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Raster Image Correlation Spectroscopy in Live cells Expressing Endothelin ET_A Receptor

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Abstract

Fluorescence spectroscopy is the most common non-radioactive technique used to study GPCR interactions with their ligands. Raster image correlation spectroscopy (RICS) exploits spatio-temporal correlation functions rather than the simple temporal correlations of conventional fluorescence correlation spectroscopy. In this paper we describe the use of RICS and the number and brightness method to determine the diffusion of a construct of endothelin ET_A receptor with EGFP and the aggregation state in the cytoplasm. Our construct seems to locate mainly in the cytoplasm where it undergoes diffusion and it appears to be monomeric. Although our construct could not fully represent the native protein, we believe that the methodology we describe in this paper could be used by anyone in this field.

Key words: Endothelin ET_A receptor, GPCR, live cell, imaging, RICS.

Introduction

Endothelins are a family of vasoconstrictive peptides expressed in different tissues (1), which maintain basal vascular tone and blood pressure (2). Endothelin 1 (ET-1), the most potent of these peptides, has been found as an etiologic or aggravating factor in a number of cardiovascular diseases, including essential hypertension, pulmonary hypertension, acute renal failure, cerebral vasospasm after subarachnoid hemorrhage, vascular remodeling, cardiac hypertrophy, and congestive heart failure (3). ET-1 mediates its effects via two G-protein coupled receptors (GPCR) namely ET_A and ET_B, which are coupled to several subfamilies of the heterotrimeric G protein family (2). It has been shown that most of the apparently deleterious effects of ET-1 are mediated through the activation of ET_A receptors (4), thus this receptor has become an important target in drug discovery of cardioprotective drugs (5,6).

Fluorescence spectroscopy is the most common non-radioactive technique used to study GPCR interactions with their ligands. However, detailed biophysical data on these interactions is scarce (7). This is the case of the ET_A receptor, which in spite of its patho-physiological relevance, it has only been studied by fluorescence resonance energy transfer (FRET) (8,9) and more recently, fluorescence correlation spectroscopy (FCS) (10).

In classical single point FCS analysis, fluctuations are measured in one volume of illumination. This analysis has the drawback that it does not provide information regarding possible correlations between adjacent (or far) volumes of observation at different time, limiting the description of the full receptor dynamics.

In order to overcome these limitations, raster image correlation spectroscopy (RICS) exploits spatio-temporal correlation functions rather than the simple temporal correlations of conventional FCS (11). RICS provides spatial information on binding and diffusion coefficients in live cells using a conventional confocal microscope (12). With this methodology, the full extent of the probability of finding a particle at a different location and at a different time is introduced into the description of the correlation of the fluctuations to capture the time and spatial evolution of the particle.

In this paper we describe the use of RICS and the number and brightness method to determine the diffusion of a construct of ET_A with enhanced green fluorescent protein (EGFP) and the aggregation state in the cytoplasm.

Materials and Methods

Cell Culture: CHO-K1 cells were cultured in Dulbecco's Modified Eagle Medium**:** Nutrient Mixture F-12 media supplemented with 10 % Fetal Bovine Serum and 1 % Penicillin-Streptomycin. They were plated on 8 well Nunc* Lab-Tek* II Chambered Coverglass overnight and transfected with eGFP ENDRA protein using the Lipofectamine 2000 transfection reagent. Experiments were also performed using NIH3T3 cells that were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum, 1 % Penicillin-Streptomycin and 0.5 % 1 M HEPES solution.

Plasmid Construction- N-terminal HA C-Terminal eGFP ENDRA: cDNA for ENDRA (Endothelin receptor type A) [Catalog Number: #EDNBL0TN00] with 3xHA-tag (N-terminus) was obtained from Missouri S&T in pcDNA3.1+ from Invitrogen. The ENDRA coding sequence including the HA-tagged N-terminus to the last codon before the stop codon was amplified by PCR using *Pfu* Ultra DNA polymerase (Stratagene). The correct size PCR product (Insert size= 1926 bp.) was purified (Qiagen) and digested using restriction enzyme sites designed into the primers (Kpn1 and Age1) (Fig. 1). Product was run on a 1% agarose gel and

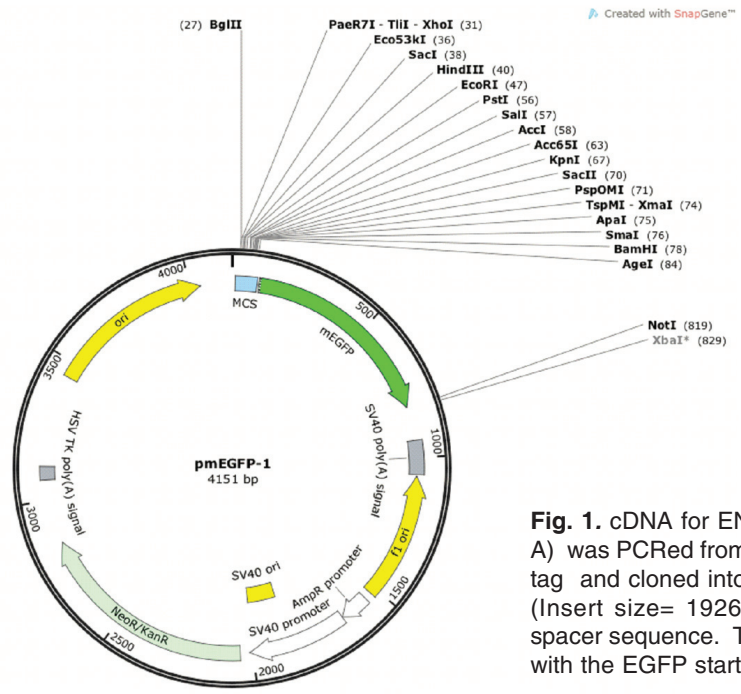

Fig. 1. cDNA for ENDRA (Endothelin receptor type A) was PCRed from pCDNA3.1 template with 3xHA-tag and cloned into pmEGFP-1 using the Kpn1(5') (Insert size= 1926 bp) and stop codon removed spacer sequence. The Age1(3') site used is in frame with the EGFP start.

excised for gel extraction (Qiagen). Digestion of the vector pmEGFP-1 (Addgene 36409: - deposited by Benjamin S. Glick, PhD from the University of Chicago) with Kpn1 –HF™ and Age1-HF™ (NEB) was performed, followed by gel purification. The PCR product was ligated into the eGFP vector. The resulting plasmid (KAN resistant) were screened by restriction digest and then verified by DNA sequencing (Retrogen). The ENDRA and the eGFP were verified by using BLAST NCBI.

***RICS measurements*:** An Olympus FV1000 confocal microscope was used for the RICS experiments. Excitation was at 488nm at a nominal power of 2%. Excitation/emission was split using the DM405/488. Emission was collected using BA505-525nm bandpass. The Objective was a UPLSAPO 60X W, NA 1.20 from Olympus. The frame size was set at 256x256 with a pixel dwell time of 10ms or 8 ms. The pixel size was 0.050mm or 0.072mm depending on the experiment, but always in the optimal range for the RICS experiments. Data were acquired using the pseudo-photon counting of the Olympus FV1000. For each experiment, 100 frames were acquired. RICS and brightness analysis was performed using the SimFCS software (www.lfd.uci.edu). For the brightness analysis the detrend option was used. This option compensate on a pixel basis for the bleaching.

RICS correlation functions: The RICS correlation function is defined in equation 1.

$$G_{RICS}(\xi,\psi) = \frac{< I(x,y) I(x+\xi, y+\psi) >}{< I(x,y) >< I(x,y) >} - 1$$

where *I* is the intensity at each pixel and the brackets indicate the average over all pixels x, y of an image. According to the definition of the correlation function, one image is sufficient to determine the RICS correlation function. However, in general, a stack of images must be collected to separate the mobile from the immobile fraction of molecules. The definition of the RICS correlation function is similar to the definition of the Image Correlation Spectroscopy (ICS) given by Petersen et al (Petersen, 1986; Petersen, 1998; Petersen NO, 1993). The difference in the RICS approach with respect to ICS is in the particular way data are collected in the raster scan confocal microscope which results in a relationship between the position of the pixel in the image and the time a specific pixel is measured.

$$time\ \ at\ pixel\, n = y \times \tau_l + x \times \tau_p$$

$$\xi = x \times \tau_p$$

$$\psi = y \times \tau_l$$

The resulting correlation function can then be expressed in terms of the pixel time t_p and the line time t_l

***Brightness analysis*:** The data set need for the Brightness analysis consists of a stack of images of the same field of view. These images generally are the same used for the RICS analysis. For each pixel of the stack of images we define

$$av = \frac{\sum_k I(x,y)}{K}$$

$$var = \frac{\sum_k (I(x,y) - av)^2}{K}$$

Where the sum is over the same pixel in each frame of the stack, *K* is the number of frames and *I* is the intensity at one pixel of each frame. The brightness B is defined by the following relationship

$$B = \frac{var}{av}$$

For a pure Poisson distribution of counts, the brightness is equal to one. If there is an excess variance (with respect to the Poisson variance) the value of B will exceed 1. It was shown that this additional variance depends only on the molecular brightness of the particles that fluctuate in number in the volume of excitation. Brightness is generally expressed as counts/pixel-dwell-time/molecule. Since brighteness depends on the laser intensity and system detection efficiency, the value of the brightness is calibrated

for a given substance. In this paper we use a monomeric EGFP construct expressed in the same type of cell to determine the "brightness of the monomer. Then the measurement of the brightness is expressed as a ratio to the brightness of the monomer. Generally a dimer will have a ration of 2 with respect to a monomer. Note that the brightness is a molecular property that cannot be derived from a simple intensity measurement since in general intensity is proportional to the product of the number of molecules in the volume of excitation times the brightness of each molecule.

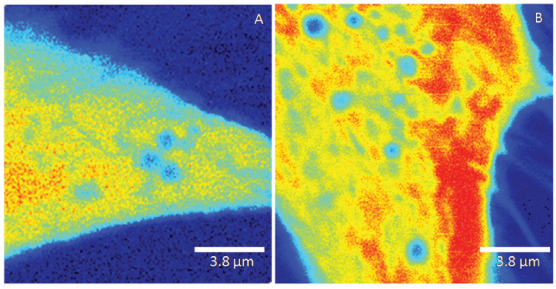

Fig. 2. A) CHO-K1 cell expressing the ET_A-EGFP construct. B) NIH 3T3 cell expressing the ET_A-EGFP construct. The focus is on the bottom part of the cell for both images.

Results and Discussion

The RICS method was applied to cells expressing the ET_A receptor. The plane of focus was set as close as possible to the bottom membrane plane. A typical image of a CHO-K1 cell expressing the ET_A-EGFP construct is shown in Fig. 2. Note that the border of the cells lack of the characteristic increase of intensity expected for a membrane bound receptor. The construct is well expressed in both cell lines.

The RICS correlation function and the fit using one component diffusion model for the CHO-K1 cell is shown in Fig. 3.

Analysis of three cells gives an average diffusion coefficient of D=11.0±0.3 µm²/s. The fits of the RICS correlation function are generally very good as judged by the relatively small residues as shown in the example of Fig. 3 (upper surface). On some of the cells, single point FCS measurements were done at selected points close to the membrane, giving comparable results for the diffusion coefficient. When the ligand was added to the cells, we found an increase in the diffusion coefficient giving D=19.8±2.8 µm²/s after about 1 min and then

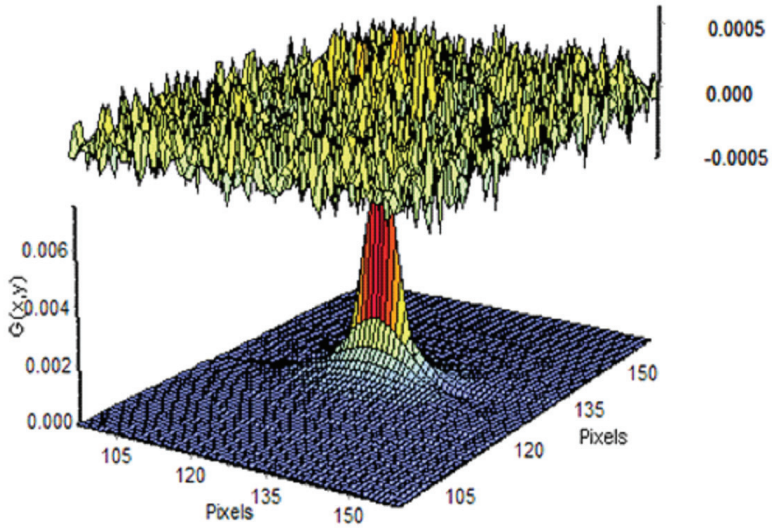

Fig. 3. Fit of the RICS correlation function. Bottom surface: RICS correlation for ET_A-EGFP in CHO-K1 cell. One component diffusion gives a value of D=10.79µm²/s. The upper surface is the residues of the fit.

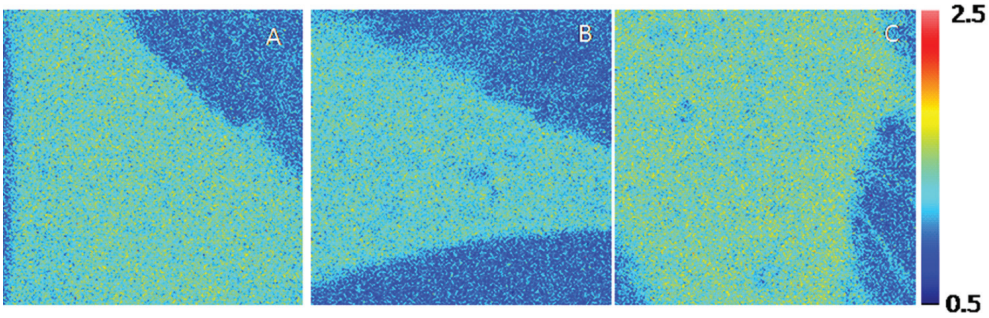

Fig. 4: Brightness map. A) CHO-K1 cell expressing monomeric EGFP; B) CHO-K1 cell expressing ET_A-EGFP, NIH 3T3 cell expressing ET_A-EGFP. The brightness is the same for all cells indicating that the protein is monomeric. In conclusion, our construct seems to locate mainly in the cytoplasm where it undergoes diffusion and it appears to be monomeric. We show that we can detect the cytoplasmic population of the ET_A receptor and accurately measure its brightness. Our specific protein seems to have lost the capability to reside at the membrane, so we were unable to study the interactions with ligand and the state of aggregation of the ET_A receptor at the membrane. Although our construct could not fully represent the native protein, we believe that the methodology we describe in this paper could be used by anyone in this field.

remained constant. Analysis of cells expressing EGFP only give a value of the diffusion coefficient of D=21.1±0.3 $\mu m^2/s$, which is typical of free EGFP in the cytoplasm of cells. For the NIH3T3 cells we found a value of D=16.3±4.2 $\mu m^2/s$.

Although we focused the plane of observation close to the bottom membrane, we believe that the ET_A molecules we observe are actually in the cytoplasm. The value of the diffusion coefficient is too large for molecules diffusing in the membrane. The prevalent localization of the mobile ET_A molecules in the cytoplasm rather than at the membrane could be due to the addition of the EGFP moiety to the receptor protein. Also the diffusion coefficient appears to be slightly smaller than the diffusion of EGFP alone, indicating that the protein is likely to be monomeric in the cytoplasm.

Brightness analysis shows that the protein is a monomer in the cytoplasm (Fig. 4). This is done by comparison with the brightness of the EGFP transfected cells (Figure 4A) with the ET_A-EGFP CHO-K1 (Fig. 4B) and NIH 3T3 (Fig. 4C) transfected cells. The ratio between the brightness of the ET_A-EGFP in both CHO-k1 and NIH 3T3 cells and monomeric EGFP was 1.03 ±0.11.

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