically bind mouse Ig

Indirect Immunofluorescence - Cell Surface

Primary Antibody Titration

Keep secondary antibody concentration consistent and titre primary antibody

Secondary Antibody Titration

Keep primary antibody concentration consistent and titre secondary antibody

Calculate Stain Index for each, use titre which gives highest value

Controls for your Immunofluorescence Staining

Negative controls

- No stain
- Non-specific antibody (isotype control)
- Secondary antibody alone for indirect immunofluorescence

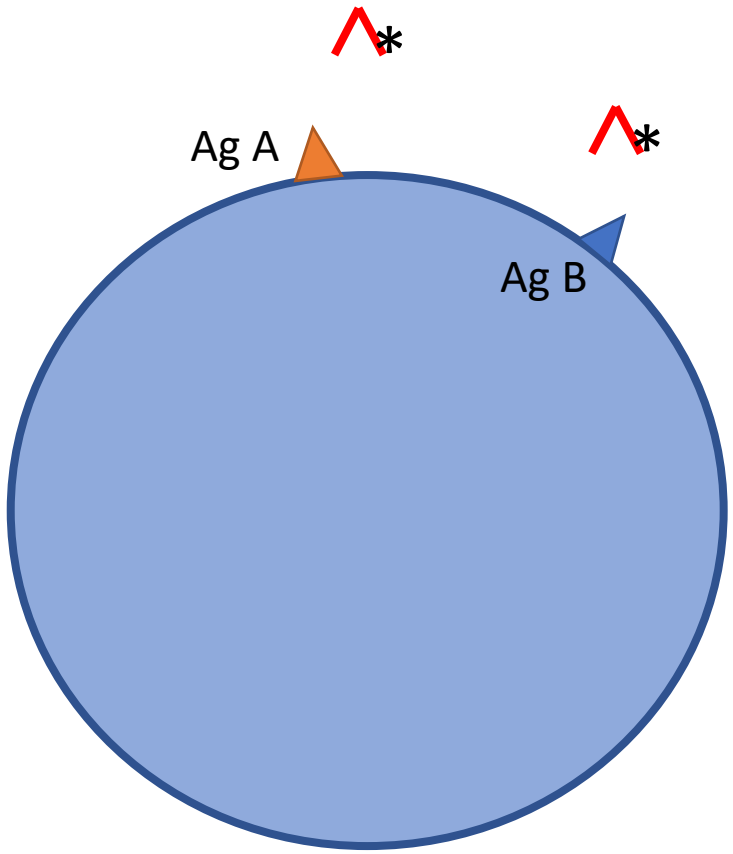
Positive controls

- An antibody directed against an antigen known to be strongly expressed by your cell of interest

Staining with more than One Antibody

- Relatively easy for direct staining as each antibody can have a particular fluorochrome
- Can be done with indirect staining but is more complicated

Indirect Immunofluorescence with Two Primary Antibodies



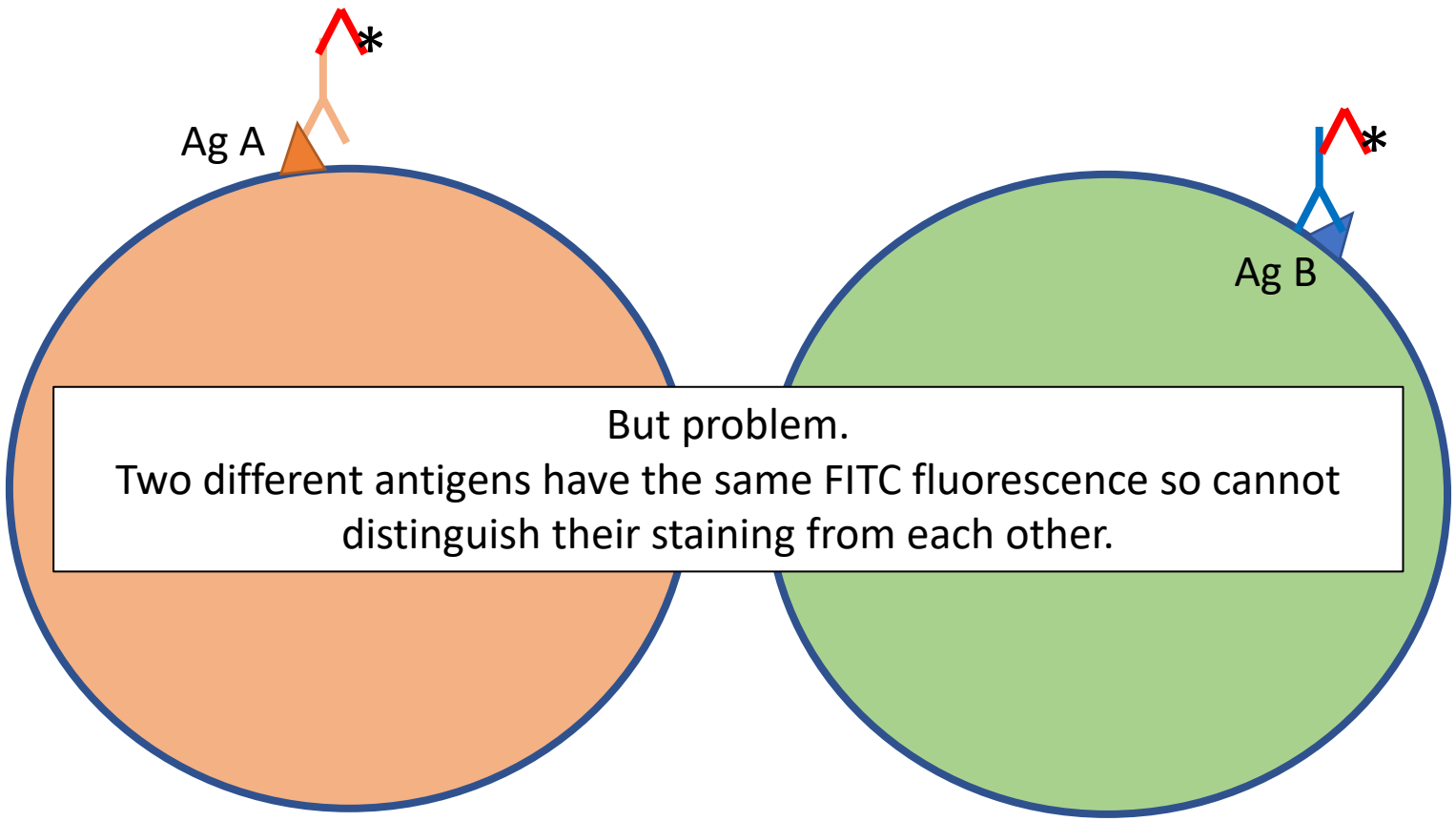
Mouse Ig anti-Ag A

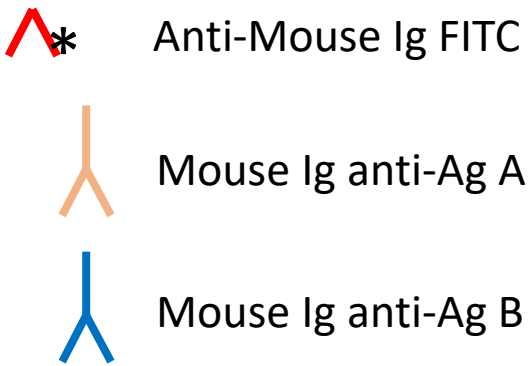
Mouse Ig anti-Ag B

Anti-Mouse Ig FITC

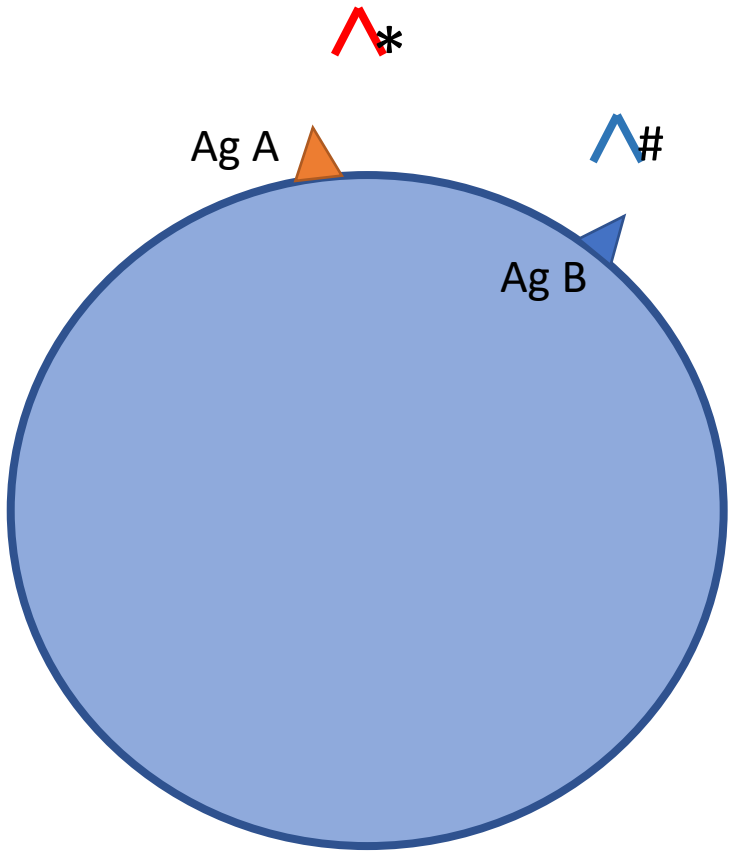
1. Add Primary antibodies first.
2. Wash unbound Primary antibodies
3. Add Secondary antibody
4. Wash unbound Secondary antibodies
5. Run on Flow Cytometer

Indirect Immunofluorescence with Two Primary Antibodies





Indirect Immunofluorescence with Two Primary Antibodies



Mouse Ig anti-Ag A

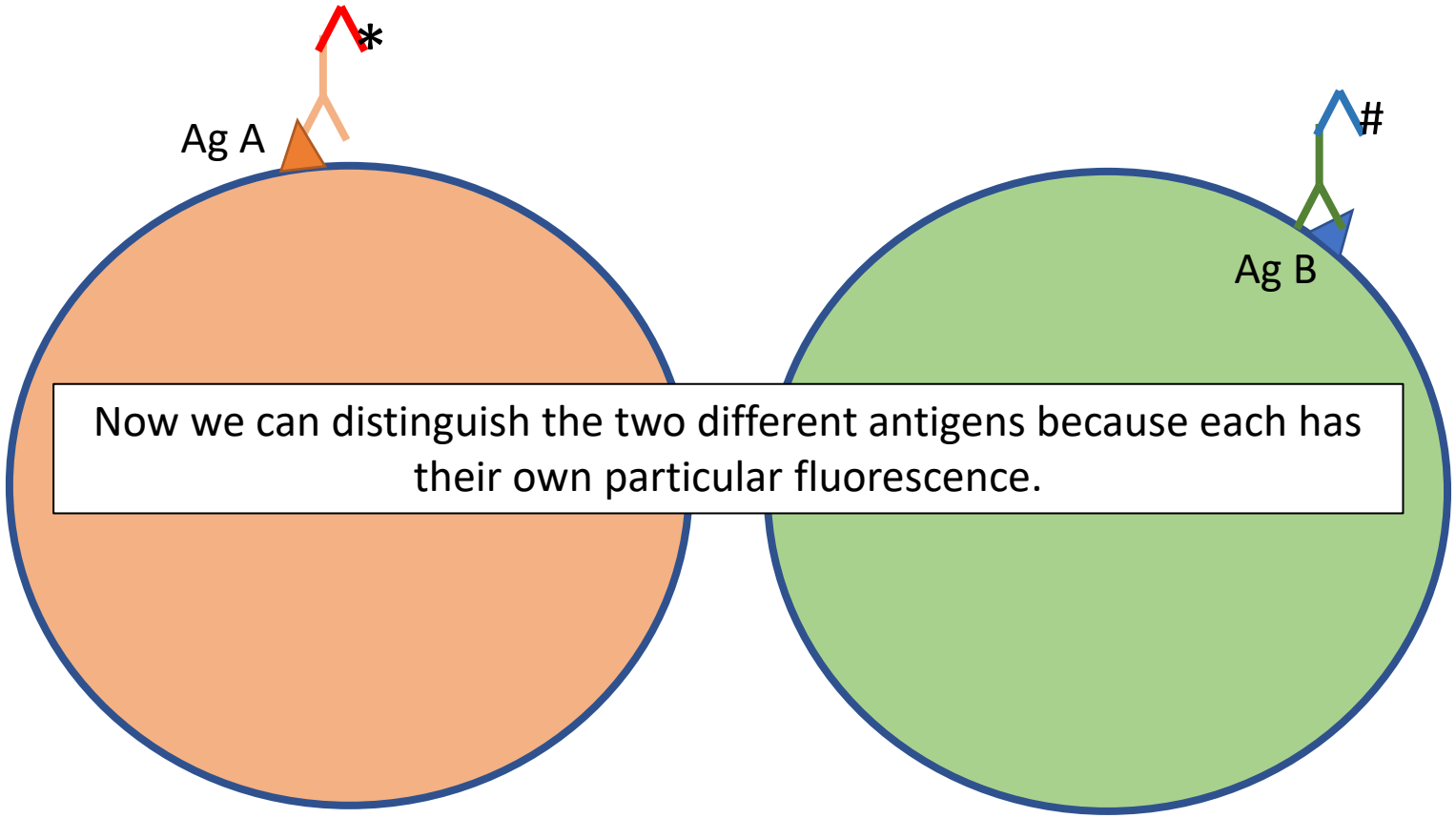
Rabbit Ig anti-Ag B

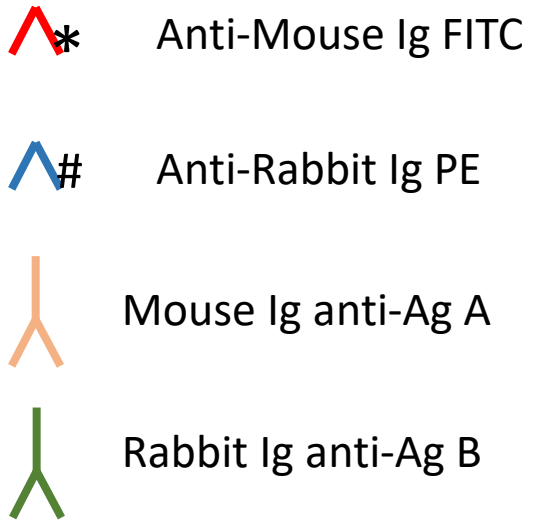
* Anti-Mouse Ig FITC

\# Anti-Rabbit Ig PE

1. Add Primary antibodies first.
2. Wash unbound Primary antibodies
3. Add Secondary antibodies
4. Wash unbound Secondary antibodies
5. Run on Flow Cytometer

Indirect Immunofluorescence with Two Primary Antibodies





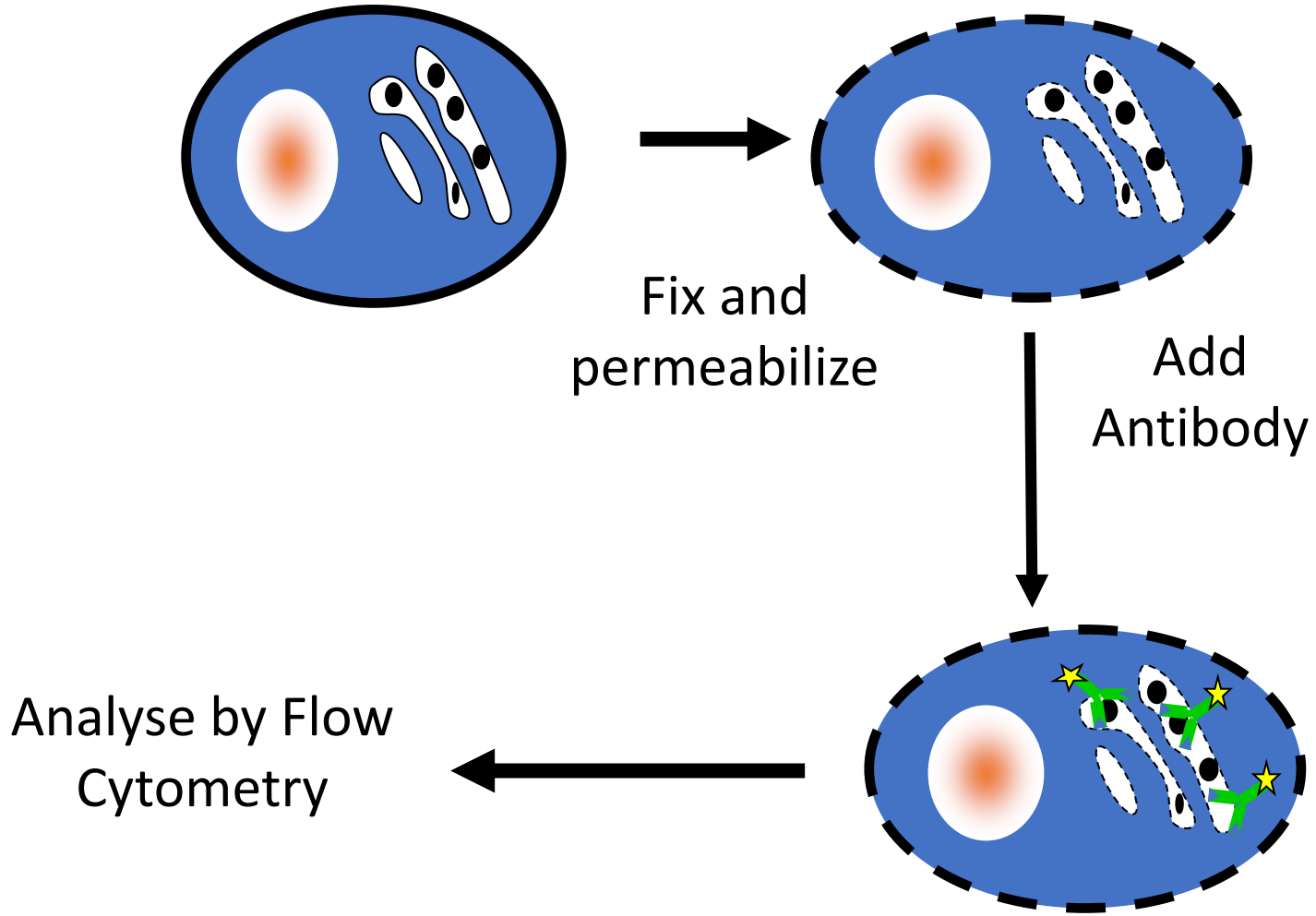
Measuring Cytoplasmic and Nuclear Antigens

- Can use flow cytometry to measure cytoplasmic and nuclear antigen expression

- Need to permeablise cell membrane before antibody labelling

Intracellular Staining

Eg Bcl-2 family members



Basic Fix and Perm method

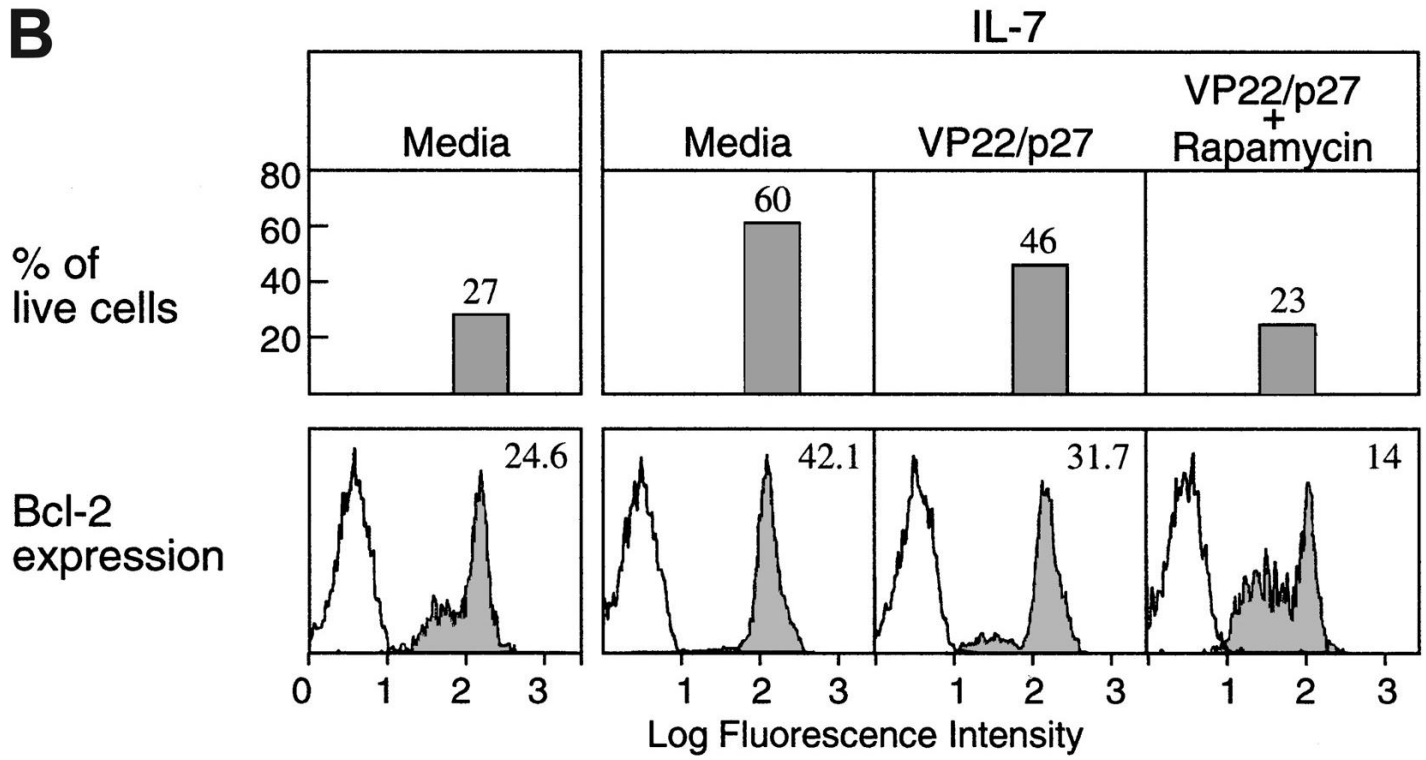
1. Cells in single cell suspension.
2. Add fixative (paraformaldehyde)
3. Incubate to allow fixation to take place
4. Wash in permeabilisation solution twice (saponin)
5. Resuspend cells in permeabilisation solution and add antibody
6. Wash out unbound antibody in permeabilisation solution
7. Resuspend cells in FACS buffer

Run on flow cytometer

Intracellular Staining

Bcl-2 family members

Bcl-2



Fix and Perm Methods

- Many different protocols that you can use. Each optimised for a particular antigens and cell types
- Basic Fix and Perm method good for most lymphoid and myeloid cytoplasmic and nuclear antigens
- May need stronger fixatives and permeabilisation solutions for other cell types and antigens

Simultaneous staining of cell cycle associated proteins (cyclins) and DNA

Prepare and fix cell suspension

1. Collect cells and resuspend in PBS at approximately 10^6 cells/ml.
2. Setup tubes with 10ml of 80% ethanol or absolute methanol fixative. Keep tubes on ice (0-4C). Setup enough tubes corresponding to the number of samples you wish to run (Neg Ctl, Cyclin A, etc..)
3. Transfer 1ml of cell suspension into each tube with the cold fixative. Incubate on ice or 4C for at least 4 hours to several days.

Darzynkiewicz et al, Current Protocols in Cell Biology, 1999

Simultaneous staining of cell cycle associated proteins (cyclins) and DNA

Label cells with anti-cyclin primary antibody

4. Centrifuge fixed cells at 300g for 5min. Remove alcohol supernatant and then resuspend cells in 5ml PBS, centrifuge as before.
5. Remove supernatant and resuspend cell pellet in 1ml of 0.25% Triton in PBS. Incubate on ice for 5mins, then add 5ml PBS and centrifuge 300g 5mins. Remove supernatant.
6. Resuspend cells in 100ul of PBS/1% bovine serum albumin (BSA). Add your primary antibodies (Cyclins, neg ctl abs). Incubate at least 60 minutes at room temperature or 4C overnight. Occasionally resuspend or gently agitate cells.
7. Add 5ml PBS/1%BSA to each tube. Spin down 300g 5mins and then remove supernatant.
8. Resuspend cells in 100ul and add secondary antibody (FITC conjugated). Incubate 30 minutes RT in dark with gentle agitation.
9. Add 5ml PBS/1%BSA to each tube. Spin down 300g 5mins and then remove supernatant.

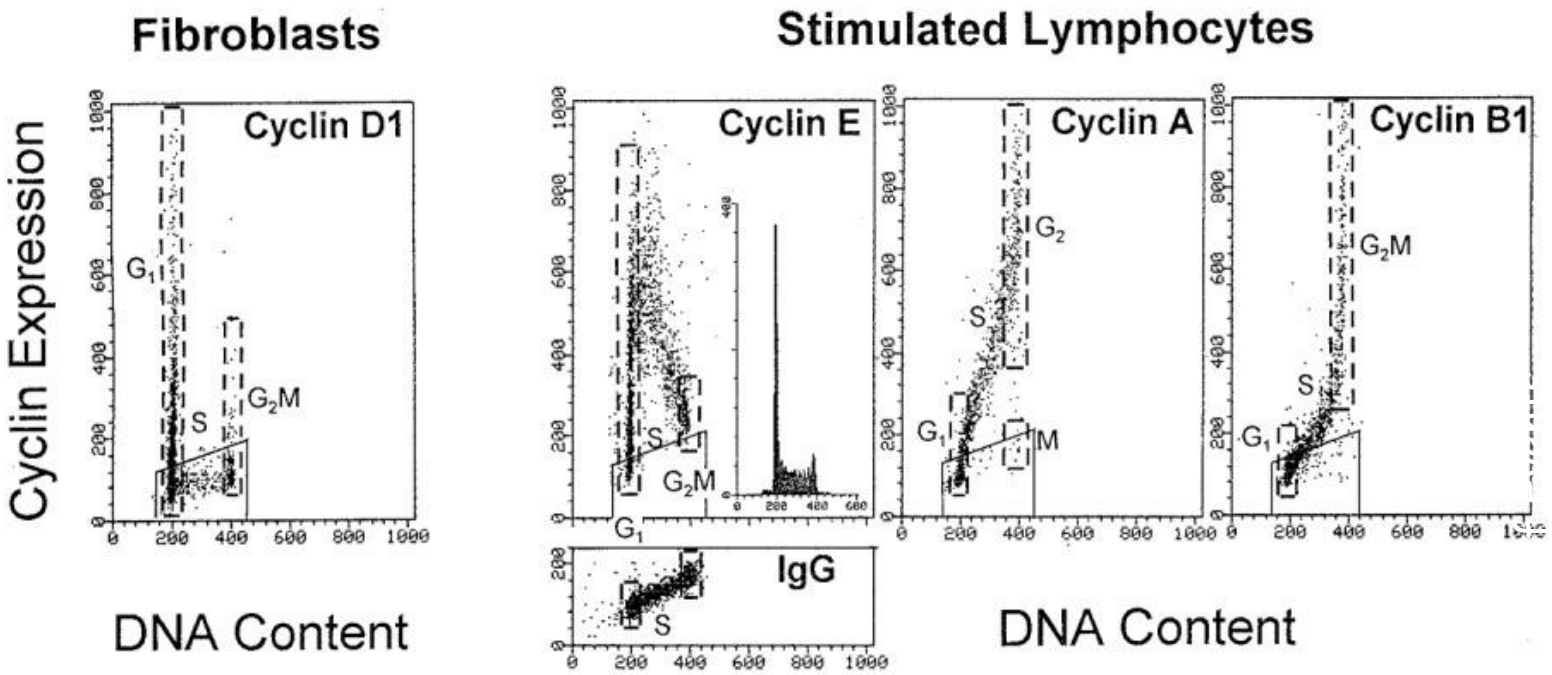
Darzynkiewicz et al, Current Protocols in Cell Biology, 1999

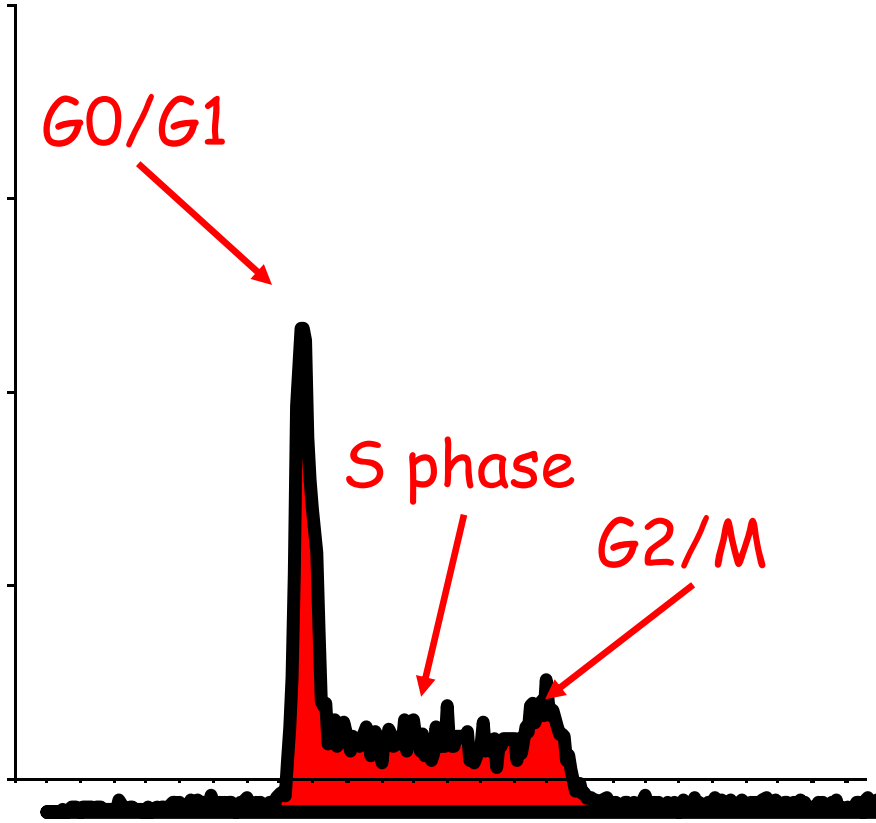
Simultaneous staining of cell cycle associated proteins (cyclins) and DNA

Stain cells with Propidium Iodide (PI) for DNA content

10. Resuspend each cell pellet in PI staining solution (5ug/ml PI, 200ug/ml DNase –free RNAse, prepare fresh). Incubate for at least 20 minutes in dark before running on flow cytometer.
11. Measure green-fluorescence (FITC) associated antibody staining and DNA-associated red fluorescence (PI) on flow cytometer.

Darzynkiewicz et al, Current Protocols in Cell Biology, 1999





Darzynkiewicz et al, Current Protocols in Cell Biology, 1999

Combining Surface and Intracellular antigen staining

A. Surface antigen staining

1. Cells in single cell suspension.
2. Add fluorochrome conjugated antibody to cells
3. Incubate to allow antibody to bind to antigen on cells
4. Wash out unbound antibody

Surface and Intracellular antigen staining

B. Intracellular antigen staining

1. Resuspend cells in fixative (paraformaldehyde)
2. Incubate to allow fixation to take place
3. Wash in permeabilisation solution twice (saponin)
4. Resuspend cells in permeabilisation solution and add intracellular antibody
5. Wash out unbound antibody in permeabilisation solution
6. Resuspend cells in FACS buffer

Intracellular Antigen Staining Tips

- Not every intracellular antigen staining protocol is the same. Different staining procedures are needed depending on whether the protein to be detected is a cytokine, a transcription factor, a phosphorylated protein (phosphoflow), or another type of protein
- The protocol fixation and permeabilization steps should be as mild as possible and the duration as short as possible, while maximising antibody staining
- The fluorophore chosen can be affected by the staining protocol. Their brightness can be reduced by the fixation or permeabilization step. In particular, PE and APC can be affected by alcohol fixation
- It is expected that background staining (autofluorescence and non specific antibody binding) will go up with intracellular staining. Need to take this into account if trying to measure weakly expressed antigens

Whole Blood Staining

Staining for blood leukocytes can be done without first isolating the cells

This is often called the ‘Stain-Lyse-(Wash)’ method

Need to lyse red blood cells before running on flow cytometer because too many of them!

<u>Example - CD4 count</u>

Measure frequency of CD4 T cells by staining blood with CD3 and CD4 antibodies

Whole Blood Staining

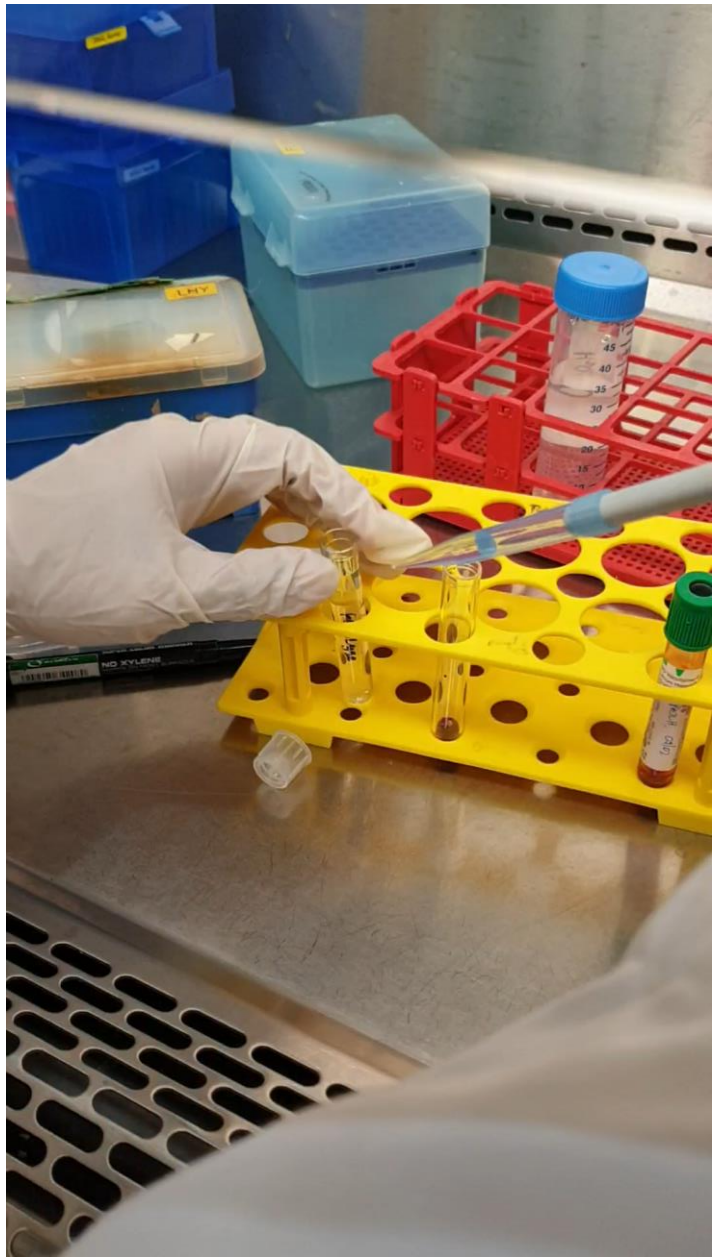
CD4 count

1. Add 100ul of anti-coagulant blood (EDTA, Heparin) to a 5ml tube
2. Add CD3 PE-Cy7 and CD4 Alexa 488 conjugated antibodies
3. Vortex to mix and incubate at room temperature
4. Add red blood cell lysis solution (including fixative)
5. Run on flow cytometer

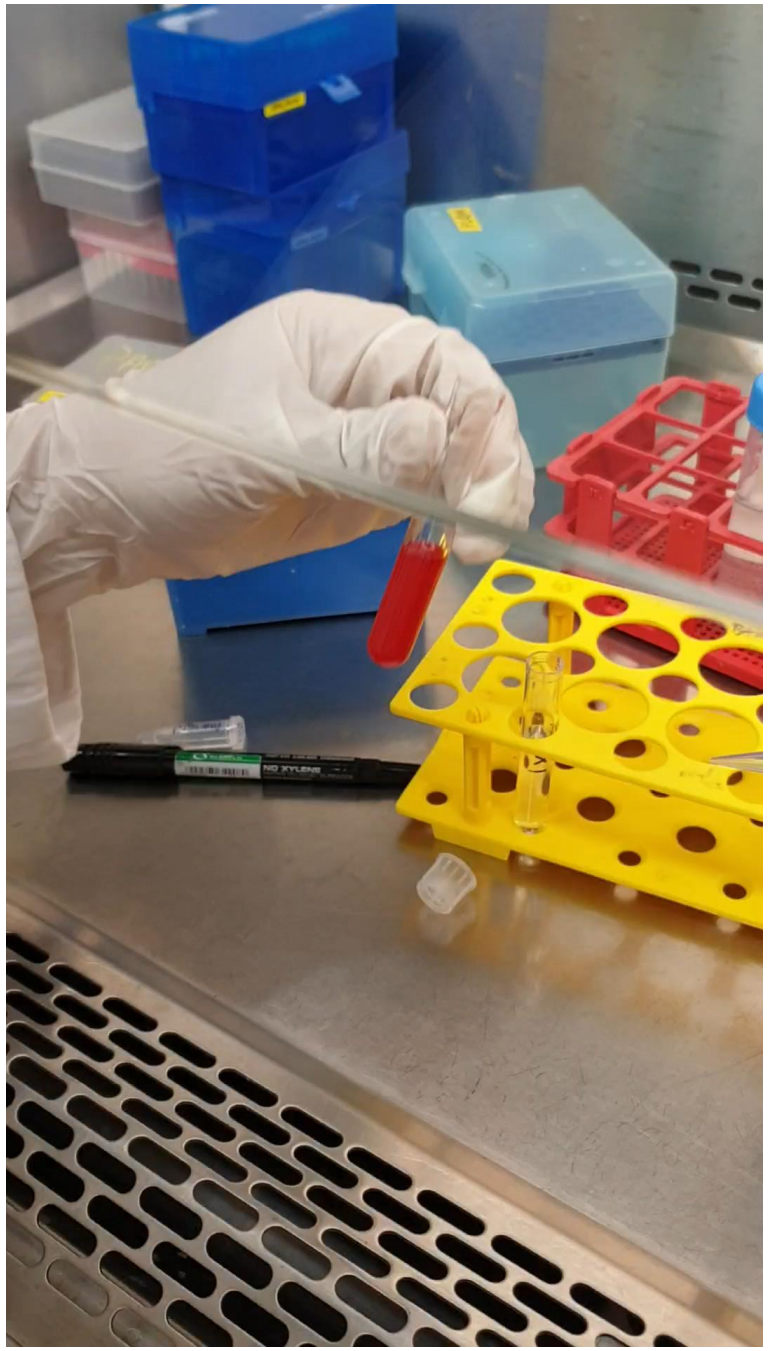
Optional: Spin down cells, remove supernatant and resuspend cells in FACS buffer

Adding blood to tube

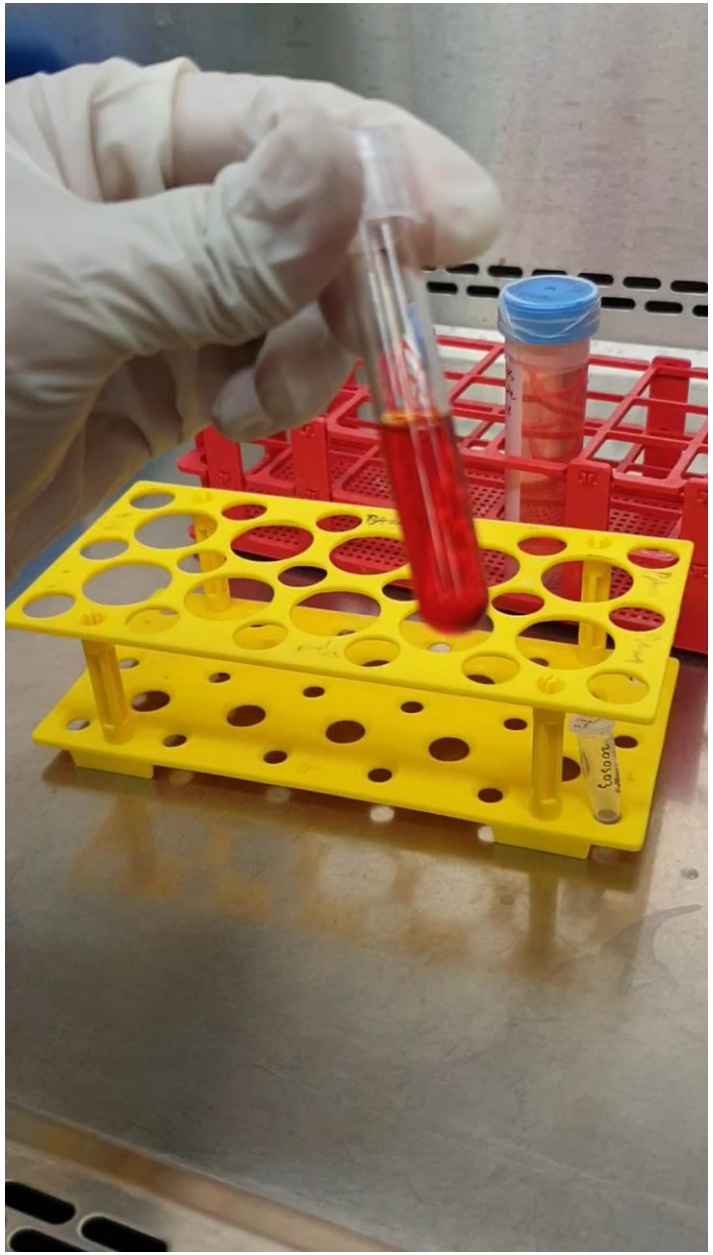
Adding antibodies to blood



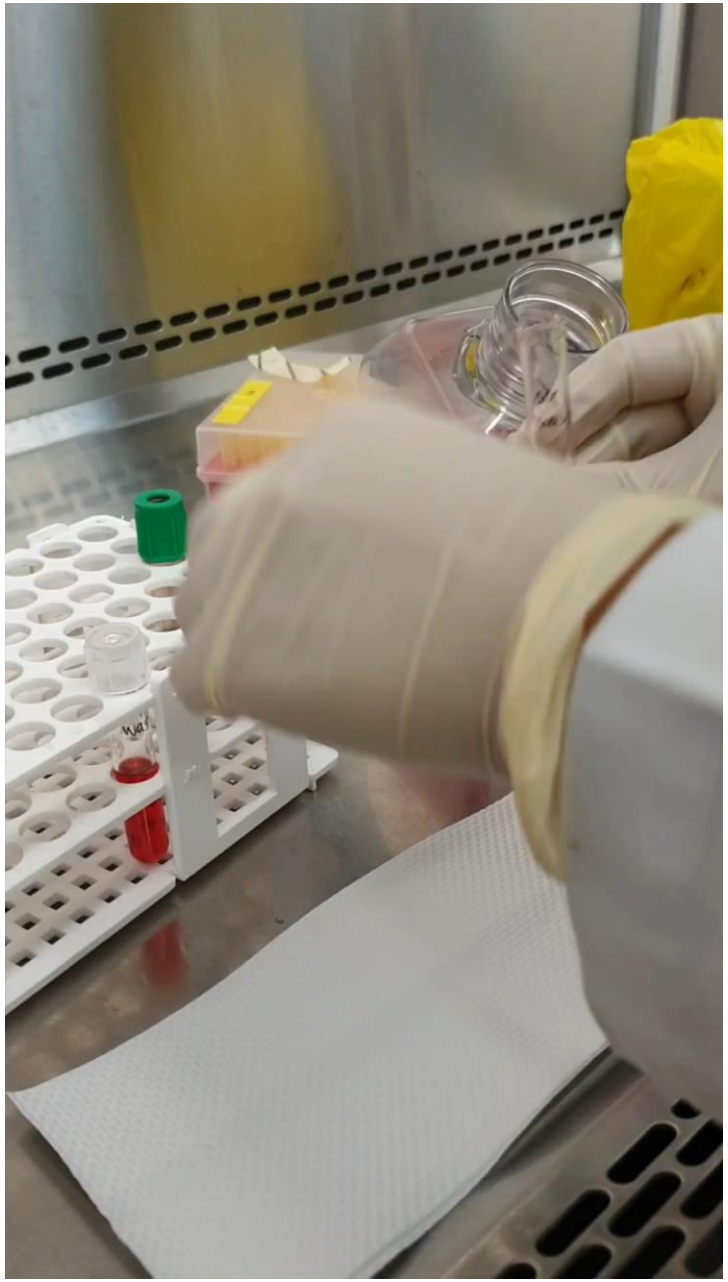
Adding lysis buffer to blood



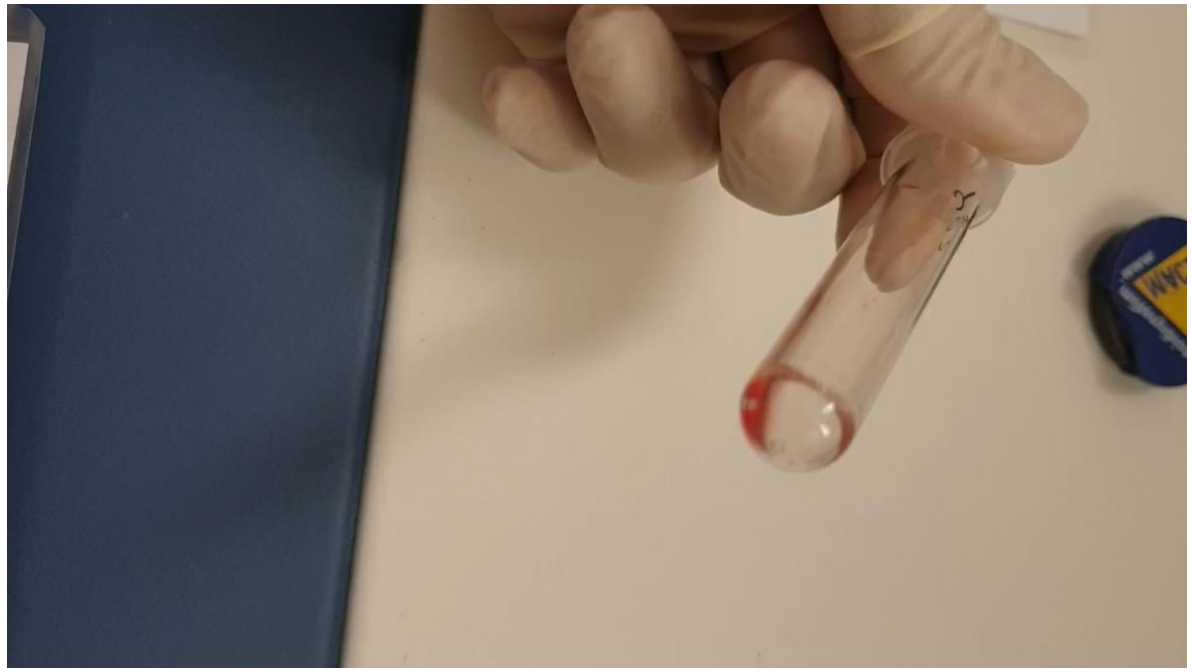
Just after lysis buffer added to blood



10 minutes after lysis buffer added to blood

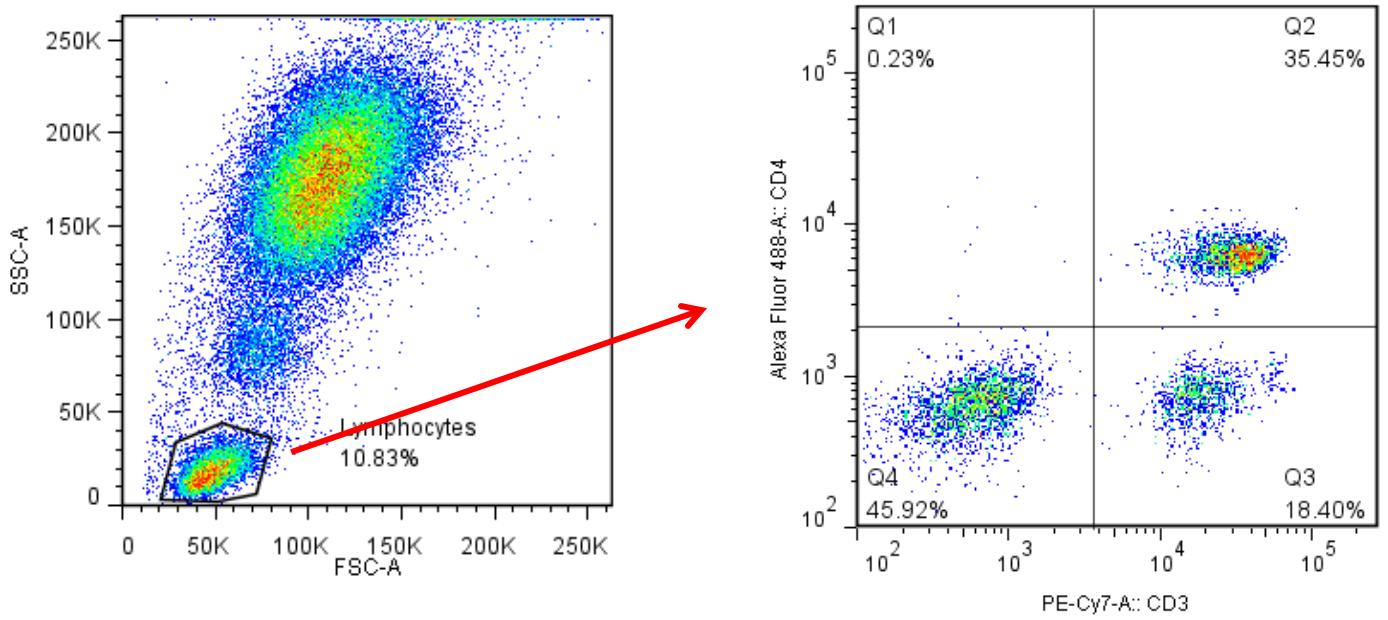


Decanting supernatant after centrifuging tube



Cell pellet after washing

CD4 Count - Final Result



1. Use the light scatter signals to identify the lymphocytes
2. Measure the percentage of them positive for CD3 and CD4 antibodies (35%)
3. Multiply against lymphocyte count to get report value

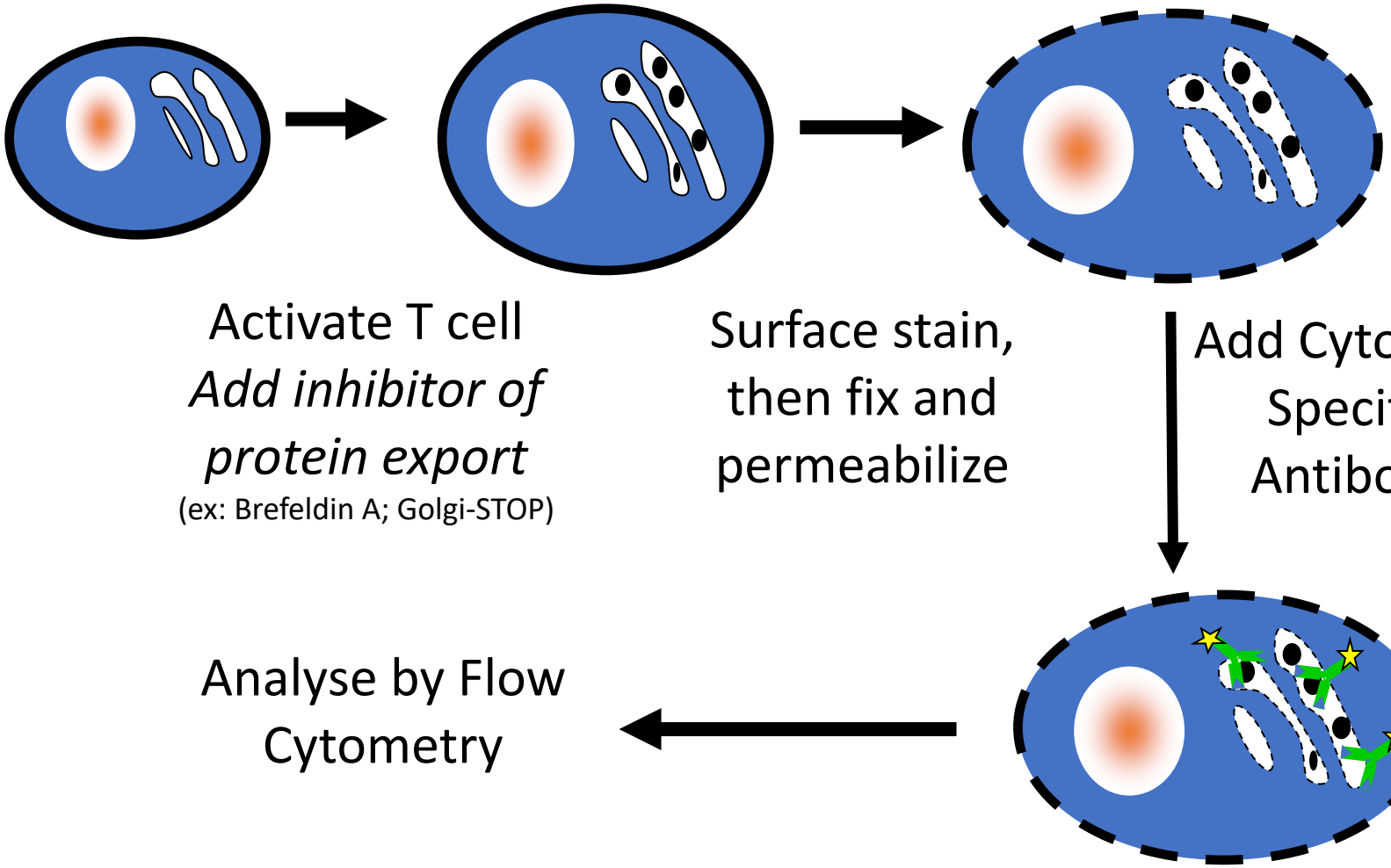
T cell Cytokine Staining

- Cytokines such as Interferon-gamma (IFNg) and Interleukin-2 are produced by T cells in response to antigen and polyclonal stimulus
- These cytokines are important for driving the immune response of the host against pathogens such as mycobacteria and viruses
- The production of these cytokines can be measured in whole blood assays utilising flow cytometry

T cell Cytokine Staining

- T cells can rapidly produce and secrete cytokines after stimulation
- To measure the cytokines by flow cytometry we use golgi transport inhibitors Monensin or Brefeldin A to trap the cytokines within the cell after their production
- Can then do surface staining followed by intracellular staining to measure which T cells are producing the cytokines following stimulus
- This technique is known as ‘Intracellular Cytokine Staining’ or ICS

Cytokine production (intracellular cytokine staining)



Whole Blood Assay for T cell Cytokine Staining

- Incubate blood with antigen or other polyclonal (PMA+Ionomycin, anti-CD3+CD28) stimuli
- Add golgi transport inhibitors to reduce secretion of cytokines from the responding cell.

- *Needs to be heparin blood. Cannot use EDTA as it will inhibit the Ca ion signalling necessary for cytokine production*

Whole Blood Assay for T cell Cytokine Staining

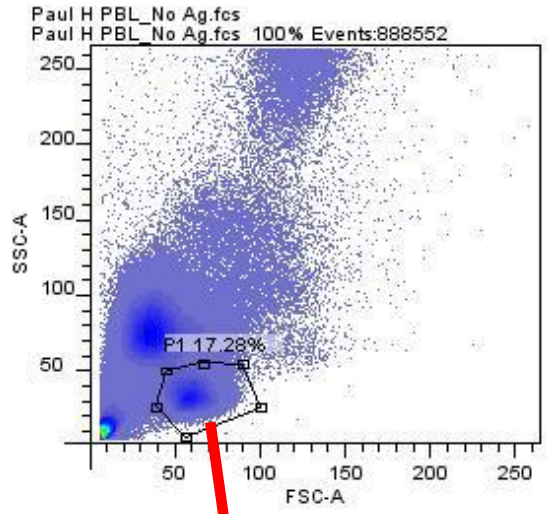
- Normally shorter stimulation time for polyclonal stimuli – 4-6 hours. Also add golgi transport inhibitors at start of culture
- Antigen stimulation normally needs longer (6-16 hours) and add golgi inhibitors 1-2 hours after initial culture to enable antigen presentation to the T cell
- Which inhibitor (Monensin or Brefeldin A) to use depends on which cytokine you want to measure and from which species the sample is from (e.g. Monensin better for murine work)
- Can also do the cytokine staining on isolated peripheral blood mononuclear cells or other leukocyte preparations

PMA+Ionomycin Polyclonal Cytokine Stimulus I

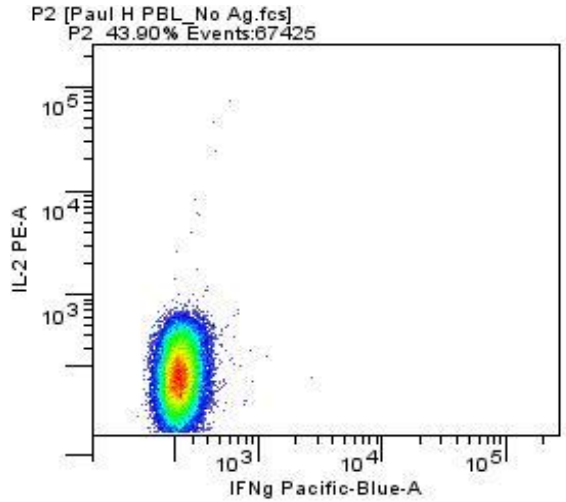
1. Add 500ul whole blood to 500ul RPMI into 24 well plate (ie 1ml per well). Two wells, one non-stimulated control and one stimulated
2. Add 10ul brefeldin A (1mg/ml) to control well
3. Add to stimulated well 10ul brefeldin A (1mg/ml), 2.5ul PMA stock (10ug/ml), and 2ul ionomycin (500ug/ml). This leads to final concentration of 25ng/ml PMA and 1ug/ml ionomycin
4. Once additions have been made to wells mix thoroughly by pipetting up and down for at least 5 times
5. Incubate at 37C and 5% CO_2 for 4 hours.

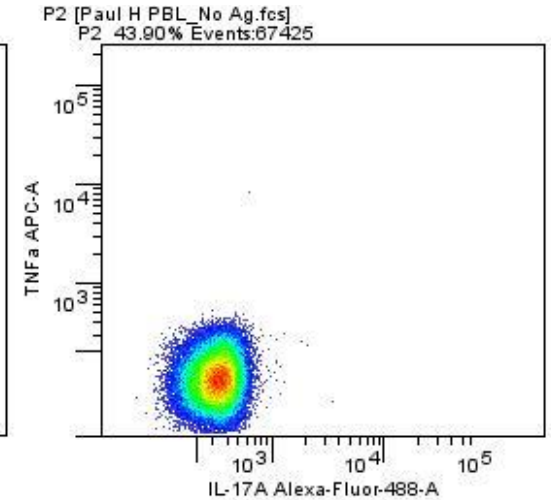
PMA+Ionomycin Polyclonal Cytokine Stimulus II

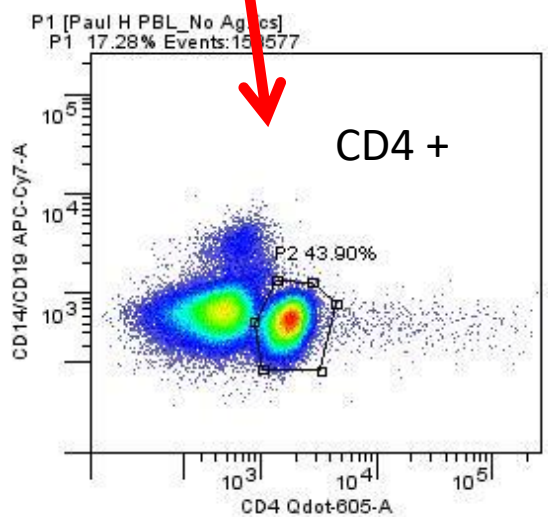
1. Add 1ml blood-RPMI to 24ml NH_4Cl red blood cell lysis solution. Leave at room temperature for 10 minutes (like other whole blood assays need to remove the overwhelming numbers of red blood cells)
2. Spin down cells and then wash again in FACS buffer
3. After last wash resuspend cells in FACS buffer and add fluorochrome conjugated antibodies directed against surface antigens. Incubate 15 minutes on ice
4. Wash out unbound antibody
5. Resuspend cells in fixative (paraformaldehyde) and incubate 15 minutes on ice
6. Wash in permeabilisation solution twice (saponin)
7. Resuspend cells in permeabilisation solution and add intracellular antibodies (cytokines)
8. Wash out unbound antibody in permeabilisation solution
9. Resuspend cells in FACS buffer and run on flow cytometer



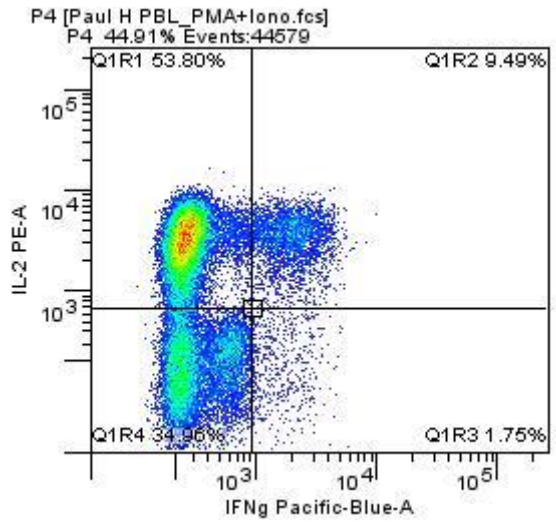
Unstimulated

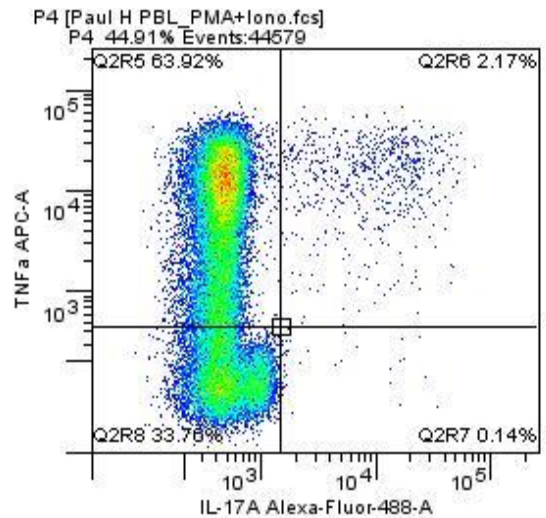






PMA+Ionomycin





Antigen Specific T cells

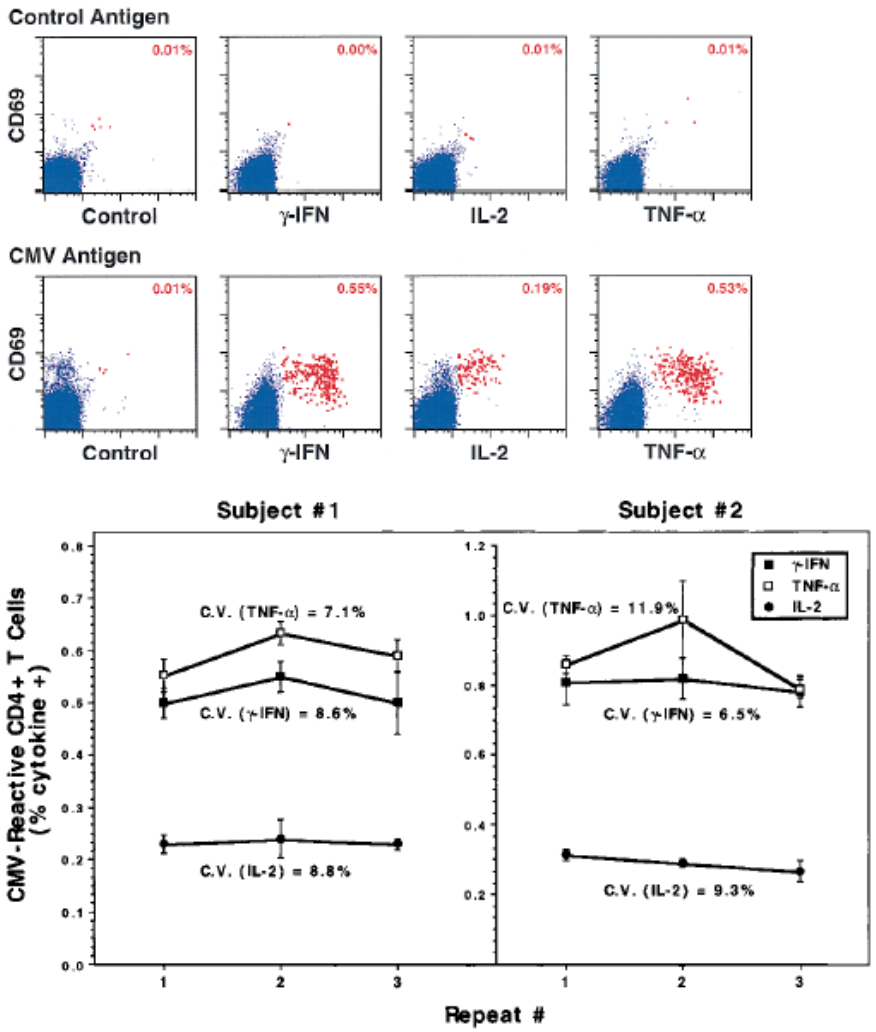
- Each T-cell possesses a receptor specific for a particular antigen.
- Initial encounter with antigen via infection or vaccination induces proliferation. Once the antigen has been cleared some of these T-cells remain as memory cells
- These memory cells drive the rapid recall immune response if this antigen is encountered again.
- Quantification and characterisation of these memory antigen specific T-cells can provide an important insight into the status of the immune system, and how it controls responses to foreign pathogens

How can you measure Antigen Specific T-cells?

Memory T-cells respond to antigen by…

- Specifically binding antigen (tetramers)
- Proliferation (CSFE)
- Cytokine production (ELISPOT and ICS)
- Activation marker expression (eg CD69, CD154, CD137)

Intra-cellular cytokine staining (ICS) to detect antigen specific T-cells by Flow Cytometry



First published in 1997 (*Wadrop et al, J. Clin Invest 99: 1739*)

Uses Brefeldin A to block secretion of cytokines by antigen activated T-cell, can then stain them directly with fluorescently conjugated antibody. Added co-stim antibody (CD28) to amplify cytokine production.

Later adapted by Suni et al to use in a whole blood assay (*J Immunol Methods 1998 212: 89*)

Variations:

- Whole protein (CD4) or peptide (CD4&CD8) antigens
- Monensin versus Brefeldin A
- Time of stimulation (6hrs-16hrs)
- Co-stim abs (CD28 and CD49d) or not

ICS FOR DETECTING ANTIGEN SPECIFIC T-CELLS

- For the ICS assay best to use whole protein antigens for detecting CD4 responses, and peptide mixes for CD8 (&CD4) measurement
- The ICS assay has been tested with viral and bacterial antigens, auto-antigens, and allergens
- Can be dealing with very low frequencies of antigen specific T-cells (down to 1 in 100 000). Need to have correct controls, and count enough cells
- Combine activation marker and cytokine staining for higher sensitivity measurements

APPLYING ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

- Commonly used tests in TB diagnosis such as the Mantoux skin test and Quantiferon assays are indirect measurements of *Mycobacterium tuberculosis* (MTB) specific T cells
- Depending on what you are trying to measure, you can use MTB specific proteins such as ESAT-6 and CFP-10, or total MTB protein preparations like PPD (skin test)
- ESAT-6 and CFP-10 stimulation can be used to identify previous exposure to MTB
- If exposure known already and you want to characterise T cell responses in active or latent TB infection, or follow the effect of anti-TB treatment, can use PPD

I. WHOLE BLOOD ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

1. Aliquot 1-2ml ml Heparin blood. Need a No Antigen negative control, plus tubes with the antigens you wish to test (eg PPD and ESAT-6)
2. To each aliquot add co-stimulatory antibodies anti-CD28 & anti-CD49d (1ug/ml) plus the antigens of interest. Add no antigen to negative control samples.
3. Vortex and incubate at 37C for 2 hours
4. Add 1ug/ml Brefeldin A to all aliquots, vortex and incubate at 37C overnight (no longer than 14 hours)

II. WHOLE BLOOD ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

1. Add 2.5mM EDTA solution to each tube and vortex for 15 seconds. Incubate for 15 minutes at room temperature (helps separate T-cell and antigen presenting cell clumps)
2. Add 25 times volume of NH_4Cl red blood cell lysis solution. Leave at room temperature for 10 minutes (like other whole blood assays need to remove the overwhelming numbers of red blood cells)
3. Spin down cells and then wash again FACS buffer

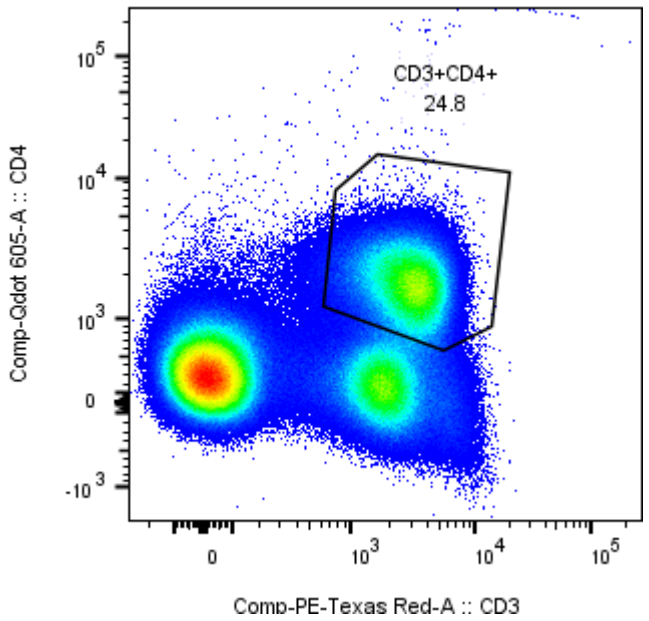
III. WHOLE BLOOD ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

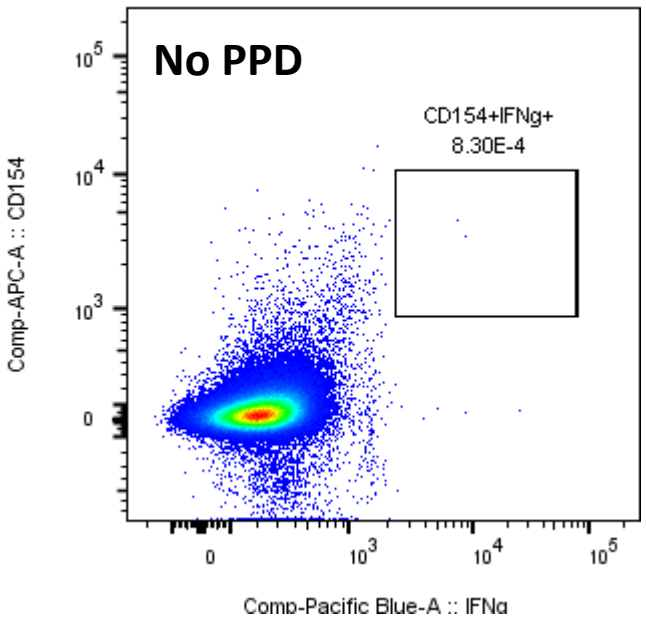
1. After last wash resuspend cells in FACS buffer and add fluorochrome conjugated antibodies directed against surface antigens. Incubate 15 minutes on ice.
2. Wash out unbound antibody
3. Resuspend cells in fixative (paraformaldehyde) and incubate 15 minutes on ice.
4. Wash in permeabilisation solution twice (saponin)
5. Resuspend cells in permeabilisation solution and add intracellular antibodies (cytokines)
6. Wash out unbound antibody in permeabilisation solution
7. Resuspend cells in FACS buffer and run on flow cytometer

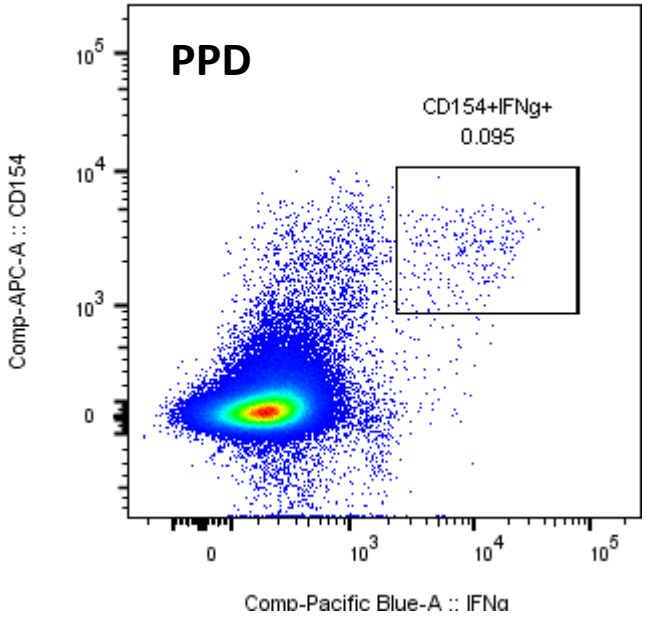
ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

Example staining…

<u>4-colour</u> CD3+CD4+CD154+IFNg

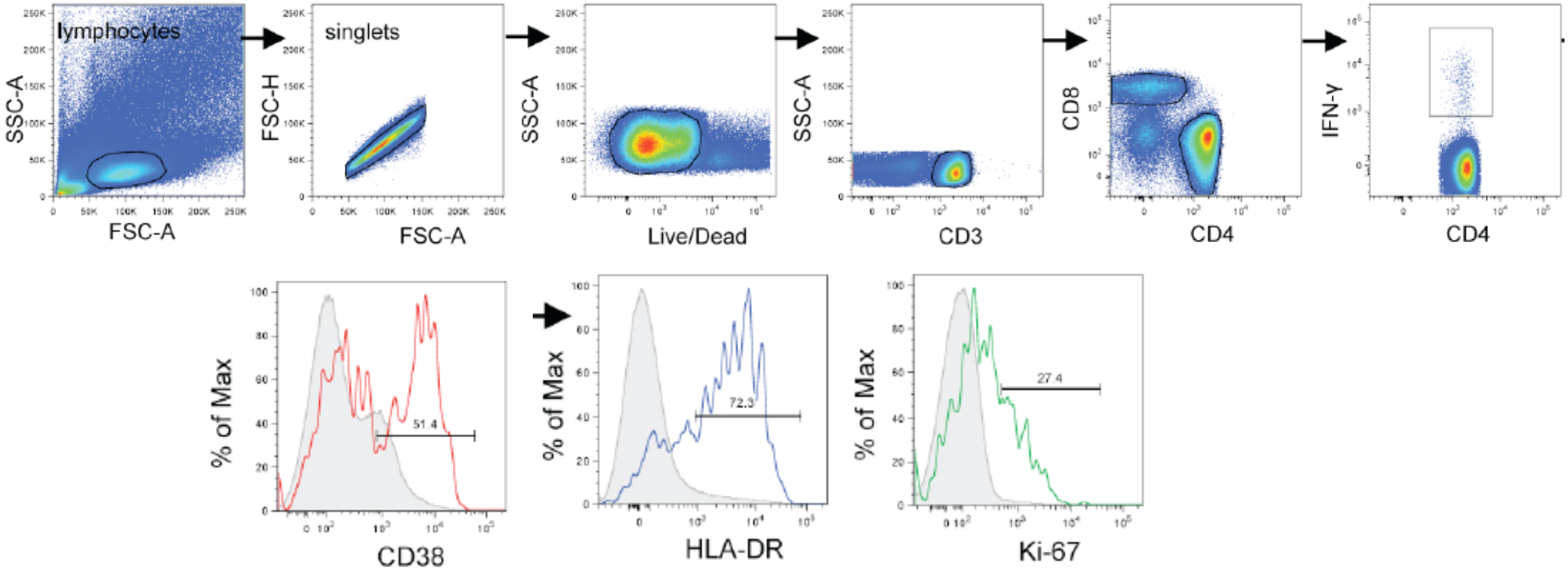




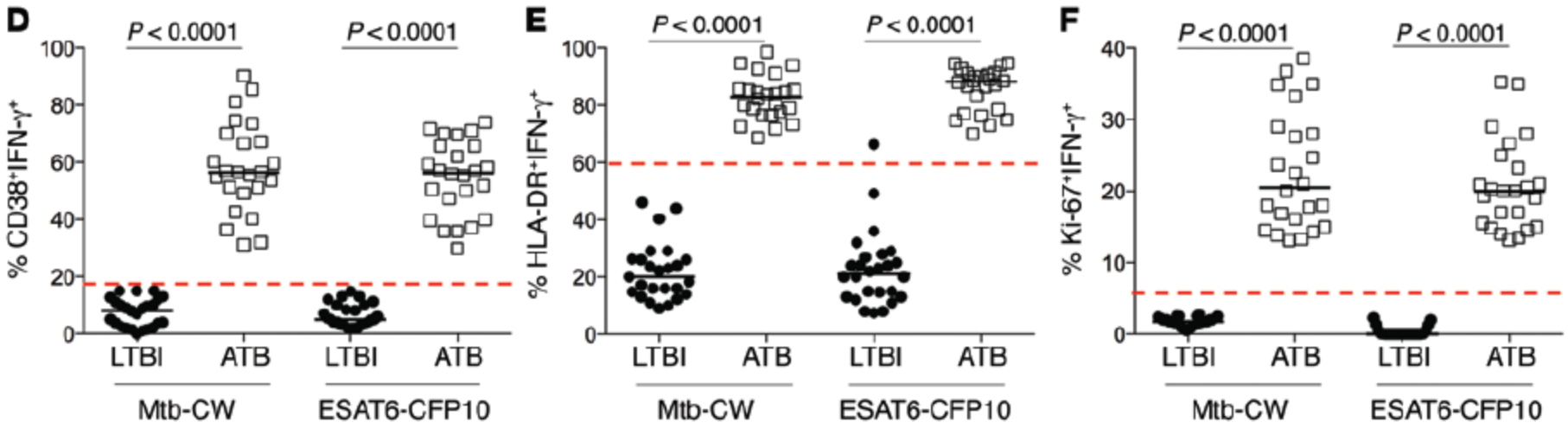


Can ICS help Discriminate Active from Latent TB?

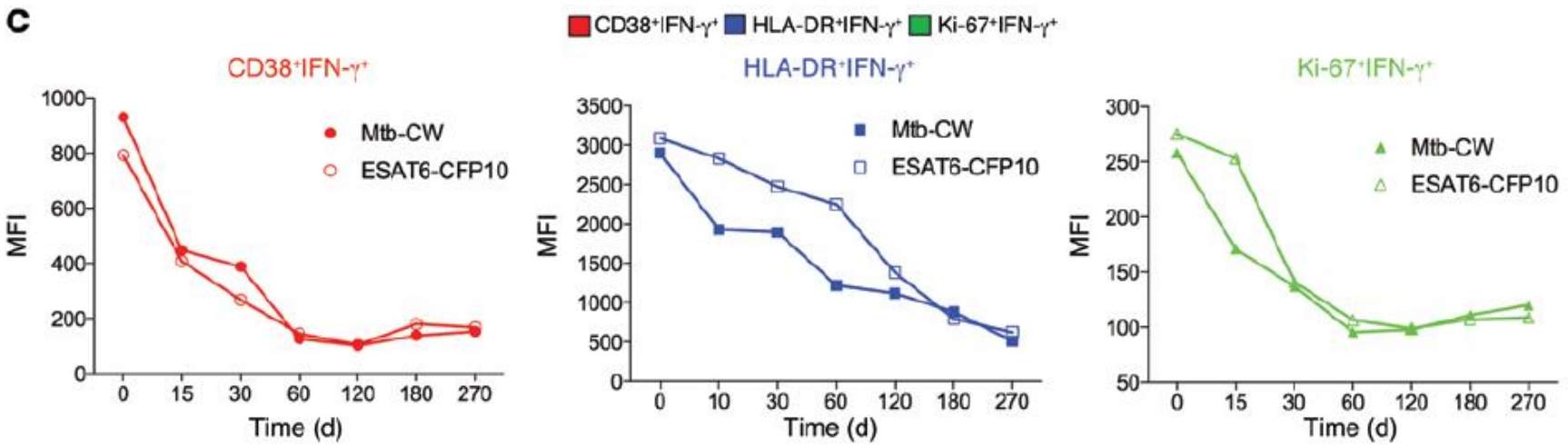
A recent study seemed to have found phenotypes of MTB specific CD4 cells that give stronger discrimination between active and latent TB



"Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response" Adekambi et al, J Clin Invest 2015



LTBI: Latent TB infection, ATB: Acute TB infection, Mtb-CW: Mtb cell wall antigens



Change in MTB specific CD4 T-cell phenotype over time of treatment.

ANTIGEN SPECIFIC T-CELL ICS IN TUBERCULOSIS

Always best to setup whole blood assay with freshly drawn blood

Can store the samples after stimulation:

1. After stimulation can put blood directly in to preservative (Streck Cell preservative) and store at RT for up to 2 weeks before processing

(Herry et al, Journal of Clinical Tuberculosis And Other Mycobacterial Diseases, 2021)

2. After EDTA treatment you can use FACSlyse solution (BD Biosciences) to lyse red blood cells and fix leukocytes. After this you can wash and then the fixed white cells can be cryopreserved in 10% DMSO/40% foetal calf/RPMI in liquid nitrogen

(Kagina et al, Journal of Immunological Methods 417 (2015) 22–33)

ANTIGEN SPECIFIC T-CELL ICS FOR COVID-19

The Journal of Clinical Investigation

RESEARCH ARTICLE

SARS-CoV-2–specific T cell responses and correlations with COVID-19 patient predisposition

Arne Sattler,[1] Stefan Angermair,[2] Helena Stockmann,[3] Katrin Moira Heim,[4] Dmytro Khadzhynov,[3] Sascha Treskatsch,[2] Fabian Halleck,[3] Martin E. Kreis,[1] and Katja Kotsch[1]

[1]Department for General, Visceral and Vascular Surgery, [2]Department of Anesthesiology and Intensive Care Medicine, [3]Department of Nephrology and Medical Intensive Care, and [4]Department of Infectiology and Pneumonology, Charité – Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany.

Coronavirus disease 2019 (COVID-19) has emerged as a global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). So far, viral targets of cellular immunity and factors determining successful mounting of T cell responses are poorly defined. We therefore analyzed cellular responses to membrane, nucleocapsid, and spike proteins in individuals suffering from moderate or severe infection and in individuals who recovered from mild disease. We demonstrate that the CoV-2–specific $CD4^+$ T helper cell response is directed against all 3 proteins with comparable magnitude, ex vivo proliferation, and portions of responding patients. However, individuals who died were more likely to have not mounted a cellular response to the proteins. Higher patient age and comorbidity index correlated with increased frequencies of CoV-2–specific $CD4^+$ T cells, harboring higher portions of IL-2–secreting, but lower portions of IFN-γ–secreting, cells. Diminished frequencies of membrane protein–reactive IFN-γ^+ T cells were particularly associated with higher acute physiology and chronic health evaluation II scores in patients admitted to intensive care. CoV-2–specific T cells exhibited elevated PD-1 expression in patients with active disease as compared with those individuals who recovered from previous mild disease. In summary, our data suggest a link between individual patient predisposition with respect to age and comorbidity and impairment of CoV-2–specific Th1-type cellular immunity, thereby supporting a concept of altered T cell function in at-risk patients.

Thank you for your attention!

Questions?

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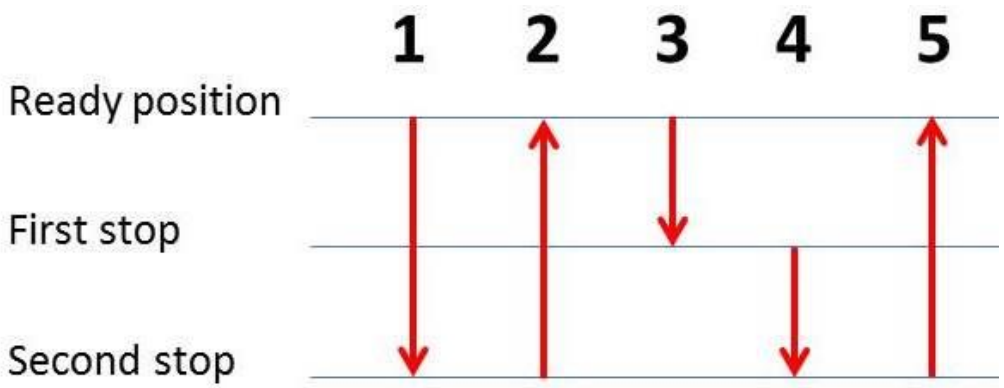
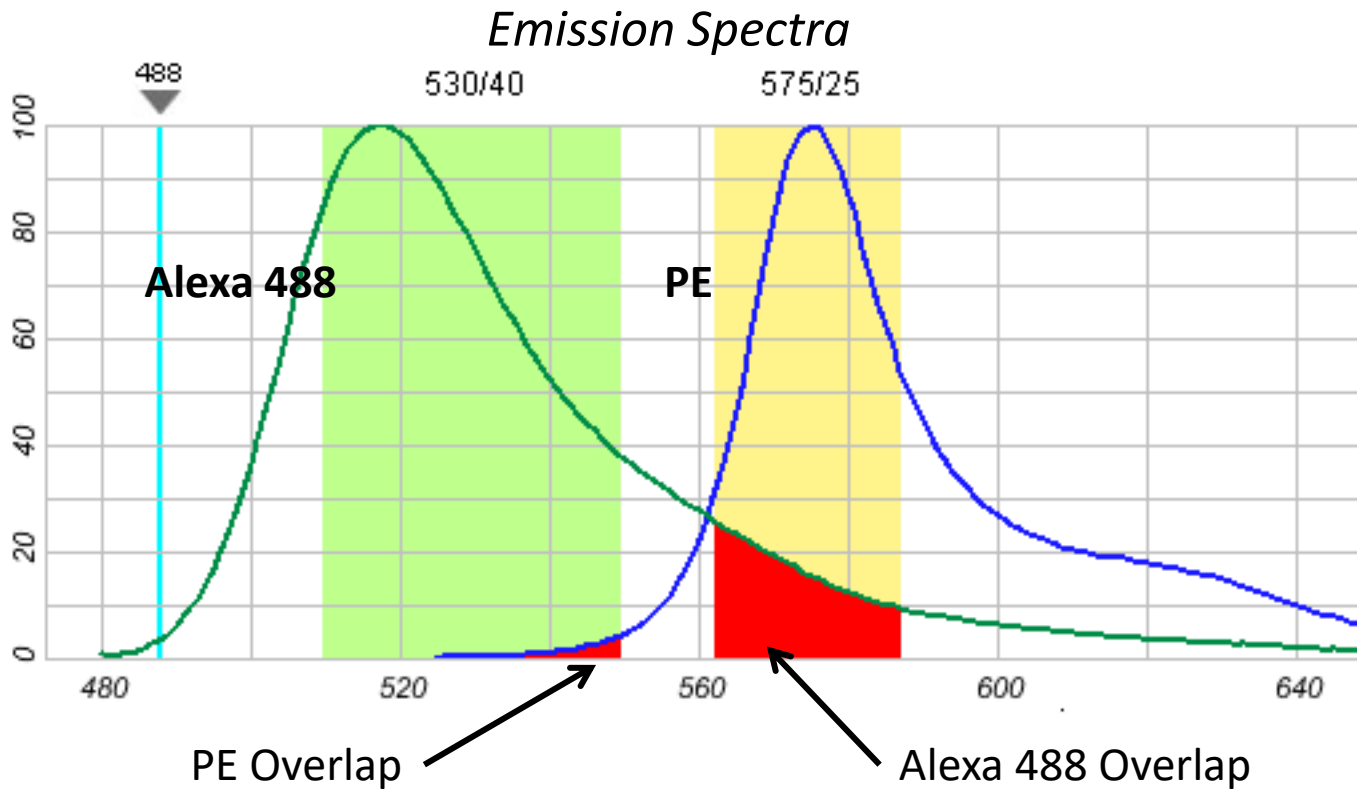
Reverse Pipetting



1. Attach tip to the pipette, press its knob to the second stop. Dip the tip into the solution to a certain depth according to the volume set.
2. Slowly release the pipette knob till the starting position. Withdraw the tip from the liquid.
3. Move the pipette to the receiving vessel and dispense the liquid by gently pressing the pipette knob to the first stop. Withdraw the tip from the liquid. Some liquid will remain inside the tip.
4. The liquid remaining in the tip can be dispensed back into the original solution or thrown away.
5. Release the pipette thumb to the ready position.

Fluorescence Compensation

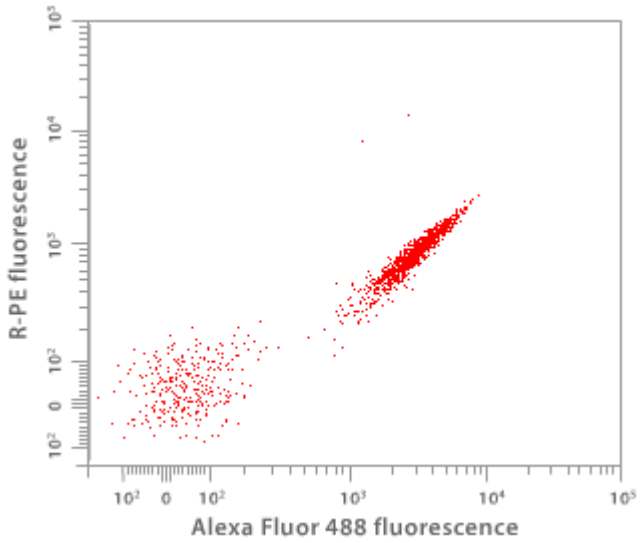


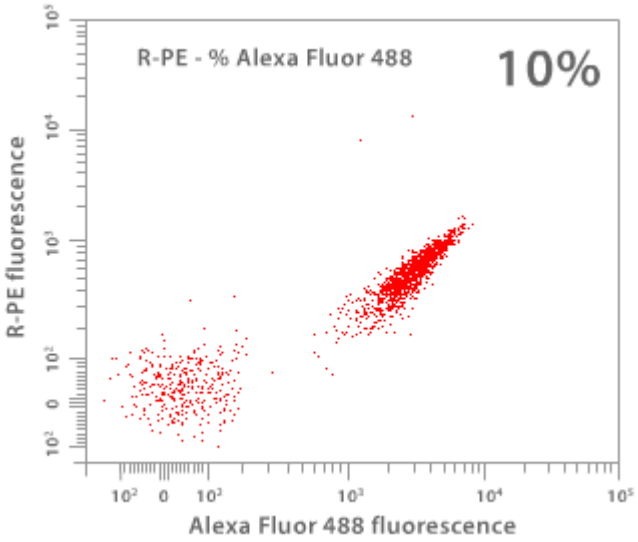
The amount of Alexa 488 signal overlapping into PE is a constant proportion of the measured Alexa 488 signal.

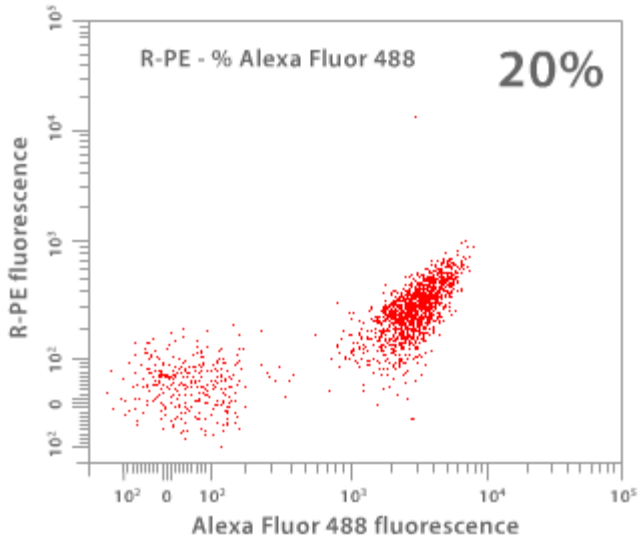
Measured PE = True PE + x%(Alexa 488) => True PE = Measured PE – x%(Alexa 488)

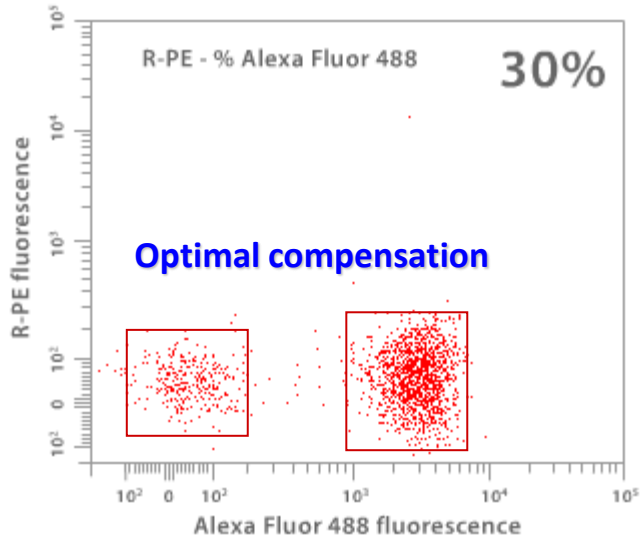
You can determine this ‘x’ percentage empirically and subtract out the Alexa 488 overlap from PE signal.

Single Colour Control…









These 2 gates should have the same mean PE intensity

Compensation in more than one fluorescence channel - PE