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The border-to-border distribution method for analysis of cytoplasmic particles and organelles

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Abstract

Comparing the distribution of cytoplasmic particles and organelles between different experimental conditions can be challenging due to the heterogeneous nature of cell morphologies. The border-to-border distribution method was created to enable the quantitative analysis of fluorescently labeled cytoplasmic particles and organelles of multiple cells from images obtained by confocal microscopy. The method consists in four steps: (1) imaging of fluorescently labeled cells, (2) division of the image of the cytoplasm into radial segments, (3) selection of segments of interest and (4) population analysis of fluorescence intensities at the pixel level either as a function of distance along the selected radial segments or as a function of angle around an annulus. The method was validated using the well-characterized effect of brefeldin A (BFA) on the distribution of the vesicular stomatitis virus G protein, in which intensely labeled Golgi membranes are redistributed within the cytoplasm. Surprisingly, in untreated cells, the distribution of fluorescence in Golgi membrane-containing radial segments was similar to the distribution of fluorescence in other G protein-containing segments, indicating that the presence of Golgi membranes did not shift the distribution of G protein towards the nucleus compared to the distribution of G protein in other regions of the cell. Treatment with BFA caused only a slight shift in the distribution of the brightest G protein-containing segments, which had a distribution similar to that in untreated cells. Instead, the major effect of BFA was to alter the annular distribution of G protein in the perinuclear region.

Keywords

Border-to-border distribution method; Brefeldin A; VSV; Glycoprotein; Fluorescence localization

Introduction

Cytoplasmic particles and organelles are never randomly distributed. Instead, they are organized into different regions of the cell that play important roles in cellular function (Rao

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and Mayor 2014). For example, the Golgi apparatus is often localized near the nucleus and the microtubule organizing center (MTOC) (Marsh et al. 2001; Sutterlin and Colanzi 2010; Zhu and Kaverina 2013), whereas the endoplasmic reticulum (ER) is more widely distributed in the cytoplasm. The localization of cellular particles and organelles is readily visualized by microscopy. However, the analysis of such data is often primarily descriptive and subjective, especially when there is substantial heterogeneity among the cells being analyzed. This limitation underscores the need for methods to quantify inherently heterogeneous microscopy data. Quantitative methods have been developed for image segmentation for object or region identification and quantification of their properties such as number, intensity and spatial position (reviewed in Hamilton 2009).

The goal of the experiments presented here was to develop methods for quantifying the distribution between the nucleus and the edge of the cell of fluorescent elements that typically are not represented as individual objects but instead are more diffusely distributed. This method will be useful for analysis of drugs or other perturbations that alter the distribution of such particles and organelles within the cytoplasm. We call this analysis the border-to-border distribution method. The goal of the border-to-border distribution method is to measure the distribution of fluorescently labeled particles and structures within individual cells and develop quantitative parameters describing this distribution, such as mean distance and skew. These parameters can then be used to compare populations of cells treated with varying conditions. As an example of the utility of the approach, we analyzed the effect of brefeldin A (BFA) on the distribution of the vesicular stomatitis virus (VSV) G protein.

BFA is an inhibitor of intracellular protein transport (Misumi et al. 1986; Takatsuki and Tamura 1985), which causes a redistribution of Golgi membranes in the cytoplasm (Doms et al. 1989; Lippincott-Schwartz 1990). BFA targets a subset of GTP-exchange factors (Donaldson et al. 1992; Helms and Rothman 1992; Peyroche et al. 1999), which activates proteins required for the recruitment of COPI to transport vesicles involved in recycling membranes from Golgi to ER. The decrease in transport of membranes from the Golgi apparatus to the ER causes a fusion of Golgi and ER membranes (Nebenfuhr et al. 2002), leading to a distribution that is more typical of ER than Golgi membranes. The redistribution of membrane proteins, such as the VSV G protein, caused by BFA has been well characterized by fluorescence microscopy (Doms et al. 1989; Barzilay et al. 2005; Goldenberg et al. 2007).

VSV G protein is a type I membrane glycoprotein that has been extensively utilized to study the secretory pathway. G protein in VSV-infected cells is intensely labeled in Golgi membranes, in what is described as a typical “perinuclear” localization (Rogalski et al. 1984; Burkhardt et al. 1989). The remainder of G protein labeling is distributed between the ER, secretory vesicles and the plasma membrane. When cells are treated with BFA, the intense “perinuclear” localization appears to vanish. Instead, G protein labeling appears to have a more diffuse pattern throughout the cytoplasm (Doms et al. 1989; Barzilay et al. 2005; Goldenberg et al. 2007). The goal of the experiments described here was to quantify this redistribution in a population of VSV-infected cells treated with BFA.

The border-to-border distribution method consists in four steps: (1) imaging of fluorescently labeled cells, (2) division of the image of the cytoplasm into radial segments, (3) selection of segments of interest and (4) population analysis of fluorescence intensities at the pixel level either as a function of distance along the selected radial segments or as a function of angle around an annulus. For analysis of Golgi membranes, the segments of interest were those containing high perinuclear fluorescence. These were selected using a “search annulus” around the nucleus. Using the search annulus, the segments in each cell were separated into a perinuclear high (PNH) population that contained the area of the cell with the brightest Golgi membranes. The remaining segments comprised the perinuclear low (PNL) population. Surprisingly, in untreated cells, the PNH and PNL segments had fluorescence distributions with very similar mean distance and skew, indicating that the presence of Golgi membranes in PNH segments did not shift the distribution of G protein towards the nucleus compared to the PNL segments. In cells treated with BFA, the PNH segments shifted slightly in distribution away from the nucleus and towards the edge of the cells as expected, whereas the distribution in PNL segments was very similar to that in untreated cells. The more striking effect of BFA was in the annular distribution of fluorescence around the nucleus, which had a pronounced peak in untreated cells that corresponded to the brightest Golgi membranes but treatment with BFA caused a redistribution that resulted in a more even distribution around the nucleus.

Materials and methods

Cells and virus

Wild-type VSV (Indiana serotype, Orsay strain) was grown at 37 °C in BHK cells in Dulbecco's modified Eagle medium containing 2 % fetal bovine serum. HeLa cells were cultured in Dulbecco's modified Eagle medium containing 7 % fetal bovine serum. Infections were carried out at a multiplicity of infection of 50 PFU per cell in order to rapidly synchronize the infection.

Antibodies

The primary antibodies used for these experiments were rabbit anti-G protein antibody (Fitzgerald Industries International) at a 1:200 dilution and Alexa Fluor 488-conjugated mouse anti-GM130 (BD Biosciences) at a 1:5 dilution. The secondary antibody used was either Alexa Fluor 488- or Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) at a 1:500 dilution.

BFA treatment and cellular imaging

HeLa cells were seeded at a density of 3×10^5 on 8-well chamber slides (Millipore) overnight. Cells were treated with BFA (10 μ g/ml; Sigma Chemical) for 1 h and were infected with VSV-Orsay at an MOI of 50 for 4 h in the presence of BFA. The cells were washed with phosphate-buffered saline, then labeled with PKH26 (1:500 in diluent C; Sigma-Aldrich). Samples were then fixed with 3.7 % paraformaldehyde (Sigma-Aldrich) for 15 min and permeabilized with buffer containing 1 % bovine serum albumin (Sigma-Aldrich) and 0.1 % saponin (Calbiochem) for 10 min. The samples were then labeled with the rabbit anti-G protein primary antibody for 45 min and then with Alexa Fluor 488-

conjugated goat anti-rabbit IgG for 45 min. Confocal images were obtained with a Nikon C1Si Confocal Microscope System with a $\times 60$ multi-immersion objective.

Data collection

Stacks containing images of the DAPI, PKH26 and G protein labels of the same field were analyzed by ImageJ, v.1.48q (Rasband 1997), with the following steps. (1) The DAPI image from an individual cell was smoothed to prevent discontinuities in the border of the nucleus; the region of interest (ROI) was defined by adjusting the threshold to include all of the nucleus and using the “Wand (tracing)” tool to define the border and the position of the centroid was calculated from the x and y values within the ROI. (2) The same process was repeated with the PKH26 image to define the border of the edge of the cell. (3) The segment with the longest distance from the centroid of the nucleus to the edge of the cell was selected and defined as segment 0, the first of n segments per cell (typically $n=360$). Segments that intersected more than two borders were discarded. Alternatively, these segments could have been included in the analysis by considering only the part from the border of the nucleus to the first border of the edge of the cell. (4) Data collected for the pixels along each segment in the G protein image were segment number (seg), angle to centroid (angle), pixel number along the segment (n), distance in native units (d), if any were associated with the image, normalized (fractional) distance between the nucleus and the edge of the cell (x), and fluorescence intensity (y). Data from each cell were recorded in a separate file whose filename included the experiment date, drug treatment and cell identity. An ImageJ macro containing all of these steps is available upon request.

Data analysis

Data analysis was performed with the R statistical package (R Core Team 2015). Fluorescence intensity measurements (y) for each cell were normalized to the median fluorescence intensity in that cell to eliminate variation in the level of G protein expression. Data from adjacent segments were pooled to create sectors to dampen large intensity variations between segments (typically 3 segments per sector). A search annulus around the nucleus was defined in order to identify the sector with the brightest perinuclear fluorescence (typically pixels with $n=2-6$). The sector with the brightest perinuclear fluorescence and the two surrounding sectors (9 segments total) were labeled as perinuclear high (PNH) and the remaining sectors were labeled perinuclear low (PNL).

Sectors were analyzed to determine the spatial distribution parameters for the fluorescence intensity as follows.

1. For each pixel, the fraction of total fluorescence in the sector:

$$f_i = y_i / \sum_i y$$

2. For each sector, mean relative distance of the fluorescence:

$$m = \sum f_i * x_i$$

3. Standard deviation in distance distribution of fluorescence:

$$s = \left(\sum (f_i * (x_i - m)^2) \right)^{1/2}$$

4. Skew in distance distribution of fluorescence (Pearson's third moment):

$$m3 = \sum \left((f_i * (x_i - m) / s)^3 \right)$$

5. Kurtosis in distance distribution of fluorescence (Pearson's fourth moment):

$$m4 = \sum \left((f_i * (x_i - m) / s)^4 \right)$$

The values obtained in these experiments were determined to be normally distributed. Consequently, differences among treatment groups (untreated vs. BFA-treated) and sector characteristics (PNH vs. PNL) were evaluated by analysis of variance with Tukey's post hoc test for multiple comparisons.

Results

The border-to-border distribution method was developed to quantify distribution of cytoplasmic particles and organelles. In the experiments described here, the two borders were the nucleus and the edge of the plasma membrane, i.e., the borders that define the cytoplasm. The goal of this approach is to provide a quantitative description of the distribution of elements in individual cells using mathematical parameters used to describe the distribution of any population (i.e., mean, standard deviation, skew, etc.). Statistical methods can then be used to combine the results from many cells to determine a "representative" distribution and to determine whether experimental perturbations have a statistically significant effect on any of the distribution parameters. To validate this method, we used the well-established phenomenon of the effect of BFA on the cellular secretory pathway to alter the localization of VSV G protein within infected cells (Fig. 1). HeLa cells were treated with BFA for 1 h before infection to inhibit the secretory pathway. The cells were infected with VSV for 4 h, fixed, permeabilized and labeled with an antibody against G protein, then imaged with confocal microscopy.

In untreated cells infected with VSV, the G protein was concentrated in focal regions near the nucleus and was also present throughout the rest of the cytoplasm in a more diffuse reticular pattern (Fig. 1a) as reported previously (Rogalski et al. 1984; Burkhardt et al. 1989; Bergmann et al. 1981). In BFA-treated cells, these focal regions near the nucleus disappeared and the G protein fluorescence was redistributed throughout the cytoplasm (Fig. 1b). The bright focal areas near the nucleus in untreated cells corresponded to the Golgi apparatus, as shown by colocalization with the cis-Golgi marker GM130 (Fig. 2a–c), whereas most of the remaining intracellular G protein that did not colocalize with GM130 corresponded to G protein in other cellular membranes. Following treatment with BFA, both the GM130 and G protein assumed a more diffuse pattern of labeling (Fig. 2d–f). These

results are consistent with the mechanism of action of BFA and its previously described effects on G protein distribution (Doms et al. 1989; Bannykh et al. 2005). The goal of the border-to-border distribution analysis presented here was to quantify the extent to which the “perinuclear” fluorescence corresponding to G protein localized in Golgi membranes was redistributed to other regions of the cytoplasm following treatment with BFA. The initial hypothesis to be tested was that Golgi-localized G protein fluorescence that was skewed toward the nucleus in control cells would be more evenly distributed between the nucleus and the edge of the cell in BFA-treated cells.

In order to define the borders of the cytoplasm, VSV-infected cells were labeled with the fluorescent membrane probe PKH26, then were fixed, permeabilized with saponin (which does not solubilize PKH26) and labeled with antibody against the VSV G protein and with DAPI to label nuclei. Images were obtained by confocal microscopy using a slice size and focal plane that captured both the plasma membrane at the bottom of the cell and a portion of the nucleus.

The borders of the nuclei and the edge of the membrane labeling were delineated using the selection tools provided by ImageJ software. Examples of imaging of the cells from Fig. 1 and definition of the borders of the cytoplasm are shown in Fig. 3a–f.

The next step in the analysis is the division of the image of the cytoplasm into segments. Figure 3g, h shows the borders of the nucleus and edge of the cell and a cross that marks the centroid of the nucleus from which segments radiate. The cytoplasm was divided into 360 segments that start at the first border (the nucleus) and end at the second border (the edge of the cell). For clarity, only 90 segments are shown in Fig. 3. The segments were numbered beginning with the longest segment. Segments that cross more than two borders were excluded from the analysis because there would be a gap in the data following the second border. The dataset for each cell consisted in the fluorescence intensity and distance of each pixel along the approximately 360 segments included in the analysis. For most of the cells analyzed here, the segment lengths varied between 5 and 50 pixels. We also found that it is often convenient to group neighboring segments together into sectors to dampen large intensity variations between segments. For the analyses shown below, sectors contained 3 segments, so that there were up to 120 sectors per cell.

A key step in the border-to-border distribution method is selection of the sectors of interest. For analysis of the effects of BFA, the sectors of interest were those that include bright perinuclear fluorescence, since this is the morphological feature affected by BFA. The approach to identify these sectors is illustrated in Fig. 4. Figure 4a shows “faux cell” images generated by using a logarithmic color scale to show fluorescence intensity of the pixels in all segments of a cell. A “search annulus” consisting in pixels 2–6 from the nucleus was used to define the perinuclear region. That region sufficed to capture most of the Golgi-like labeling in the cell (Fig. 4b). Fig. 4c shows histograms of the fluorescence intensities in the portion of sectors contained within the annulus of untreated and BFA-treated cells in four independent experiments. The fluorescence intensities on the x-axis were normalized to the median intensity within the annulus for each cell in order to correct for cell-to-cell variation in G protein expression. A minority of sectors contained high fluorescence. To compare the

brightest sectors of the cell to the sectors that had low fluorescence, the sectors within each cell were divided into two groups. To define perinuclear high (PNH) sectors, the sector with the highest intensity in the annulus was chosen, along with two flanking sectors on each side for a total of 9 segments (Fig. 4d). This was large enough to account for the size of the Golgi apparatus in most cells. The remaining sectors were considered the perinuclear low (PNL) group.

Pixel intensities were analyzed as a function of radial distance along the PNH and PNL segments in a population of cells. Figure 5 shows results for 4 independent experiments with approximately 10 cells analyzed in each experiment. For each cell, the mean fluorescence intensity in the PNH and PNL segments was determined at the specified relative distance interval between the nucleus and the edge of the cell (0–0.1, 0.1–0.2, etc.). The data shown are means $\pm$ SD for the approximately 10 cells in each experiment. In the untreated cells, the PNH segments had higher normalized fluorescence intensities than the PNL segments, as expected based on the way they were selected. The PNH segments also had more variability among individual cells, indicated by the higher standard deviations. In the BFA-treated cells, the differences in the normalized fluorescence intensities between PNH and PNL segments and the variability among cells were reduced, reflecting more uniformity in G protein distribution in cells treated with BFA. The data in Fig. 5 also show the reproducibility of the distributions among the four experiments.

It is obvious from Fig. 5 that the PNH segments in untreated cells had higher fluorescence intensity than PNL segments and that the difference was reduced in BFA-treated cells. It is also clear from Fig. 5 that the radial fluorescence distributions in both PNL and PNH segments in untreated and BFA-treated cells were skewed with higher intensity towards the nucleus. However, it is not obvious whether the distributions differ to the extent to which the fluorescence is skewed towards the nucleus. For this analysis, the data from all cells in the four experiments in Fig. 5 were combined to give the fluorescence distributions shown in Fig. 6a. Figure 6b shows the mean relative distances of the fluorescence intensity in PNH and PNL sectors of untreated and BFA-treated cells. Figure 6c shows the skew calculated for these distributions. By convention, a positive skew would have a higher intensity toward the nucleus and a negative skew would have a higher intensity toward the edge of the cell. Unexpectedly, the mean relative distance and skew in PNH sectors were not significantly different from those in PNL sectors in untreated cells. This indicates that the presence of Golgi membranes in PNH sectors results in a mean distance and skew that is similar to that of other G protein-containing membranes in the PNL sectors. Treatment with BFA did not affect the distance or skew of PNL sectors. However, the mean distance and skew of PNH sectors in BFA-treated cells were shifted away from the nucleus towards the edge of the cell as expected, although the differences in the radial distribution parameters were rather small considering the dramatic change in morphology following BFA treatment.

The effect of BFA was more pronounced when the annular distribution around the nucleus was analyzed. This is illustrated in Fig. 7. Figure 7a shows 18 search annuli of untreated and BFA-treated cells sorted by brightest PNH intensity and oriented with the brightest sector up, which was defined as an angle $\theta = 0^\circ$. Sectors to the right of 0° are positive and to the left of 0° are negative. The normalized fluorescence intensity in each sector was averaged across

all cells and is shown as a function of in Fig. 7b. The peak in normalized fluorescence intensity was substantially higher in untreated cells than in BFA-treated cells. Collectively, the data in Figs. 6 and 7 show that the major effect of BFA on the distribution of G protein is not so much on its proximity to the nucleus but instead it is no longer concentrated in a limited focal region around the nucleus.

Discussion

The border-to-border distribution method is a process for quantifying the localization of cytoplasmic particles and organelles so that the results from multiple cells can be combined for statistical comparison between treatment groups. The approach is similar to those described previously for analysis of the radial distribution of individual objects in the nucleus, such as signals from fluorescence in situ hybridization (Gue et al. 2005), or centromeres, telomeres and PML nuclear bodies (Vermolen et al. 2008).

Our method was developed to quantify the position of cytoplasmic elements whose position is more accurately represented by a distribution rather than a discrete position. Cells can be analyzed in multiple ways. The radial distribution parameters can include mean distance, standard deviation, skew and kurtosis. For example, the radial distribution of fluorescence between the nucleus and the edge of the cell was analyzed by determining mean distance and skew (Figs. 5, 6). The annular distribution can also be analyzed using parameters such as peak height and width in an annulus at any specified distance from either border, for example, the annular distribution of fluorescence in the perinuclear region of a cell (Figs. 4c, 7).

For the experiments presented here, the radial distribution data of cells were analyzed based on the level of fluorescence intensity near the nucleus, with the PNH sectors corresponding to those containing concentrated Golgi membranes. The PNL sectors contained multiple types of membranes including the ER, plasma membrane, transport vesicles and Golgi membranes not captured in the PNH sectors. Many cells had one major region of Golgi membranes but others had multiple regions that were not captured in the PNH sectors. However, one of the surprising results of this analysis was that, in untreated cells, the fluorescence in PNH sectors was collectively no more perinuclear than that in PNL sectors, because both groups of sectors had the same mean distance and skew in their distributions (Figs. 5, 6). Instead, the only difference in radial distribution between PNH and PNL segments was in the overall intensity, reflecting a higher level of G protein in most of the membranes captured in the PNH segments. This higher intensity along the entire length of the PNH segments is not readily visualized by eye from the images but was a consistent and reproducible feature of these cells (Fig. 5). Such a preferential localization has been observed previously as a higher level of G protein at the cell surface in regions of the plasma membrane juxtaposed to the Golgi apparatus (Rogalski et al. 1984).

In cells treated with BFA, the fluorescence distribution in PNH sectors was shifted towards the edge of the cell (Figs. 5, 6). Since BFA primarily affects the Golgi membranes, the distribution of G protein within the remainder of the endomembrane system represented by the PNL sectors remained unchanged. The change in radial distribution of fluorescence in

PNH sectors, although statistically significant, was relatively small compared to what might be expected from the dramatic change in morphology (Fig. 1). This change was better quantified by analysis of the annular distribution (Fig. 7). In untreated cells, the Golgi membranes have an asymmetric distribution around the annulus. This is represented by the peak height in the annular distribution (Fig. 7). In cells treated with BFA, the G protein had a more symmetrical distribution around the nucleus, represented by the reduction in the peak height in the annular distribution.

The location of cellular membranes is driven by transport along the cytoskeleton (Wozniak and Allen 2009). In general, minus end-directed motors drive retrograde transport along both microtubules and actin filaments, resulting in the accumulation of membranes near the nucleus, whereas plus end-directed motors tend to drive transport toward the edge of the cell. In the case of Golgi membranes, the minus end-directed motors that drive accumulation around the nucleus are primarily members of the dynein family that transport membranes along microtubules (Yadav and Linstedt 2011).

The ER of mammalian cells maintains its widespread distribution throughout the cytoplasm via its interaction with plus end-directed motors, such as kinesins that transport ER membranes along microtubules in an anterograde direction (Waterman-Storer and Salmon 1998; Terasaki and Reese 1994). In contrast to Golgi membranes, the ER is transported in a retrograde fashion through its association with actin-based minus end-directed motors (Waterman-Storer and Salmon 1998; Terasaki and Reese 1994). For this reason, it is not concentrated around the MTOC but instead is more symmetrically distributed around the nucleus. In cells treated with BFA, Golgi membranes assume an ER-like distribution, because they fuse with the ER (Nebenfuhr et al. 2002). Thus, the high peak height in the annular distribution in untreated cells decreased after treatment with BFA because of the different motors that drive their perinuclear distribution.

The border-to-border distribution approach can be used to analyze fluorescence distributions among a variety of experimental conditions, such as treatment with drugs that inhibit cellular processes, like BFA, or genetic changes such as gene deletion or RNA silencing. This method can also be used to analyze changes as a function of time, such as the movement of cargo through the endocytic pathway or the movement of viral proteins during the replication cycle. This method can also be used to determine differences between cell types, such as transformed cells versus primary cells. There are cell types in which the method is better suited than the HeLa cells studied here, such as cells with a larger cytoplasm to nucleus ratio. Conversely, there are also examples of cell types in which the border-to-border distribution is not well suited, such as cells with small cytoplasm to nucleus ratios or experimental perturbations in which the shape of the cells changes dramatically. Cells that are rounded or columnar also present a special challenge. In these cases, the method will need to be expanded to analyze these cells in three dimensions; for example, to analyze the effects of separation of the basolateral and apical membranes in polarized cells on the distribution of elements in the underlying cytoplasm. These are only a few examples of how this analytical method can have broader applicability in cell biology.

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Abbreviations

BFA	Brefeldin A
BTBDM	Border-to-border distribution method
ER	Endoplasmic reticulum
MTOC	Microtubule organizing center
PNH	Perinuclear high
PNL	Perinuclear low
VSV	Vesicular stomatitis virus

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Fig. 1. Effect of BFA on distribution of VSV G protein. Untreated HeLa cells (**a**) or cells treated with BFA (**b**) were infected with VSV for 4 h, fixed, permeabilized and labeled with antibody against G protein (*green*) and DAPI (*blue*), then imaged by confocal fluorescence microscopy

Fig. 2. Colocalization of intense G protein labeling with cis-Golgi marker GM130 is disrupted by BFA treatment. Untreated HeLa cells (**a–c**) or cells treated with BFA (**d–f**) were infected with VSV for 4 h, fixed, permeabilized and labeled with antibody against G protein (**a, d**), GM130 (**b, e**) and DAPI (*blue*, merge **c, f**). *Inset* shows a higher magnification of the selected cell

Fig. 3.

Labels used for the border-to-border distribution method. Untreated HeLa cells (**a–c, g**) or cells treated with BFA (**d–f, h**) were infected with VSV for 4 h, labeled with PKH26 (**b, e**), then fixed, permeabilized and labeled with antibody against G protein (**c, f**) and DAPI (**a, d**). Borders defined by DAPI and PKH26 labeling are shown for the cell in the upper left of each image. Cell borders with 90 segments (yellow) drawn from the border of the nucleus to the plasma membrane superimposed on the G protein label are shown in (**g**) and (**h**)

Fig. 4.

Selection of segments containing high perinuclear fluorescence. **(a)** "Faux cell" diagrams of untreated and BFA-treated cells. *Purple* indicates regions of high fluorescence, while *teal* indicates lower fluorescence. **(b)** "Faux cells" with annulus at pixels 2–6 from the nucleus. **(c)** Percent of segments as a function of their normalized fluorescence intensities in perinuclear sectors. **(d)** "Faux cells" with annulus and perinuclear high (PNH) segments shown

Fig. 5.

Distribution of fluorescence intensity in sectors containing high versus low perinuclear (PN) G protein labeling in untreated and BFA-treated cells infected with VSV. The relative distance from the nucleus to the edge of the cell (x) was calculated for each pixel by dividing the pixel position within its segment by the total number of pixels in the segment. The data were binned according to their x values and the mean fluorescence intensity $\pm$ SD was calculated for each bin. Note that each bin contained a different number of pixels because of the variation in segment length. Data shown are from 4 independent experiments

Fig. 6.

PNH and PNL sectors have similar distributions that are only slightly altered in PNH sectors of cells treated with BFA. **a** Fluorescence intensity distribution in untreated and BFA-treated cells from all experiments. *Closed circles* are PNH sectors and *open circles* PNL sectors. **b** Average relative distance of fluorescence from the nucleus of PNH and PNL sectors in untreated and BFA treated cells from all experiments. **c** Skew of the fluorescence distribution of PNH and PNL sectors from untreated and BFA treated cells. The *box* in the box and whisker plots in (b) and (c) represent the values of the first, second (median) and third quartiles. The *whiskers* extend to the most extreme data point, which is no more than

1.5 times the length of the box away from the box and the *circles* represent outlying data points. Only PNH sectors in BFA-treated cells were significantly different ($p<0.05$) from all other comparisons in **(b)** and **(c)**

Fig. 7. Regions of high fluorescence intensity in the perinuclear annulus of cells infected with VSV are reduced after BFA treatment. **a** Selected annuli from untreated and BFA-treated cells, sorted by highest fluorescence at $\theta = 0^\circ$. **b** Normalized fluorescence intensity in the annulus of untreated and BFA-treated cells as a function of θ . *Black lines* are LOESS curves fit to the data