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Visualization of Lipid–Receptor Interactions on Single Cells by Time-Resolved Imaging Fluorescence Microscopy

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The physical interaction between plasma-membrane lipids and the epidermal growth factor (EGF)-receptor was investigated on single A431 human epidermoid carcinoma cells by monitoring fluorescence resonance energy transfer (FRET) between exogeneously added fluorescein-EGF (donor) and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (Bodipy-PC, acceptor) using donor-photobleaching FRET-microscopy. The measured mean FRET-efficiency of 13% is indicative of such a physical interaction and exemplifies the great potential and sensitivity of time-resolved imaging fluorescence microscopy techniques for the study of lipid-receptor interactions on single cells.

KEY WORDS: Epidermal growth factor; EGF-receptor; lipid-protein interaction; photobleaching.

INTRODUCTION

Digital imaging optical microscopy offers a vehicle for adapting the classical "cuvette" methods of spectroscopic analysis to the needs of the cell biologist. We have undertaken the systematic implementation of spectral and temporal resolution in the light emission microscope [1–8] and are currently able to image living and fixed cells and subcellular structures on the basis of the following photophysical modalities: phosphorescence and delayed fluorescence using a CCD camera-based system and synchronized mechanical excitation and emission choppers [1]; fluorescence resonance energy transfer (FRET) by the usual donor–acceptor emission combinations and by a new technique, donor photobleaching kinetics (pb-FRET-microscopy) [2–6]; photobleaching kinetics of intrinsic and extrinsic probes [2–6]; and fluorescence lifetimes, one of the most recent developments [7,8].

Numerous chemical and biochemical processes taking place in living cells are reflected in changes perceived by time-resolved processes. The determinations of rates and lifetimes are unique in that they provide quantitative measures that are transferable from one system to another, unlike signals and parameters based on absolute or even relative light intensities.

These microscope techniques can be readily applied to the study of lipid distribution and metabolism in cells [9]. Our most recent efforts in this area have been based on pb-FRET and fluorescence lifetime-imaging microscopy (FLIM) [8]. FLIM has proven to be very useful for obtaining images of lipid distributions on boar spermatozoa corrected for dynamic quenching [8]. In this report we focus on the use of pb-FRET-microscopy for monitoring the interaction between the epidermal growth factor (EGF) receptor and plasma membrane phospholipids on single A431 human epidermoid carcinoma cells. Receptor–lipid interactions are of great interest in view of their possible intervention in signal transduction. Most studies in this area have been based on partially purified

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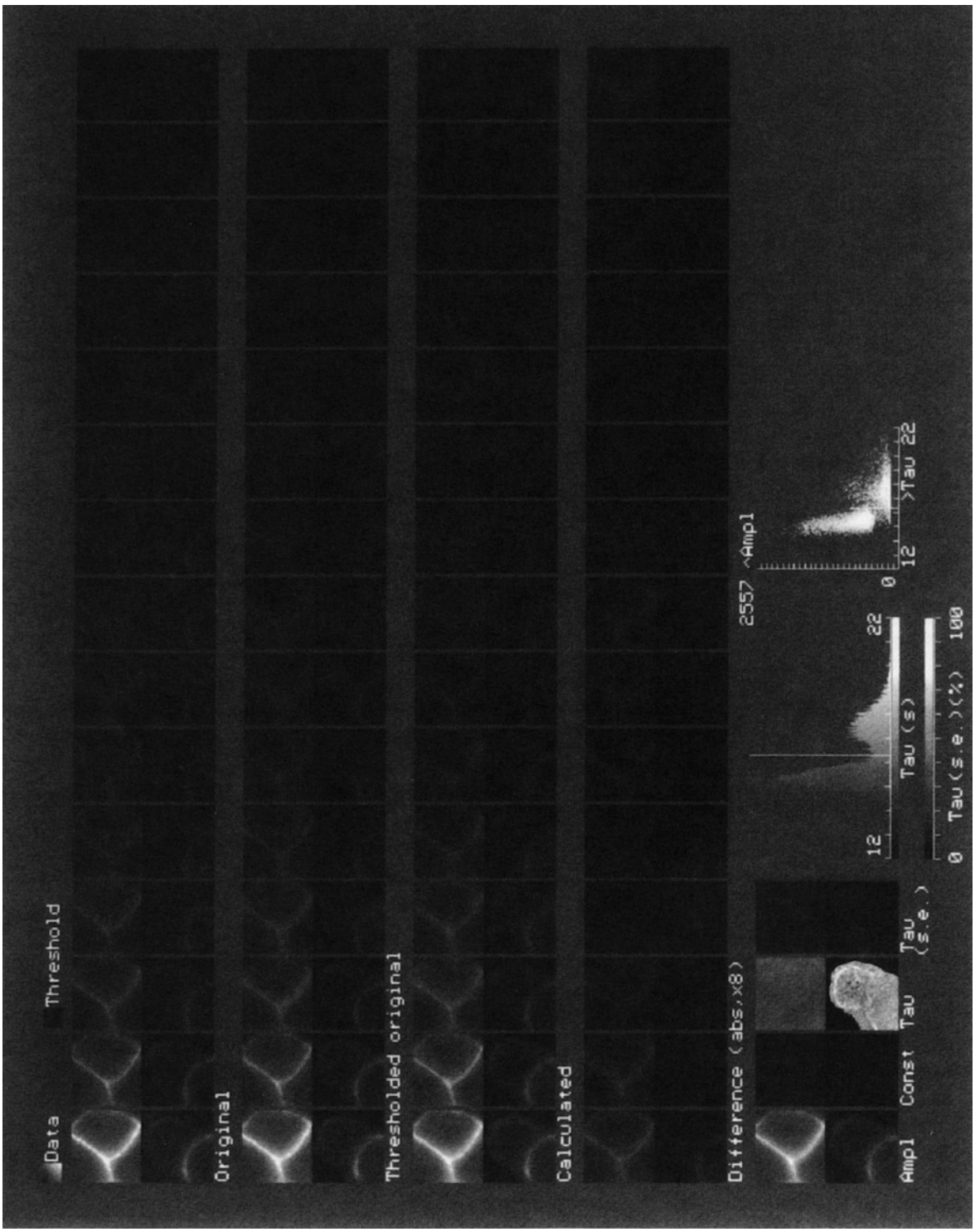
Data
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12
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12
22
Tau (s)
0
Tau(s.e.)(%)
100

Fig. 1. *Pb-FRET microscopy for studying the interaction of the EGF receptor with plasma membrane PC.* A431-cells (cultivated as described in Ref. 10) were incubated for 75 min at 4°C with 50 n*M* Fl-EGF (prepared as in Ref. 10), in phosphate-buffered saline (PBS) supplemented with 8.7 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 50 m*M* glucose, 1% (w/v) bovine serum albumin (BSA), and either 1 μ*M* 1-palmitoyl-2-pentanoyl-[5-(Bodipy 530/550)]-*sn*-3-phosphocholine (Bodipy-PC; Molecular Probes, Eugene, OR) or 1 μ*M* egg PC (Sigma, St. Louis, MO). After washing and fixation with 4% (w/v) paraformaldehyde in PBS, the cells were mounted on microscope slides and used for pb-FRET microscopy. Our CCD camera–microscope system is described in Ref. 11. The excitation source was an HBO 100-W Hg lamp (Osram, Germany). For specific imaging of fluorescein fluorescence (Fl-EGF), we used a Zeiss (Oberkochen, Germany) Plan-neofluar 40× NA 1.4 oil-immersion objective and a Zeiss 450- to 490-nm excitation filter, an Omega (Omega Optical, Brattleboro, VT) FT 500 dichroic mirror, and a Corion (Holliston, MA) BP 510 (10-nm-bandpass) emission filter. A sequence of 31 images was obtained for each data set, the first without illumination (blank) and the others with an exposure of 3 s to the excitation light; the camera was binned 2×2, such that an image pixel corresponded to a 0.32×0.32-μm^2 area in the object plane. Data analysis and image processing were carried out on a DEC MicroVaxII system using programs in Fortran and TCL-Image (TPD; Technical University of Delft, Delft, The Netherlands) ([4]; Gadella and Jovin, manuscript in preparation). For further explanation see the text.

reconstituted systems. Here we demonstrate that by implementation of photophysical techniques in the microscope these interactions can be visualized on single cells as well.

RESULTS AND DISCUSSION

The interaction of lipids with the EGF receptor was assessed by FRET using EGF labeled with fluorescein isothiocyanate (Fl-EGF) as the donor and phospholipid phosphatidylcholine (PC) labeled with a Bodipy moiety in the *sn*-2 acyl chain (Bodipy-PC) as the acceptor. The Fl-EGF was bound to the receptor on A431 cells and the Bodipy-PC was incorporated into the plasma membrane by simultaneous incubation with ethanol-injected Bodipy-PC vesicles. In the pb-FRET technique, use is made of the fact that the photobleaching time constant (τ_{bl}) is inversely correlated with the lifetime of the excited state (τ) of a fluorophore [3]. Since FRET shortens the τ of the donor, measuring the τ_{bl} of the donor in the absence and presence of the acceptor provides a means for determining FRET. The results of a pb-FRET experiment using the above donor–acceptor pair on A431-cells are given in Fig. 1. The two separate photobleach experiments, with Fl-EGF alone or in the presence of Bodipy-PC, are merged together in the top and bottom parts, respectively, of all the subimages shown. The composite image shows the original bleach data (row 1); the thresholded original data (row 2); the calculated data based on a single-exponential pixel-by-pixel fit (row 3); the difference between calculated and observed data (row 4); and in row 5 the images of the bleachable and nonbleachable part of the initial fluorescence image (Ampl and Const), the τ_{bl} image (Tau), its relative standard error [Tau (s.e.)], the temporal histogram of Tau, and a two-dimensional histogram with the correlation between Tau and Ampl. The Tau subimage clearly shows that the τ_{bl} of Fl-EGF is increased upon the addition of Bodipy-PC. The change in photobleaching kinetics is also apparent from the temporal and two-dimensional τ_{bl} histograms. The increase in τ_{bl} is indicative of FRET from fluorescein to Bodipy and is correlated to the transfer efficiency E by the relation $E = 1 - \tau_{bl}/\tau'_{bl}$, where τ_{bl} and τ'_{bl} are the photobleaching time constants of the donor (fluorescein) in the absence and presence of the acceptor (Bodipy) [2–6]. From this relationship, an average $E = 13\%$ was determined for the experiment in Fig. 1. Together with the R_o for the fluorescein–Bodipy pair of 5.5 nm, this implies an average distance between Fl-EGF and Bodipy-PC of ~ 7–8 nm, assuming a 1:1 complex. This study reveals a direct physical interaction between plasma membrane phospholipids (PC) and the EGF receptor on single cells and exemplifies the great potential use of time-resolved fluorescence microscope techniques. The latter have also been applied to the study of ligand (EGF)-induced receptor aggregation, an essential step in the signal transduction pathway.

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