

Quantitative Fluorescence Symposium

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Quantitating Immunofluorescence

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Immunohistochemical techniques have become a standard method to detect the presence, and to localise the position of molecules in cells of tissues.

Detection by immunohistochemistry has become important in diagnostic pathology and research laboratories.

Experimental conditions or disease may change the amount of certain molecules within cells and this can inform about normal biological or pathological processes.

Beyond detection, can immunohistochemistry be used to determine the amount of molecules within tissue?

That is, is quantitative immunohistochemistry feasible?

Not yet feasible to detect absolute amount of molecules in tissue

We are able to determine relative changes in amount of substances – **semi-quantitative** measurements

However, to perform these experiments successfully there are a number of conditions which must be met, so that **a typical approach might be.....**

1. Optimise and standardise the immunohistochemistry

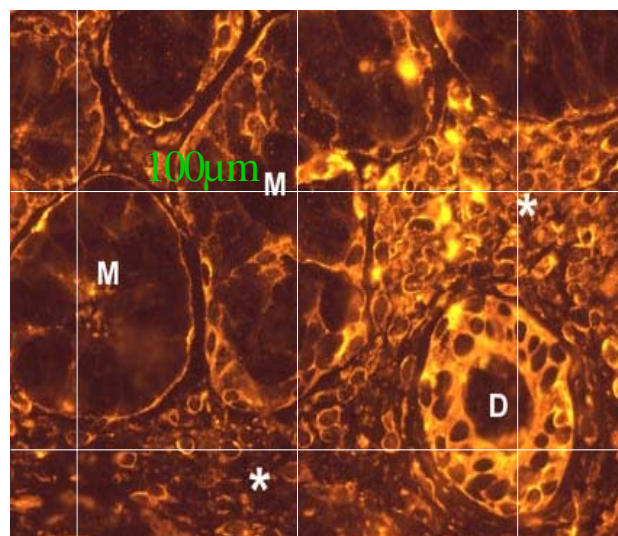
- perform ALL incubations at the same time using same batches of Ab and labels

2. Choose fluorophores carefully for good spectral separation

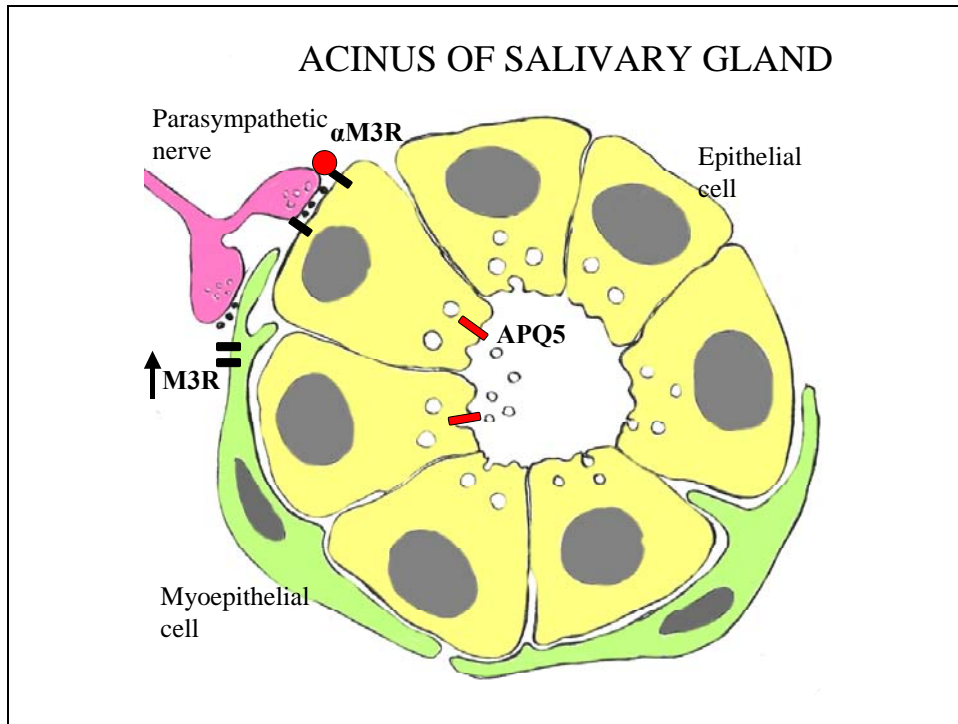
3. Use unbiased sampling technique

- random sampling of tissue, use grid to ensure systematic selection within sections (using motorised stage)

A grid can be used to systematically sample tissue using random start-point and then sampling particular structures in regular way using grid intersects



Human salivary gland – acinus. Labelled for muscarinic receptor (M3)
D – duct



Method For Sampling Tissue Sections

Each sample was a **random section**

The first acinus was chosen randomly.
Subsequent acini were chosen systematically in
a grid of $100\mu\text{m} \times 100\mu\text{m}$

Two regions of the section were sampled with 5 images

A total of 10 images per sample were obtained.

In this case, images were taken of acini membrane with
antibody labelling with 100x objective and zoom of 3.5

4. Collect images so that results can be quantitatively assessed/measured

Procedures to Obtain Comparable Images for Quantitation of Fluorescence Intensity

All antibody incubations were performed at the **same time**

Samples from controls and patients were coded to ensure unbiased data collection – **blind testing**

All samples were viewed and **confocal settings were determined for the brightest sample**

All images collected at the **same confocal settings**

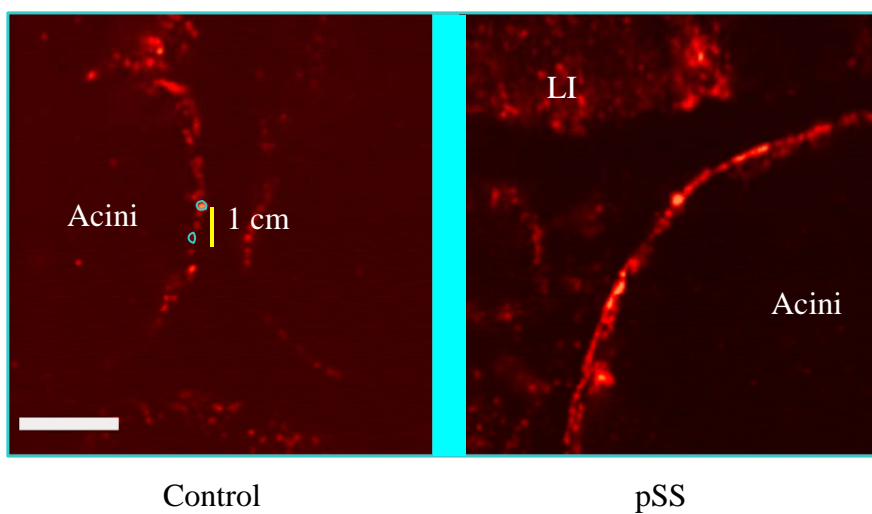
Collect images using consistent confocal settings

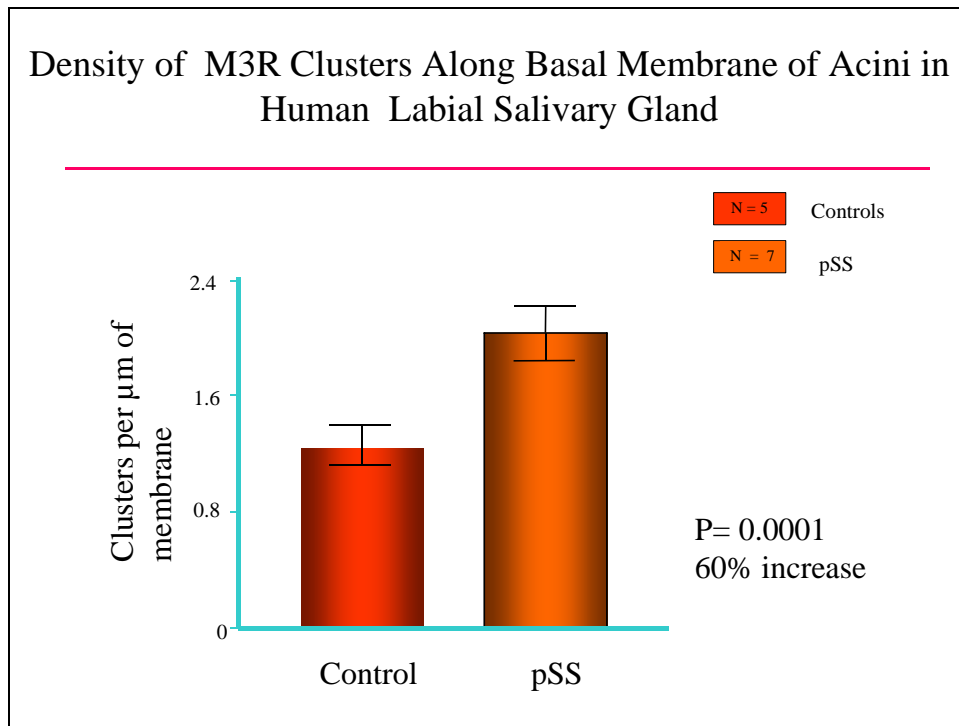
- standardise settings on the brightest sample
- collect all images using same settings
- collect all images on same day, including controls

5. Optical section thickness

6. Aim for precise localisation - appropriate resolution to subcellular level

Count of M3R clusters along membrane in Labial Salivary Gland





Remember Controls:

<http://www.aecom.yu.edu/aif/instructions/immunofluor/controls/abodies.htm>

CELL AND TISSUE LABELLING – CHOICES, CHOICES, CHOICES

1. Choice of primary antibodies #1:

A specific, high affinity, well-characterised Ab!

For qualitative immunofluorescence.....

BEST



WORST

Affinity purified Polyclonal IgG to entire native protein
 Monoclonal IgG purified from Ascites to native antigen
 Affinity purified Polyclonal IgG to unique peptide sequence
 Purified Monoclonal IgG to unique peptide sequence
 Non-purified polyclonal serum to any antigen

NOTE: Polyclonal antibody to unique peptide antigen – in this case, generally only a short peptide (20-30 amino acids) and **appropriate protein folding is rare**. Antibodies are rarely specific even after affinity purification. They often work well on Western blot, where protein is linearised but not in immunohistochemistry.

2. Choice of primary antibodies #2

For **quantitative immunofluorescence** - generally want one epitope, one molecule, therefore use a monoclonal Ab

BEST



WORST

Monoclonal IgG to purified from Ascites to native antigen
 Monoclonal Culture Supernate to native antigen

Purified Monoclonal IgG to unique peptide sequence

ie, indirect labelling using secondary uses a polyclonal fluorescent conjugate to detect the primary antibody. Therefore generally use **directly conjugated primary monoclonal antibodies => signal likely to be very limited.**

However, you may also need to consider the **detection system.....**

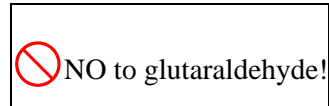
3. FIXATION

Needs to be **fast**, to preserve **structure**, be **non-fluorescent** and to **preserve antigenicity**

Two main types:

Cross-linkers (aldehydes) and dehydrators (coagulant fixatives)

Paraformaldehyde preferred – make up fresh from paraformaldehyde powder, remember 3mm/hr penetration. Fixation from 10 mins (cells) to several hours for tissues



Other options: cold methanol, cold acetone (solubilises lipid, acts as coagulant and can change protein shape)

4. IMMUNOLABELLING OF LIVE CELLS

Method is quite straight forward for labelling **cell surface markers**

-also used to label internal compartments by allowing **endocytosis** of the antibody to occur or by using Streptolysin-O to semi-permeabilise the cells

- all in the timing!

Cell surface labelling

Incubate in media containing the antibody.

Sequentially label with fluorescent secondary

May only need to label for as short period as 1-2 mins!

To internalise cell surface receptors

Fluorescently labelled antibody may be internalised by receptor-mediated endocytosis (may require 37°C) or if internalisation NOT required (at or below 4°C).

Streptolysin-O (bacterial toxin) forms proteinaceous pores within cellular membranes, so Ab pass through without loss of subcellular organelles.

Need to optimise conditions for concentration, temperature and time.

5. ARTIFACTS

Two causes: **Fixation:** Aldehydes are autofluorescent

Autofluorescence: Need to check fluorescence levels of unlabelled cells/tissue **BEFORE YOU START!**

Image in each of the channels corresponding to fluorescence spectra of secondary antibodies

There are ways to remove autofluorescence – bleaching, quenching etc

6. IMAGING ISSUES

- many issues common to other fluorescence imaging situations

- use fluorophores that **resist bleaching** (avoid Phycoerythrin!)

- avoid **bleedthrough** by setting appropriate emission settings and imaging sequentially

- check **registration errors** (may be due to fractional changes in angle of dichroic mirrors, or low NA lens with chromatic aberrations)

SOME GENERAL REFERENCES:

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<http://www.jhc.org>