Video Article

Quantitative Immunofluorescence Assay to Measure the Variation in Protein Levels at Centrosomes

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Abstract

Centrosomes are small but important organelles that serve as the poles of mitotic spindle to maintain genomic integrity or assemble primary cilia to facilitate sensory functions in cells. The level of a protein may be regulated differently at centrosomes than at other .cellular locations, and the variation in the centrosomal level of several proteins at different points of the cell cycle appears to be crucial for the proper regulation of centriole assembly. We developed a quantitative fluorescence microscopy assay that measures relative changes in the level of a protein at centrosomes in fixed cells from different samples, such as at different phases of the cell cycle or after treatment with various reagents. The principle of this assay lies in measuring the background corrected fluorescent intensity corresponding to a protein at a small region, and normalize that measurement against the same for another protein that does not vary under the chosen experimental condition. Utilizing this assay in combination with BrdU pulse and chase strategy to study unperturbed cell cycles, we have quantitatively validated our recent observation that the centrosomal pool of VDAC3 is regulated at centrosomes during the cell cycle, likely by proteasome-mediated degradation specifically at centrosomes.

Video Link

The video component of this article can be found at https://www.jove.com/video/52030/

Introduction

Centrosomes consist of a pair of centrioles surrounded by pericentriolar material (PCM). Being the major microtubule organizing centers (MTOCs) in mammalian cells, centrosomes serve as the two poles of mitotic spindles in dividing cells, and thus help maintain genomic integrity[1]. In quiescent cells (*e.g.*, during G0 phase), one of the two centrioles of the centrosome, namely the mother centriole, is transformed into a basal body to assemble the primary cilium, a sensory organelle protruding out from the cell surface[2]. Once the cells re-enter the cell cycle, primary cilia are disassembled and each centriole directs the assembly of a procentriole at its proximal end that gradually elongates to form a mature centriole[3]. At the onset of S-phase, a cartwheel-like structure that provides the 9-fold symmetry to the centriole is formed on the surface of each existing centriole and will become the base of each procentriole. Sas6 that is indispensable for centriole assembly is recruited to form the hub of the cartwheel[4-6]. Other centriolar proteins are then assembled onto the cartwheel in a highly regulated, proximal to distal manner[7]. After precisely completing centriole duplication, cells assemble additional pericentriolar materials to build two functional centrosomes by the end of G2 phase[8]. In addition to the core centriolar components[9-11], several other proteins including kinases, phosphatases, chaperones, scaffold components, membrane associated proteins and degradation machinery are associated with centrioles, basal bodies and PCM at different times of the cell cycle[12-16]. It is often noted that the centrosomal levels of many proteins are temporally regulated by centrosomal targeting mechanisms and/or proteasomal degradation at centrosomes. Importantly, the fluctuations in the centrosomal level of several proteins such as Plk4, Mps1, Sas6, and CP110 at different points of the cell cycle appears to be crucial to regulate centriole assembly[5,17-22], and in the case of Mps1 preventing this centrosomal degradation leads to the formation of excess centrioles[19]. On the other hand, the centrosomal fractions of several proteins are less labile compared to cytosolic pools. For example siRNA-mediated down-regulation of Centrin 2 (Cetn2) led to only a moderate decrease of the protein level at the centrioles despite great reduction in its whole cell levels[23]. It is therefore crucial to measure the changes in the level of centrosomal proteins at the centrosome rather than measuring the whole cell protein levels when assessing their centrosome-specific functions.

In this study we have developed an assay using indirect immunofluorescence (IIF) to quantify the relative level of a protein at centrosomes. This assay is developed particularly to analyze cells that are from different samples and thus can not be imaged at the same time. These samples can be cells that were treated with different reagents (*i.e.*, drug versus control), collected at different time points (*i.e.*, pulse versus chase), or are in different phases of the cell cycle. The principle of this assay lies in measuring the background corrected fluorescent intensity corresponding to a protein at a small region and to normalize that value against the same for another protein whose levels do not vary under the chosen experimental conditions. Several studies in centrosome biology have recently utilized various quantitative microscopy techniques, in both live or fixed cells, to determine the centrosome-specific function of candidate proteins[24-27]. Similar to those assays, the present technique also measures the background corrected fluorescence intensity of the test protein. However, the inclusion of the normalization using an internal standard in this assay would likely offer greater accuracy and confidence in analyzing two different samples that are on two different coverslips.

Moreover, in addition to examining the protein level at centrosomes, with minor adjustments this method can be applied to a diverse set of experimental conditions or at other cellular sites.

Here, we combine our quantitative microscopy assay with a BrdU pulse-chase strategy to compare cells from different cell cycle stages. Instead of using standard cell cycle arrest and release techniques to study various cell cycle time points, asynchronously growing cells are incubated with BrdU to label cells in S-phase, and the labeled cells are chased for various times (typically 4-6 hr). Most of the labeled cells will be in S-phase immediately after the pulse. The length of the chase is chosen so that after the chase, labeled cells will be in late S, G2, or mitosis, which can be verified by morphological characteristics such as- position of centrosomes with respect to nuclei, distance between centrosomes, condensation of chromosomes *etc.* Thus, the length of the chase depends on the average duration of S, G2 and M phase of a particular cell type. Since this approach avoids cell cycle inhibitors such as hydroxyurea, aphidicolin, nocodazole, *etc.*, it allows a more physiologically relevant cell cycle analysis.

Thus, we demonstrate here that the quantitative fluorescence microscopy assay alone, or in combination with BrdU pulse-chase assay, is a simple yet powerful technique to accurately measure the relative changes in the centrosomal level of a candidate protein during an unperturbed cell cycle. We measured the centrosomal level of VDAC3, a protein that we recently identified at centrosomes in addition to mitochondria[16,28], using these assays. Results obtained here verify our previous observation that the centrosomal pool of VDAC3 is regulated by degradation, and also varies in a cell cycle dependent manner[16], furthermore validating the applicability of this method.

Protocol

1. Cell Culture

1. Use human telomerase reverse transcriptase (hTERT) immortalized retinal pigment epithelial cells (hTERT-RPE1; referred to here as RPE1). NOTE: RPE1 cells are near-diploid, non-transformed human cells that are commonly used to study centriole assembly and ciliogenesis. These cells follow a normal centriole duplication cycle coordinated with a regulated cell cycle.
2. Passage a near-confluent 100 mm cell culture dish of RPE1 cells at 1:5 dilution of the original culture into a fresh 100 mm dish containing 10 ml of DME/F-12 (1:1) medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin G and 100 µg/ml streptomycin (the complete medium is referred as DME/F-12 medium). Grow cells at 37 °C in the presence of 5% CO_2.
    1. Incubate 12 mm round glass coverslips in 50 ml of 1 N HCl in a covered glass beaker, O/N without shaking, at 60 °C in a water bath or incubator.
    2. After discarding the acid solution, wash the coverslips three times in 100 ml distilled water by incubating for 15 min with occasional shaking. Repeat the washing similarly in 70% Ethanol and 95% Ethanol.
    3. Dry the coverslips by individually spreading them out on a laboratory blotting paper in a biosafety cabinet, followed by sterilization with UV radiation for 60 min.
3. Place 2-4 dry coverslips in a 35 mm cell culture dish. Dilute the solution of fibronectin or fibronectin like engineered polymer (stock concentration of 1 mg/ml) to a concentration of 25 µg/ml in cell culture PBS.
    1. Place 80-100 µl of the diluted solution onto each coverslip and incubate for 60 min to coat the top side of the coverslip. Wash the coverslips thrice using PBS before adding complete medium.

2. Growing Cells and Treating Cells with Proteasome Inhibitors

1. Passage 2 x 10^5 asynchronously growing RPE1 cells in each 35 mm dish containing coverslips and grow the cells in 2 ml complete medium. Replace the culture medium every 24 hr with fresh pre-warmed complete medium.
    1. To analyze the effect of proteasome inhibition on the centrosomal level of the test protein (here VDAC3 or γ-tubulin), grow the cells in two 35 mm dishes for 44 hr. Replace the culture medium with complete medium and add either MG115 or DMSO as the control solvent at a final concentration of 5 µM or 0.05% respectively. At the same time, add BrdU to the cells at a final concentration of 40 µM and incubate cells for 4 hr.
    2. Transfer each coverslip in a 24-well plate. Add 500 µl chilled methanol to each well and incubate the plate at -20 °C for 10 min to fix the cells on the coverslips. Immediately wash the coverslips three times with 500 µl wash buffer (1x PBS containing 0.5 mM $MgCl_2$ and 0.05% Triton-X 100).
2. Harvest the residual cells from the 35 mm dish and centrifuge the cells at 1,000 x g for 5 min. Use the cell pellet to analyze the total protein by western blotting (step 6).

3. BrdU Pulse and Chase Assay to Analyze Protein Levels in Different Cell Cycle Phases

1. To analyze the variation of the level of a protein (here VDAC3, Sas6 or Cep135) at centrosomes at different phases of the cell cycle, grow cells in two 35 mm dishes containing coverslips for 44 hr. Replace the culture medium with complete medium containing 40 µM BrdU and incubate the cells for 4 hr in the cell culture incubator.
2. From one dish, transfer the coverslips to a 24-well plate to fix the cells using chilled methanol as described in step 2.1.2.
3. After the 4 hr BrdU pulse, remove the BrdU-containing medium, wash the cells once with PBS and once with complete medium, add fresh medium, and then grow the cells in the absence of BrdU for various times (typically another 4 hr for RPE1 cells) before fixing the cells as described in step 2.1.2.
   NOTE: For RPE1 cells, the majority of the BrdU-positive cells are in late S or G2-phase after a 4 hr chase. This must be determined independently for each cell type.

4. Immunostaining

1. Incubate the fixed cells on coverslips in 200 µl blocking buffer (2% BSA and 0.1% TritonX-100 in 1x PBS) for 30 min.
    1. Incubate the cells with a mix of primary antibodies (typically one raised in rabbit and another raised in mouse) diluted in blocking buffer O/N at 4 °C in a humidified chamber.
    2. To make a humidified chamber, put a wet paper towel in the bottom half of an empty 1,000 µl pipette tip box. Lay a strip of Parafilm on the rack surface, and spot a droplet (15-20 µl) of the antibody solution onto the Parafilm for each coverslip to be incubated. Invert a coverslip onto a droplet of antibody solution (so that cells are immersed) and close the lid of the tip box.
    3. Invert coverslips again and return them back to the 24-well dish. Wash coverslips and then incubate them in 150 µl secondary antibody mixture (here green-fluorescent dye conjugated anti-rabbit and red-fluorescent dye conjugated anti-mouse diluted in blocking buffer) for 1 hr at RT. Wash the coverslips three times.
    NOTE: The fluorophore-conjugated Secondary antibodies are light sensitive. Protect the samples from light during steps 4.1.3-5.1.2.

2. Prepare for anti-BrdU staining by fixing the stained RPE1 cells again with 500 µl chilled methanol at -20 °C for 10 min, as described in step 2.1.2. Wash the coverslips thrice.
NOTE: This fixation secures the primary and secondary antibody labeling of the protein(s) of interest during the acid hydrolysis process in the following step.
    1. Incubate the cells in 200 µl of 2 N HCl for 30 min at RT. Neutralize with 300 µl of 1 M Tris-Cl, pH 8 and wash the cells three times using wash buffer.
    2. Block the cells again using 200 µl blocking buffer for 30 min at RT.
    3. Incubate the cells with rat anti-BrdU antibody (diluted in blocking buffer) for 45 min at 37 °C in a humidified chamber. Return the coverslips back to the 24-well dish, wash them and then incubate with blue-fluorescent dye conjugated anti-rat secondary antibody (diluted in 150 µl blocking buffer in 24-well dish for 1 hr at RT.

3. Wash the coverslips three times. Spot a droplet (roughly 3-6 µl) of a mounting solution containing antifade reagent on a glass microscope slide. Invert a coverslip, with the cell-side facing down, onto the mounting solution. Wipe excess liquid by gently pressing the coverslip against the slide using soft cleaning tissue. Apply transparent nail polish along the edge of the coverslip to seal it onto the microscope slide.

5. Immunofluorescence Image Acquisition and Analysis

1. Use a 100X Plan Apo oil immersion objective (with a 1.4 numerical aperture) to acquire the images of BrdU-positive RPE1 cells at ambient temperature.
    1. Put a slide on the microscope (see equipment) attached with a camera capable of digital imaging. For each fluorophore, determine the appropriate exposure time (typically between 300-1,000 msec) by manually examining all samples of an experiment. Before acquiring images of a cell, manually determine the appropriate top and bottom focal plane along the Z-axis (typically 0.2 µm step size). Acquire images of different samples that are on separate coverslips using identical exposure time for different fluorophores along the Z-axis using a digital microscopy imaging software package.

2. Perform deconvolution (in these cases using No neighbor algorithm) of all image stacks acquired along the Z-axis.
    1. Obtain the total intensity projection of each image stack along the Z-axis.

3. In cells where centrosomes are close (distance between two centrosomes is less than 2 µm), draw a small square (typically 20-30 pixels per side) around both centrosomes and mark the selected area.
    1. Draw a larger square (typically 24-35 pixels per side) surrounding the first square and mark the selected area of the large square.
    2. Obtain the area (A) and the total fluorescence intensity (F) of each fluorophore in each box (S denotes small box and L denotes large box).
    3. Analyze the background corrected fluorescence intensity of each fluorophore using the formula described by Howell *et al.*[29]: $F = F_S - [(F_L - F_S) \times A_S / (A_L - A_S)]$.
    4. Obtain the normalized fluorescence intensity of the protein of interest (here VDAC3 or Sas6) by calculating the ratio of the background-corrected fluorescence intensity of its fluorophore to that of the fluorophore used for the chosen internal standard (here γ-tubulin, Sas6 or Cep135).

4. In the case of analyzing cells where centrosomes are well separated (distance between two centrosomes is greater than 2 µm), analyze each centrosome separately by drawing two separate sets of boxes. Draw a small square (typically 15-25 pixels per side) around each centrosome and mark the selected area. Draw a larger square (20-30 pixels per side) surrounding the first square and mark the selected area.
    1. Obtain the area (A) and the total fluorescence intensity (F) of each fluorophore in each box, and calculate the background corrected fluorescence intensities of each fluorophore, separately for each centrosome, as described in step 5.3.3.
    2. Combine the background corrected fluorescence intensities of each protein from the two centrosomes to obtain the total centrosomal level of that protein in that cell.
    3. Obtain the normalized intensity for the protein of interest by calculating the ratio of its total centrosomal intensity to the total centrosomal intensity of the internal standard.

5. Analyze at least 15-25 cells for each experimental condition and plot the graph in a spreadsheet.

6. Analyzing the Total Protein Using Western Blotting

1. Resuspend the cells in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and incubate for 10 min on ice to lyse the cells.

1. Centrifuge the mixture at 10,000 x g for 10 min, separate the lysate from the pellet fraction and measure the total protein concentration of each lysate using a bicinchoninic acid (BCA) assay kit.

2. Mix 40 µg of lysate with 4x SDS-PAGE loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 100 mM Dithiothreitol, 0.1% Bromophenol blue).
    1. Boil the samples for 10 min and run the samples on a 12.5% denaturing SDS-PAGE at a constant voltage of 200 V.

3. Transfer the proteins separated on SDS-PAGE onto a nitrocellulose membrane using western blotting technique (at a constant voltage of 90 V for 1 hr in cold).
    1. Block the membrane with 3% non-fat milk in 1x PBS, and then incubate the membrane with solutions of diluted primary antibodies (in this case rabbit anti-VDAC3, rabbit anti-γ-tubulin and mouse anti-α-tubulin) for 1 hr at RT.
    2. After washing the membrane three times (each 5 min incubation under shaking) using PBS-T buffer (1x PBS and 0.2% Tween-20), incubate the membrane in a mixture of near-Infrared (IR) fluorescent dye conjugated anti-rabbit and IR fluorescent dye conjugated anti-mouse secondary antibodies (diluted in PBS-T) for 1 hr.
    3. Wash the membrane three times and then scan the membrane to analyze the bands using an infrared fluorescence imaging system suitable for immunoblot detection.

Representative Results

Our recent studies identified novel centrosomal localization and function of VDAC3, one of the mitochondrial porins[16,28]. Immunostaining of several mammalian cells including RPE1 cells using a VDAC3-specific antibody showed prominent centrosomal staining and comparatively weak mitochondrial staining. We also showed that centrosomal VDAC3 is preferentially associated with the mother centriole, and the centrosomal pool of both the endogenous and ectopically expressed VDAC3 is regulated by degradation[16]. In order to validate the quantitative IIF assay we examined the changes in the centrosome-associated VDAC3 pool in cells that are in S-phase.

Here, asynchronously growing RPE1 cells were treated with either the proteasome inhibitor MG115 or the control solvent DMSO for 4 hr. To restrict our analysis to cells that that are in S-phase and undergoing centrosome assembly, we only examined cells that incorporated BrdU during the same 4 hr and had two closely spaced centrosomes (spaced within 2 µm of each other). We examined multiple random fields of cells stained for BrdU and nuclei from both control and MG115 treatment (**Figure 1A**) to conclude that the brief treatment of MG115 did not affect the BrdU incorporation (roughly 58%) compared to control treatment (roughly 56%) in RPE1 cells. As we were comparing only cells that were in S-phase (as judged by the presence of two γ-tubulin foci spaced within 2 µm of each other), we considered γ-tubulin as a potential internal standard for normalization. Thus, we first examined if centrosomal γ-tubulin levels in BrdU-positive cells vary upon proteasome inhibition. Not surprisingly, there was considerable cell-to-cell variation in γ-tubulin fluorescence intensity from one cell to another, and we found that this variation was best presented in box and whisker diagrams that present the data set as a whole available. In box and whisker diagrams, boxes represent the lower and upper quartile, the marker in the box indicates the median of each series, and the whiskers represent minimum and maximum values, that are often (though not always) outliers. As seen in **Figure 1D**, the centrosomal γ-tubulin level was not significantly different between BrdU-positive cells treated with either MG115 or DMSO, thus validating γ-tubulin as an internal standard for this experiment. In contrast to γ-tubulin, the background-corrected fluorescence intensity corresponding to centrosomal VDAC3 was roughly 2.5-fold higher in MG115-treated cells than in control cells (**Figure 1C**). When we normalized total VDAC3 fluorescence against that of γ-tubulin, the fold increase dropped slightly to roughly two-fold (**Figure 1E**). Although the fold change was greater without normalization, we have more confidence in the accuracy of the normalized data, which we feel better controls for any general effect of MG115 treatment, as well as variation due to sample processing. VDAC3 staining at non-centrosomal sites (**Figure 1B**) or the total cellular level of VDAC3 (**Figure 1F**) was not affected by proteasome inhibition. Thus, we quantitatively verify our previous observation that the centrosomal pool of VDAC3 is regulated by proteasome-mediated degradation.

In order to measure the change in centrosomal VDAC3 during the cell cycle we have labeled the cells with a 4 hr BrdU pulse followed by a 4 hr chase of the labeled cells. Since only S-phase cells will incorporate BrdU during the pulse (average distance between two centrosomes is 1.35 ± 0.16 µm), the majority of BrdU-positive RPE1 cells after 4 hr chase will be at either late S-phase or early G2 phase (average distance between two centrosomes is 1.55 ± 0.22 µm), with a minor population at late G2 phase where the centrosome pair has significantly separated (average distance between two centrosomes > 2 µm)[30]. γ-tubulin, being a major PCM component greatly accumulates at centrosomes during centrosome maturation that takes place at G2 phase[31,32], and this increase makes it a poor internal standard for assessing cell cycle variations of other centrosomal proteins. On the other hand, Sas6 is recruited to procentrioles during early S-phase to stimulate assembly of the cartwheels[4,5,7], and remains at the base of the newly formed centrioles until cells enter mitosis when it begins to be degraded (**Figure 2** and reference[5]). However, in HeLa or U2OS cells, the centriolar level of Sas6 gradually increases as cells progress from S-phase to G2 phase[5]. Therefore, we first measured the centrosomal Sas6 level with respect to that of Cep135, another core centriolar protein that localizes to the proximal ends of centrioles throughout the cell cycle[33]. We did not observe any significant difference in the background-corrected total fluorescence intensities of Cep135 or Sas6 in BrdU-positive RPE1 cells from pulse or chase, when centrosomes are closely spaced (**Figure 3A-D**; 'close' cells where average distance between two centrosomes is less than 2 µm). Accordingly, the normalized Sas6 level remained similar in these cells (**Figure 3E**). However, we observed a modest increase in the overall fluorescence intensity values of Sas6 and a slight increase of the same for Cep135 in cells where centrosomes are well separated (distance between two centrosomes> 2 µm; 'distant' cells). Accordingly, the normalized total centrosomal Sas6 level in cells with two well-separated centrosomes was slightly, but statistically significantly higher than that in cells with closely spaced centrosomes. This is likely due to the inherent regulation of Sas6 level with cell cycle progression[5]. However, it is also possible that the slight increases (which or of lower statistical significance) are due to adding fluorescence intensities of two centrosomes that were analyzed separately, as compared to analyzing two centrosomes at the same time in cells with closely spaced centrosomes. Nonetheless, when the fluorescence intensity of VDAC3 was normalized to that of Sas6 alone, the VDAC3 level at two closely spaced centrosomes was increased roughly 1.5 fold in cells that are in late S-/early G2-phase, compared to the early S-phase cells (**Figure 4A-C, F**; 'close' cells). When these cells progress to late G2 phase, the normalized VDAC3 level at two centrosomes was reduced by 2.4-fold compared to that in late S-phase (**Figure 4C, F**).

Because the Sas6 levels increase slightly from late S-phase to G2, using Sas6 as an internal standard leads to a slight overestimation of the decrease in VDAC3 levels in late G2-phase cells (centrosomes are separated by more than 2 µm). While our data suggest that Cep135 is a better choice as an internal standard for assessing cell cycle variations, we cannot use it for VDAC3 because the VDAC3 and Cep135 antibodies available to us are both from rabbit. However, multiplying the median value for Sas6-normalized VDAC3 (F_{VDAC3}/F_{Sas6}) by the median value of Cep135-normalized Sas6 gives us an approximate value for Cep135-normalized VDAC3 [$(F_{VDAC3}/F_{Sas6}) \times (F_{Sas6}/F_{Cep135}) = F_{VDAC3}/F_{Cep135}$]. When we performed this analysis, the change in VDAC3 levels between late S/early G2 and late G2 drops slightly to 2-fold. BrdU-positive cells that are in any stage of mitosis as judged by condensed chromosomes and weak Sas6 staining are excluded from our analysis, due to the dramatic drop in Sas6 levels. However, we noted that the centrosomal VDAC3 level remained low during mitosis (data not shown). Because Cep135 levels do not drop during mitosis (data not shown), we could in principle repeat the re-normalization procedure to estimate the Cep135-normalized VDAC3 levels in mitosis. However, in reality centrosomal Sas6 levels in mitosis were too variable to allow for an accurate determination of the Sas6-normalized VDAC3 levels in the first place. Regardless, overall, our data verified that the centrosomal pool of VDAC3 is regulated at centrosomes during the cell cycle.

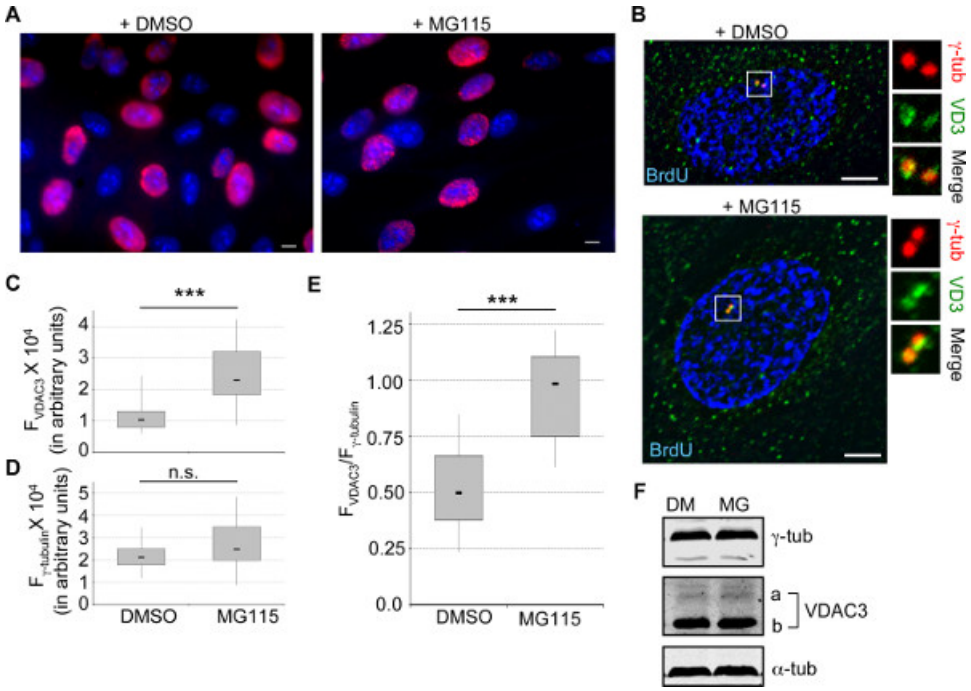

Figure 1. Asynchronously growing RPE1 cells were incubated with BrdU and MG115 (MG) or DMSO (DM) for 4 hr. (**A**) Shown are random fields of DMSO or MG115 treated cells stained with anti-BrdU antibody (red) and Hoechst (DNA; blue). Here and in all other images the bar represent 5 µm. (**B-E**) DMSO or MG115 treated cells were stained with antibodies against VDAC3, γ-tubulin and BrdU. BrdU-positive cells were imaged under identical imaging conditions (exposure times- 400 msec for blue fluorophore/BrdU; 500 msec for green fluorophore/VDAC3; 300 msec for red fluorophore/γ-tubulin), and the background corrected fluorescence intensities of VDAC3 and γ-tubulin were determined. Representative images showing VDAC3 (VD3; green), γ-tubulin (γ-tub; red) and BrdU (blue) are shown in (**B**). Here and in all other images, insets show digitally magnified centrosomes as indicated by the squares. The background corrected fluorescence intensities (**F**; in arbitrary units) corresponding to VDAC3 and γ-tubulin from twenty-five cells are plotted in box and whisker diagram in (**C**) and (**D**) respectively. Here and in all other cases, boxes represent lower and upper quartile, the marker in the box (-) indicates the median of each series, and the whiskers represent minimum and maximum values. Normalized fluorescence intensity values of VDAC3 from those twenty-five cells are plotted in (**E**). For (**C-E**), p value is derived from unpaired T-test. *** indicates $p < 10^{-5}$, and n.s. indicates $p > 0.05$. (**F**) Immunoblots showing whole-cell levels of VDAC3 (both VDAC3a and VDAC3b[16]) and γ-tubulin (γ-tub) where α-tubulin (α-tub) is loading control. Please click here to view a larger version of this figure.

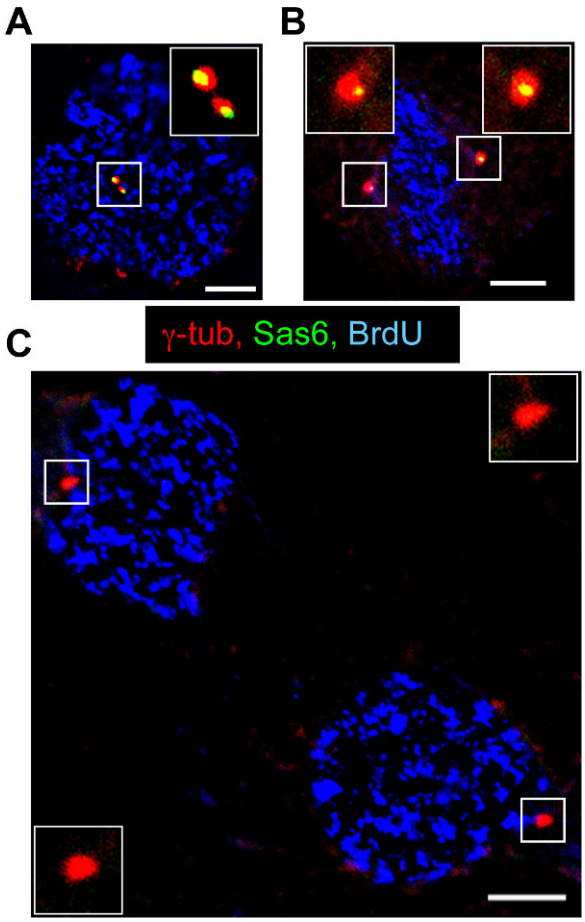

Figure 2. Asynchronously growing RPE1 cells that were labeled with a 4 hr BrdU pulse and chased for another 4 hr in the absence of BrdU were stained with antibodies against Sas6 (green) and γ-tubulin (γ-tub; red). Representative images show Sas6 staining during (**A**) S-phase (with two close Sas6 foci), (**B**) metaphase (two weak Sas6 foci at the poles) and (**C**) telophase (Sas6 foci are lost from the poles). Please click here to view a larger version of this figure.

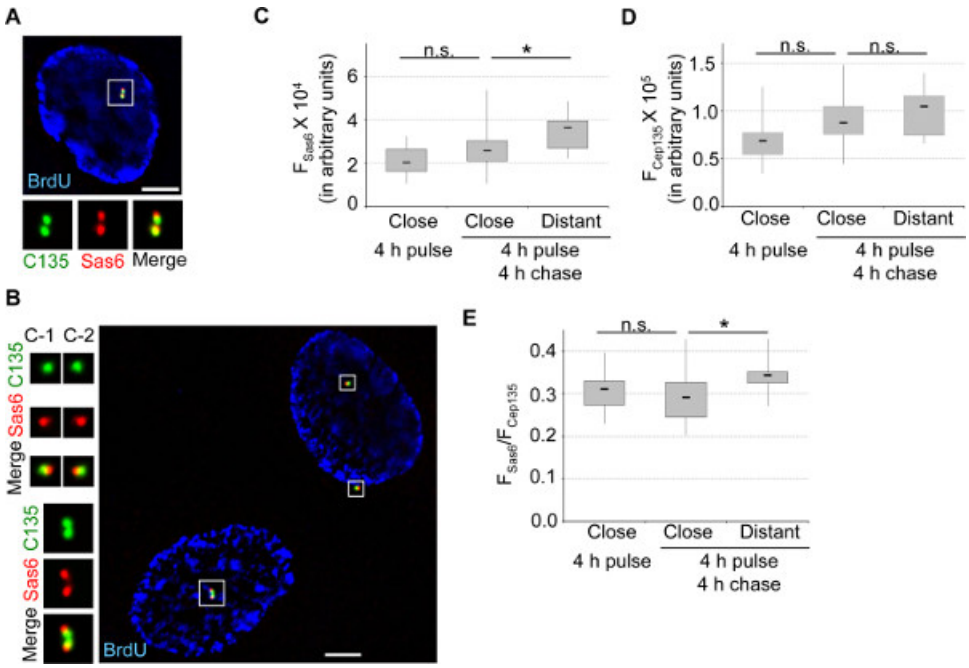

Figure 3. Asynchronously growing RPE1 cells were labeled with a 4 hr BrdU pulse and chased for another 4 hr in the absence of BrdU. BrdU-positive cells stained for Cep135 and Sas6 were imaged under identical imaging conditions (exposure times- 400 msec for blue fluorophore/ BrdU; 400 msec for green fluorophore/Cep135; 500 msec for red fluorophore/ Sas6), and the background corrected fluorescence intensities of Sas6 and Cep135 were determined. The distance between two centrosomes was also measured in all cells. A cell where the distance between two centrosomes is less than 2 µm was considered as 'close' and where the value is more than 2 µm was categorized as 'distant'. (**A-B**) Representative images showing Cep135 (C135; green), Sas6 (red) and BrdU (blue) are shown. In (**B**), C1 and C2 are the two centrosomes of the representative 'distant' cell. (**C-D**) The background corrected fluorescence intensities (F; in arbitrary units) corresponding to Sas6 (**C**) and Cep135 (**D**) from fifteen cells of each category are plotted in box and whisker diagram. (**E**) Normalized intensities of Sas6 from those cells are plotted in box and whisker diagram. For (**C-E**), p value is derived from unpaired T-test. * indicates 0.001 < p < 0.05, and n.s. indicates p > 0.05. Please click here to view a larger version of this figure.

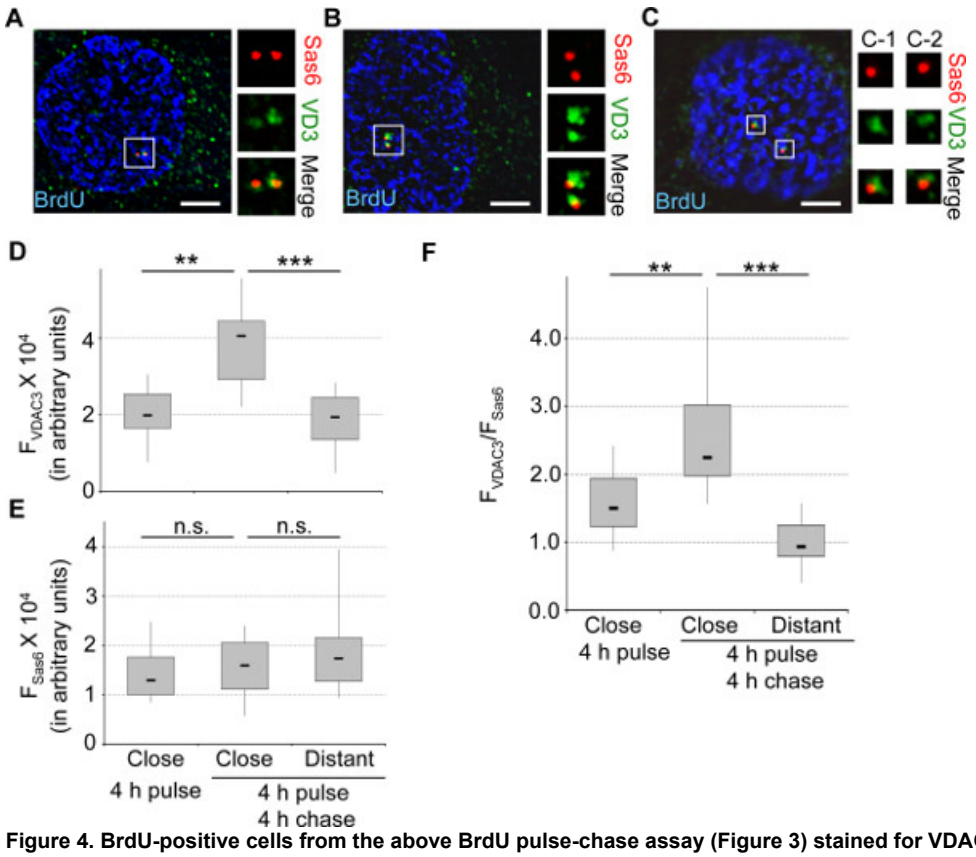

Figure 4. BrdU-positive cells from the above BrdU pulse-chase assay (Figure 3) stained for VDAC3 and Sas6 were imaged under identical imaging conditions (exposure times- 400 msec for blue fluorophore/ BrdU; 500 msec for green fluorophore/Sas6; 1,000 msec for red fluorophore/VDAC3), and the background corrected fluorescence intensities of Sas6 and VDAC3 were determined. The distance between two centrosomes was measured in all cells, and cells were categorized as 'close' (the distance between two centrosomes < 2 µm) and 'distant' (the distance between two centrosomes > 2 µm), as in **Figure 3**. (**A-C**) Representative images of 'close' cell in BrdU pulse (**A**), in BrdU chase (**B**), and 'distant' cell in BrdU chase (**C**) stained for VDAC3 (VD3; green), Sas6 (red) and BrdU (blue) are shown. In (**C**), C1 and C2 are the two centrosomes. [**D-E**] The background corrected fluorescence intensities (F; in arbitrary units) corresponding to VDAC3 (**D**) and Sas6 (**E**) from fifteen cells of each category are plotted in box and whisker diagram. (**F**) Normalized intensities of VDAC3 from those cells are plotted in box and whisker diagram. For (**D-F**), p value is derived from unpaired T-test. *** indicates $p < 10^{-5}$, ** indicates $10^{-5} < p < 0.001$, and n.s. indicates $p > 0.05$. Please click here to view a larger version of this figure.

Discussion

Quantitative microscopy in cell biology is commonly associated with live-cell imaging assays such as Fluorescence Resonance Energy Transfer (FRET), Fluorescence Recovery After Photobleaching (FRAP), *etc.* However, there are growing examples of cell biologists developing different quantitative microscopy assays for fixed cells in recent years[27,34-36]. Importantly, progress in understanding centrosome biology often requires understanding of the centrosome-specific function of proteins whose centrosomal pools may be regulated differently than other pools. Thus, we developed a quantitative IIF assay to specifically analyze the relative centrosomal level of a protein in two differently treated samples. Because this requires that cells are fixed and processed on separate coverslips, we therefore introduced an internal standard for the purpose of normalization, to better control for possible artifacts due to the necessity of handling the two experimental cell populations separately, and increase the accuracy of the assay. There are only a few previous studies[37] that have used such an internal standard to measure the relative fluorescence intensity of a test protein.

While choosing an internal standard is crucial for the accuracy of our assay, it is arguably the most challenging aspect. Ideally, the centriole pair duplicates during S-phase, and the duplicated centrioles then accumulate additional PCM components including γ-tubulin during G2 so that the two mature centrosomes can become the poles of mitotic spindle. Therefore, we assume that the centrosomal level of γ-tubulin would not change significantly in cells that are strictly in S-phase. This seems to be the case in RPE1 cells where S-phase cells are marked by a brief BrdU labeling. However, the validity of an internal standard must be tested for each cell type and experimental condition. For example, while the background-corrected VDAC3 fluorescence increased more than two fold in response to proteasome inhibition in aphidicolin treated (S-phase arrested) U2OS cells, there was also a small but statistically significant ($0.001 < p\text{-value} < 0.05$) increase in centrosomal γ-tubulin (supplementary Figure). Thus, γ-tubulin is not always an appropriate internal control, even for cells that are arrested in the cell cycle.

Similar to all other IIF microscopy assay, one of the drawbacks of this assay is that antibodies against the test protein and the internal standard must be raised in different host species. While our data suggest that Cep135 is a better internal standard than Sas6, we could not normalize VDAC3 levels against Cep135 as the available antibodies against these proteins were both raised in rabbit. Thus, we instead normalized against Sas6, which caused an overestimation of the decrease in VDAC3 levels between late S-phase and G2. In a situation where the internal standard

varies modestly, one can introduce another normalization factor to account for that variation. For example, to correct for the variation in Sas6, we multiplied the Sas6-normalized VDAC3 intensity by the Cep135-normalized Sas6 intensity.

In cases where choosing an appropriate internal standard is difficult, fluorescent dye coated microspheres (0.02-0.04 µm diameter) may provide an alternative strategy. The signal of a fluorescence microsphere present in the same field as the cell of interest can be used to normalize the fluorescence signal corresponding to the protein of interest. On the other hand, if the test and control cells are imaged together, one may not need such an internal standard for normalization. We have previously used a version of this assay to compare the centrosomal level of Mps1 in cells overexpressing either cyclin A[19] or Antizyme[20], which either prevent[19] or promote[20] the degradation of Mps1 at centrosomes, respectively, to that in adjacent untransfected cells imaged at the same time.

In order to examine the effect of centrosomal VDAC3 upon proteasome inhibition, we used BrdU labeling to identify and compare cells that are in S-phase. While MG115 treatment may inhibit cell cycle progression, this inhibition requires a significantly higher concentration[38] than that used in this study. At the concentrations used here MG115 can block cell cycle checkpoints and transitions, but does not block cell cycle progression. However, that must be verified for each cell type, as we did here by showing that MG115 did not alter the percentage of BrdU positive cells compared to DMSO. However, arresting cells in S-phase using aphidicolin or hydroxyurea (HU) or synchronizing cells using mitotic 'shake-off' and release or double thymidine block and release could be used as alternative approaches to generating equivalent populations of cells in S-phase.

The BrdU pulse-chase strategy to study cell cycle time points will be very useful in many functional assays that require an unperturbed cell cycle. This is a biologically more significant assay that might be able to replace cell cycle arrest techniques using various cell cycle inhibitors. Use of these inhibitors may often cause different types of physiological stress in the cells resulting in aberrant changes in the cell cycle phenotypes. Use of the pulse-chase approach enabled us to demonstrate that depletion of Cetn2 only delayed centriole assembly[39] as opposed to causing a complete block as suggested by previous studies[23]. Detecting BrdU-positive cells requires acid hydrolysis of the samples. We and many other researchers are using a technique that re-fixes the cells after primary and secondary staining for test proteins, prior to acid hydrolysis, with insignificant loss of staining intensities for different centrosome proteins[40]. However, a potential alternative could be to use 5-ethynyl-2′-deoxyuridine (EdU), another thymidine analog that is, similar to BrdU, incorporated efficiently in replicating DNA[41]. Visualization of EdU using 'click chemistry" does not require acid hydrolysis[42], and may therefore more suitable for some antibodies.

In addition, this assay can easily be used to measure the change in post-translational modifications of a particular protein at centrosomes. For example, the centrosomal level of phosphorylation can be normalized against the total level of the protein itself, provided that phosphorylation site-specific antibodies exist. This assay could be immediately applied to assessing relative levels of phosphorylation between experimental conditions or cell cycle phases, but could potentially be adapted to assessing total levels of phosphorylation if critical parameters of antibody affinities are known or can be determined. This assay should also be applicable for study of proteins at any other site in cells, although the region of interest should not be too large. The assay will be particularly useful to measure the location-specific modulation of a protein that is localized at multiple cellular sites.

There are a handful of technical parameters such as differential photobleaching, the effect of digital imaging, linearity of the fluorescence intensities of fluorophores, background noise *etc.* that must be taken in consideration to maximize the accuracy of the measurement[43]. For example, one can switch the fluorochrome associated with each secondary antibody to assess the contribution of photobleaching. Many other parameters can be well controlled if one uses identical imaging conditions (exposure time, gain, step size, number of Z-stacks, *etc.*) and the same imaging software in each imaging run. Although we used the No-neighbor algorithm throughout our assay to perform image deconvolution, this assay can be combined with other deconvolution algorithm such as constrained iterative. We also note that it should be easy to adapt this assay to any number of different imaging software packages, as most commonly available image analysis software has tools for the analysis of fluorescence intensity and distances.

This assay should be applicable to any cell type, and our choice of cells here is purely based on their relevance in centrosome biology research, and their use in our previous examinations of centrosomal VDAC3 function[16,28]. However, the validity of internal standards and appropriate times for pulse-chase to identify cells in S-phase, G2, or mitosis must be determined for each cell type. Overall, our newly developed quantitative fluorescence technique in combination with the BrdU pulse-chase assay will be a very useful method to resolve several intricate mechanisms in centrosome biology and in many broader aspects of cell biology.

Disclosures

The authors declare that they have no competing financial interests.

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