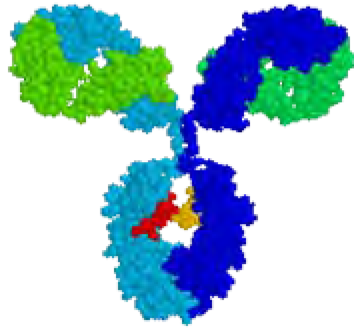
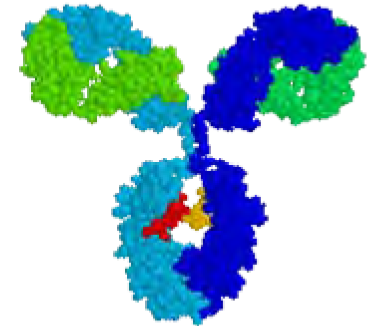
Immunophenotyping:

How distinguish cells from one another



Simon Monard

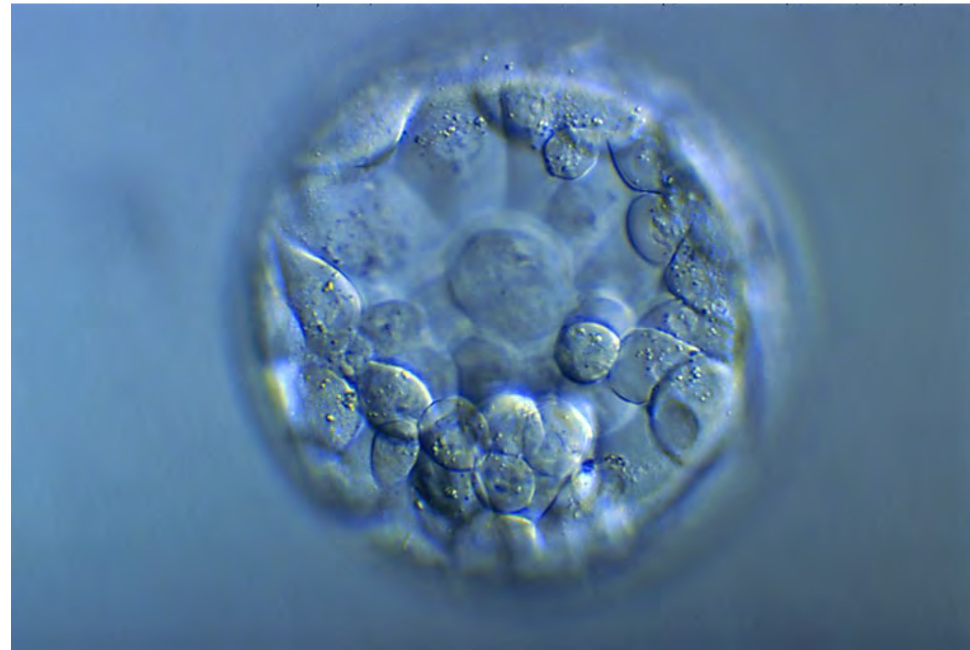
smonard@staffmail.ed.ac.uk



MRC Centre for Regenerative Medicine

The Challenge

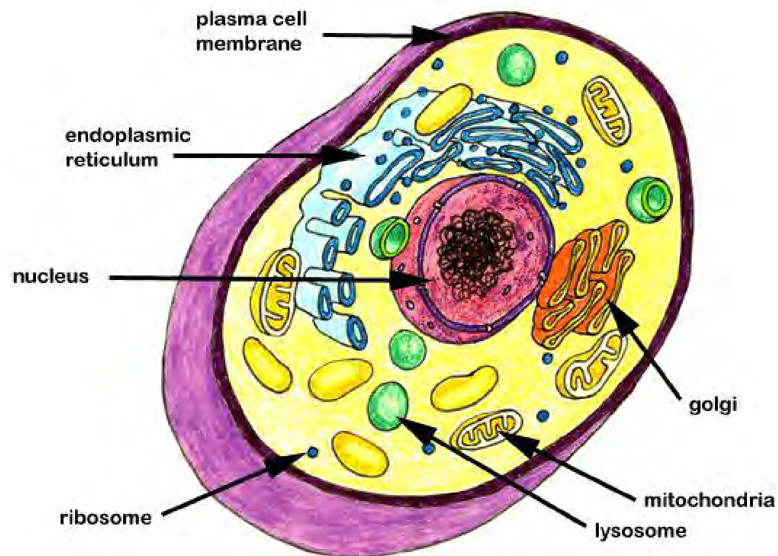
Biologists who study multicellular organisms often want to distinguish different types of cells from one another or identify cells at different stages of development, activation or differentiation. They may also want to physically separate such cells



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Cells



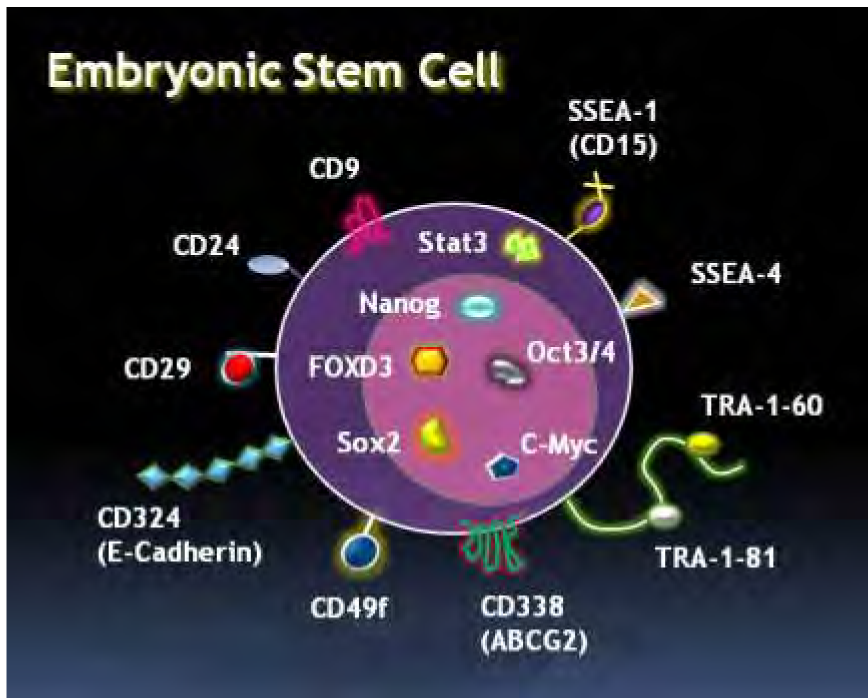
The different types of cells in our bodies are genetically identical but express different genes. The products of some of these genes will be proteins on the cell surface, some will be secreted and others will be proteins within the cell.

They dictate the function of the cell.

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Markers



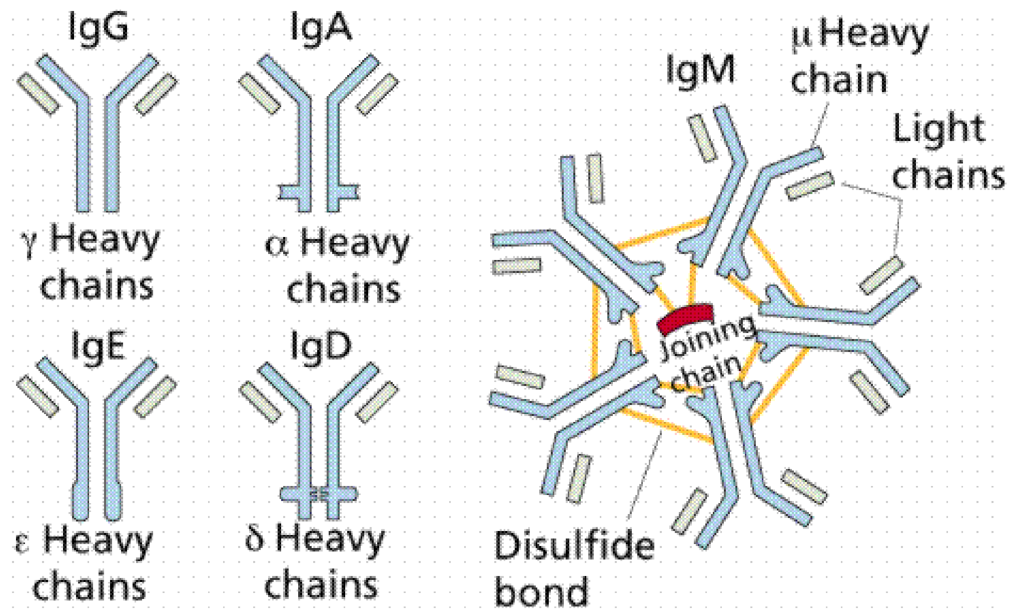
Identifiable proteins on the on or within cells are known as markers.

Mostly they are not unique to one cell type but certain combinations are used to confirm the identity of cells

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

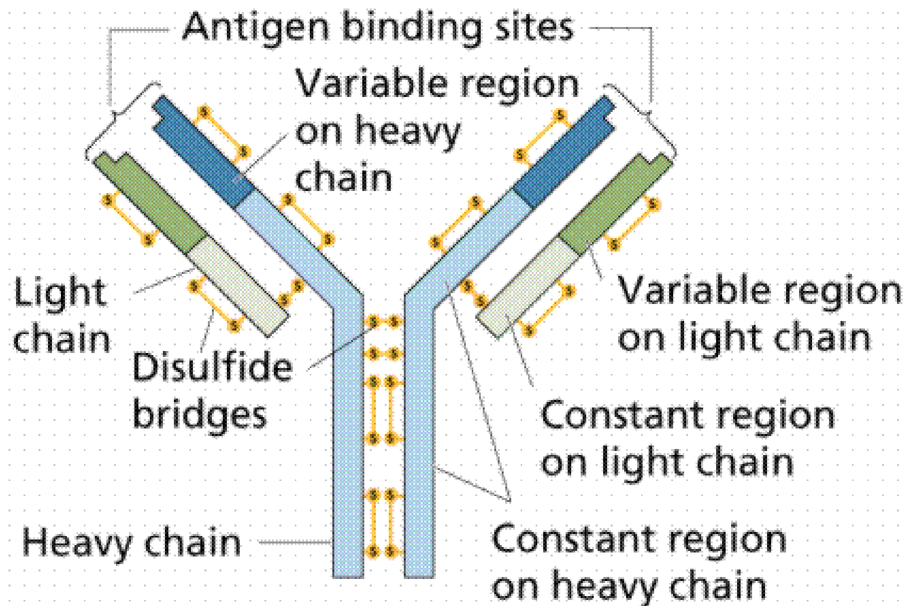
Antibodies (Immunoglobulins)



Centre for Regenerative Medicine
MRC

THE UNIVERSITY OF EDINBURGH

Antibodies (Immunoglobulins)



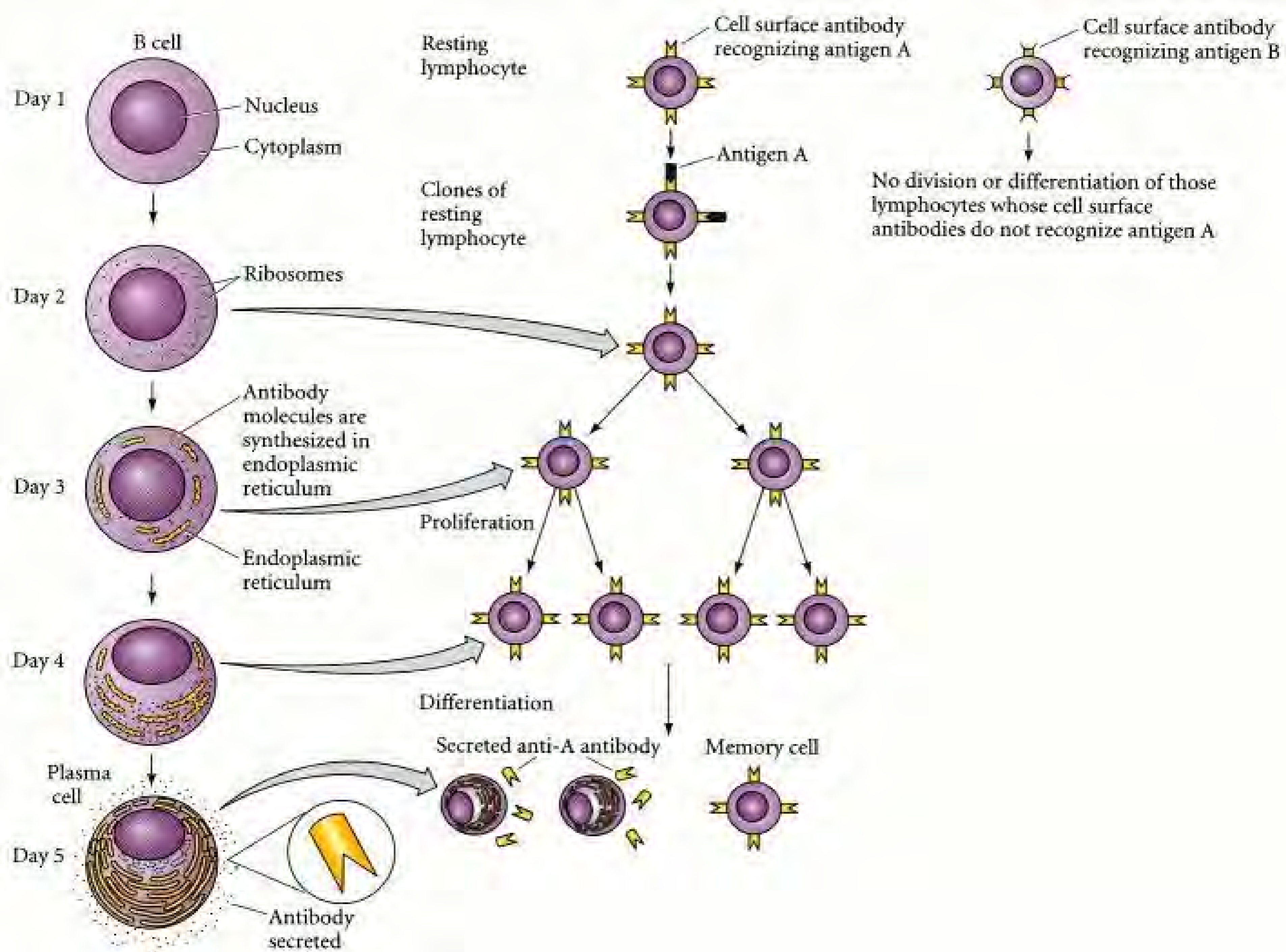
The genes that encode antibodies can be spliced together in various different ways giving a staggering 18 billion possible binding sites

MRC Centre for Regenerative Medicine

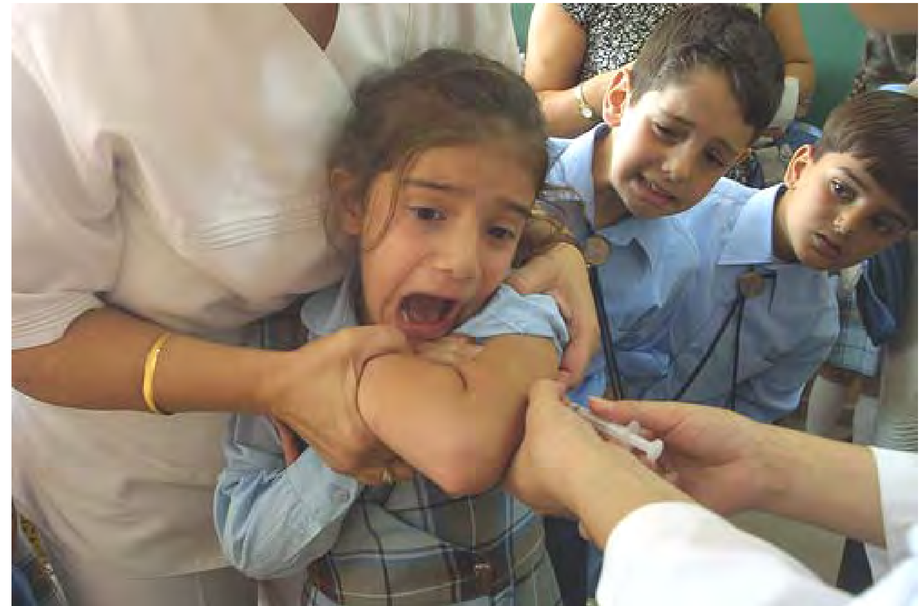
THE UNIVERSITY OF EDINBURGH

Antigens

An antigen is anything that can illicit an immune response, it could be bacterial, viral, a toxin, food, another persons cells, anything that isn't you and ends up inside you…
Many different antibodies may bind to one antigen, the precise bit they stick to is called an epitope. So an epitope fits snuggly into the binding site of an antibody.


B cell
Day 1
Nucleus
Cytoplasm
Day 2
Ribosomes
Day 3
Antibody molecules are synthesized in endoplasmic reticulum
Endoplasmic reticulum
Day 4
Plasma cell
Day 5
Antibody secreted
Resting lymphocyte
Cell surface antibody recognizing antigen A
Cell surface antibody recognizing antigen B
Antigen A
Clones of resting lymphocyte
No division or differentiation of those lymphocytes whose cell surface antibodies do not recognize antigen A
Proliferation
Differentiation
Secreted anti-A antibody
Memory cell

Production of Antibodies



MRC Centre for Regenerative Medicine

Nature. 1975 Aug 7;256(5517):495-7.

Continuous cultures of fused cells secreting antibody of predefined specificity.

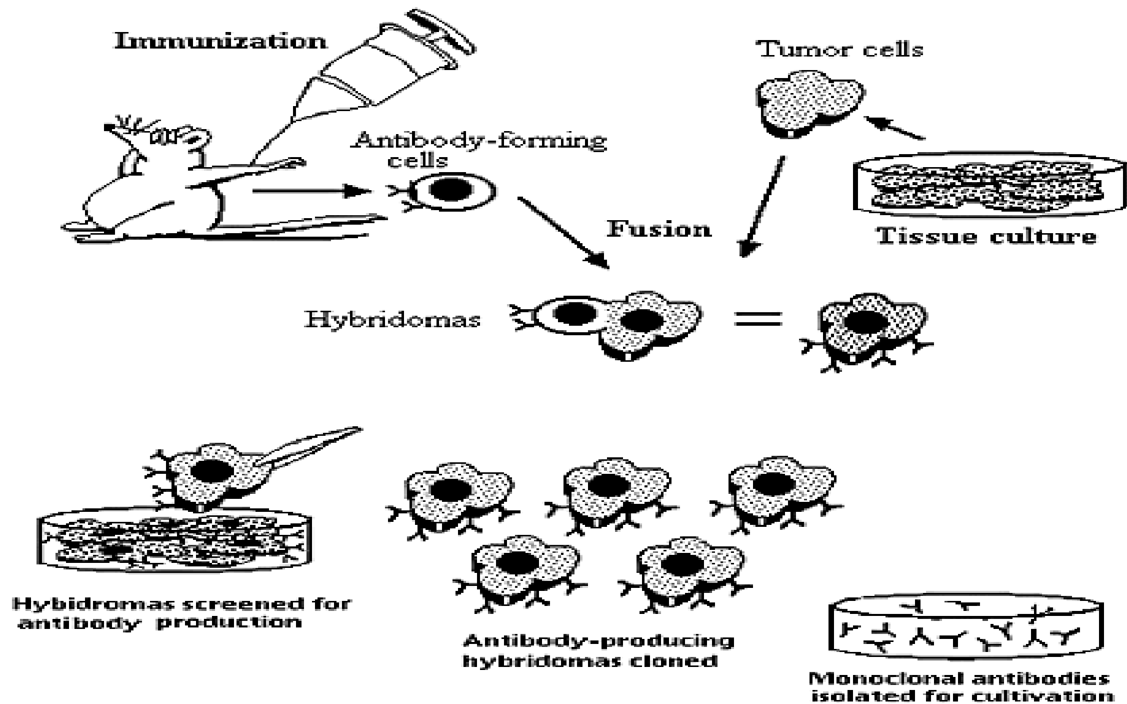
Köhler G, Milstein C.

Continuous cultures of fused cells secreting antibody of predefined specificity

THE manufacture of predefined specific antibodies by means of
permanent tissue culture cell lines
are at present a considerable numb
myeloma cells[1,2] and screening pr
reveal antibody activity in some
not a satisfactory source of monocl
specificity. We describe here the
tissue culture cell lines which se
cell (SRBC) antibodies. The cell li
mouse myeloma and mouse splee
donor. To understand the express
Ig chains from the parental lines,
two known mouse myeloma lines
Each immunoglobulin chain r
expression of one of several *V* and
for its variable and constant sectio
one of the two possible alleles (al
ref. 3). When two antibody-proc
products of both parental lines ar
the light and heavy chains of both
joined, no evidence of scramblin

The protein secreted (MOPC 21) is an IgG1 (κ) which has been fully sequenced[7,8]. Equal numbers of cells from each parental line were fused using inactivated Sendai virus[9] and samples contining 2×10^5 cells were grown in selective medium in separate dishes. Four out of ten dishes showed growth in selective medium and these were taken as independent hybrid
karyotype
under the
shows the
roducts of
1) give a
nd *b*) or a
ure of the
, arrows).
result of
the IEF
the heavy
ins of the
f the IEF
with no
previously
cules are
heavy and
tracellular
ch hybrid
cells must

Monoclonal Antibodies



MRC Centre for Regenerative Medicine

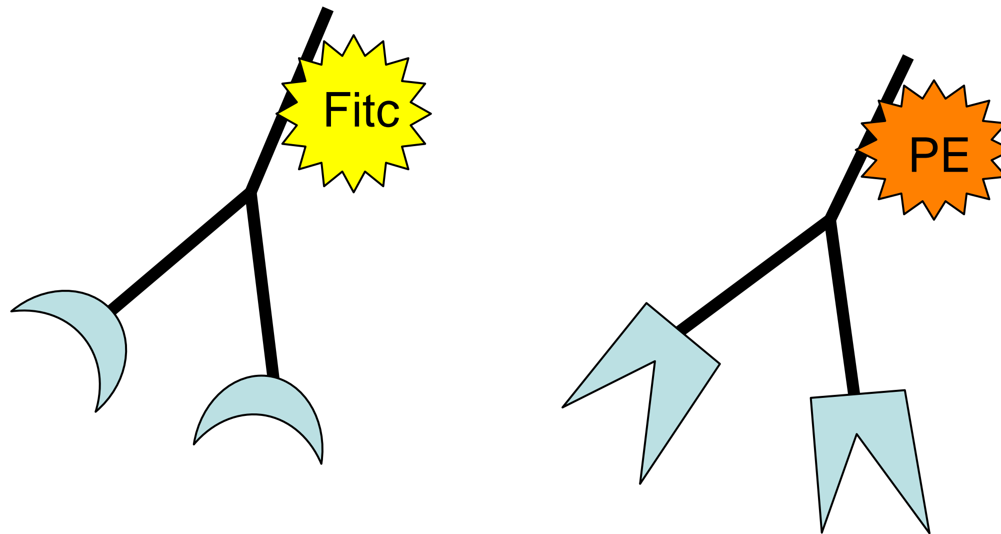
THE UNIVERSITY OF EDINBURGH

Fluorescent Dyes for Labeling Antibodies

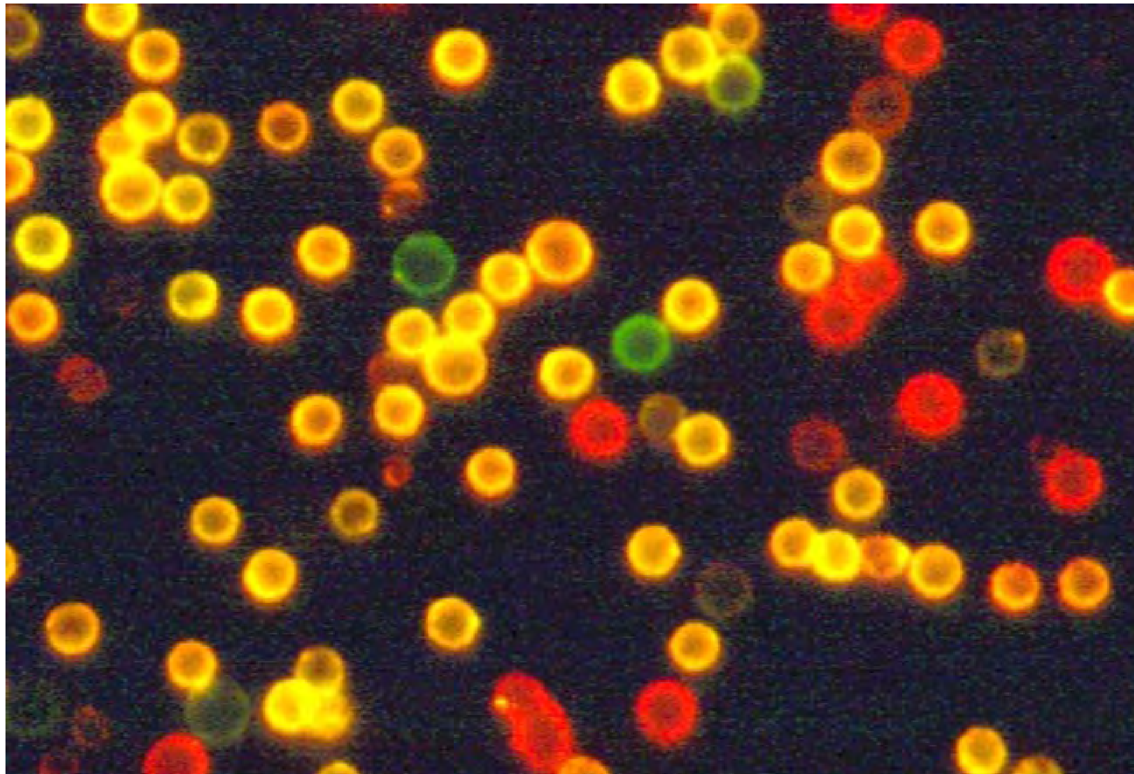
Fluorescein(Fitc)
Phycoerythrin(PE)
Allophycocyanin(APC)
Peridinin chlorophyll(PerCp)
PE Tandems
APC Tandems
Nano Crystalls (q-dots)
Pacific blue
Alexa family
E-fluor family
Brilliant violets etc

MRC | Centre for Regenerative Medicine

Labeling Antibodies



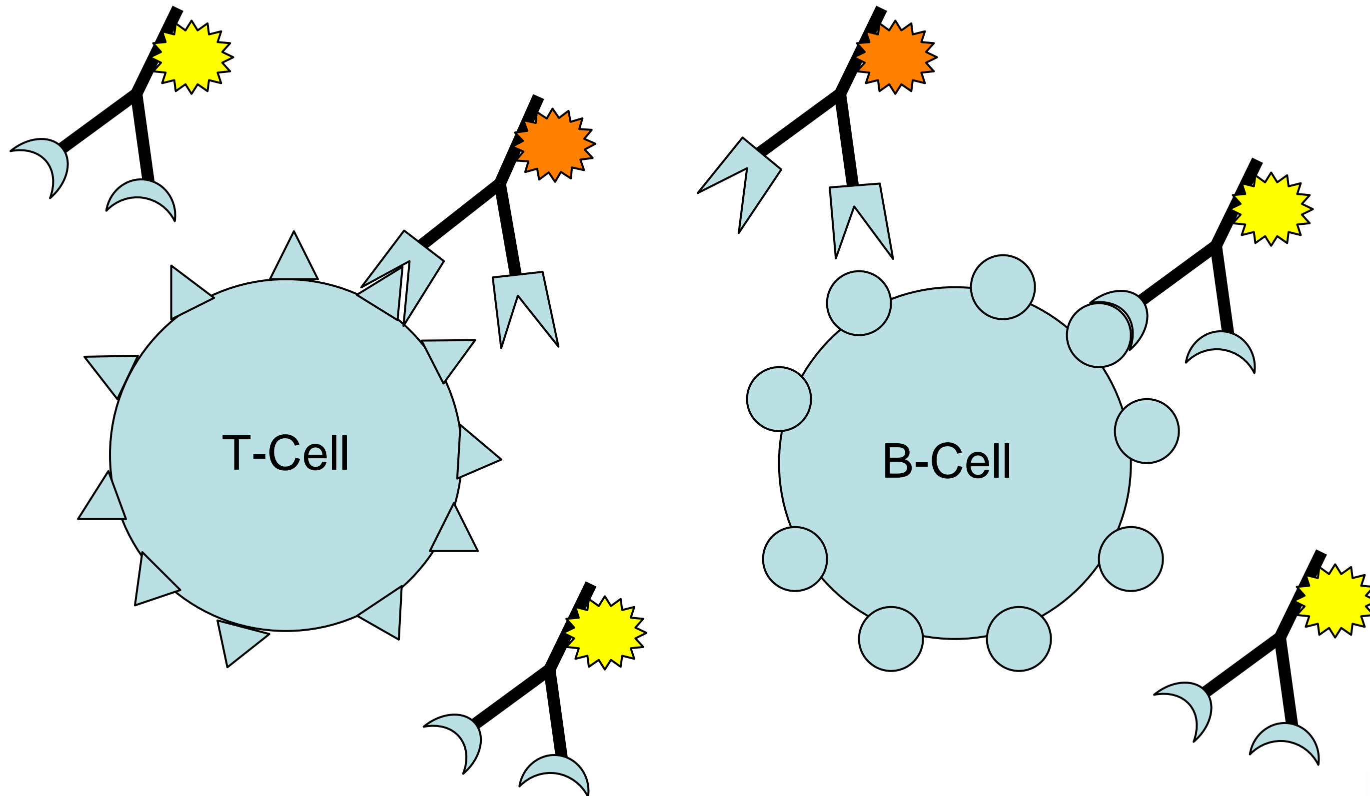
Labeled antibodies



Centre for Regenerative Medicine
MRC

THE UNIVERSITY OF EDINBURGH

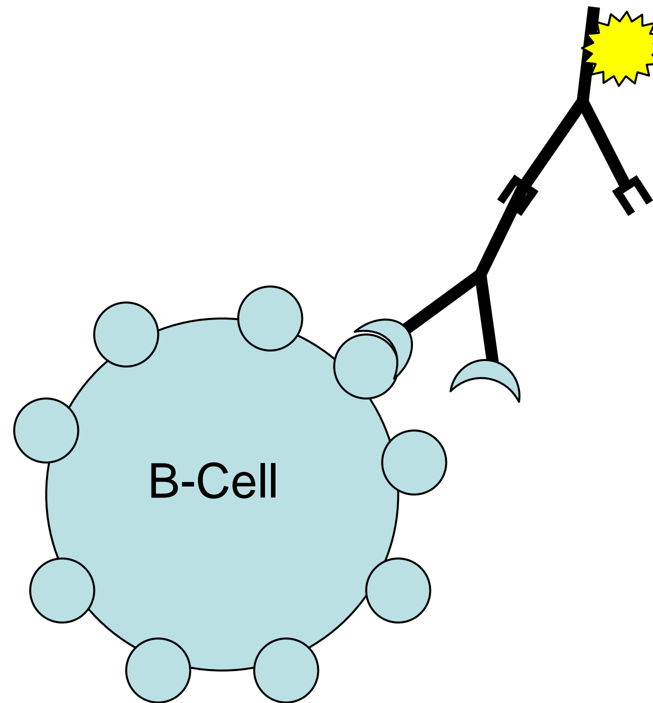
Labelled antibodies
(Direct Immunofluorescence)



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

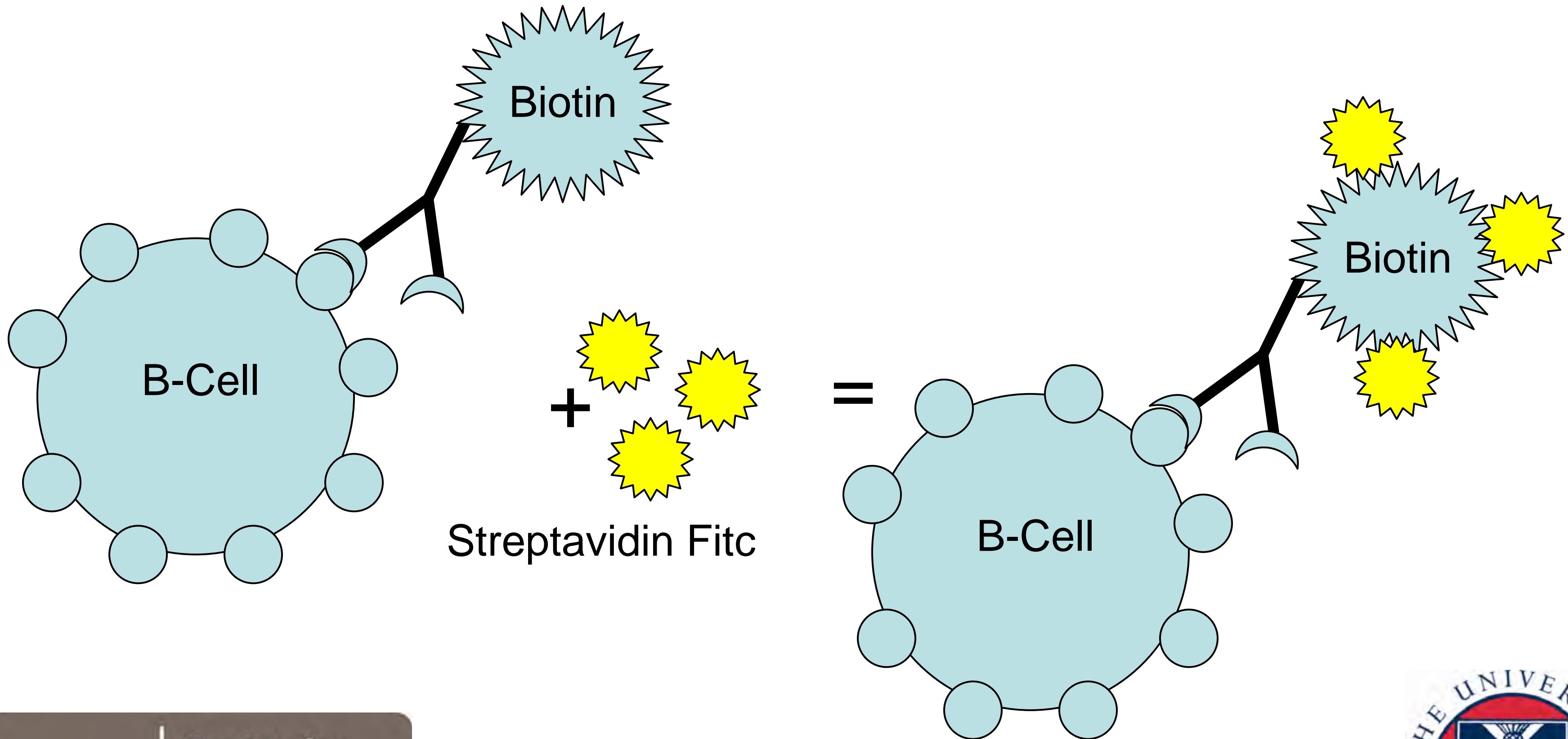
Unlabelled antibodies
(indirect immunofluorescence)



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

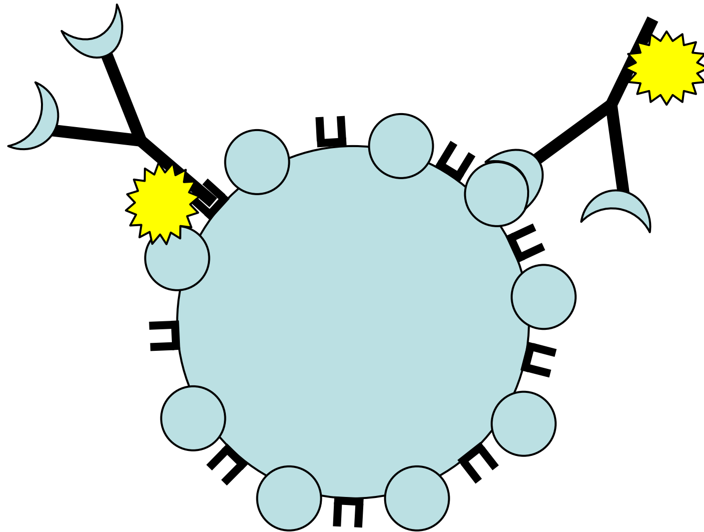
Biotinylated antibodies



Centre for Regenerative Medicine
MRC

THE UNIVERSITY OF EDINBURGH

Non Specific Binding, Fc receptors

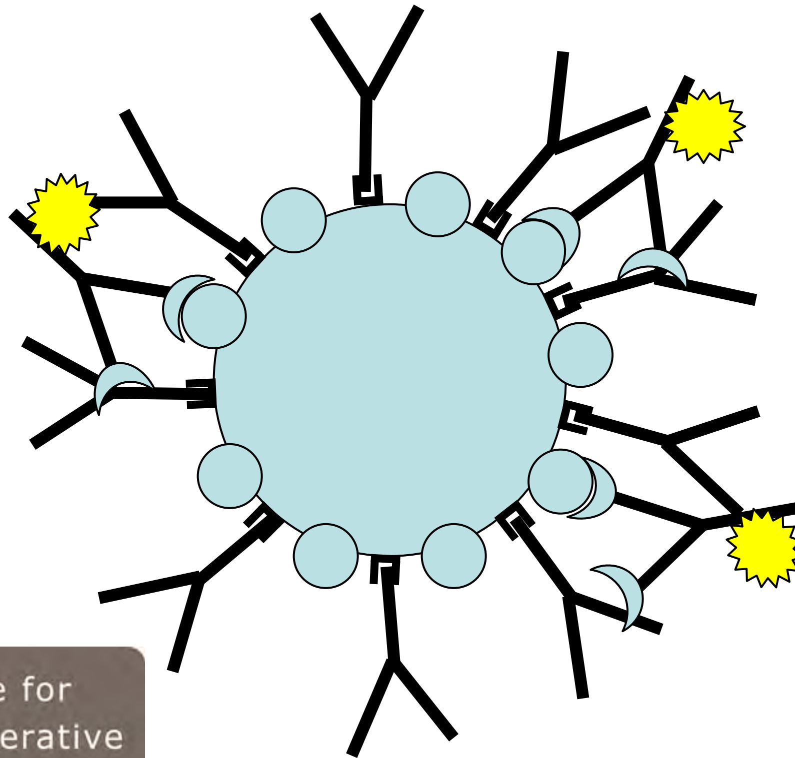


Centre for Regenerative Medicine

MRC

THE UNIVERSITY OF EDINBURGH

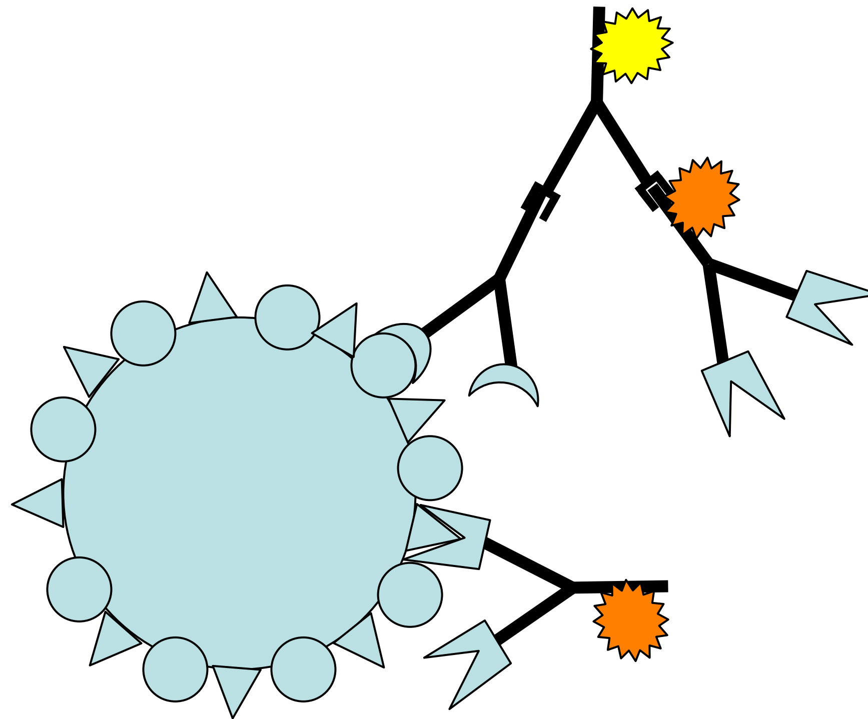
Non Specific Binding, Fc receptors



Centre for Regenerative Medicine
MRC

THE UNIVERSITY OF EDINBURGH

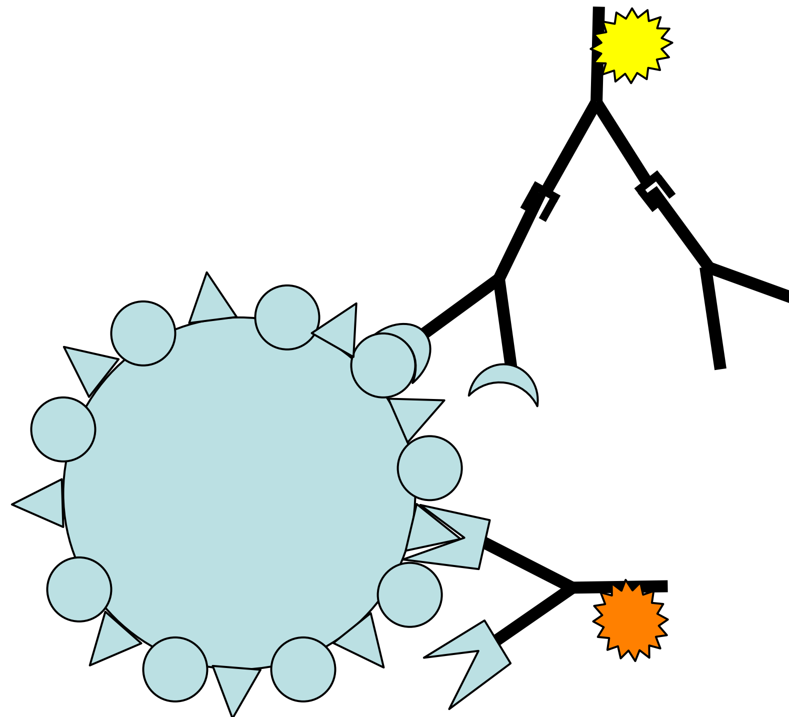
Unlabelled and directly labeled antibodies



MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Unlabelled and directly labeled antibodies

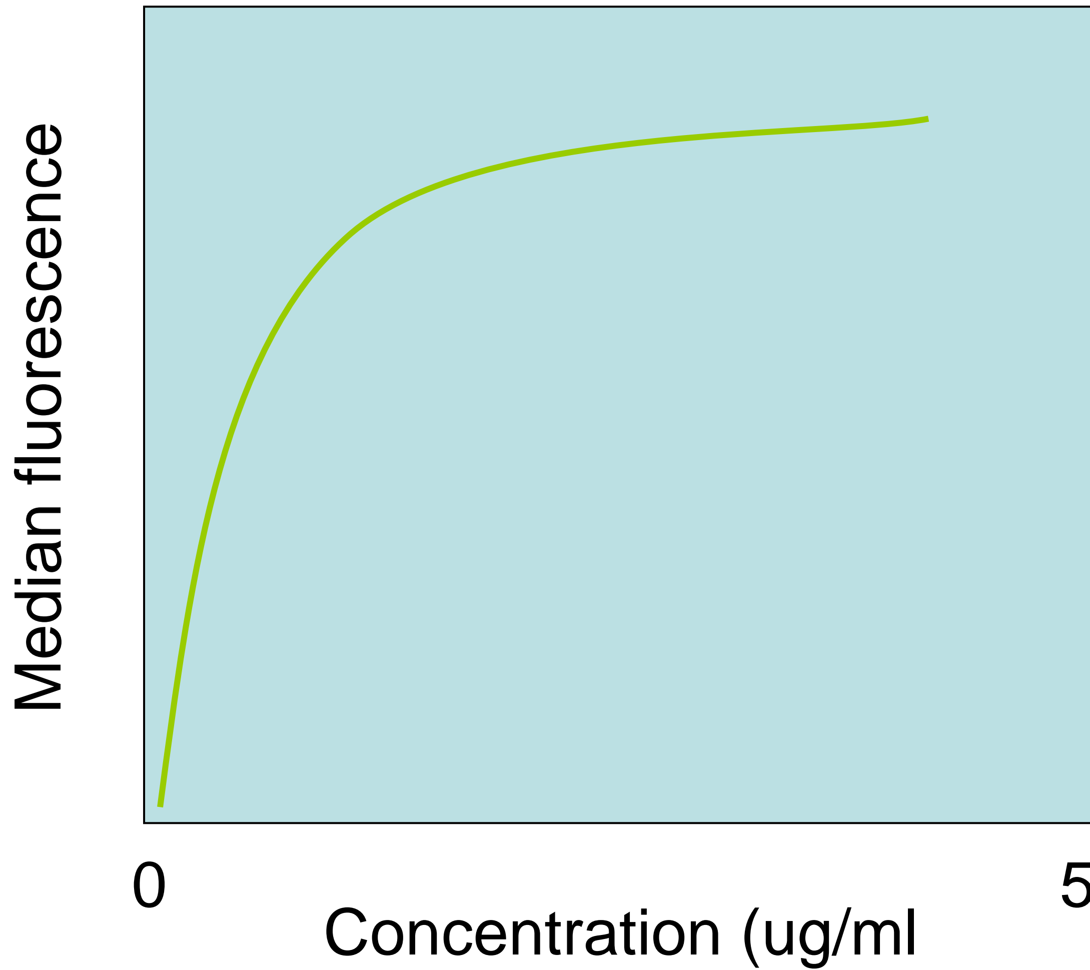


MRC Centre for Regenerative Medicine

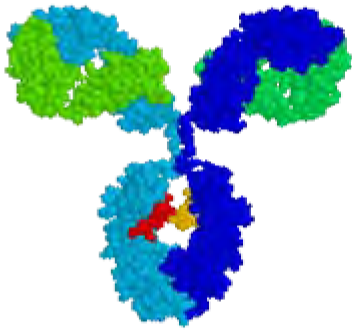
THE UNIVERSITY OF EDINBURGH

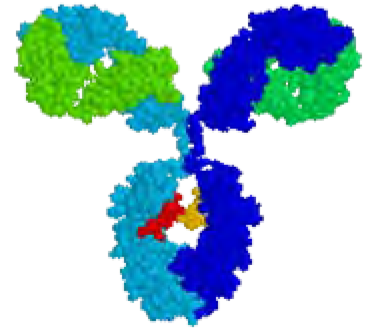
ISCR
INSTITUTE FOR STEM CELL RESEARCH

Titration Curve



Immunophenotyping Controls





Controls

"Negative Control"
Positive Control
Controls for Spectral Overlap
FMO Controls
Biological controls

MRC | Centre for Regenerative Medicine

Biological Controls

- This could be untreated cells, uninfected animals, wild type animals etc

MRC Centre for Regenerative Medicine
THE UNIVERSITY OF EDINBURGH

The “Negative” Control

Unstained cells

Isotype matched antibodies of no know specificity..

Both

Other

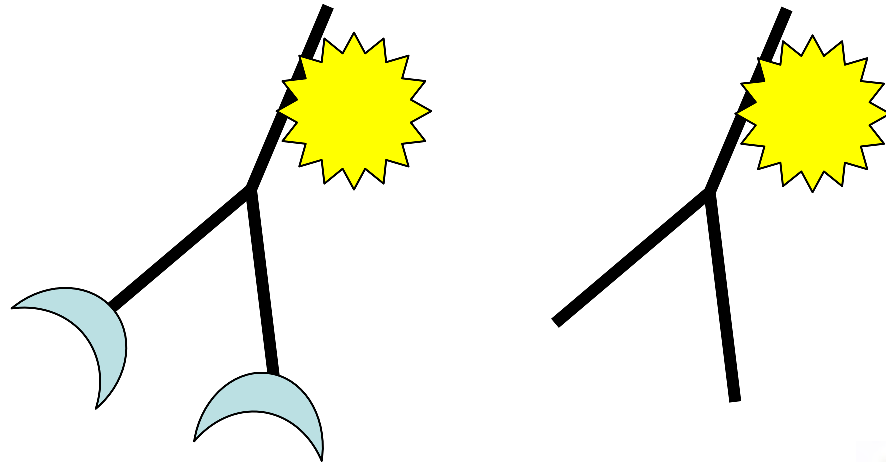
MRC | Centre for Regenerative Medicine

Isotype Controls

Their use is controversial

Difficult to match the F:P ratio

Reviewers like them

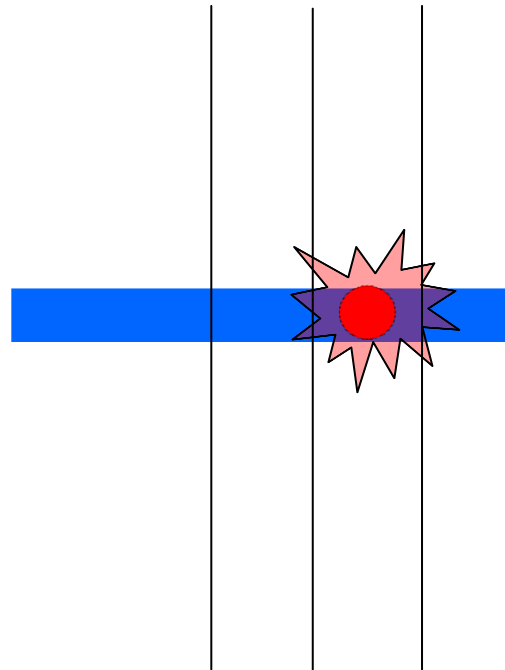


MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Non-specific fluorescence

When cells appear to bind antibody "non-specifically" People blame Fc receptors or "stickiness" of cells. Its often unbound antibody

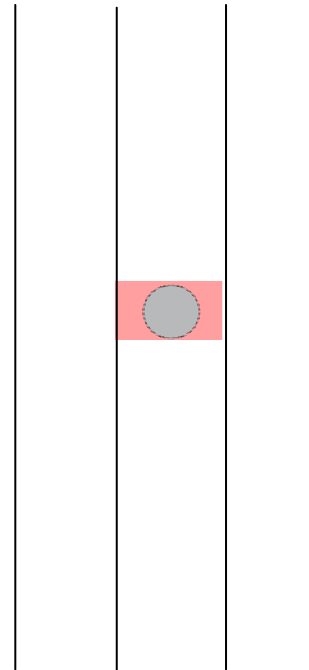


MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Non-specific fluorescence

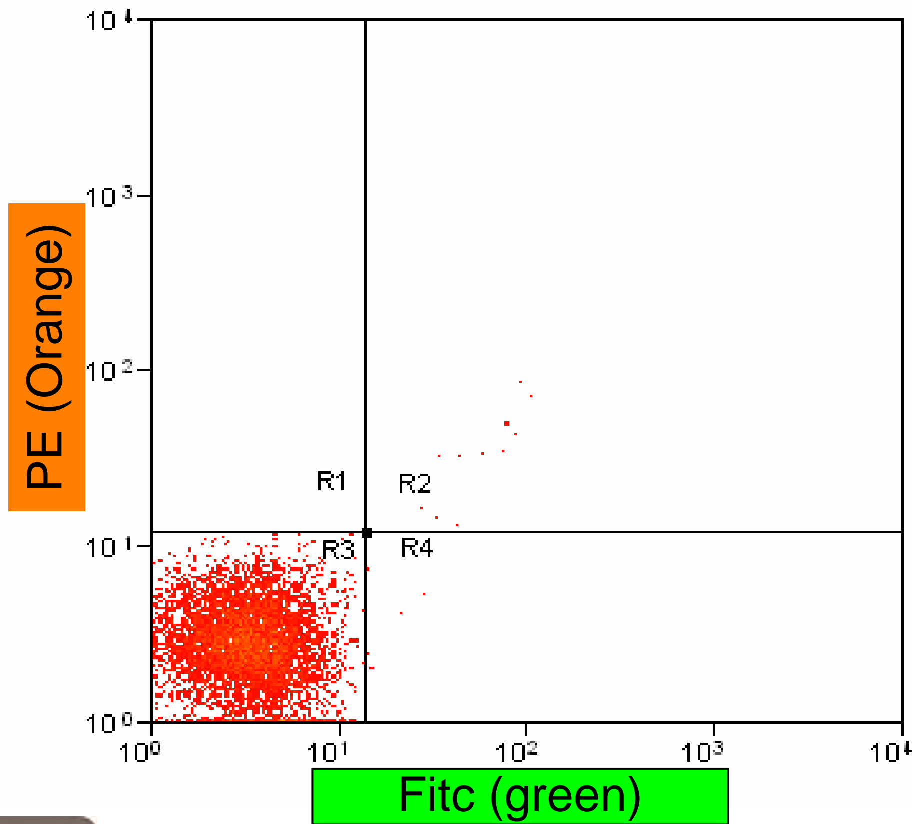
When cells appear to bind antibody "non-specifically" People blame Fc receptors or "stickiness" of cells. Its often unbound antibody



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

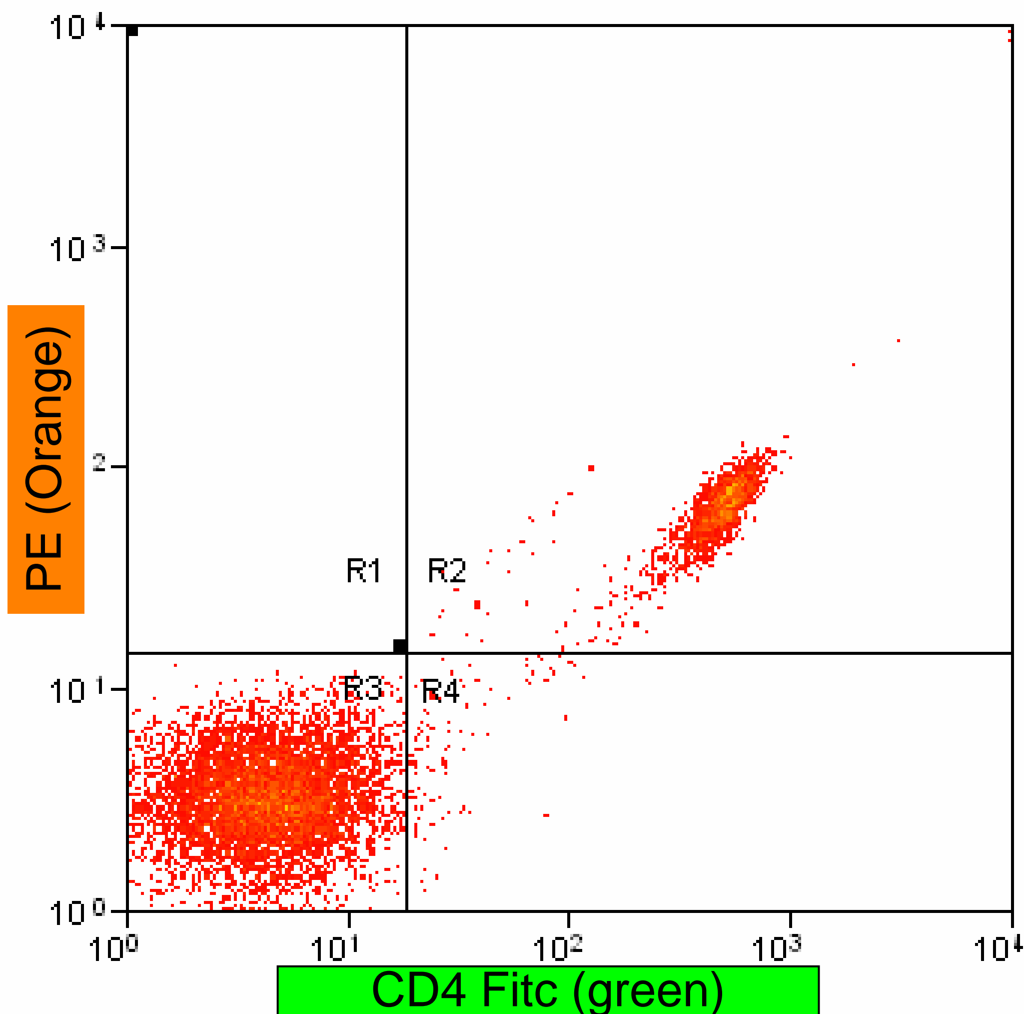
Negative control



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Spectral Overlap

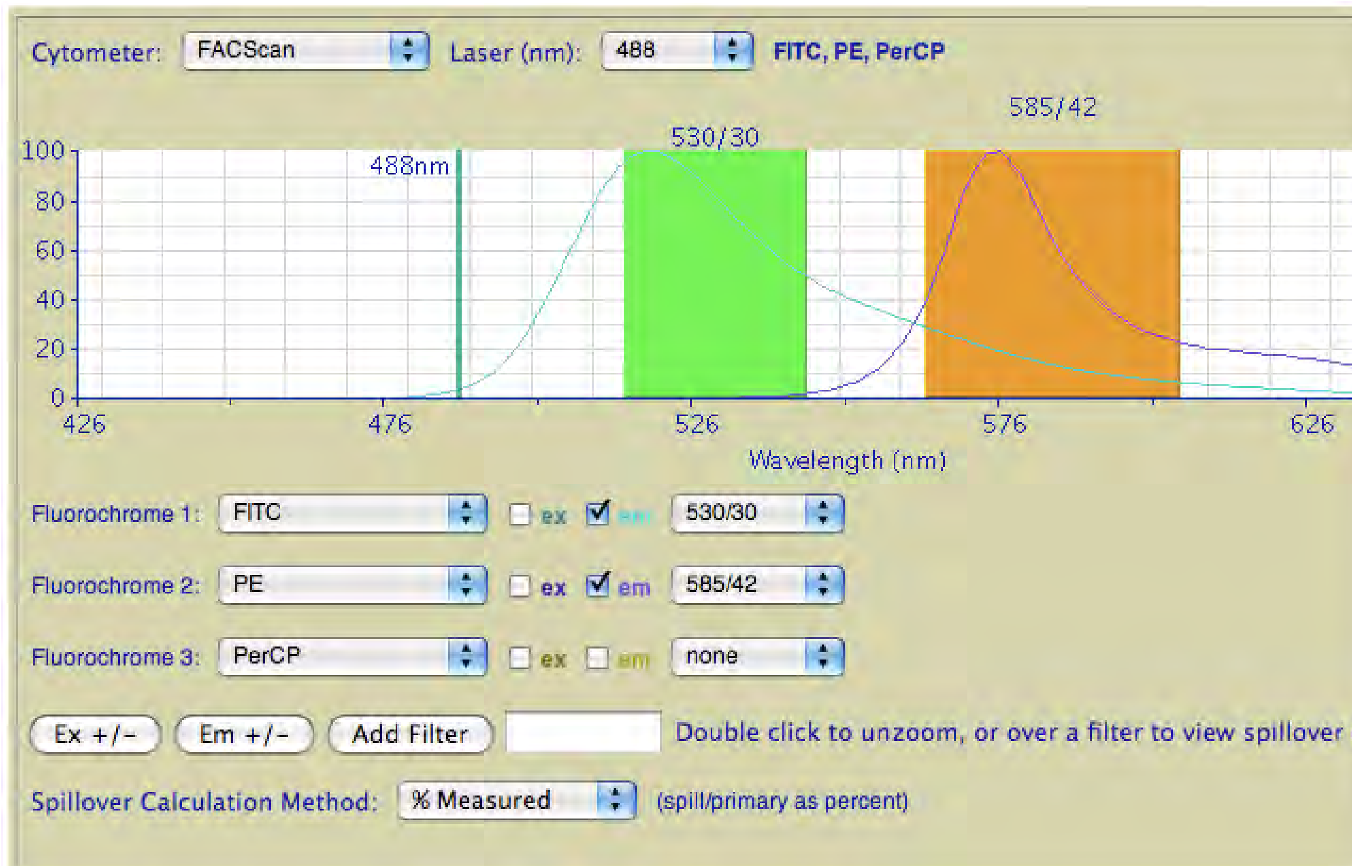


Re...	Mean	Median
Total	99.81, 17.21	5.27, 3.54
R1	0.00, 0.00	0.00, 0.00
R2	503.28, 75.74	498.93, 66.01
R3	5.09, 3.49	4.40, 3.18
R4	33.38, 6.21	24.01, 5.27

MRC Centre for Regenerative Medicine

The University of Edinburgh

Spectral Overlap



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

The Solution: Colour Compensation

In the example on the previous slide, some of the PE signal is actually from the Fitc fluorescence.

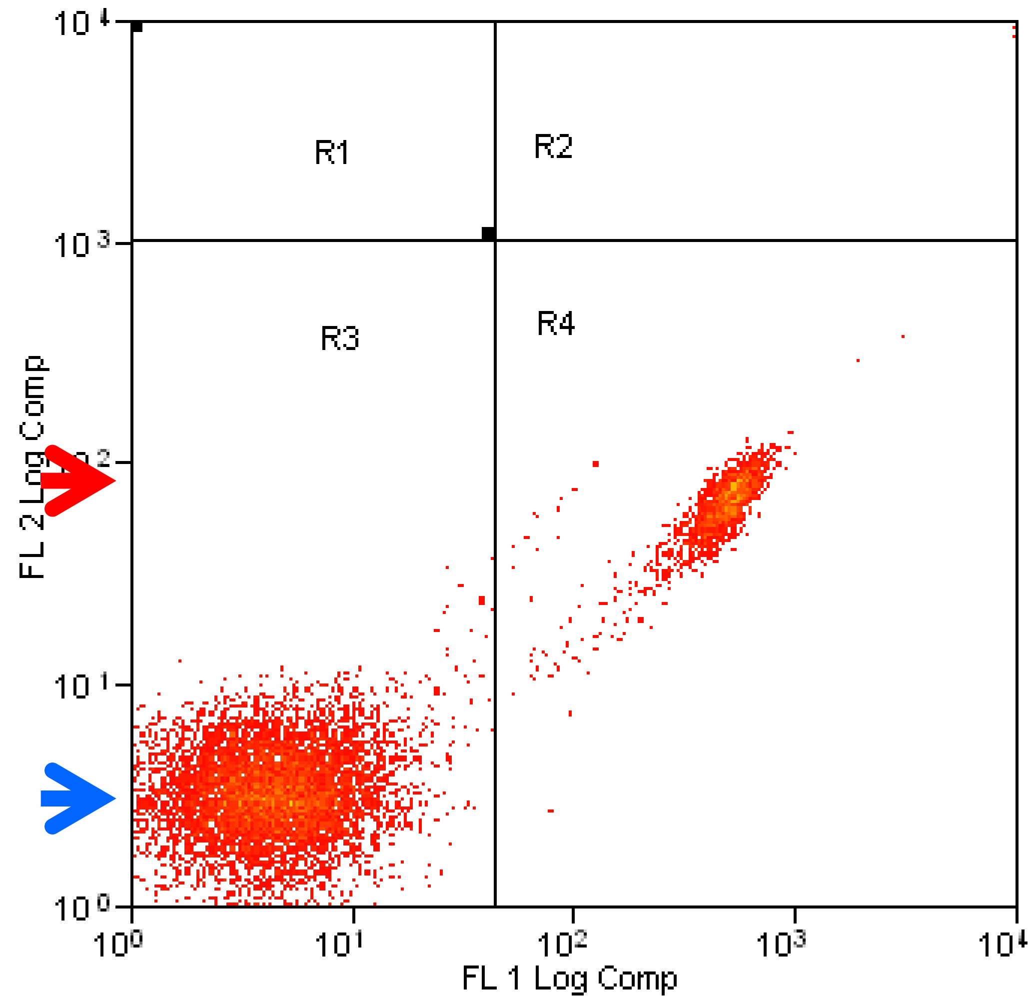
So, we subtract about 20% of the FITC signal from the PE signal.

Written FL2-%FL1 so in this case its FL2-20%FL1

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Spectral Overlap

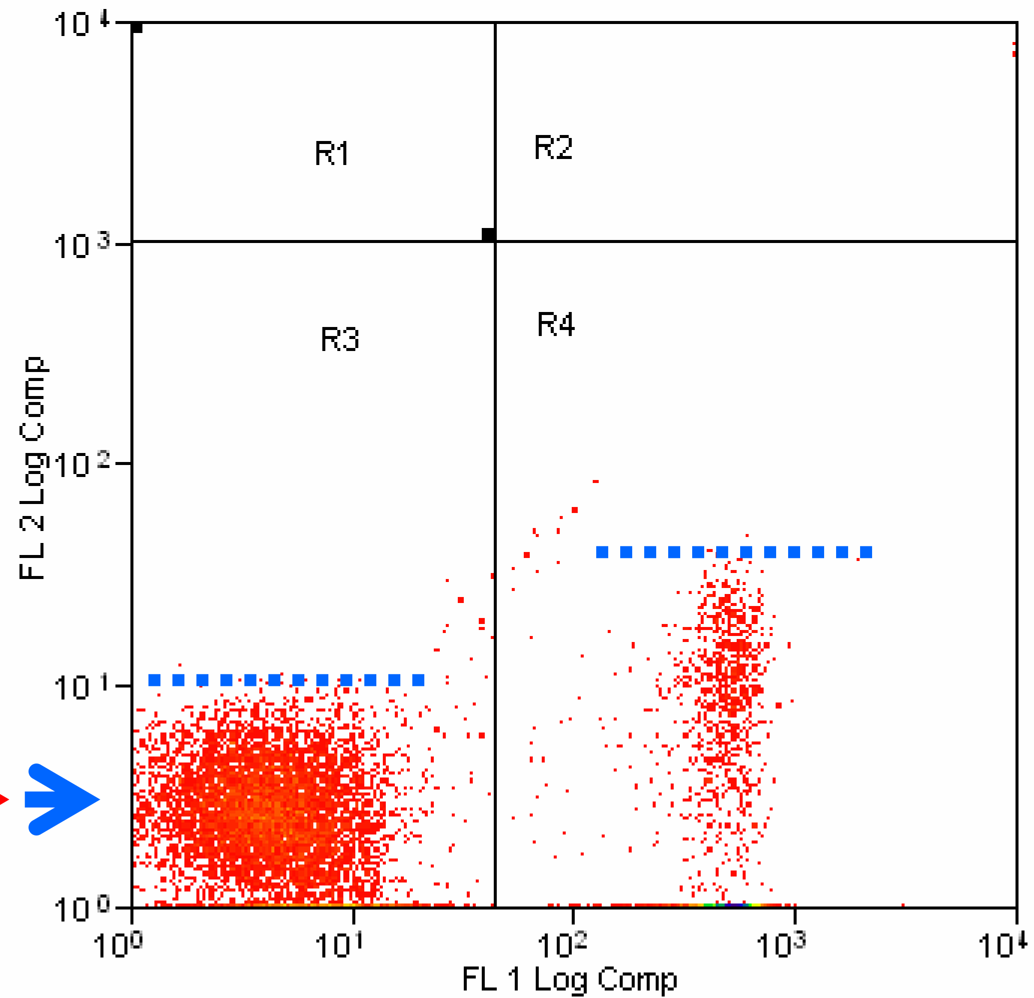


Re...	Mean	Median
Total	99.81, 17.21	5.27, 3.54
R1	0.00, 0.00	0.00, 0.00
R2	10000.01, 8978.93	10000.01, 8654.76
R3	5.37, 3.55	4.40, 3.18
R4	492.00, 65.50	498.93, 66.01

MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Real World



Re...	Mean	Median
Total	99.81, 5.23	5.27, 2.56
R1	0.00, 0.00	0.00, 0.00
R2	10000.01, 7638.19	10000.01, 7224.77
R3	5.37, 2.90	4.40, 2.56
R4	492.00, 6.61	498.93, 2.56

Spread

- The more compensation required and the brighter the signal the greater the spread.
- Spread is a result of errors incurred with photon counting statistics and cannot be corrected for using compensation

Compensation

- With a multicolour experiment it is essential that the colour compensation is done correctly to avoid:
- Incorrect interpretation of data
- Scorn of those who know how to do it correctly

The Three Commandments

1. The positive and negative particles should have the same background fluorescence.

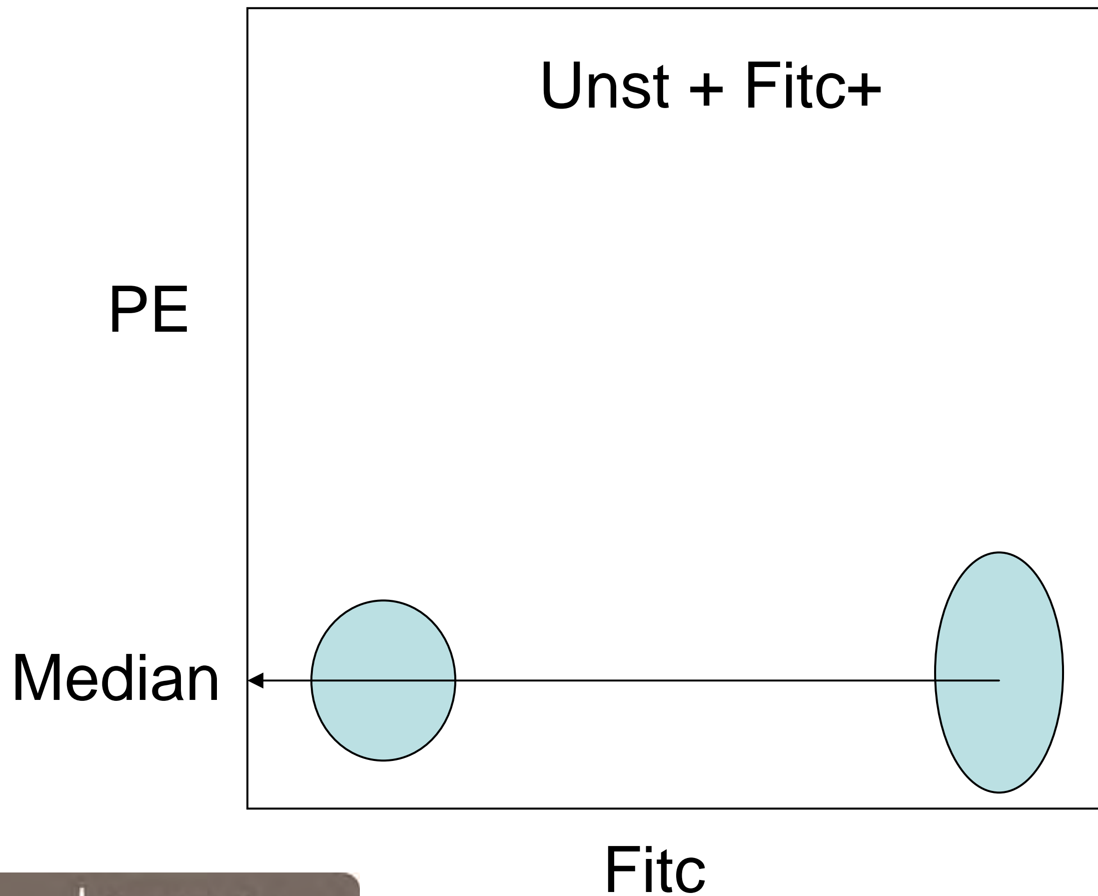
2. The positive particles should be at least as bright as anything in your samples

3. The fluorochrome should be exactly the same as that used in your experiment. Tandem dyes should be same batch.

MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

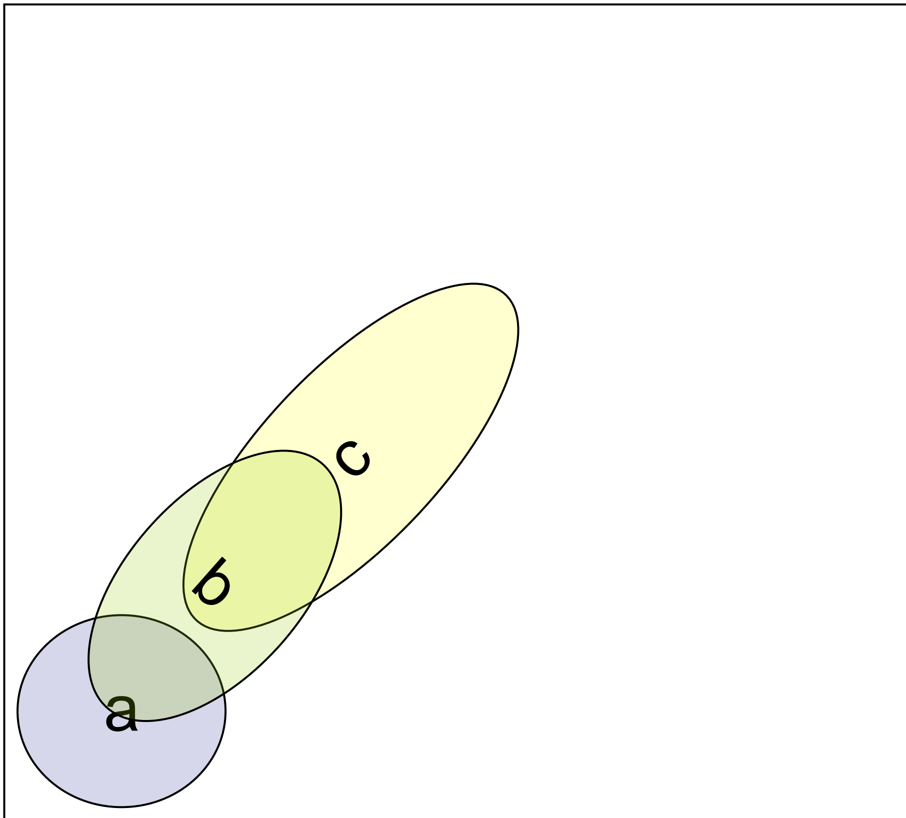
Simple Compensation



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Autofluorescence

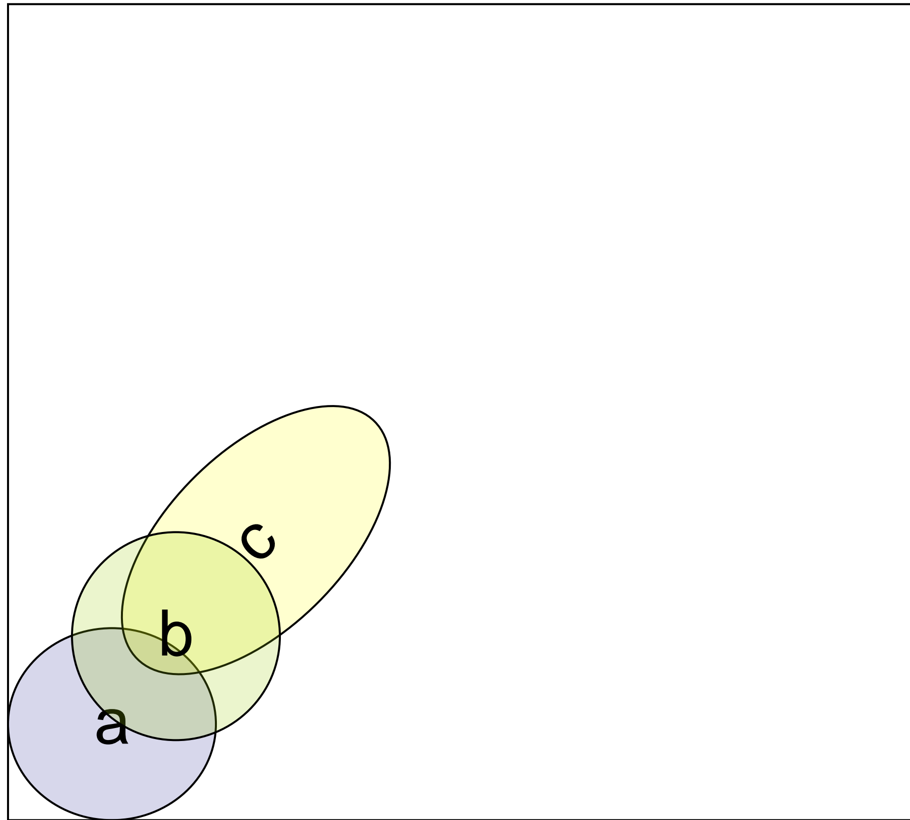


Different cell types may have different levels of autofluorescence. In general the bigger they are the more they have

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

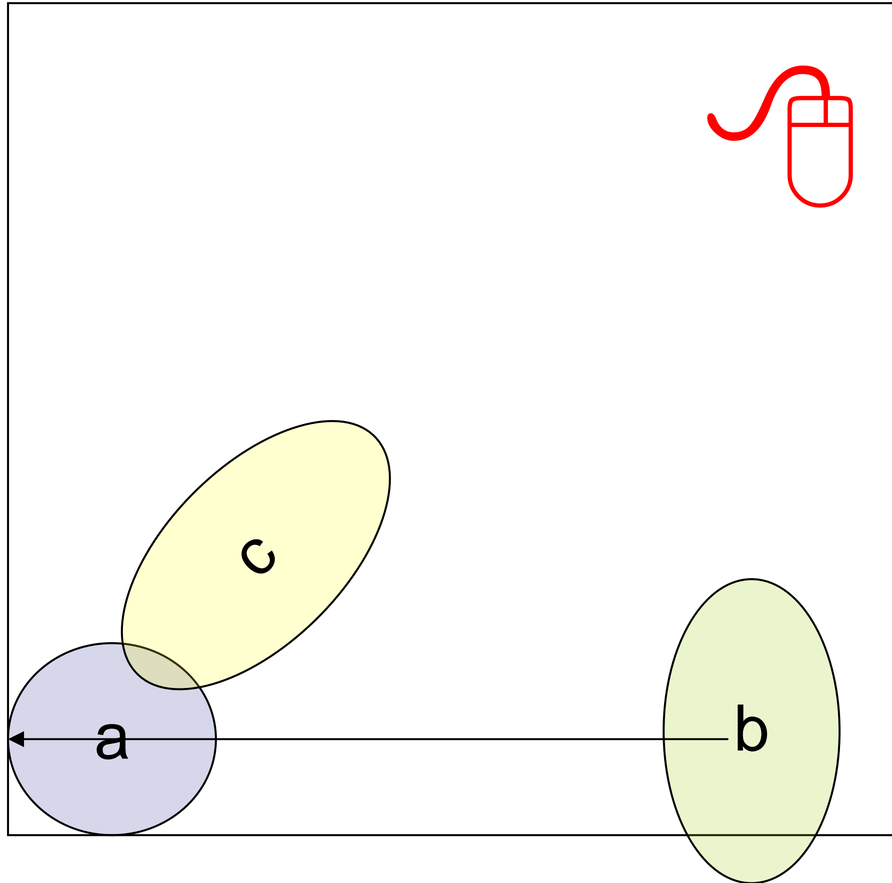
Cell Mixture Negative



MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

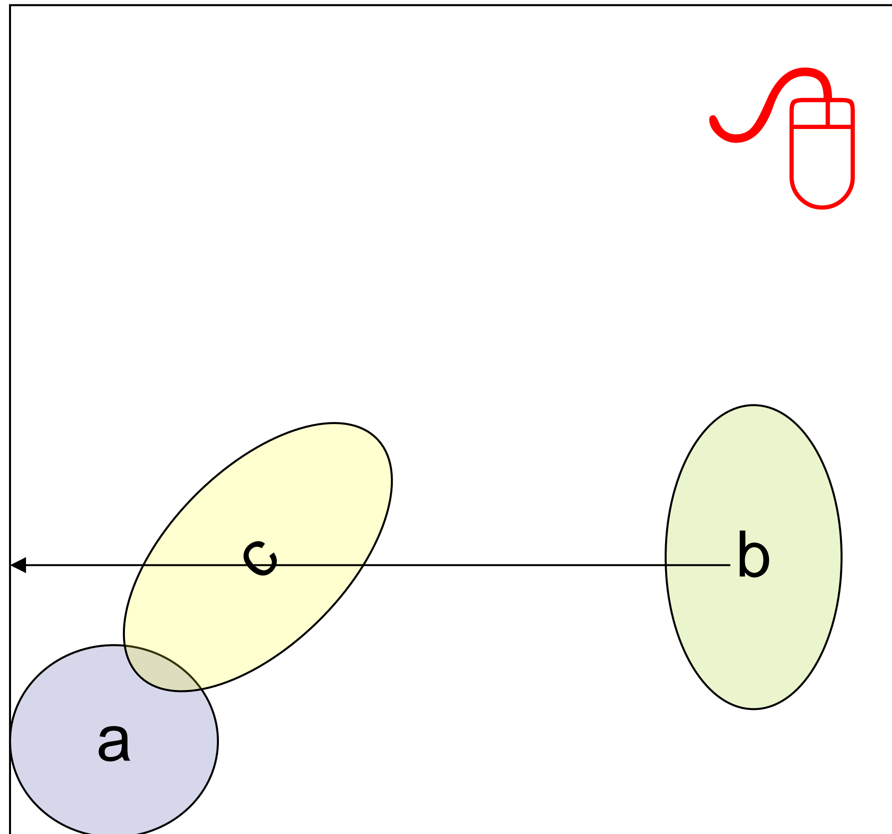
Compensated?



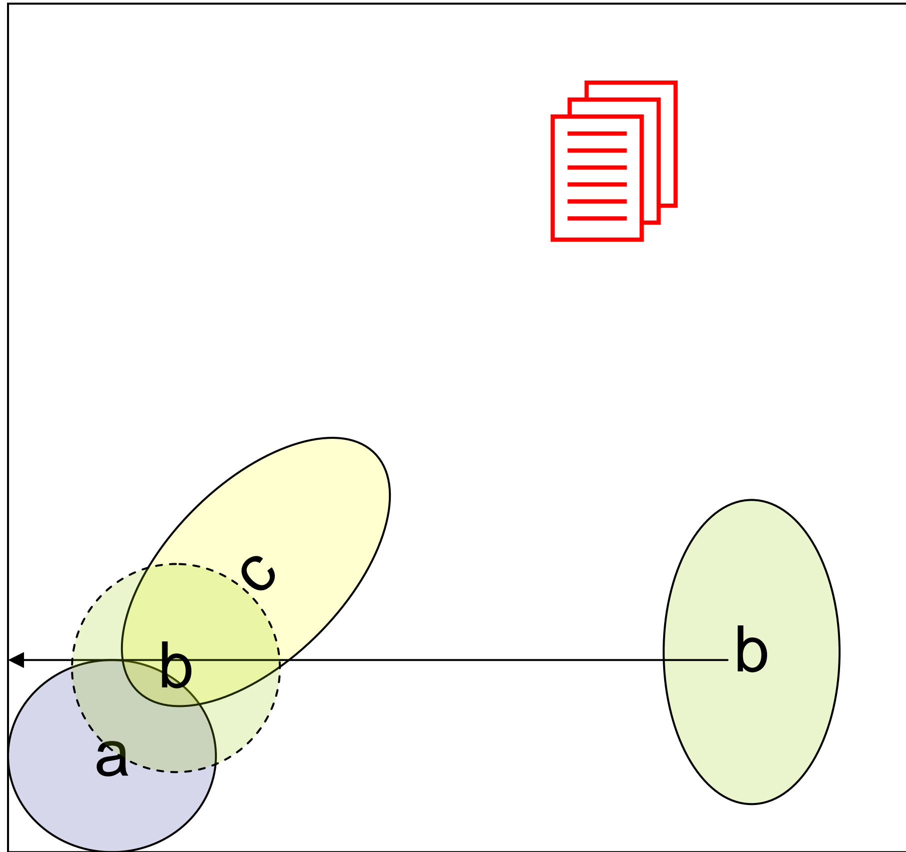
MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Compensated?



Compensated



MRC | Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

So:

You really can't use stained cells as compensation controls when you have a mixture of cell types.

Also the cells stained with your antibodies may be very infrequent.

Antibody Capture beads

The solution to this problem is to use antibody capture beads.

They are available against mouse, rat and hamster Igs

Some are against the kappa chain which means they bind most but not all antibodies.

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Antibody Capture beads

They provide a clear negative and positive population.

They are bright and easy to use.

Saves sample.

MRC | Centre for Regenerative Medicine

Polystyrene beads

Carboxylated polystyrene beads are available in a variety of sizes.

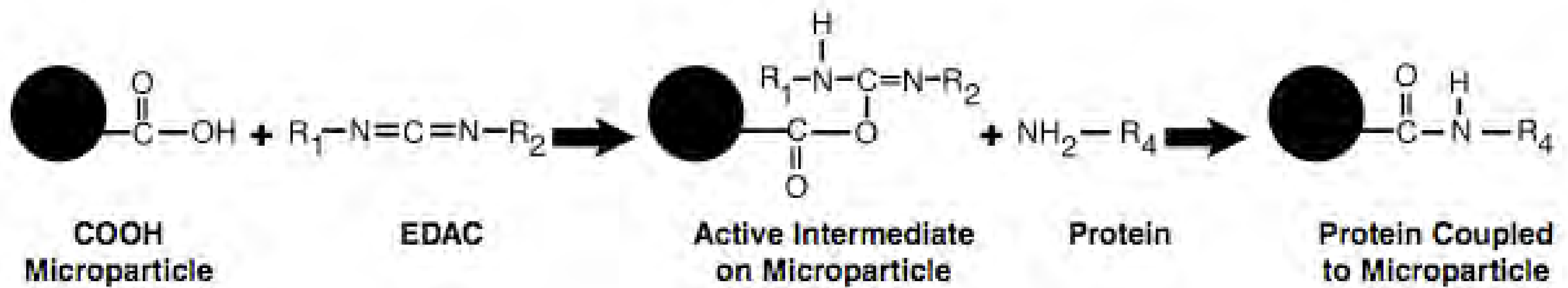
They can be bound to any protein with a simple reaction.

The whole procedure takes about 2 hours.

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

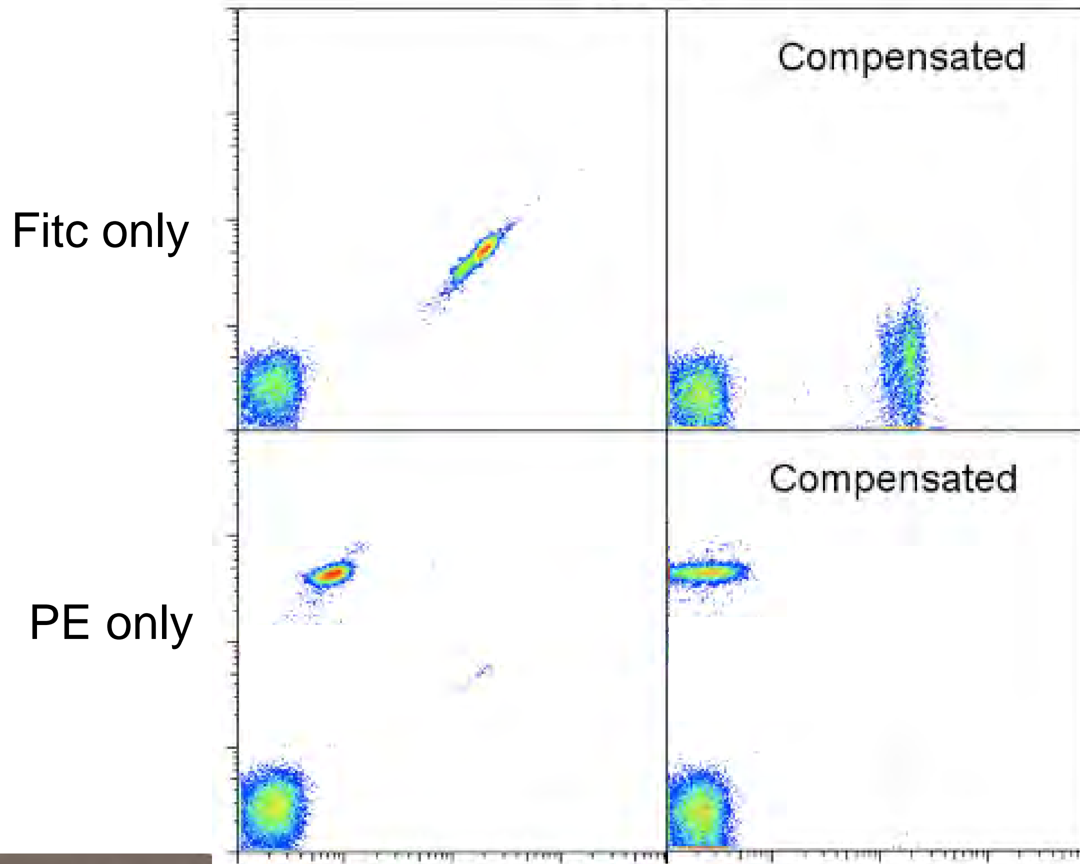
Protein Coupling



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Anti-Rat Capture beads



MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Compensation Controls For Fluorescent Proteins

Same cell type without the FP?

Polystyrene particles

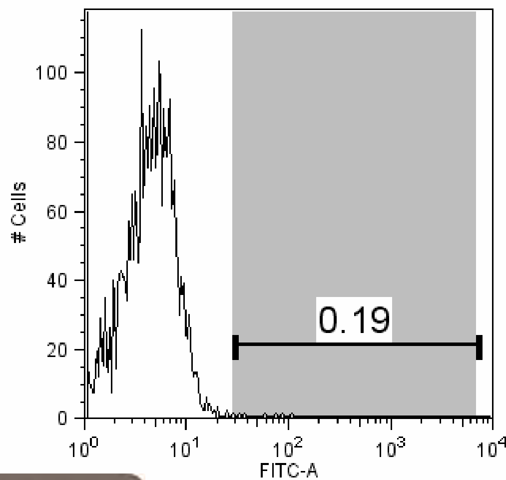
MRC | Centre for Regenerative Medicine

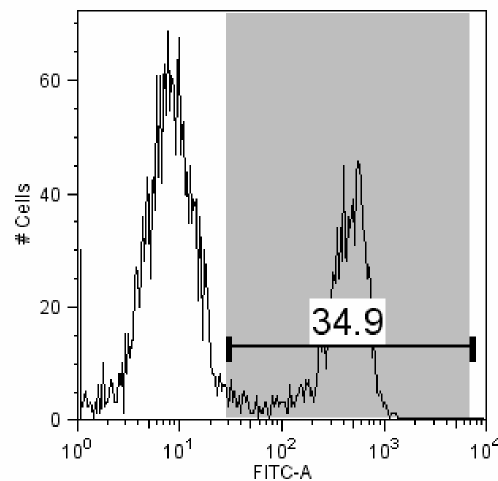
What Is Positive

- By positive for a certain antibody we mean those cells are expressing the antigen that is recognized by that antibody
- One of the consequences of the compensation spread is that one cannot use a negative control to determine positivity in multi-colour experiments.
- How do we determine what is positive?

What Is Positive

- With a single colour experiment we can simply compare our test sample to an appropriate negative control

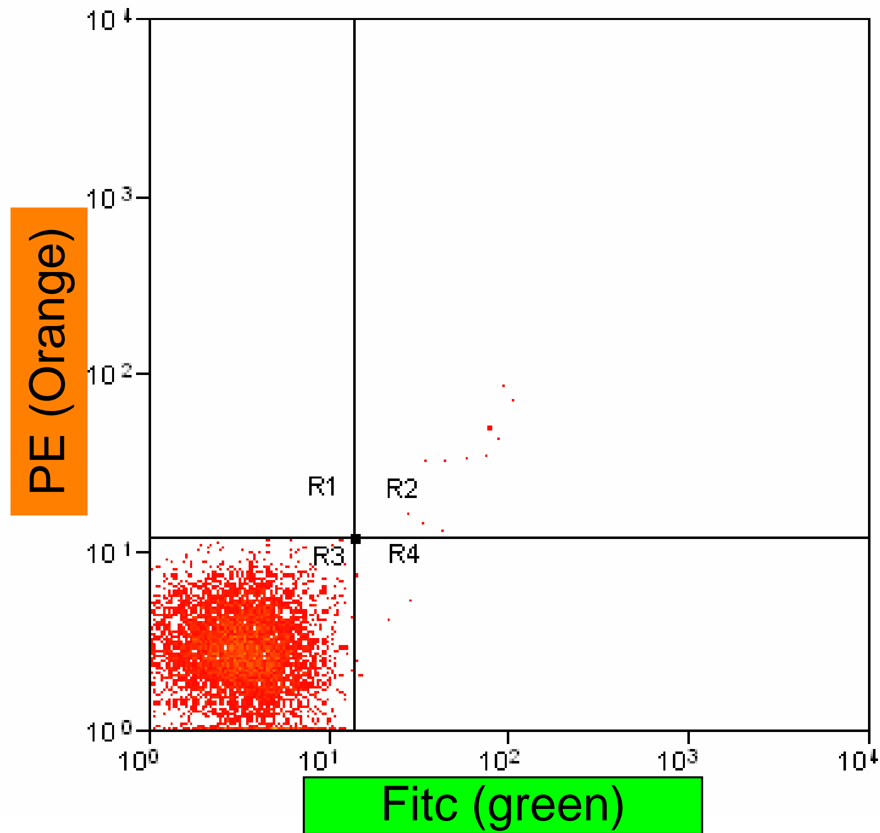


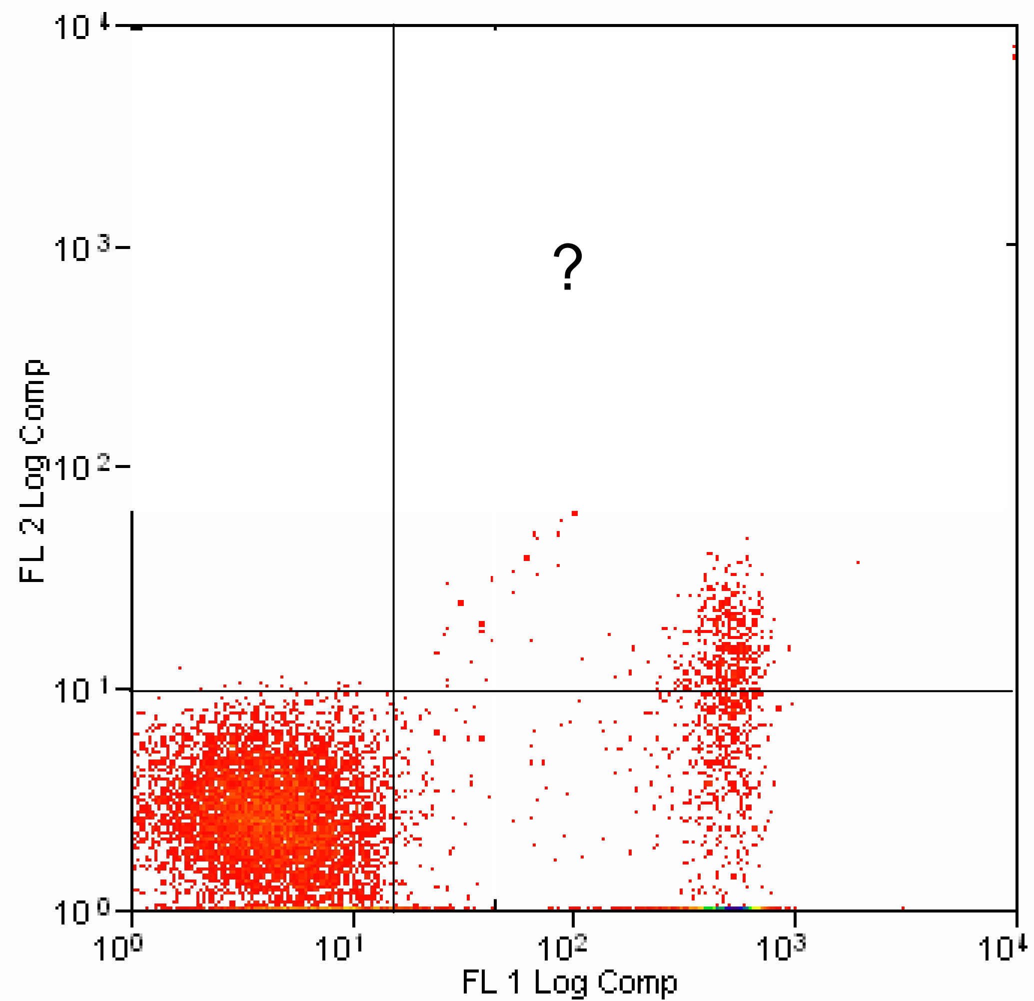


MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

What Is Positive 2 colour



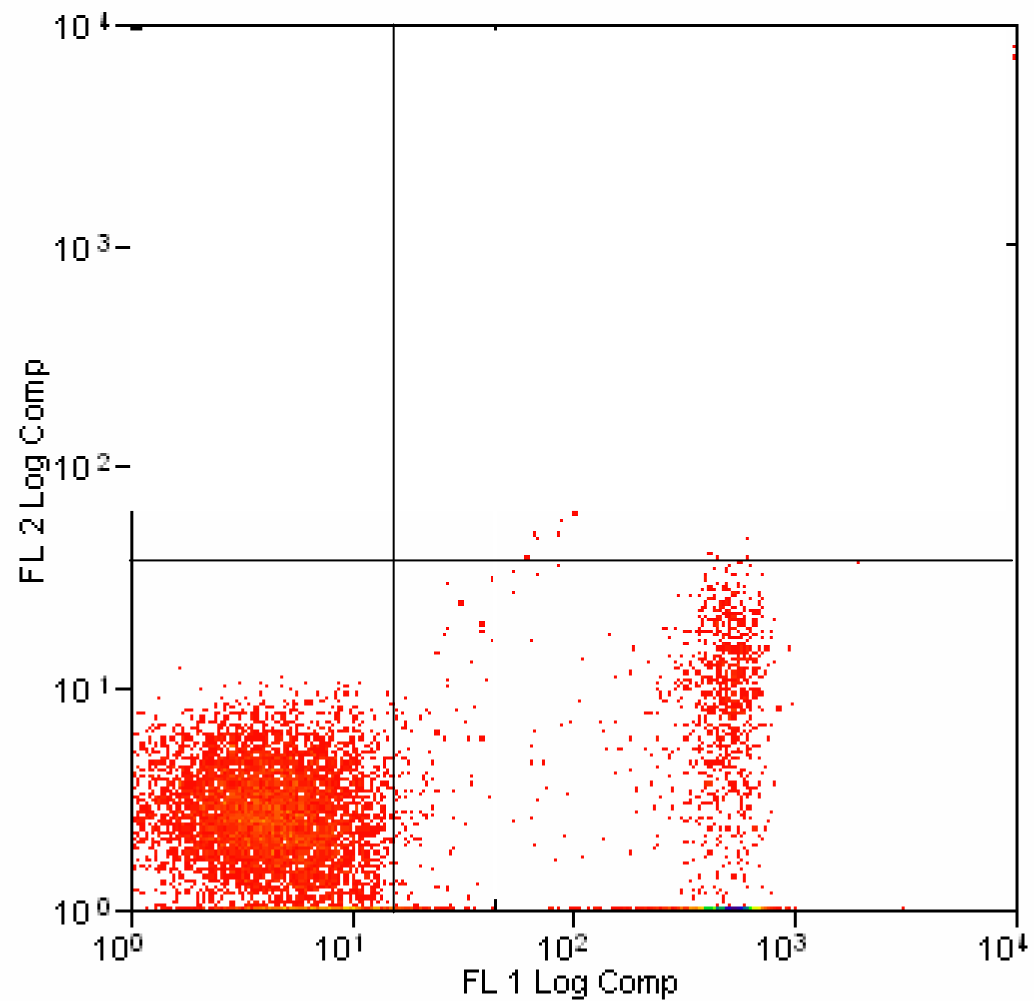


Re...	Mean	Median
Total	99.81, 5.23	5.27, 2.56
R1	0.00, 0.00	0.00, 0.00
R2	10000.01, 7638.19	10000.01, 7224.77
R3	5.37, 2.90	4.40, 2.56
R4	492.00, 6.61	498.93, 2.56

MRC | Centre for Regenerative Medicine

FMO

- FMO=(Fluorescence Minus One)
- By leaving out one antibody at a time we can better determine the contribution of the test antibody in that channel.



Re...	Mean	Median
Total	99.81, 5.23	5.27, 2.56
R1	0.00, 0.00	0.00, 0.00
R2	10000.01, 7638.19	10000.01, 7224.77
R3	5.37, 2.90	4.40, 2.56
R4	492.00, 6.61	498.93, 2.56

MRC Centre for Regenerative Medicine

FMO

- FMO controls are a much better way to identify positive vs. negative cells

- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH

Summary Controls

- Controls are essential
  - Negative
  - Compensation
  - FMOs

MRC Centre for Regenerative Medicine

THE UNIVERSITY OF EDINBURGH