Confocal FRET microscopy to measure clustering of receptor-ligand complexes in endocytic membranes

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Running Title: Receptor clustering measured by FRET

Keywords: MDCK cells, pIgA-R, energy transfer efficiency (E%), FRET correction algorithm, membrane microdomains, apical endocytic compartments

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ABSTRACT

Our main objective is to address the molecular mechanisms underlying the dynamics of protein distribution within membranes, which is involved in several cellular processes, such as protein sorting, organelle and membrane microdomain biogenesis, protein-protein interactions, receptor function and spatial organization of signal transduction. Quantitative methods that require advanced mathematical modeling are necessary to further our understanding of the dynamics of protein organization within cellular membranes. An assay based on Fluorescence Resonance Energy Microscopy (FRET) was developed to differentiate between the clustered and random distribution of membrane-bound fluorophore-labeled receptor-ligand complexes. Furthermore, we have developed a novel mathematical model, which is tailored to large, tightly packed molecular clusters. Our results demonstrate that polymeric IgA-receptor-ligand complexes are organized in clusters within apical endocytic membranes of polarized MDCK cells, since energy transfer efficiency (E%) levels are independent from acceptor fluorescence, a standard parameter to confirm clustered distribution. Here we describe a second parameter: With increasing unquenched donor fluorescence and unquenched donor : acceptor ratios, E% decreases. Our mathematical model explores this phenomenon by describing how some donors are prevented from interacting with an acceptor by the presence of other donors or unknown molecules. We call this effect ‘donor geometric exclusion’. In summary, we present a new sensitive FRET-based method to quantify the colocalization and distribution of receptor-ligand complexes in endocytic membranes of polarized cells.
INTRODUCTION

In the context of protein sorting and trafficking, it is of interest to know which specific cellular components are distributed in close proximity within the membrane. The sorting of membrane proteins is central to biosynthetic and endocytic trafficking, to endosomal organization, and in polarized cells to the additional task of maintaining cell polarity. We are proposing that in the process of sorting out membrane proteins and forming transient microdomains, a clustering process occurs. Our main objective is to test whether complexes between polymeric IgA receptor (pIgA-R) and its ligands organize in a clustered manner in the apical endocytic compartments of polarized MDCK cells.

MDCK cells provide an ideal biological system to study the transport, sorting and signaling in fully polarized epithelial cells. Endocytosis, transcytosis and exocytosis are complex and highly regulated transport mechanisms used by these cells to move fluids, proteins, lipids, nutrients and other materials into, across and out of the cell, respectively (Figure 1). Polarized epithelial cells have two distinct plasma membranes (PMs), apical and basolateral PMs, separated by tight junctions. It is mainly in these cells that transcytosis takes place: the internalization of extracellular components - e.g. pIgA by the pIgA-R - at the basolateral PM and transport to the apical PM. Transcytosis is facilitated by a network of vesicles and endosomes that are partially shared with other endocytic pathways. Polarized epithelial MDCK cells stably transfected with pIgA-R are one of the best studied transcytotic models, and yet, there are still questions concerning the morphology and/or organization and regulation of the endocytic compartments involved in the transcytotic pathway.
Apical trafficking via endocytic compartments is of particular interest in polarized epithelial cells, since it includes the critical last sorting step of basolateral-to-apical transcytotic cargo, before release to the apical PM. Several lines of evidence indicate that the apical endocytic compartments of polarized epithelial MDCK cells are an excellent model to study endosomal protein sorting. These compartments include the apical early endosome (AEE), the apical recycling endosome (ARE) and to lesser extent the common endosome (CE), but not the basolateral early endosome (BEE) (Barroso and Sztul, 1994; Brown et al., 2000; Gibson et al., 1998). First, the apical endocytic compartments are clearly compartmentalized into vacuolar areas containing fluid-phase components and tubulo-vesicular structures, which are devoid of fluid-phase components and contain only membrane-bound cargo (Leung et al., 2000; Barroso and Sztul, 1994). Second, the dynamics of the apical endocytic compartments can be imaged by following the trafficking of pIgA-R, a well-known marker for basolateral-to-apical transcytosis and because they are localized proximally to the apical PM (Apodaca et al., 1994; Barroso and Sztul, 1994). Fourth, trafficking through apical endocytic compartments is regulated by signaling molecules, providing an additional level of molecular control to the apical targeting pathway (Hansen and Casanova, 1994; Huttner and Zimmerberg, 2001; Van IJzendoorn et al., 2000; Winckler and Mellman, 1999).

For our quantitative analysis of receptor clustering events in the apical endocytic compartments, we used laser scanning confocal microscopy and fluorescence resonance energy transfer (FRET), in particular, the parameter of energy transfer efficiency (E%) (Wu and Brand, 1994; Periasamy and Day, 1999; Day et al., 2001; Kenworthy, 2001). As a first step we have internalized pIgA-R ligands - labeled with different fluorophores (‘donor and acceptor’) - from opposite PMs in
MDCK cells, which are stably transfected with rabbit pIgA-R. On binding, the basolaterally internalized pIgA-R-ligand complexes are transported to the apical PM, while the apically internalized receptor-ligand complexes are endocytosed from the apical PM and recycled back to the apical PM (Figure 1). Basolaterally and apically internalized pIgA-R-ligand complexes will eventually co-localize and accumulate in apical endocytic compartments, just below the apical PM, upon internalization at 17°C, which blocks delivery to the apical PM (Figure 1 shows an example of co-localization in the ARE) (Barroso and Sztul, 1994; Apodaca et al., 1994). It is the purpose of our experiments to use FRET microscopy to determine whether clustering precedes their forward transport from apical endocytic compartments to the apical PM.

To calculate E% using fluorescence imaging microscopy requires a reliable method to remove spillover contamination from the FRET signal. FRET occurs when donor and acceptor fluorophores have sufficiently large spectral overlap, favorable dipole-dipole orientation, proximity of 1-10nm and large enough quantum yield (Lakowicz, 1999). The very spectral overlap, however, is the cause of the contamination due to the overlap of the emission spectra of donor and acceptor (donor cross-talk) and that part of the acceptor absorption spectrum which is excited by the donor wavelength (acceptor bleed-through). There are a number of methods to avoid, minimize or correct the contamination, each with certain limitations depending on the level of sensitivity desired (Chamberlain et al., 2000; Gordon et al., 1998; Bastiaens and Jovin, 1996; Wouters et al., 1998). The method used in our experiments, which is based on an algorithm correcting the spillover contamination in a pixel-by-pixel manner, using single-labeled reference specimens, is favorable to obtain highly sensitive corrected FRET signals (Elangovan et al, 2002).
The data presented here demonstrate a clustered distribution of pIgA-R-ligand complexes in the membranes of apical endocytic compartments, using FRET confocal microscopy. The relationship between E% and acceptor and unquenched donor (uD) levels was used to determine that differently labeled receptor-ligand complexes are distributed in a clustered manner in apical endocytic membranes. As modeled previously, E% being independent of acceptor levels is one indicator of a clustered assembly (Kenworthy and Edidin, 1998); another indicator demonstrated in this paper is the decrease of E% with increasing uD levels and uD : acceptor (uD:A) ratios, which is especially useful when acceptor data is not available or when donor and acceptor expression levels are not easily modulated. For the mathematical analysis, we have developed a stochastic model, which describes E% levels in large and dense clusters as a function of the concentration of donor- and acceptor-labeled receptor-ligand complexes. This model explores the ‘donor geometric exclusion’ phenomenon, in which increasing donor densities prevent some donors from interacting with a potential acceptor. In clusters, the existence of donors that can participate in FRET events (FRET donors) and donors that cannot (non-FRET donors) was validated by donor bleaching experiments. In summary, our results implicate the clustering of receptor-ligand complexes in protein sorting and transport within apical endocytic compartments. Furthermore, our analysis of receptor distribution in membranes should be readily applicable to other examples of clustering of membrane components.
MATERIALS AND METHODS

Culture of MDCK Cells on Filter Inserts. MDCK cells stably transfected with pIgA-R were grown to confluence in 100mm cell culture dishes for four days, trypsinized, centrifuged and resuspended in DMEM/10% FBS/Pen-Strep (Barroso and Sztul, 1994). 120µl of the cell suspension were placed on top of an inverted Transwell Clear insert (Corning Costar, Cambridge, MA), i.e. on the outside of the membrane, allowing the visualization of the MDCK cells directly through a coverslip using an inverted microscope (Brown et al., 2000). After 3 days in culture these cells are fully polarized and are used immediately according to specific internalization protocols (Barroso and Sztul, 1994).

Internalization of Fluorophore-Labeled Ligands. We have previously shown that polarized MDCK cells transfected with rabbit pIgA-R, internalize apically and basolaterally added pIgA-R-ligands at 17°C to transport the receptor-ligand complexes to apical endocytic compartments, (Barroso and Sztul, 1994). The inserts are washed with PBS and equilibrated with DMEM/HEPES/BSA at 17°C. 160 µg/ml pIgA-R pseudo-ligands ([Fab]2 fragments of antibodies raised against the extracellular domain of the rabbit pIgA-R) conjugated to Alexa488 (Molecular Probes, Eugene, OR) or Cy3 (Amersham Life Science, Pittsburgh, PA) are applied to the apical and basolateral PM, respectively, which is eight times that of our original protocol, to minimize the presence of empty receptor units. These pIgA-R ligands were shown to be transported across the polarized MDCK cells in a manner similar to dIgA, the physiological ligand of pIgA-R, as described in Barroso and Sztul (1994). Cells are incubated at 17°C for four hours to allow internalization of pIgA-R-ligand complexes into the sub-apical region by transcytosis from the basolateral towards the apical PM and by endocytosis from the apical PM (Figure 1). At this
temperature, delivery to the apical PM is blocked and both receptor-ligand complexes predominantly localize in apical endocytic compartments, located approximately 3.5 µm below the apical PM (Barroso and Sztul, 1994). Visualization of all our images and data presented takes place at this focal plane. Then, the cells are washed with PBS to remove unbound ligands and immediately fixed with 4% paraformaldehyde/PBS.

In all, three different samples were used: The double-labeled specimen, containing apically internalized Alexa488-pIgA-R-ligand complexes (donor) and basolaterally internalized Cy3-pIgA-R-ligand complexes (acceptor), plus corresponding single-label reference samples containing either Alexa488 or Cy3, which were used to establish the contamination levels.

**Laser Scanning FRET Microscopy.** For data collection, we used a Nikon PCM 2000 laser scanning confocal microscope, equipped with a 60x water immersion lens 1.2 NA, Argon (488nm) and Green HeNe (543nm) laser, emission filters 515/50nm and 590nm LP, respectively. SimplePCI software (Compix, Cranberry Township, PA) was used to drive the hardware, image acquisition and processing. Bleaching is undetectable when the argon laser is only used for one scan to collect the final image - see below. PCM is set to collect data simultaneously in both channels at a 1024x1024 pixel image.

**Data Collection.** The specimen is positioned in a small chamber created by a coverslip between two metal rings, filled with a small amount of PBS and placed on the microscope stage. We first select an appropriate area of the specimen, check the cell height (15-20 µm) and a focal plane of 3.5 µm below the apical PM. This is done with only the Green HeNe laser in operation. Optimal
PMT settings are also established in this pre-image-acquisition phase. With the zoom setting at 2.3 and without any image processing, a one-scan image of the double-labeled specimen is taken with only the Green HeNe laser/acceptor excitation (the argon laser is blocked), followed by a one-scan image with only the argon laser/donor excitation (Green HeNe laser blocked). The single-labeled acceptor specimen follows the same protocol; the single-labeled donor specimen is only scanned with the argon laser, as we do not observe fluorescence when subjected to the acceptor excitation. Images of all three types of specimen are taken under the exact same conditions: 60x water immersion lens, PCM 1024 color, 2.3 zoom, no processing.

**Bleaching experiments:** Imaging conditions are precisely the same as described above taking two single scans with the acceptor/donor excitation respectively, at time ‘0’ for the double-label, single-label donor and acceptor. For the ‘bleaching-the-donor’ experiments, this is followed by 30sec of bleaching with the argon laser (donor excitation - both donor channel and acceptor channel fluorescence is collected simultaneously), switching to the acceptor excitation and taking a one-scan image. Another period of 30sec of argon laser bleaching is then performed until a total of 5 minutes of bleaching time has been accumulated. The ‘bleaching-the-acceptor’ experiments were conducted following the principles established in the literature (Gadella and Jovin, 1995; Bastiaens and Jovin, 1996; Kenworthy and Edidin, 1998; Jovin and Arndt-Jovin 1989). After surveying the cells as described above, the zoom is changed to 10x, which results in the capture of only the centrally located cells of interest. The HeNe laser is now allowed to scan continuously until the acceptor is bleached, which takes approximately 10min. The zoom is changed back to 2.3x and new one-scan images are taken separately with the HeNe (acceptor) and Argon (donor) lasers. Under acceptor excitation in the acceptor channel, the bleached
'window’ is clearly visible allowing us to establish the pixel coordinates and the registration of different images thus correcting any slippage. The donor fluorescence (donor excitation/donor channel) within this bleached window, before and after bleaching the acceptor corresponding to the quenched and uD fluorescence, forms the basis of calculation for the energy transfer.

**Post-Acquisition Data Generation.** There are two contaminants in the FRET signal: donor cross-talk and acceptor bleed-through. We have developed a novel algorithm, which removes these contaminants pixel-by-pixel on the basis of matched fluorescence levels between the double-label specimen and a single-label reference specimen, using seven images: two single-label donor reference images (donor excitation/donor channel and acceptor channel); two single-label acceptor reference images (donor and acceptor excitation, both in the acceptor channel); three double-label images (donor excitation/donor and acceptor channel, acceptor excitation/acceptor channel) (Elangovan et al, 2002). The donor excitation/acceptor channel corresponds to the uncorrected FRET (uFRET) image.

The pixel-by-pixel correction used to generate the corrected FRET (cFRET) image is actually based on the average value of narrow fluorescence ranges, for more efficient running of the correction algorithm (Elangovan et al, 2002). In our case, we chose the average of 12 fluorescence units, i.e. 0-12, 13-24 etc continuing to the highest fluorescent units in the image. Using the average of even narrower ranges did not improve the sensitivity.

**Post-Acquisition Data Analysis.** The single-number Förster- type energy transfer $E$ is described as the ratio of energy transfer to the total sum of rates for all processes by which the excited
donor can return to its ground state. Thus, $E$ is based on the energy that is transferred from the donor to the acceptor and is dependent on the distance between donor and acceptor fluorophores and the geometry of binding of the donor/acceptor pair (Wouters et al., 2001; Lakowicz, 1999).

In contrast, ‘apparent’ $E\%$ is not only dependent on $E$, but is also influenced by the concentrations of free and bound donor or acceptor molecules. By this definition, most references in the literature, including our measurements, fall into the category of ‘apparent’ $E\%$, which, for brevity we will continue to call $E\%$ in this paper.

$E\%$ is an expression of the energy transfer as a percentage of $uD$, as described in Eq. 1 (Lakowicz, 1999). There are different methods to establish $E\%$, the most widely used being ‘bleaching-the- acceptor’ (Bastiaens and Jovin, 1996; Wouters et al., 1998). To avoid the potentially negative results of photobleaching, we have pursued an alternative algorithm-based approach which allows us to establish an $uD$ value by adding the $cFRET$ value - representing total energy transfer - to the quenched donor ($qD$) fluorescence and thus to calculate $E\%$:

$$E\% = 100 \times \frac{cFRET}{(qD + cFRET = uD)} \quad \text{Eq. 1}$$

As a first step, we visually select appropriate regions of interest (ROIs - usually one complete cell) from the uFRET image and note their pixel coordinates. These pixel locations are applied to the other images and fluorescence values are extracted. A custom-written analysis program selects pixels between 10 and 254 arbitrary units in the uFRET image. Eliminating values below 10 arbitrary units removes background noise, which we previously established to be on average 8 arbitrary units (data not shown). By not considering pixels at 255 units (the maximum of the
range) we eliminate saturated pixels. The selected uFRET pixel locations are transferred to the qD image (donor excitation/donor channel) and pixels containing saturated donor fluorescence are eliminated (this is a precaution to avoid a potentially misleading calculation of the uD value; in actuality, there are very few saturated donor pixels). This final pixel selection becomes the template for all calculations. The final numbers are transferred to Excel spreadsheets for calculation of the various parameters. This analysis allows us to estimate E% by using Eq. 1 and plot its relationship to actual acceptor and uD levels as well as actual uD:A ratios. Here, we use E%, acceptor and uD values averaged over an ROI. These average values based on the original pixel-by-pixel analysis are used to compare different ROIs. It is important to stress that such a comparison is only possible since the excitation efficiencies (ε), quantum yields of the fluorophore molecules and the detection efficiencies (Q) are maintained constant throughout the experiments.
RESULTS

Here, we show that pIgA-R-ligand complexes, internalized from opposite PMs, co-localize in clusters in apical endocytic membranes of polarized epithelial MDCK cells. To demonstrate this clustering phenomenon, we have used laser scanning confocal FRET microscopy and shown the independence of E% from the acceptor, a standard for a clustered distribution pattern (Kenworthy and Edidin, 1998). We have also identified a new parameter indicative of clustered distribution, namely the E% decrease with rising uD levels and resulting uD:A ratios. To explore this phenomenon, we have developed a mathematical model to describe the effect of ‘donor geometric exclusion’, which we propose is due to the presence of donors not participating in energy transfer, because they are prevented from interacting with an acceptor by other donors or unknown molecules.

FRET assay. In our biological system, fluorophore pairs are not always separated by a consistent distance and FRET occurs over a wide range of fluorescence intensities at a membrane plane, making it extremely important to use a sensitive and finely tuned FRET assay with a signal spillover correction system. We have developed an algorithm method that correct FRET contamination (donor cross-talk and acceptor bleed-through) in highly sensitive manner, as described in Elangovan et al. (2002). Seven images are taken to make the correction of the double-label FRET signal. Those include images of single-label reference donor and acceptor, as well as double-labeled specimens with comparable fluorescence ranges taken under identical imaging conditions (Figure 2). Applying the algorithm with several different single-label reference specimens produces near-identical results, indicating that different single label controls do not change the correction level (data not shown). Since single and double-label specimens are
distinct, only fluorescence levels are matched, not their pixel locations. The single-label donor specimen under donor excitation in the donor channel represents the baseline uD fluorescence (Figure 2A) and in the acceptor channel shows the corresponding donor cross-talk contamination (Figure 2B). Similarly, using a single-label acceptor specimen, Figure 2C accounts for the baseline acceptor fluorescence under acceptor excitation/acceptor channel conditions, and in Figure 2D the corresponding acceptor bleed-through with donor excitation in the acceptor channel. In the double-label specimen, we collect 3 images: Acceptor excitation/acceptor channel (Figure 2E) representing acceptor fluorescence, donor excitation/donor channel (Figure 2F), showing the qD and finally, the uFRET image (Figure 3G). Having deducted the bleed-through and cross-talk contaminations from the uFRET in a pixel-by-pixel manner using our algorithm-based correction method, the final cFRET image emerges (Figure 3H). Comparing Figure 2G and H it is clearly visible where contamination has been removed. Three typical ROIs, each including one cell, are shown in Figure 2G-H. In both uncorrected and corrected images, the punctate pattern of apical endocytic membranes located at the level of the apical apex is clearly visible (Figure 2).

Another important point to make is that we have increased the sensitivity of our FRET-based assay in two ways: (1) by using the ‘actual’ donor and acceptor gray-level intensity values to calculate uD:A ratios, instead of the internalized D:A ratios and (2) by thresholding our results, so that we only use pixels for evaluation, which have participated in energy transfer based on the uFRET image. Since fluorescence intensity is proportional to concentration, local concentrations of labeled proteins can be assayed by fluorescence microscopy of the selected ROIs. Different cells internalized with different donor and acceptor concentrations of receptor-ligand complexes
show significant variability in their actual uD:A ratio as observed by FRET confocal microscopy. Variability between different cells can be caused by different expression levels and different rates of transcytosis. The 'actual’ ratio, then, is based on uD and acceptor levels in those pixels, which are selected for analysis - as described earlier. This ratio becomes a reliable parameter across experiments reflecting the circumstances under which potential FRET takes place, regardless of the internalization ratio.

**Pixel-by-pixel visualization of uFRET, cFRET, uD and E% values.** Figure 3 shows false-color images depicting fluorescence intensity pixel by pixel of the three ROIs (each ROI corresponds to one cell) indicated in Figure 2. These false-color images show a two-dimensional Z-section (i.e. in the xy plane) at ~3.5µm below the apical PM for the uFRET (Figure 3A), cFRET (Figure 3B), uD (Figure 3C) and E% (Figure 3D) images. These are resolved pixels of 88 nm x 88nm. Generally, the typical irregular and punctate endosomal pattern of the apical endocytic membranes is seen across all images (the acceptor image follows the same pattern – data not shown). Removal of spectral spillover is clearly noticeable, when comparing uFRET versus cFRET (Figure 3A-B), as are areas of higher concentration of E% (Figure 3D). The uD:A ratio pixel information was used as a template to construct the E% image, by selecting ratio values of >0 and ≤10, which had the effect of isolating pixels with - for FRET - relevant presence of donor and acceptor levels. A number of observations can be made concerning the E% image presentation: There is a higher level of sensitivity apparent than in the other images, without losing the overall endosomal morphology. Pixel locations with high uD levels (uD image) show a lower E%, a phenomenon that is also clearly seen when using the average fluorescence/pixel of the total ROI (Figure 3). Higher donor concentrations/lower E% occur
more in the center of the punctate structures, whereas the higher E% pixels are mainly concentrated on the periphery. Also, individual as well as groups of 2-3 pixels are detected showing higher E%, which could represent individual vesicles budding from, or on their way to dock onto, the endosomal membrane.

**pIgA-R-ligand complexes are organized in a clustered distribution in apical endocytic membranes.** Theories of how to distinguish between clustered and a random distribution have been described in the literature, most recently applied to the biological field by Kenworthy and Edidin (1998). According to these concepts, energy transfer between donor and acceptor molecules is governed by different dynamics with respect to the relationship of E% to acceptor levels, when the distribution is random or clustered. In the random situation the likelihood of an acceptor colocalizing with a given, equally random donor population increases with increasing acceptor fluorescence and leads to an increase in E%. In contrast, in clusters that by definition have molecules in proximity, increasing acceptor fluorescence does not increase E%, as a donor molecule can only transfer its energy to one acceptor at a time. A slight E% increase may be seen in the presence of high levels of acceptor since these should increase the chances of favorable dipole-dipole orientation between donor and acceptor molecules. E% is therefore largely independent of the acceptor, which has been used as the main indicator for a clustered distribution pattern.

To determine whether receptor-ligand complexes are randomly distributed or clustered in apical endocytic membranes, we chose a large number of ROIs (147), each representing one complete cell, and a wide range of uD and acceptor values to create as broad a data base as possible. We
show that E% decreases with rising uD levels (Figure 4A), decreases with rising uD:A ratios (Figure 4B) and is independent of acceptor levels (Figure 4C). This fits the model of a clustered organization.

To find out how our algorithm-based energy transfer efficiency results compare with the standard method of ‘bleaching-the-acceptor’, we conducted an experiment according to this method. Confirming our previous results, the ‘bleaching-the-acceptor’ E% results are also independent of acceptor fluorescence levels indicating a clustered distribution (Figure 5A). E% values fall within the same ranges (20-40%), as the majority of ROIs do in experiments using the algorithm correction method (35-50%). As expected in a direct comparison between the two systems, standard deviation ranges (error bars) overlap (Figure 5B). Furthermore, correlation analysis between acceptor fluorescence and E% for the algorithm method has a coefficient of 0.13 and for the ‘bleaching-the-acceptor’ –0.07, both indicating that the two parameters are unrelated, i.e. E% is largely independent of the acceptor.

Positive controls included co-internalizing both differently labeled ligands from the same PM for 4 hours at 17°C. As expected, FRET occurred at every stage of the transcytotic/endocytotic pathways starting at the PM (data not shown). Single labeled specimen serve as negative controls, where at the donor excitation wavelength, the acceptor signal in the FRET channel represents bleed-through, that of the donor is cross-talk (Figure 2B/D).

We examined different cohorts based on ranges of E% and found statistically different levels of uD levels and uD:A ratios within these groups. Figure 4D examines the average values of the
different parameters of four groups based on different energy transfer efficiency ranges. We arbitrarily created 4 groups based on E\%: group 1 (E=55-65%; N=15), group 2 (E=45-55%; N=29), group 3 (E=35-45%; N=70), group 4 (E=25-35%; N=33) and then examined the uD, uD:A ratio and acceptor between the groups on their statistical difference. The negative dependence of E\% on uD and uD:A ratio is statistically significant (note non-overlapping error bars between groups 1&3, 1&4, 2&4 for uD, and between 1&4 for uD:A ratio), but that acceptor fluorescence does not differ significantly between the groups (all error bars overlap). The P-values [P(T<=t)two-tail] between these groups for the uD are No. 1 vs. No.2: P= 3.08E-08; No. 2 vs. No. 3: P= 1.59E-13; No. 3 vs. No. 4: P= 2.5E-07. For the uD:A ratio: No. 1 vs. No.2: P= 0.00078; No. 2 vs. No. 3: P= 3.72E-06; No. 3 vs. No. 4: P= 0.0022. For the acceptor: No. 1 vs. No.2: P= 0.068; No. 2 vs. No. 3: P= 0.29; No. 3 vs. No. 4: P= 0.745. The existence of these different groups suggests the presence of different stages of sorting and organization of clusters. In summary, we confirm that E\% is indeed independent of the acceptor and we conclude that using this parameter we can establish a clustered distribution for pIgA-R-ligand complexes in apical endocytic membranes.

**Establishing clustered distribution using E\% vs. uD – A novel parameter.** We propose that clustered distribution of labeled ligands in apical endocytic compartments can be identified by a decrease of E\% with increasing uD:A ratios and uD levels. This criterion is most useful when acceptor fluorescence data is not available or when acceptor and donor levels are not easily modulated as for example in our receptor-ligand experiments and in experiments in which donor and/or acceptor expression are generated by transient transfection. At low uD:A ratios, every donor has ample opportunity to transfer energy to one of the many acceptors. Increasing the
uD:A ratio has the possible effect of some donors preventing others from being in FRET distance with an acceptor, causing $E\%$ to decrease. We refer to this phenomenon as ‘donor geometric exclusion’. Currently, we cannot exclude that other membrane components also prevent a given donor from transferring energy to an acceptor. Thus, there are three possible ways for a labeled pIgA-R-ligand donor complex to be geometrically/spatially prevented from transferring energy to an acceptor in a clustered situation: (a) by some unknown membrane component (b) by an unlabeled receptor-ligand complex, or (c) by another labeled complex. Since we are saturating the ligands with excess dye during the conjugation phase and using high labeled ligand concentration levels during internalization, we expect a reduced number of unlabeled receptor-ligand complexes or free receptors to be present in apical endocytic membranes. Furthermore, since uD levels is directly proportional to the donor amount present in apical endocytic membranes and increased uD levels leads to a significant decrease in $E\%$, we suggest that donor-labeled receptor-ligand complexes are a highly likely source for blocking other donors from transferring energy to potential acceptors.

To investigate the mechanism of the negative dependence of $E\%$ on uD:A ratios, we introduce a novel model, which is suitable for the description of large, tightly packed clusters (see detailed description in the Appendix). This model takes into account fully labeled complexes, extensive clusters of colocalized, differently labeled pIgA-R-ligands, and makes certain geometric assumptions to account for ‘donor geometric exclusion' effects but not competition between donors for an acceptor, which could also play a role.
To distinguish between clustered and random distributions of labeled receptor-ligand complexes, we compare our experimental data with the predictions of our ‘donor geometric exclusion’ model and of three other models for different distributions of complexes. One model describes a situation in which randomly placed acceptor molecules interact with a low density of donor molecules (Dewey and Hammes, 1980), while another model described a situation in which dimers are distributed at low density (Kenworthy and Edidin, 1998). We also constructed a model for randomly placed molecules following the assumptions of the ‘donor geometric exclusion’ model.

The four models express the predicted E% as a function of several parameters, including the Förster distance R₀, the distance of closest possible approach R, and the surface densities of the acceptor and donor fluorophores s_A and s_D, respectively. In our experiments, R₀ and R are known quantities, and the acceptor and donor fluorescence determine the respective surface densities up to a factor given by the total membrane surface area in apical endocytic membranes over which the receptors are distributed. Though we currently have no estimate for the apical endocytic membrane surface area containing receptor-ligand complexes, we will argue that the random distribution models contradict our experimental results both qualitatively and quantitatively. On the other hand, we will show that the predictions of both the Kenworthy-Edidin dimer model and our model of an infinite, perfect cluster agree qualitatively with our experiments. Quantitatively, our results lie between the predictions of the two clustering models.

Our experimental data point to a clustered, rather than a random distribution in three different ways: (1) Decrease of E% with donor surface density and uD:A ratios: In both the
approximation of Dewey and Hammes and our model for randomly distributed labeled receptor-ligand complexes, E% depends only on acceptor density (Figure 6B), but not on donor surface density (Figure 6A). In both the Kenworthy-Edidin dimer model and our model of a perfect cluster, E% decreases with increasing uD:A ratios (Figure 6C). This agrees well with the experimental data shown in Figures 4B/D. The experimental results in Figures 4A/B/D show a significant decrease of E% with donor surface density and uD:A ratios, strongly indicating a clustered distribution; (2) **No significant dependence of E% on acceptor surface density:** In both the approximation of Dewey and Hammes and our model for randomly distributed labeled receptor-ligand complexes, E% increases with increasing acceptor surface density and decreases to zero if acceptor surface density is taken to zero (Figure 6B). The experimental data in Figures 4C/D and 5A shows no significant dependence of E% on acceptor fluorescence (correlation coefficient 0.13 and 0.07). Most markedly, E% does not vanish as acceptor fluorescence becomes small. This again indicates a clustered distribution; (3) **Rough quantitative estimate:** A data point close to the median of the distribution in Figure 4B is given by uD:A = 2.5, E = 40%. For a completely random distribution of labeled complexes, an E% ~40% would correspond to an acceptor surface density of approximately 13% according to our model, and 20% in the model of Dewey and Hammes (see Figure 6B). At a uD:A ratio of 2.5, this would correspond to a fraction between 45% (our model) and 70% (Dewey and Hammes) of the entire apical endocytic membrane surface covered by labeled receptor-ligand complexes. In view of the many functions performed by the apical endocytic compartments, one would expect the actual percentage of the surface to be much lower. Since the Kenworthy-Edidin dimer model and our model of a perfect cluster contain no adjustable parameters, we may directly compare Figure
6C with Figure 4B. The experimental data shown in Figures 4C and 5A fall between the predictions of the two clustering models shown in Figure 8C.

In summary, our experimental data cannot be reconciled with a perfectly random arrangement of labeled receptor-ligand complexes, but fits the predictions of the two clustered models at least qualitatively. The above computation seems to indicate that the ‘local’ surface density of labeled complexes near a typical reference donor-labeled ligand should be around 45-70%, suggesting similar values for the density of labeled ligands within a cluster. In other words, the immediate neighborhood of a reference donor should contain 3-4 labeled complexes, contrary to the assumptions of the dimer model. We expect actual cluster densities to be noticeably higher, since our model neglects two effects that negatively influence E%, namely finite cluster size and donor-donor competition. In the future we expect to be able to estimate typical cluster density, which will require an understanding of the fluctuations of the apical endocytic membrane surface area and its correlation with the acceptor and donor surface densities.

**Donor photobleaching leads to increased E%**. E% is the total energy transfer from the donor to the acceptor expressed as a percentage of total uD levels. Total uD levels includes all donors, i.e. those that participate in FRET (FRET donors) and those, which do not (non-FRET donors). Equation 1 can therefore be restated thus:

\[
E% = \frac{100 \times \text{total energy transfer (cFRET)}}{[\text{uD (FRET)} + \text{uD (non-FRET)}]} \quad \text{Eq. 2}
\]
We hypothesized that by preferentially removing non-FRET donor, E% should increase. Such a preferential elimination is possible by bleaching the specimen with short periods of donor excitation and taking measurements after each bleaching period. Since the degree of bleaching of a fluorophore depends on the time the molecule spends in the excited state, participating in energy transfer constitutes an additional and rapid pathway for de-excitation, resulting in less time spent by a donor molecule in the excited state. Thus, FRET and non-FRET donors exhibit different rate constants, i.e., the non-FRET donors bleach first and at a faster rate. To demonstrate this effect, we used donor excitation to bleach single and double-labeled specimens for 10 periods of 30sec (Figure 7). Then, we selected five ROIs with an average of ~1.5 uD:A ratio and corresponding E% ~ 21% at time 0. Comparing single-label uD (non-FRET donors) with double-label uD levels shows a statistically different rate of bleaching at each time point (Figure 7A), which demonstrates the presence of FRET donors in the double-label samples, since their absence should result in identical rates of bleaching in double- and single-label samples. The acceptor molecules in both single- and double-labeled specimens are only partially excited by the donor laser wavelength and show no statistical difference between the double and single-labeled experiments (Figure 7B). In Figure 7C, we have compared average normalized uD levels vs. average normalized E% at cumulative bleaching time points for five ROIs. Having demonstrated in Figure 7A that non-FRET donors bleach at a faster rate, we are suggesting that if non-FRET donors in the double-label are being bleached first, then E% should increase. As shown in Figure 7C, after a period of 300s of bleaching, E% increases 1.5-2x as uD levels decreases dramatically by >80%, demonstrating the presence of non-FRET donors in double-labeled clusters.
It has been shown before that donor photobleaching is an alternative way to measure FRET (Gadella and Jovin, 1995; Wouters et al., 1998; Schmid et al., 2001; Glauner et al., 1999). The photobleaching rate of the donor decreases proportionally to the reduction of the lifetime of the donor’s excited state that is generated from the occurrence of a FRET event. However, this method to measure E% can only applied correctly to donor-acceptor pairs, which are separated by a fixed distance. Considering our biological situation, i.e. clusters of receptor-ligand complexes in endocytic membranes, we can assume that our donor-acceptor pairs are not separated by a fixed distance. Nevertheless, the average E% using the donor photobleaching technique was ~25%, which is comparable to the E% determined by our algorithm at similar uD:A ratios.
DISCUSSION

Membrane components can either be organized in clusters, distributed randomly or show a mixture of these two situations. Assembly of membrane-bound cellular components into patches, microdomains, rafts or clusters prior to transport seems to be a ubiquitous sorting mechanism employed by the cell for many different pathways and has been described by many authors (Pentcheva and Edidin, 2001; Galbiati et al., 2001; Ikonen, 2001; Kobayashi et al., 2001; Maier et al., 2001; Mukherjee and Maxfield, 2000; Woodman, 2000). However, the presence of microdomains in endosomal membranes is still controversial (Hansen et al., 1999; Kobayashi et al., 2001; Sarnataro et al., 2000), possibly because of the transient nature and size of these domains (Tang and Edidin, 2001; Brown and Jacobson, 2001). We define ‘microdomains’ as a concentration of clustered molecules within a membrane plane. Our main hypothesis is that protein sorting requires clustering of cargo molecules during the formation of transport intermediates.

To test this hypothesis, we have developed a FRET microscopy-based assay to determine whether receptor-ligand complexes are clustered in sub-pixel domains in the apical endocytic compartments of polarized MDCK cells. FRET can be used to measure molecular proximity (<10nm) as well as to discriminate between clustered donor and acceptor molecules and high amounts of donor and acceptor molecules randomly distributed on membranes. As proposed earlier by Kenworthy and Edidin, if all donor and acceptor fluorophore molecules are clustered, E% should be independent of acceptor concentration (Kenworthy and Edidin, 1998). Quantitative analysis of E% was carried out using a custom algorithm that removes spectral
contamination. Average E% is ~40% and it is independent of acceptor levels. Our results were confirmed by the standard FRET technique of total bleaching of the acceptor fluorochrome. Since a random distribution of donor and acceptor molecules results in E% increasing with rising acceptor levels, our results indicate a clustered distribution of receptor-ligand complexes in the apical endocytic compartments of polarized MDCK cells.

A model for E% in membrane protein clusters. We have presented a new model, which may be regarded as an extension and improvement of the Kenworthy and Edidin model, which is based on dimers. We submit that under our experimental conditions and generally under conditions where microdomains are formed, clusters based on dimers would be an unrealistic assumption. Our ‘perfect’, i.e. large and dense cluster assumption goes to the other extreme and it is therefore not surprising that the actual experimental results fall between the two models. This model describes the ‘donor geometric exclusion’ phenomenon, which is due to some donors or unknown molecules preventing other donors from participating in energy transfer. Two lines of evidence demonstrate the existence of non-FRET donors and their negative effect on E%. First, we have shown that increasing uD levels result in E% decrease both in a pixel-by-pixel and ROI average analysis (Figure 3-4). Second, the coexistence of donors that participate in FRET events (FRET donors) and donors that do not participate in FRET (non-FRET donors), was validated by donor bleaching experiments, in which non-FRET donors are bleached before FRET donors leading to an increase in E%, on the basis that these two different species of donors exhibit distinct energy rate constants. Our model attributes the decline of E% with increasing donor fluorescence to geometric exclusion only, and neglects donor-donor competition events, which has a similar effect. However, since donors involved in donor-donor competition should
be able to alternately transfer energy to the closest acceptor during the relatively long laser dwell time, we propose that ‘donor geometric exclusion’ is significantly more important for the donor-induced E% decrease than donor-donor competition.

It may be said that an attempt should be made to reduce donor fluorescence to a level where the phenomenon of ‘geometric exclusion’ does not occur, and donor competition can be neglected. This, in our view, is undesirable and unrealistic. If a sufficient number of donors are not available for energy transfer, FRET may never take place and the results may be confused with random or mixed distribution. Furthermore, once the labeled complexes are internalized there is no control over their distribution and colocalization within a cluster and future improvements in methodology must concentrate on increasing the sensitivity of the assay, while incorporating the phenomena, rather than ignoring them. Figure 6 and the detailed appendix, as well as the schematic view of the above clustered distribution model (Figure 8) visualizes our experimental findings that E% is dependent on uD levels as well as uD:A ratio. In this simplified example, we are comparing uD:A ratios of 1:1, 2:1 and 3:1– each with N= 12 molecules. As the uD:A ratios increase, the likelihood of ‘donor geometric exclusion’ and associated drop in E% increases. For uD:A ratios below 1:1, the differences are probably not significant, as increasing the number of acceptor molecules relative to donors in a cluster, offers more opportunities to transfer energy. At the other extreme, at uD:A ratios >3 the effects of the rising donor fluorescence per pixel are less apparent and thus cannot be used to distinguish a clustered from a random distribution.

**Biological implications.** For membrane-bound protein complexes to traverse the cell, they have to be internalized, pass through different endosomal distribution points and sorted to their correct
destination (Barroso and Sztul, 1994; Brown et al., 2000; Leung et al., 2000; Gibson et al.,
1998). The formation of transient membrane microdomains is thought to be an important element
of this trafficking process (Galbiati et al., 2001; Ikonen, 2001; Kobayashi et al., 2001; Maier et
al., 2001; Mukherjee and Maxfield, 2000; Woodman, 2000). We have postulated that protein
clustering is one determinant for the existence of membrane microdomains, which most likely
involves other processes and effectors such as coat-proteins, the cytoskeleton, signal sequences
and specific affinities between proteins and membrane lipids (Mukherjee and Maxfield, 2000;
Woodman, 2000; Simons and Ikonen, 1997; Verkade et al., 2000; Sonnichsen et al., 2000). Here
we demonstrate that receptor-ligand complexes are distributed in a clustered manner in apical
endocytic membranes. These results directly implicate the clustering of membrane components
in protein sorting and transport in polarized cells. Furthermore, we propose that differences in
energy transfer between ROIs or even between pixels within an ROI could represent different
densities of acceptor and donor labeled molecules within clusters. Such different densities may
reflect progressive sorting stages of receptor-ligand complexes within apical endocytic
membranes, which could involved in the ability of proteins to be specifically incorporated into
transport vesicles budding off from apical endocytic compartments.

The ability to visualize E% distributions within cells poses some intriguing questions. As each
of the images shown in Figure 3 present a section in the xy-plane of a complete cell at steady-
state, the larger punctate structures undoubtedly represent the irregular endosomal morphology,
and the small structures (1-3 pixels) may be vesicles proceeding from or towards the apical
endocytic compartments. The effect of the proposed ‘donor geometric exclusion’ is visible,
where pixel locations of high uD levels exhibit lower E%. There may also be a biological
significance in the spatial distribution of higher E% levels on the periphery of the punctate structures, which may represent vesicles in the process of budding or fusion. As mentioned above, the E% distribution in a spatially organized manner might reflect a continuum of different levels of local cluster densities, i.e. protein sorting, caught at steady-state. Nevertheless, the FRET-based assay described in this paper can also be applied to investigate the distribution of other membrane-bound receptors as well as the organization of membrane proteins and lipids into membrane microdomains such as rafts or patches, by quantitating donor and/or acceptor levels and E% data.

We have also used the same methodology for two-photon (2-P) FRET microscopy (Periasamy, 1999; Periasamy, 2000) with comparable results (data not shown). 2-P microscopy has certain advantages, such as its application for thicker specimens and the lack of out-of-focal plane photo damage. In our particular case, where the 2-P donor excitation wavelength did not excite the acceptor (no acceptor bleed-through), no single-label acceptor information needed to be collected, and in the absence of this information, the new donor based parameter for clustering became particularly valuable.

In summary, 1-P or 2-P FRET microscopy can be used to calculate E% and determine quantitatively the clustering patterns of membrane-bound protein complexes. Here we have shown that uD-induced E% decrease can be used as a new parameter for membrane protein clustering. A novel mathematical model, which largely explains the E% decrease with increasing uD levels by accounting for the ability of donors and other unknown molecules to prevent other
donors from transferring energy to potential acceptors (‘donor geometric exclusion’), has been presented to describe large, tightly packed clusters of membrane-bound proteins.

ACKNOWLEDGEMENTS

We thank Sarah Smith for her assistance with the experiments and data analysis. We would like to thank Jeff Larson from Nikon for his help with confocal microscopy. We thank Dr. Guy C. Cox, University of Sydney (Australia) and Dr. J. N. Demas (Department of Chemistry, University of Virginia) for their helpful comments concerning donor bleaching. We thank Dr. James Casanova (Department of Cell Biology, University of Virginia Medical School) and Dr. Philippe I.H. Bastiaens (European Molecular Biology Laboratory, Heidelberg, Germany) for their helpful comments. We would also like to thank the members of the Keck Center for Cellular Imaging, Dr. Lance Davidson (Department of Biology, University of Virginia) as well as members of the laboratories of Dr. Barroso and Dr. Bloom (Department of Biology, University of Virginia) for helpful discussions.
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APPENDIX

A model for FRET efficiency in membrane clusters

Models for small oligomers, and in particular dimers, have been used to analyze FRET efficiency (E%) as a function of both acceptor surface density and donor : acceptor ratio. The predictions of the dimer model are expected to be valid so long as acceptor and donor surface densities are low and typical cluster sizes are small. We present here a new clustering model, which is tailored to large clusters or high acceptor and donor surface densities. Our model can be viewed as an extension of the Kenworthy-Edidin dimer model to larger cluster sizes.

1. Physical assumptions

Consider a single excited donor molecule in the presence of a number (k) acceptor molecules, located at distances $r_1, \ldots, r_k$ from the donor. The Förster model predicts that the probability of the excited donor to return to the ground state via FRET is given by

$$E = 1 - \left\{ 1 + \sum_{i=1}^{k} \left( \frac{R_o}{r_i} \right)^6 \right\}^{-1}$$

where $R_o$ is the Förster distance, a physical constant of the involved donor and acceptor fluorophores. By definition, the probability that an excited donor molecule returns to the ground state through FRET is 50%, if a single acceptor molecule is located at a distance $R_o$. In order to analyze the dependence of FRET on experimental parameters, we combine Eq. (1) with a model for the geometric distribution of fluorophores.

The relevant parameter values in our system are as follows:

**Förster distance.** $R_o = 67.5$ Å

**Protein shape and size.** The acceptor and donor fluorophores are attached to identical pseudo-ligands of a membrane receptor (polymeric IgA-receptor), which are internalized from opposite membranes. The pIgA-R ligands occupy a cylindrical space with circular cross section of diameter $R = 80$ Å.

**Receptor occupancy.** In our experimental approach, we attempt to saturate the receptors with increasing amounts of pIgA-R ligands to reduce the number of empty receptors.

**Number of fluorophores.** An average of 5 per pIgA-R ligand, as indicated by the manufacturer. Experimentally, we saturate with fluorophores and hence assume that an average of 5 binding sites are occupied.
2. The geometric model

We model the membrane as a two-dimensional surface, and we visualize the proteins of interest as disks in a plane directly above the membrane. The possible locations of proteins are discretized by covering the surface with discs in tight packing (see Figure 9). Each disc is either vacant (representing an unlabeled receptor or an unknown protein), occupied by A (representing an acceptor-labeled pseudo-ligand) or by D (representing a donor-labeled pseudo-ligand).

Supposing that a given disc is labeled by D and considering the neighboring discs, we distinguish two extreme cases:

- **Perfectly clustered.** All discs in the neighborhood of a given donor are labeled by either A or D. We assume that the probability of each disc in the cluster to be labeled by A is independent of all other discs. It is given by the fraction of acceptor-labeled proteins within the cluster,

\[
\tilde{f}_A = \frac{[D]}{[A+D]} = \frac{1+[D:A]}{1+[A]}^{-1} \tag{Eq. A2}
\]

- **Perfectly random.** Here, the neighboring discs can be either labeled or vacant. The probability that a disc is labeled by A is determined by the fraction \(s_A\) of the membrane surface covered by acceptor-labeled proteins.

Thus, the distribution of the number \(N\) of neighbors of a given disc that are labeled by A is a \((6,p)\)-binomial random variable, where \(p=\tilde{f}_A\) in a perfect cluster, and \(p=s_A\) in a perfectly random arrangement.

To simplify computations, we replace the actual locations of the fluorophores in each disc by the average location at the center of the disc. Similarly, we replace the actual orientation of the fluorophores by an average over all possible orientations, corresponding to \(\kappa^2 = 2/3\) (Lakowicz, 1999). Since \(R > R_o\), the contribution of all but adjacent discs to the right hand side of Eq. (1) can be neglected. If \(N\) of the six neighbors of a D-labeled reference disc are labeled by A, then each donor fluorophore in the reference disc interacts with 5N acceptor fluorophores whose average distance is approximately \(R\). By Eq. (1) (with \(k=5N\) and \(r_i = R\) for \(i = 1, \ldots, k\)), it contributes

\[
E \approx 1 - \left\{1 + 5N(R_o/R)^6 \right\}^{-1} \tag{Eq. A3}
\]

to the FRET signal. We treat \(E\) and \(N\) as random variables, and compute \(E\%\) as an expected value

\[
E\% \approx \left\langle 1 - \left\{1 + 5N(R_o/R)^6 \right\}^{-1} \right\rangle \tag{Eq. A4}
\]
For comparison, we also consider the following models:

- **Dimer model (Kenworthy and Edidin, 1998).** The apparent E% in dimers is given by $E% = E_{dimer} f_A$. Using Eq. (3) with N=1 we compute

  $$E_{dimer} = 1 - \left\{ 1 + 5 \cdot \left( \frac{R_o}{R} \right)^6 \right\}^{-1} \approx 0.64$$

  Eq. A5

- **Rational approximant (Dewey and Hammes, 1980).** The first in a sequence of rational approximations is given by

  $$E\%_{random} = \left\{ 1 + \frac{\pi c_A}{2} \left( \frac{R_o}{R} \right)^4 \right\}^{-1} = \left\{ 1 + 9.07 \left( \frac{R_o}{R} \right)^6 \right\}^{-1}$$

  Eq. A6

Here, $c_A$ is the so-called reduced surface density, which is related with our parameter $s_A$ by $c_A = 5(1.54)(R_o/R)^2 s_A$. In each case, the factor 5 accounts for the 5 fluorophores per labeled pseudo-ligand.

The predictions of the four models are shown in Figure 6
Figure Legends

**Figure 1.** Endocytic trafficking pathways in polarized epithelial MDCK cells and sub-apical co-localization of differently labeled pIgA-R ligands internalized from opposite PM’s. Different membrane trafficking pathways exist – partially shared – for different internalized components. Arrows 1-3 and 1,4 and 5 show basolateral receptor recycling (e.g. LDL-R and Tf-R) respectively. Arrow 1-2 is the basolateral-lysosomal pathway (e.g. LDL). Arrows 1, 4 and 6-7 represent basolateral-to-apical transcytosis, arrows 6-11 apical receptor recycling, both utilized by our internalized receptor-ligand complexes (except that internalization at 17°C results in sub-apical accumulation of receptor-ligand complexes by blocking delivery to the apical PM; Hunziker et al., 1990; Barroso and Sztul, 1994). The closed star represents the basolaterally internalized Cy3-pIgA-R-ligand complex (Acceptor). The open star is the apically internalized Alexa 488-pIgA-R-ligand complex (Donor). In this Figure, we show an example in which both complexes first cross in the CE and then co-localize and accumulate in the ARE, where FRET may occur (Barroso and Sztul, 1994).

**Figure 2.** Seven images are needed to process a corrected FRET image. All images are taken at a focal plane ~ 3.5µm below the apical PM under the same imaging conditions. They were modified in Photoshop at the same rate to a higher level of contrast for better visualization. Images shown (overall size 26.4µm x 26.4µm) contain several ROIs. Pixel gray level intensities in the single-label and double-label specimens are matched over a wide range of fluorescence values for the algorithm to pick matching intensities for the double-label correction.

A. Single labeled donor/donor excitation/donor channel: shows a broad range of fluorescence levels that can be matched with the double-labeled specimen (F)
B. Single labeled donor/donor excitation/acceptor channel: represents donor ‘cross-talk’, i.e. the spillover of donor excitation into the acceptor channel. Areas of higher donor intensity in ‘A’ show greater level of cross-talk in ‘B’.

C. Single labeled acceptor/acceptor excitation/acceptor channel: shows a broad range of fluorescence levels that can be matched with the double-labeled specimen (E)

D. Single labeled acceptor/donor excitation/acceptor channel: acceptor ‘bleed-through’, i.e. the amount of acceptor fluorophore that is excited by the donor wavelength. Again, the higher the level of acceptor fluorescence in ‘D’, the greater the bleed-through in ‘C’.

E. Double labeled specimen/acceptor excitation/acceptor channel: showing sufficient level of acceptor fluorescence for FRET to occur and displaying the same ROIs as ‘F’, ‘G’ and ‘H’.

F. Double-labeled specimen/donor excitation/donor channel: representing the quenched donor (qD) level in several ROIs, each one complete cell.

G. Double labeled specimen/donor excitation/acceptor channel: ‘uncorrected’ FRET (uFRET), i.e. FRET energy transfer plus donor cross-talk (based on donor levels in ‘E’) and acceptor bleed-through (based on acceptor levels in ‘G’). By way of example, three typical ROIs are identified, each representing one complete cell that will be used for data analysis (a false-colored rendering of these ROIs is shown in Figure 3).

H. Corrected FRET (cFRET) image: image G was processed by our custom algorithm, which removes donor cross-talk and acceptor bleed-through. The resulting image represents the actual energy transfer. The same ROIs as in ‘G’ are indicated.

Figure 3. False-colored images of 3 ROIs showing uFRET, cFRET, uD and E% distributions. The three ROIs indicated in Figure 3G&H were false-color processed to show
pixel-by-pixel distribution of uFRET, cFRET, uD and E%. Each represents a Z-section (xy-plane) of one complete cell taken 3.5 μm below the apical plasma membrane at the level of the apical endocytic compartments. Each image is 100 x 100 resolved pixels of 88 x 88nm equaling an image size of 8.8 x 8.8 μm. Removal of spectral spillover is clearly seen when comparing cFRET with uFRET. Furthermore, higher levels of uD correlate with lower E% levels. Arrows show high level of E%/low uD, located in the periphery of punctate structures. Arrowheads show low level of E%/high uD in the middle of punctate structures.

**Figure 4.** E% decreases with increasing uD levels and uD:A ratios and is independent of acceptor levels. Donor and acceptor labeled pIgA-R-ligand complexes were internalized from opposite PMs for four hours at 17° C at different molar ligand concentrations. E% was determined against this wide-ranging group of ROIs (147) with respect to uD levels (panel A), actual uD:A ratios (panel B) and acceptor levels (panel C). When arbitrarily creating 4 groups (panel D) based on E% (25-35, 35-45, 45-55, 55-65), the average values in these groups with respect to uD levels and uD:A ratios show that they are statistically different, whereas confirming that E% is independent of the acceptor.

**Figure 5.** E% is independent of acceptor levels by the ‘bleaching the acceptor’ technique. The standard method of ‘bleaching-the-acceptor’ was used to validate our algorithm approach. With this method, a double-labeled specimen is sequentially imaged with both acceptor and donor excitation (donor excitation in the donor channel represents qD). A ROI is subsequently bleached with the acceptor excitation alone until all acceptor fluorescence is removed. The specimen is again imaged; the acceptor excitation/acceptor channel image confirms the existence
of the bleached ROI, the donor excitation/donor channel image represents the uD within the ROI. The difference between the qD and uD fluorescence is the energy transfer. The results fall within the same range as those produced by our algorithm with overlapping standard deviations.

**Figure 6. A & B: E% predictions of two models for a perfectly random arrangement of labeled receptor-ligand complexes.** The lower curves show the first rational approximant of Dewey and Hammes (1980), and the upper curves show Eq. A4 (Appendix), with p=s_A. A: E% as a function of uD:A ratio for a fixed acceptor surface density (shown for s_A=10%). B: E% as a function of acceptor surface density s_A (in %) for arbitrary uD:A ratio. In both models, E% is independent of uD:A ratio, if acceptor surface density is kept fixed. It increases with acceptor surface density, and vanishes for small acceptor surface density.

**Figure 6 C & D: E% predictions of two models for clustering of labeled receptor-ligand complexes.** The lower curves were computed with the Kenworthy-Edidin dimer model and the upper curves were computed with our model of a perfect cluster, using Eq. 3 (Appendix) with the p=f_A. C: E% as a function of uD:A ratio. D: E% as a function of acceptor surface density s_A (in %) for fixed uD:A ratio (shown for uD:A=2). In both models, E% decreases with increasing uD:A ratio, and is independent of acceptor surface density.

**Figure 7. Donor excitation bleaching leads to increased E%.** 5 ROIs of single-donor, single-acceptor and double-labeled specimens were subjected to continuous donor excitation for periods of 30sec for a total of 5min, with data points taken at each 30sec interval. Single-label donor molecules and those double-label donor molecules not involved in FRET bleach faster than those
involved in FRET, because of their different rate-constants – being longer in the excited state.  

**Panel A** shows the averages of the ROIs; the single-label uD bleaches faster than the double-label, while there is no statistical difference in the effect on the acceptor levels (**Panel B**). **Panel C** displays the normalized uD and E% levels over bleaching time demonstrating the existence of donors not involved in the FRET: the decrease in uD levels by photobleaching leads to an increase in E% as non-FRET donors bleach faster. Overall, E% increases 1.2 to 2 times, whereas uD decreases by >80%.

**Figure 8.** Simplified model to demonstrate decreasing E% with rising uD intensity and uD:A ratio as a novel parameter for clustered distribution (see text and appendix for full description). A simplified model to support our experimental findings of increasing uD levels and uD:A ratio causing a decrease in E%. Different number of donor and acceptor molecules (resulting in three different uD:A ratios) for the total number of molecules N=12 are considered in this schematic to illustrate the dynamics of increasing donor molecule presence (and uD:A ratios) and their effect on E%. The increasing uD:A ratios result in to lower E%, due to the increasing likelihood of donors blocking other donors to be in proximity to an acceptor and preventing the transfer of potential energy, a phenomenon we have termed ‘donor geometric exclusion’. This phenomenon can only occur in a clustered distribution where molecules are by definition in proximity and therefore serves as a novel indicator for clusters.

**Figure 9 [Appendix]:** Two examples of different configurations of donor-labeled (D), acceptor-labeled (A) and vacant discs (empty). The reference donor disc is shaded.
Apical PM

Basolateral PM

Nucleus

15-20 μm

Alexa 488-plgA-R-ligand

Cy3-plgA-R-ligand

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

B

Algorithm

Acceptor

Bleaching
Figure 6
A

Normalized uDonor intensity

Cumulative bleaching time (s)

B

Normalized acceptor levels

Cumulative bleaching time (s)

C

Normalized uD levels & E%

Cumulative bleaching time (s)

Figure 7
Increasing uD and uD : A ratios

N=12
uD= 6
uD:A = 1:1

N=12
uD= 8
uD:A =

N=12
uD= 9
uD:A =

Acceptors
Donors
Donors involved in FRET

Increasing uD and uD : A ratios

Figure 8
low D:A ratio/higher E%

high D:A ratio/lower E%

D = Donor
A = Acceptor

Figure 9 [Appendix]