Immunocytochemistry

Part 1

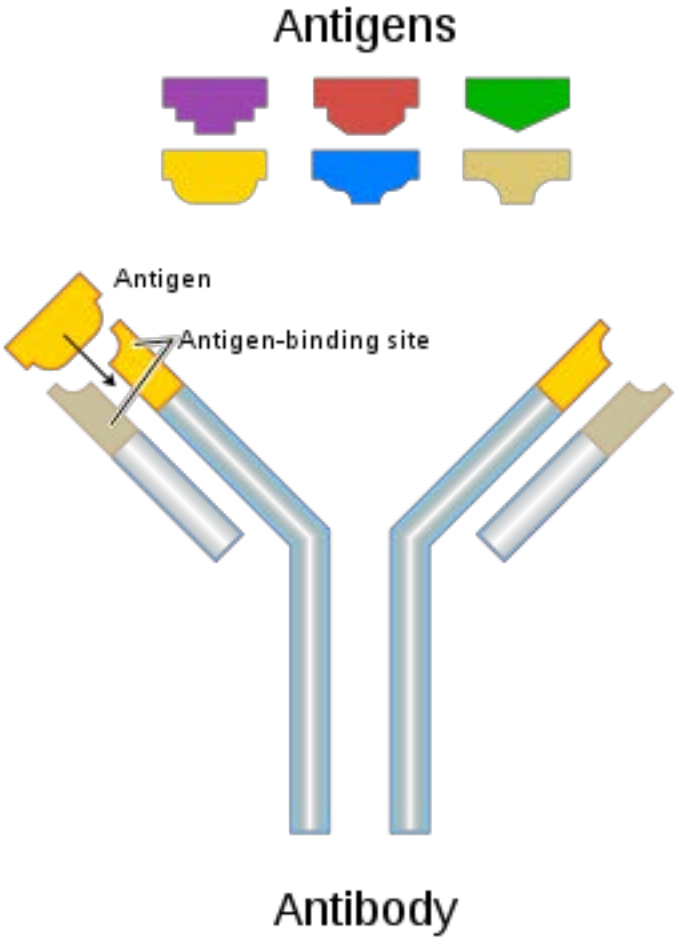
Definition

- Immunocytochemistry (ICC) is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in or on the cell via specific epitopes.

Antibodies

- Antibodies (also known as immunoglobulins[1], abbreviated Ig) are gamma globulin proteins that are found in blood or other bodily fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses.

- Antibodies are generated as part of the immune repsonse.



Antigen

- An antigen is a substance or molecule that when introduced into the body triggers the production of an antibody by the immune system which will then kill or neutralize the antigen that is recognized as a foreign and potentially harmful invader.

Epitope

- An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. The part of an antibody that recognizes the epitope is called a paratope.

Fixation

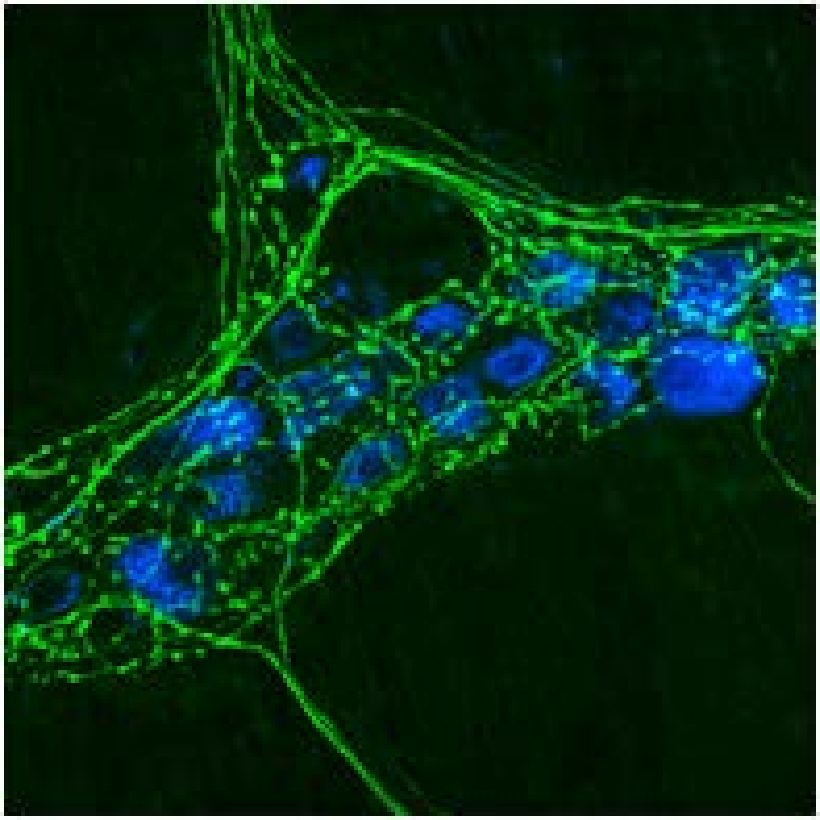
- To ensure free access of the antibody to its antigen (if located *within* the cell), the cells must be fixed and permeabilized. In general, fixation strengths and times are considerably shorter for cells than on the thicker, structurally complex tissue sections. For immunocytochemistry, sample preparation essentially entails fixing the target cells to the slide.

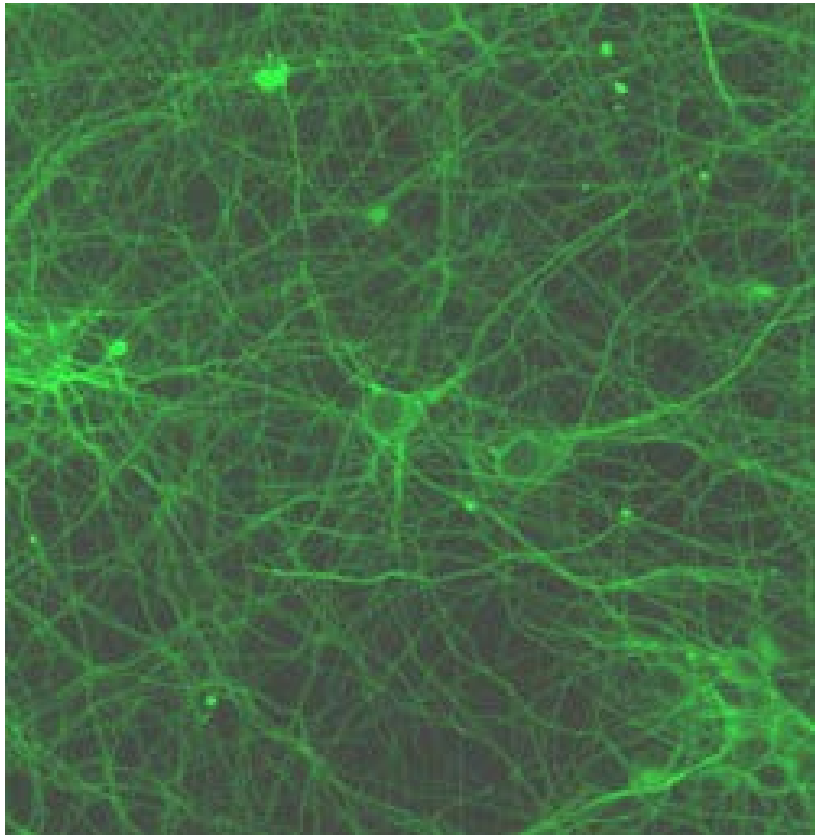
Fixation continued

- Fixation should immobilize antigens while retaining cellular and subcellular structure. It should also allow for access of antibodies to all cells and subcellular compartments. The fixation and permeablization method used will depend on the sensitivity of the epitope and antibody themselves, and may require some optimization.

- Fixation can be done using **crosslinking reagents**, such as paraformaldehyde (PFA). Crosslinkers are better at preserving cell structure, but may reduce the antigenicity of some cell components as the crosslinking will obstruct antibody binding. For this reason, antigen retrieval techniques may be required, particularly if there is a long fixation incubation or if a high percentage of crosslinking fixative is used. Another option is to use **organic solvents**. These remove lipids while dehydrating the cells. They also precipitate proteins on the cellular architecture.

Sample ICC Images





Immunocytochemistry

Part 2

Blocking Nonspecific Sites

- Antibodies show preferential avidity for specific epitopes
- However, antibodies may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen.
- nonspecific binding causes high background staining that can mask the detection of the target antigen.
- To reduce background staining in IHC, ICC and any other immunostaining application, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind
- Common blocking agents:
  - *normal serum*
  - *non-fat dry milk*
  - *BSA or gelatin*
  - *Commercial blocking buffers with proprietary formulations are available for greater efficiency*

General Blocking Procedures

- The blocking step for ICC is performed after fixation just prior to incubating the sample with the primary antibody

- The general protocol is to incubate the fixed sample with the appropriate blocking buffer for a time period from 30 minutes to overnight at either ambient temperature or 4ºC based on the optimized protocol specific to each antibody and target antigen

- *Sufficient washing after the blocking step is CRITICAL to remove excess protein that may prevent detection of the target antigen*

Blocking Agents

- **Normal serum** is a common blocking reagent, because the serum carries antibodies that bind to reactive sites and thus prevents the nonspecific binding of the secondary antibodies used in the assay. **A critical factor, though, is to use serum from the species that the secondary antibody was generated in, as opposed to the species of the primary antibody**. Serum from the primary antibody species would bind to reactive sites, but the secondary antibody would recognize those nonspecifically-bound antibodies along with the antibodies bound to the target antigen.

- Besides serum, high-protein buffers made with **0.1 to 5% bovine serum albumin (BSA), gelatin or nonfat dry milk** is often used to coat all proteins in a sample. This approach essentially forces primary antibodies to out-compete the blocking protein for binding to cognate ligands while reducing nonspecific binding because the antibodies have no greater binding affinity for nonspecific epitopes than do the buffer proteins. While these buffers can be easily made in the lab, for best results they must be made fresh prior to use, which increases the time and workload of the IHC staining.

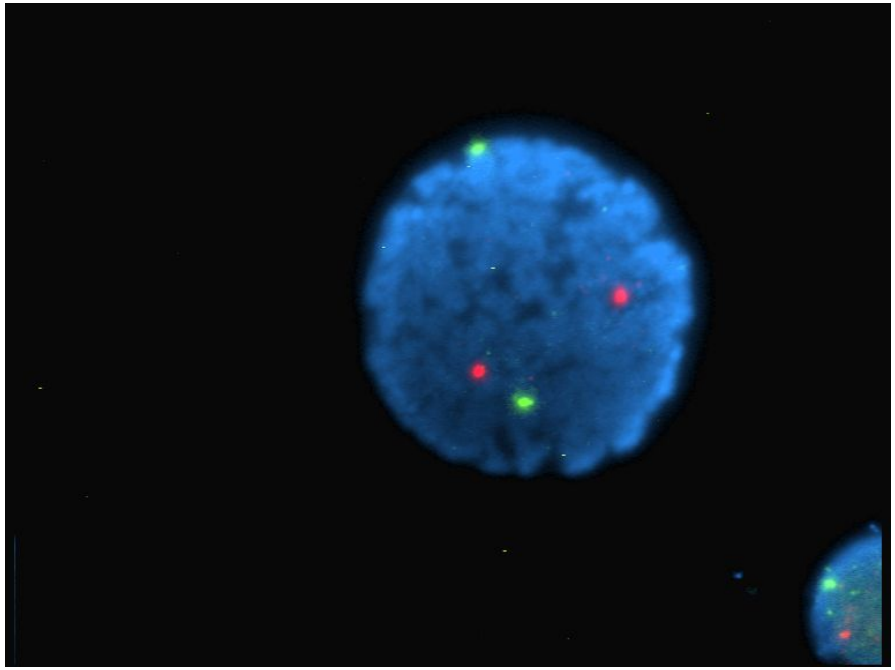
- **Commercial buffers** are also available to block samples in preparation for antibody treatment. These buffers can contain highly-purified concentrations of single proteins or proprietary protein-free compounds. A benefit of using commercial blockers is that there are many available options that perform better than gelatin or casein and have improved an shelf life.

Immunocytochemistry

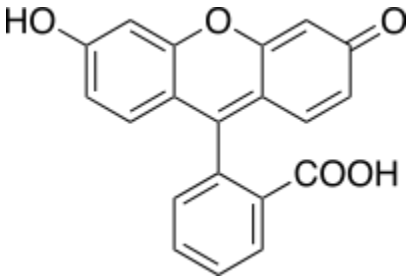
Part 3

Fluorophore

- It is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore and the chemical environment of the fluorophore.



Flourescein



- Fluorescein is a synthetic organic fluorophore commonly used in microscopy
- Fluorescein has an absorption maximum at 494 nm and emission maximum of 521 nm (in water)
- The major derivative is fluorescein isothiocyanate (FITC)

FITC - fluorescein isothiocyanate

- first synthesis in the 1870s

- multitude of applications, including biological research
-
- visible light excitation and emission, a high quantum yield, and pH tolerance in the physiological range

- Limitations
  - Although protein conjugates can be made using fluorescein, the fluorophore has a tendency to **quench** after only a few dyes are attached.
  - These conjugates also tend to photo-bleach quickly, making it difficult to obtain images requiring longer exposure times.

Alexa Fluor® 488

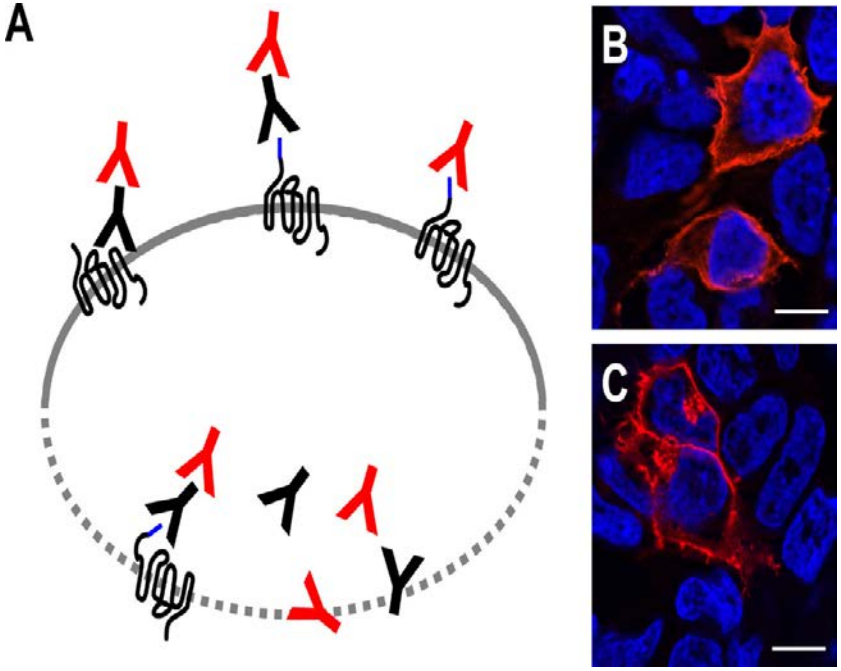
- **The Alexa Fluor® 488 dye**—with nearly identical spectral properties and quantum yield as fluorescein isothiocyanate (FITC)—produces brighter, more photo-stable conjugates.

- Introduced in 1997, the Alexa Fluor® 488 dye is the first dye in 100 years to seriously challenge fluorescein's dominance as the green-fluorescent dye of choice.

  A perfect spectral match for FITC filters—absorption—and emission profiles of Alexa Fluor® 488 and fluorescein are nearly identical, so there's no need to change equipment, settings, or filters
  .
- **Brighter conjugate fluorescence**—fluorescein conjugates rapidly quench as more fluorophores are added. Alexa Fluor® 488 dye allows more fluorophores to attached before self-quenching. This produces brighter conjugates which means you can add less antibody and still get optimal results.

- **Superior photo-stability**—allows more time for image observation and capture, permitting greater sensitivity and simplifying low-abundance target detection.

ICC Example: GPCRs



Visualization of GPCRs by fluorescent antibodies. Antibodies against extracellular receptor regions or N-terminal epitope tags can be used to visualize GPCRs in non-permeabilized cells (A, above), intracellularly located receptors, intracellular receptor segments or C-terminal epitope tags are only recognized after cell permeabilization (A, below). Either the primary antibody is fluorescent or a secondary antibody carrying a fluorophore is applied in a second step. Localization of the N-terminally HA-tagged human Y_1 receptor in HEK293 cells (B and C; HA-tagged receptor in red, nuclei in blue, bar represents 10 µm). The receptor can be visualized in the cell membrane of both non-permeabilized (B) and permeabilized cells (C) and intracellularly only within permeabilized cells (C).

Böhme and Beck-Sickinger *Cell Communication and Signaling* 2009 **7**:16 doi:10.1186/1478-811X-7-16

FLUOROPHORE TABLE

Dye	Absorbance Wavelength	Emission Wavelength	Visible color
Hydroxycoumarin	325	386	blue
methoxycoumarin	360	410	blue
Alexa fluor	345	442	blue
aminocoumarin	350	445	blue
Cy2	490	510	green (dark)
FAM	495	516	green (dark)
Alexa fluor 488	494	517	green (light)
Fluorescein FITC	495	518	green (light)
Alexa fluor 430	430	545	green (light)
Alexa fluor 532	530	555	green (light)
HEX	535	556	green (light)
Cy3	550	570	yellow
TRITC	547	572	yellow
Alexa fluor 546	556	573	yellow
Alexa fluor 555	556	573	yellow
R-phycoerythrin (PE)	480;565	578	yellow
Rhodamine Red-X	560	580	orange
Tamara	565	580	red
Cy3.5 581	581	596	red
Rox	575	602	red
Alexa fluor 568	578	603	red
Red 613	480;565	613	red
Texas Red	615	615	red
Alexa fluor 594	590	617	red
Alexa fluor 633	621	639	red
Allophycocyanin	650	660	red
Alexa fluor 633	650	668	red
Cy5	650	670	red
Alexa fluor 660	663	690	red
Cy5.5	675	694	red
TruRed	490;675	695	red
Alexa fluor 680	679	702	red
Cy7	743	770	red

Nucleic acid probes:

Dye	Absorbance Wavelength	Emission Wavelength	Visible color
DAPI	345	455	blue
Hoechst 33258	345	478	blue
SYTOX blue	431	480	blue
Hoechst 33342	343	483	blue
YOYO-1	509	509	green
SYTOX green	504	533	green
TOTO 1, TO-PRO-1	509	533	green
SYTOX orange	547	570	yellow
Chromomycin A3	445	575	yellow
Mithramycin	445	575	yellow
Propidium iodide	536	617	red
Ethidium bromide	493	620	red

ICC Protocol

- **Wash and Fix Cells**
- 1. Aspirate the growth media and wash 3 times with PBS.
- 2. Fix with 4% paraformaldehyde for 10 minutes.
- 3. Remove the 4% paraformaldehyde to a hazardous waste receptical.
- 4. Wash cells with PBST (PBS + tween-20).
- **Blocking and Incubation:**
- 5. Incubate cells with 1% BSA in PBST for 30 min to block non-specific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species that the secondary antibody was raised in).
- 6. Incubate cells in the diluted primary antibody in 1% BSA in PBST in a humidified chamber for 1 hr at room temperature or overnight at 4° C.
- 7. Aspirate the solution and wash the cells three times in PBS, 5 min each wash.
- 8. Incubate cells with the secondary antibody in 1% BSA for 1 hr at room temperature in dark.
- 9. Aspirate the secondary antibody solution and wash three times with PBS for 5 min each in dark.
- **Counter staining:**
- 10. Incubate cells in 0.1-1 µg/ml Hoechst or DAPI (DNA stain) for 1 min.
- 11. Rinse with PBS.