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Visualisation and Analysis of Vascular Receptors Using Confocal Laser Scanning Microscopy and Fluorescent Ligands

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Abstract

The use of fluorescent ligands to analyse receptor distribution is increasing in popularity. This is due to the ever growing number of fluorescent ligands and the increased sensitivity of microscope-based technologies. Image-analysis methods have advanced to a stage where quantification of fluorescent signals is relatively simple (if used appropriately). In this chapter we describe a method of analysing the 2D and 3D distribution of fluorescent ligands in segments of blood vessels. In addition, we introduce the issues surrounding the accurate analysis of colocalization of two different fluorescent ligands.

Key Words: Image analysis, Fluorescent ligands, Blood vessels, Colocalization, Adrenoceptors, Cannabinoid receptors

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1. Introduction

Our current knowledge of the distribution and function of hormone receptors has been advanced through the constant development of new techniques. Knowing the distribution and cellular location of receptors in native tissues is essential to fully understand the biological function of the tissue. Over the years there have been many different techniques aimed at 'mapping' receptors.

The early developmental work of Berson and Yalow (1957-1966) was crucial in the development of the radioimmunoassay (1). Whilst their work concerned the measurement of hormone levels in plasma, the general principle would soon be applied to the measurement of receptors. Whole mount autoradiographs of the mouse diaphragm showing curare- and decamethonium-binding sites were first presented by Waser (2). The early

1980s through to the early 1990s saw a growth in the number of radioligand binding studies and *in vitro* autoradiography of tissue slices. In particular, a significant effort was made to map the distribution of β -adrenoceptors on a variety of tissues including the heart (3) and blood vessels (4). However, the resolution of autoradiography does not permit accurate analysis of binding at a cellular level and the time taken for development of the plates is prohibitive.

Antibodies, often harbouring fluorescent tags, targeted at cell surface receptors (and receptor subtypes) have also been employed. However, the specificity of antibodies, particularly the commercially available adrenoceptor antibodies, has been criticized (5). Even if antibodies were highly specific they generally target the intracellular portion of the receptor and thus can only be used on nonliving permeabilized tissue.

It is clear from above that the ideal scenario is to have a fluorescent marker that is relatively quick and easy to use and can be visualised on live tissue, at high resolution. Fluorescent ligands offer several advantages in this respect (6-8) but must be used with caution and an appreciation of the basic pharmacological principals of drug action (see **Note 1**).

The following methods describe the use of fluorescent ligands to visualise α -adrenoceptors, β -adrenoceptors and cannabinoid receptors on segments of live blood vessels. The techniques described will work for fixed tissue and with some adjustments should be appropriate for studying other multicellular tissue types. The methods in this chapter describe the preparation of tissues, the setup of the confocal laser scanning microscope, the image capture and subsequent 2D and 3D analysis of receptor binding using histograms and colocalization.

2. Materials

For confocal microscopy studies it is best to use vessels which have a relatively thin wall (i.e. $<100\mu\text{m}$ thick) such as the smallest branches of the mesenteric vasculature or cerebral vasculature. Thicker-walled vessels such as aorta and carotid artery can be used but may require to be cut open in order to clearly visualise the endothelium and innermost layers of smooth muscle. In all blood vessels the vascular adventitia can be easily visualized and is now proving to be of considerable importance in modulating normal vascular function. A range of fluorescent ligands can be used to visualize receptors within the blood vessel wall (see **Note 2**).

1. Prepare a suitable physiological salt solution (PSS) for tissue incubation using distilled or purified water (e. g. Krebs of the following composition: 119 mM NaCl, 24.9 mM NaHCO_3 , 4.7 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2.5 mM CaCl_2 , 11.1 mM glucose).

2. Oxygenate the Krebs solution with 95% O₂, 5% CO₂ to bring to pH 7.4. Oxygenate vigorously for 5-10 min before adding the CaCl₂ (*see Note 3*).
3. Remove tissue samples (e. g. blood vessels) and transfer to PSS as quickly as possible.
4. Store segments of blood vessels in a fridge for up to 24 h. However, for best results use the segments immediately.

3. Methods

3.1 Vessel Preparation

1. Pin excised vessel in a gel-filled Petri dish and cover with PSS (room temperature is acceptable). In some cases ice-cold PSS may be preferred (*see Note 4*).
2. For thick -walled vessels (i.e., carotid and aorta), cut open with the tips of fine spring scissors, taking care not to damage the endothelial cell layer
3. Carefully remove the peri-vascular adipose tissue (PVAT; *see Note 5*).
4. Avoid excessive stretching of the artery and try to grasp with forceps only at the edges where cells will already be damaged from dissection.

3.2 Vessel Incubation

1. Add one or more vessel segments to a 1-ml polypropylene tube containing ~50 to 100 μ l PSS. The volume should be kept to a minimum as fluorescent ligands can be costly.
2. Add the chosen nuclear stain (e.g., SYTO 62) at a concentration between 0.1, and 1 μ g/ml depending on tissue type. Concentration is not essential as this is only a marker. An incubation time of 30-60 minutes will be sufficient for penetration through the vascular wall.
3. Add the fluorescent ligands immediately after addition of the nuclear stain. You do not need to wait. Incubate with fluorescent ligands for 30-60 minutes to achieve equilibrium throughout the vascular wall. There is no need to shake or stir the tube. The concentration of fluorescent ligand used is entirely dependent of the pharmacology of the compound (*see Note 1b*).
4. If a non-fluorescent (receptor subtype selective) antagonist is being used to compete with the fluorescent ligand, this should be added at least 30 minutes prior to addition of the fluorescent ligand (*see Note 1c*).

5. Tissues can be fixed before or after incubation with a non-formalin-based fixative (formalin has fluorescent properties). However, fixation is not necessary and should, where possible, be avoided.
6. Take a regular glass microscope slide and make a well using vacuum grease. This is best achieved by coating a needle with grease and making a 'naughts & crosses' grid on the slide. The centre of the grid makes a convenient tissue well.
7. Following incubation, carefully place the vessel in the tissue well and cover with the incubation medium. No washing ensures that the ligand concentration is maintained. Seal the sample using a glass coverslip. Number 1.5 coverslips are the most suitable.
8. Slides are now ready for CLSM inspection or storage in a fridge.

3.3 CLSM Calibration

1. Ideally, a fluorescent bead for each fluorescence channel should be imaged at the beginning of each set of experiments. It is not necessary to do this on a daily basis. However, a monthly check will provide a useful 'health' check for laser performance and optical alignment (*see Note 6*).
2. Colocalization requires that the images are aligned, i.e. that an object containing both fluorophores appears in the same place in the two images. Invitrogen provides Tetraspec microspheres for alignment purposes.
3. If significant variations exist between current and previous calibrations the laser power should be checked using a power meter. Each CLSM system will have a particular way of monitoring and adjusting the laser power.

3.4 Image Collection

1. Mount the slide (or preparation) on the CLSM and focus on the specimen using the bright field illumination.
2. Select an appropriate set of excitation lines and emission filters for the sample.
3. Start the image capture sequence on the CLSM.
4. Select a 'range' look-up-table (LUT). This will normally be set to show the darkest pixels in blue and the brightest pixels in red. This will normally be a two colour palette. Set the laser intensity, brightness (offset), and contrast (gain) such that only a few, but preferably none, red and blue pixels are showing. This ensures that 99.9% of the image falls within the intensity range of the detector and that there is no cut-off and hence loss of image data.

5. Check the quality of the images – if the image change appreciably when the image is rescanned, adjust scanning to reduce the changes.
6. Collect single and serial optical sections (stacks) as per the instructions of your particular system.
7. Avoid using extremely high gain which can introduce noise and high laser power which will photobleach many fluorophores (see **Note 7**).
8. Collect duplicate images/stacks if colocalization of two or more fluorescent ligands is required (see below).

3.5 Image Analysis I (Histogram Analysis)

There are many image analysis programs available from commercial suppliers. These vary in both ease of use and cost. For the purposes of a general article such as this we will use the freely available (open source) ImageJ, which is an excellent starting platform for anyone interested in image processing and analysis (see **Note 8**).

1. Open an image.
2. Select Analyze/Histogram.
3. The software displays the histogram of the selected image and reports the minimum, maximum and average values in the image (see Fig. 1).
4. If an image stack (serial z-series) is open then select 'include all images' to obtain a histogram of the entire image series (see Fig. 2e).

3.6 Image Analysis II (3D Visualization)

Fully functional 3D visualization software can be extremely expensive. However, Image J offers a free plugin (see **Note 9**).

1. Open an image stack (series of images).
2. Select Plugins/3D/Volume Viewer.
3. Select viewing mode and orthogonal positions as required.

3.7 Image Analysis III (Colocalization)

ImageJ provides (via Analyze/Plugins) a selection of colocalization algorithms. Perhaps the most comprehensive of these is the JACoP plugin (see **Note 10**).

1. Open image A.
2. Open image B.
3. Select Analyze/Plugin/JACoP.
4. Assign images as requested.

Fig. 1. Histogram analysis of fluorescent ligand binding in the tunica adventitia (outer surface) of a segment of mouse mesenteric artery. (a) BODIPY FL-prazosin (QAPB, 0.1 μ M) binding to α_1 -adrenoceptors. (b) T1117 (0.1 μ M) binding to cannabinoid-like (GPR55) receptors. (c) Cell nuclei labelled with Syto 62 (0.1 μ g/ml). (d) The merged image shows that adventitial cells do not express equal quantities of α_1 - and CB-like receptors. The inset histograms describe the distribution of fluorescent pixels in each image. The left-shifted histogram in image c is useful in demonstrating the characteristics of a relatively dark image (i.e., when the majority of the pixels have background, low-level, intensity values). Image histogram (b) stretches further to the right indicating the presence of a greater number of high-intensity pixels.

3.8 Image analysis IV; Replicate-Based Noise Corrected Correlation (RBNCC) (Image Quality and Colocalization)

This method requires the collection of duplicate images (see **Note 11** and Fig. 4).

1. Acquire two images of each fluorophore, images A1 & A2 and B1 & B2.
2. Measure the quality (see **Note 12**) by measuring the Pearson's correlation coefficient (r) between the two replicate images, r_{AA} from A1 with A2 and r_{BB} from B1 and B2.

Fig. 2. 3D visualization and histogram construction. A segment of mouse mesenteric artery incubated with the fluorescent cannabinoid ligand (T1117, 0.1 μ M) has been visualised using CLSM. A z-series of 43 images (1 μ m axial distance) was collected. Orthogonal sections in xy (a), yz (b) and xz (c) can be examined using the Volume Viewer plugin. A fully rendered 3D view (d) is displayed in the main window. A histogram of the full fluorescent volume is shown and describes the relative distribution of pixels in the z-series. Volume Viewer is an ImageJ plugin.

3. Combine the two self-correlation coefficients to obtain a correction factor

$$C_{AB} = \frac{1}{\sqrt{r_{AA} \cdot r_{BB}}}$$

4. Make the initial colocalization measurement by measuring Pearson's correlation coefficients between A1- B1, A1-B2, A2-B1, and A2-B2.
5. Calculate the arithmetic mean of the four between fluorophore correlations (r_{AB}).
6. Use the correction factor to adjust the measured mean correlation coefficient and obtain a Pearson's correlation coefficient (R_{AB}) that is independent of the quality of the images.

$$R_{AB} = \frac{r_{AB}}{\sqrt{R_{AA} \cdot R_{BB}}}$$

Fig. 3. Colocalization of two fluorescent ligands and scatter analysis. The *upper image* shows a merge of three fluorescent images (*red*, *green*, and *blue*). The green fluorescence represents binding of QAPB ($0.1\ \mu\text{m}$) to α_1 -adrenoceptors. Red fluorescence represents binding of BODIPY TMR-CGP12177 to β -adrenoceptors. Cell nuclei (shown in *blue*) are labelled with SYTO 62 and are excluded from any colocalization analysis. *Yellow* indicates roughly similar intensities of red and green fluorescence. The *lower panel* shows the types of scatter graphs displayed by the JACoP plugin. A *scatter graph* shows the corresponding intensities in the same pixel from the two images, using the intensities as coordinates. For two perfectly matched images the points would fall along a line from the *bottom left* to the *top right* of the graph. *Two scatter graphs* are shown, on the *left* a dot plot, where each point shows the combination of intensities occurs in one or more pixel and, on the *right* a density plot where the color scale indicates how frequently each combination occurs. When large numbers of pixels are represented, the density plot is preferable.

Quality			Between Fluorophore				Final	
A1-A2 r_{AA}	B1-B2 r_{BB}	Correction C_{AB}	A1-B1	A1-B2	A2-B1	A2-B2	Mean A-B r_{AB}	Corrected R_{AB}
0.955	0.8	1.144	0.741	0.713	0.732	0.744	0.732	0.837

Fig. 4. RBNCC, noise-free measurement of correlation. A pair of images of each fluorophore (A and B) can be used to measure the quality of the images and the two quality measures can be combined into a “quality” correction factor that can be applied to the measured correlation between the fluorophores. Note that the measures correlation is the arithmetic mean for the four combinations that are possible between two pairs of images. Correcting the Pearson’s correlation coefficient (r) between two fluorophores A and B. For each fluorophore there are two replicate images acquired under the same conditions. Therefore any difference is due to noise. Noise is assessed for each fluorophore by measuring the PCC between the replicate images, image A is of high quality ($r_{AA} = 0.955$) while image B is of good quality ($r_{BB} = 0.800$). A correction (C_{AB}) is generated using both the quality measurements (r_{AA} and r_{BB}). The between fluorophore r is initially measured between the four possible combinations of the two pairs of replicate images and the mean correlation is calculated ($r_{AB} = 0.732$). Finally the correction factor ($C_{AB}=1.144$) is multiplied by the mean correlation ($r_{AB} = 0.732$) producing the corrected r ($R_{AB} = 0.837$).

4. Notes

1. (a) The reader is referred to Rang & Dale's Pharmacology. The early chapters provide an excellent introduction to the principals of drug action, which is outwith the scope of this methods chapter. It is of prime importance to check that the pharmacological properties of any fluorescent ligand are retained after addition of the fluorescent molecule. (b) With regard to the concentration of a ligand it is important not to saturate the receptors. As a rule of thumb, if the fluorescence is still increasing at 100x greater than the K_D then the binding is probably not specific. In practice this will probably produce a low level of fluorescence when visualised on the CLSM. To combat this, a higher CLSM detection setting can be used or ligand concentration can be increased. In general we would recommend not exceeding $1\ \mu\text{M}$ which is a threshold concentration for selectivity of many drugs. $0.01\text{--}0.1\ \mu\text{M}$ is an ideal concentration range for most fluorescent ligands we have examined. (c) The concentration of a competitor ligand (nonfluorescent antagonist) should be chosen on the basis of the ratio of its K_D to that of the fluo-ligand and then increased (e.g., if the competitor has the same affinity as the fluo-ligand a 100x increase should be applied. If it has a 10x greater affinity then a 10x increase if enough, and so on).
2. Many fluorescent agonists and antagonists are available and are supplied with a variety of fluorophores (8). In this chapter we describe the use of a fluorescent α_1 -adrenoceptor antagonist (BODIPY FL-prazosin, which we refer to as QAPB (9), a fluorescent β -adrenoceptor antagonist (and partial agonist; BODIPY TMR CGP12177) (10), and a fluorescent cannabinoid ligand (T1117) (9). In most fluorescent ligand examinations it will be beneficial to have a fluorescent nuclear stain present to enable identification of cell orientation, number, organization and type (11). Nuclear stains are available across the full fluorescence spectrum and can be chosen to compliment the fluorescent ligands being used. We have found the far red stains SYTO 61 & 62 to be particularly bright, stable and reliable.
3. If CaCl_2 is added too early it will precipitate but can sometimes be recovered by continued (vigorous) gassing.
4. Receptor internalisation has been shown to be temperature dependent (12). Therefore, ice-cold PSS may inhibit constitutive receptor internalization of cell membrane receptors.

5. PVAT is known to release a wide range of adipokines believed to affect vascular function (13). The PVAT contains many receptor types, binds fluorescent ligands and can be easily visualized.
6. Fluorescent beads (i.e. 'FocalCheckTM') come in a variety of diameters (6-15 μm), often with a fluorescent core and surface which have different emission wavelengths. These are ideal for alignment in both the xy and z axes. Smaller fluorescent beads ($\sim 0.17 \mu\text{m}$) can be used to measure the point-spread-function (PSF) of the CLSM (i.e. PS-SpekTM), which is essential for accurate image correction and deconvolution.
7. In some cases photobleaching can be used as an experimental tool. If the tissue being examined is incubated in media containing the fluorescent drug (i.e., no wash prior to viewing) then fluorescence may recover following photobleaching as new drug molecules begin to bind. Thus, Fluorescence Recovery After Photobleaching (FRAP) can be used to examine ligand binding in real time.
8. ImageJ is a Java-program and is constantly being updated. There are many plugins available which can be used to customize your own version of the software. In addition, the source code is available to those able to write their own specialized routines. The software can be downloaded from <http://rsbweb.nih.gov/ij/>. An 8-bit image comprising 512 x 512 pixels comprises 262,144 pixels which each have a value (intensity) between 0 and 255. Thus, a 2D image is no more than an array of numbers. It is said that a 'picture is worth a thousand words'. In biology a number is worth a thousand pictures – or at least we could *express* a thousand pictures as a limited set of numbers (i.e. graphically). The simplest way to represent the 262,144 pixels is in a histogram which shows the number of pixels which have each of the 256 possible intensities in an 8-bit image (0 is counted as a value). This can be easily achieved using ImageJ software.
9. ImageJ provides a 3D volume viewer as a "plugin". The 3D viewer provides a full 3D view in addition to orthogonal xz and yz sectioning. If a full suite of 3D measurement tools is required it is advisable to trial the range of specialist software available (e.g., Imaris, Amira, and Volocity). Figure 2 demonstrates what can be achieved quickly using imageJ. In this image series, a z-series of 43 (512x512) images was rendered for 3D visualization. A histogram of the 11,010,048 pixels is shown in Fig. 2e. A histogram which lies to the right (i.e. stretches into the brighter regions) is indicative of a highly fluorescent image or volume. Thus, comparisons of the relative positions of the histograms indicate differences in overall intensity (14).

10. JACoP generates the most commonly required coefficients (Pearson's, Manders M1 and M2). Operation is simple and results in the generation of scatter graphs like those shown in Fig. 3. While the ImageJ plugins are predesigned and easy to use, we believe that there are certain deficiencies in the general overlap method for colocalization (15). The method RBNCC (replicate based noise corrected correlation) (16) only applies to colocalization using the Pearson's correlation and Spearman rank correlation coefficients. Measurement using the two Manders coefficients, M1 and M2, is affected both by noise and by the pixel size, and it is therefore important to acquire images of high quality and only make comparisons between images with the same pixel size.
11. The accuracy of a measurement reflects the quality of the original data, in this instance the digital images. While resolution depends upon the microscope, principally the objective and its numerical aperture, quality also depends upon the amount of light detected. The quality of a digital image can be assessed by looking at it – do individual pixels differ from their neighbors, do pixels change as an image is rescanned? A sound basis for assessing quality is to make use of scattergrams (see Fig. 3) and, instead of comparing the relationship between two different fluorophores, comparing the correlation of an image of a single fluorophore with a second image of the same fluorophore. Assuming the specimen does not move, the scatter graphs shows how similar the two nominally identical, images actually are – a good measure of image quality. A quantitative measure of quality can be made using the Pearson correlation coefficient, as mentioned above.
12. The measured colocalization between two fluorophores depends upon both the quality of the images and on the underlying relationship between the fluorophores. This is an unsatisfactory situation, as we want a measure of colocalization that depends exclusively on the underlying relationship. The trick is to utilize the measure of quality outlined in the previous paragraph as a correction to the measured colocalization. Measurements should exclude areas outside the region of interest. It may be useful to define regions of interest within cells or tissues – for instance the colocalization in the nucleus and the cytoplasm may be completely different. Correlation measurements should only be made between pixels that include both fluorophores, and a threshold that differentiates between fluorescence and background is required for both fluorophores. The six required correlation measurements can be made using the JACoP plugin from ImageJ. Alternatively, RBNCC (replicate-based noise corrected correlation) has been implemented in Huygens (SVI, Netherlands).

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