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Fluorescence-based Quantification of Nucleocytoplasmic Transport

Joshua B. Kelley¹, Bryce M. Paschal²

¹Department of Molecular and Biomedical Sciences, University of Maine

²Center for Cell Signaling, Department of Biochemistry and Molecular Genetics, University of Virginia

Abstract

The sequestration of DNA within the membrane-bound nucleus is a defining characteristic of eukaryotic cells. Replication and transcription are therefore restricted to the nucleus, however, the regulation of these events relies on cytoplasmic processes including protein synthesis and signal transduction pathways. Because a variety of cellular activities depend on nuclear transport, researchers from diverse fields have found it useful to examine the nuclear localization of proteins of interest. Here we present some important technical considerations for studying nuclear and cytoplasmic localization, and provide guidance for quantifying protein levels using fluorescence microscopy and ImageJ software. We include discussion of the use of regions of interest and image segmentation for quantification of protein localization. Nucleocytoplasmic transport is fundamentally important for controlling protein levels and activity in the nucleus or cytoplasm, and quantitative analysis can provide insight into how biological output is achieved.

1. Introduction

1.1 Nucleocytoplasmic Trafficking

The defining characteristic of eukaryotes is the presence of a nucleus and other membrane bound organelles. The cytoplasm is separated from the genome by the double bilayer - the nuclear envelope - which is spanned by large macromolecular structures called nuclear pore complexes (NPCs) (Wente and Rout, 2010). While small molecules and proteins less than ~60 kDa diffuse through the NPC, larger molecules must undergo active transport into and out of the nucleus (Paine et al., 1975; Pemberton and Paschal, 2005). Import is carried out by proteins called importins, which bind to nuclear localization signal (NLS) containing cargo in the cytoplasm, carry them through the NPC and release the cargo inside the nucleus. This release is stimulated by the primary regulator of nuclear transport, the small G-protein Ran. Export is carried out by proteins called exportins, which form a heterotrimeric complex with Ran and nuclear export signal (NES) containing cargo proteins in the nucleoplasm, carry them through the NPC, and release them into the cytoplasm. Nucleocytoplasmic

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transport plays a key role in numerous cellular pathways, typically because the pathways modulate or respond to changes in transcription (Kau et al., 2004; Komeili and O'Shea, 2000; Poon and Jans, 2005).

The separation of the cytoplasm from the nucleus allows for modes of regulation based on sequestration and control of localization. Transcription factors can be retained in the cytoplasm until needed, reducing the basal level of transcription that would occur if the transcription factor was in the same compartment as its target promoter. Processing of mRNA can be completed in the nucleus, away from ribosomes which might otherwise initiate translation of unspliced transcripts.

Researchers interested in either nuclear transport or transport dependent signaling events may find themselves performing experiments to evaluate the subcellular distribution of proteins that undergo nuclear trafficking. While it is easy to view at an image and declare (qualitatively) that a protein is either in the nucleus or the cytoplasm, assessment of changes in the localization of a molecule to the nucleus or cytoplasm is more subtle, and the effect should be assessed in an unbiased and reproducible manner. Quantitation of microscopy images will help remove bias from the interpretation of data, and increase rigor by allowing larger data sets to be viewed and analyzed in the form of graphs, rather than the few representative images which can be shown in a figure (Hamilton, 2009). Increasing rigor of experiments should, in turn, improve reproducibility and provide a better basis for hypothesis generation and testing.

Careful quantitation of nuclear localization has, for example, helped advance our understanding of Ran regulation in response to cellular stress (Chatterjee and Paschal, 2015; Czubryt et al., 2000; Datta et al., 2014; Kelley and Paschal, 2007) and in premature ageing (Datta et al., 2014; Kelley et al., 2011; Snow et al., 2013). Measurements of nuclear translocation of transcription factors have improved the understanding of general stress responses (Hao and O'Shea, 2011). Recently, nuclear transport defects have been identified in neurodegenerative diseases, such as ALS and frontotemporal dementia (Chou et al., 2018; Dormann et al., 2010; Gasset-Rosa et al., 2017; Nagara et al., 2013). In each of these cases, careful quantitation of the nucleocytoplasmic localization of a variety of different cargoes has been instrumental in helping define the underlying biology.

1.2 Microscopy vs Biochemical Fractionation

There are two basic ways to assess the nuclear localization of a protein, microscopy and biochemical fractionation. The microscopy approach may entail immunofluorescence, immunohistochemistry, or fluorescent-protein tagging using transmitted light, epifluorescence, or confocal imaging. Biochemical fractionation involves the separation of nuclear and cytoplasmic fractions, and can be achieved with mechanical disruption of the plasma membrane (Dimauro et al., 2012). Unfortunately, this is known to cause disruption of the nucleus (Newmeyer et al., 1986). Another common biochemical approach to nuclear fractionation is to treat cells with a mild detergent such as Triton X-100, and define the Triton-resistant fraction as the nuclear fraction (Hymer and Kuff, 1964). This results in the recovery of many nuclear proteins in the insoluble fraction, but Triton permeabilizes the nuclear envelope such that soluble nuclear proteins are released (Adam et al., 1992). A

gentler biochemical fractionation approach is to use low concentrations of the detergent Digitonin (Adam et al., 1992), which will maintain the nuclear envelope integrity, thereby releasing soluble, cytoplasmic proteins. Nuclear proteins are retained in the nucleus and can be solubilized in a subsequent step. The nuclear derived fraction and cytoplasmic derived fractions are then examined by SDS PAGE and Western blotting, with suitable antibodies.

Microscopy has several advantages over biochemical fractionation.

1. Microscopy can be performed in live cells, or in cells which have been rapidly fixed, maintaining protein localization in the correct compartment.
2. Microscopy allows for single cell analysis. Population-level analysis can mask actual outcomes when there is cell-to-cell variability in expression or phenotype. Microscopy has the advantage of giving the researcher an accurate view of cell-to-cell variability. This can be especially important in experiments using transfection to express ectopic proteins, as there are large differences between cells in both transfection state and in expression levels within transfected cells.
3. Microscopy allows single cells to be followed over time, so kinetics of transport can be studied.
4. The rate of biochemical reactions is driven by the concentration of reactants and products. The volume of the compartment where the reaction takes place is therefore critical to understanding the biochemistry of the system. The amount of protein in the nucleus of a cell does not translate directly to knowing the concentration of the protein in the nucleus, as the concentrations of a molecule in the nucleus or the cytoplasm are dependent upon the volume of those compartments. Volume information is lost by biochemical fractionation, but maintained with microscopy. Even a 2D microscopy image has volume information, as the fluorescence in each 2D pixel represents fluorescence from above and below that point, with an effective Z dimension that is determined by the point spread function of the microscope under those imaging conditions. The point spread function describes the way that light is diffracted from a point source, and is unique to the optics and imaging conditions (Cole et al., 2011). When using the same imaging conditions, this z-dimension will be the same, and so the 2D images can be compared to each other.

Localization of a molecule can be visualized through fluorescence or colorimetric assay. Fluorescence localization can be achieved through immunofluorescence with fluorescently labeled antibodies, or through the use of fluorescent-protein chimeras. Immunohistochemistry using colorimetric reactions can also be used for localization, but because the production of the color is a chemical reaction, the time of the reaction must be carefully monitored to maintain linearity. The imaging conditions (excitation intensity, exposure time) for fluorescence microscopy can be adjusted for the sample prior to recording data such that the images are being measured within a linear range, and thus fluorescence microscopy is preferable for quantitation. The high sensitivity of modern detectors also means that fluorescence microscopy is

sensitive enough to detect single particles. Because of these advantages, we will discuss methods to quantify the nucleocytoplasmic localization of proteins by fluorescence microscopy.

2 Materials and Methods:

2.1 Microscope: Epifluorescent (widefield) or confocal microscope.

While any fluorescence microscope can be effectively used to analyze N/C levels of a protein, a confocal microscope is preferable. Because the confocal microscope limits the amount of out-of-focus light and the image is effectively a slice through the specimen, variations in cell thickness will have less effect on the apparent intensity of the protein being studied when imaged with a confocal microscope. For confocal microscopy, the pinhole size should be adjusted to minimize the depth of the focal plane while still ensuring adequate signal for both the nuclear and cytoplasmic fluorescence. Rejecting out of focus light is important, but not as important as achieving adequate detection of the protein of interest.

A microscope equipped for epifluorescence will still work well for generating data, and deconvolution algorithms can be applied to mathematically diminish the out-of-focus light. The most important consideration, over all, is that experiments that are to be compared to each other are performed on the same instrument, and that the same post-processing is performed on all of the images. Quantification of an experiment using images from a confocal microscope should not be directly compared with quantification of an experiment using images from an epifluorescence microscope, although the quantitation will be expected to have similar values, and trends in the data should be consistent. For example, if protein X becomes more nuclear over time on a confocal microscope, protein X should also become more nuclear over time when imaged with an epifluorescence microscope.

Other Imaging considerations

Focal plane: When choosing your focal plane, select the middle of the nucleus, rather than getting the periphery of the cell in focus, these positions will often be different with adherent cells, unless it is an exceedingly flat cell.

2D vs 3D imaging: Taking multiple images at different focal planes (the z dimension) allows one to capture 3 dimensions of data. This 3D data set provides the data used by deconvolution algorithms that attempt to iteratively reconstruct the 3D images by assigning out of focus light to its correct plane and point of origin. It also allows one to measure the complete cell, rather than a single data point. This paper will focus on quantifying 2D data, but a brief description of 3D quantitation is included.

The example images in figures 4 through 7 are *Saccharomyces cerevisiae* expressing Gsp1-GFP (yeast Ran) with Hoechst staining of the DNA. The cells were imaged as a z-stack and deconvolved using Huygens (Scientific Volume Imaging B. V., Netherlands). We then chose the z-position closest to the center of the cell to quantify. If you have 3 dimensional data, we recommend using the 3D information for deconvolution if available, and performing the quantitation on a 2D slice (not a z-projection) at the center of the nucleus. In the absence of

the ability to take z-stacks or deconvolved images, single focal plane images from an epifluorescence microscope will still be sufficient to quantify.

2.2 ImageJ/FIJI

ImageJ is a free, open source image analysis software developed by Wayne Rasband at the NIH (Rueden et al., 2017; Schneider et al., 2012). ImageJ is written in Java, and therefore can be run on Windows, Mac, and Linux operating systems. FIJI is an ImageJ distribution (FIJI stands for **FIJI is just ImageJ**) which includes ImageJ as well as many preinstalled plugins (Schindelin et al., 2012). For ease of use, we recommend FIJI, as it will automatically maintain the software with updates and already has many of the plugins commonly used by biologists. FIJI also has the advantage of using the new version of the core ImageJ program, ImageJ2. There are a host of changes in ImageJ2, but the most obvious changes to the average user are the increased ability to deal with large, N-dimensional data sets, and the SCientific Image Format Input and Output (SCIFIO) components that facilitate reading and writing many different image formats (Rueden et al., 2017). Throughout the methods described here, we will indicate the functions to use in ImageJ/FIJI in the form [Menu: Submenu: Command]. If you find you need more information about these functions or ImageJ in general, please read the ImageJ documentation (<https://imagej.nih.gov/ij/docs/guide/index.html>) as it explains what each command does and what the options mean, often with examples.

2.3 Excel/Sheets/Environment for Calculations

The calculations that are required can be carried out in any environment suited to performing mathematical operations, but most people have access to Microsoft Excel or Google Sheets, which are adequate for the quantitation we will describe here.

3 Calculations

3.1 Which measurement to use: Mean Fluorescence, N/C, or % Nuclear?

Digital images are a matrix of light intensity values (Figure 1). When portions of images are measured, the value of each pixel being measured is recorded, and the number of pixels that were measured is recorded. As mentioned above, concentration of reactants is what drives biochemical processes forward. Because of this, it is best to measure parameters that are related to concentration. When measuring an image, the most common data to record is the area measured, the mean fluorescence value in that area, and the total fluorescence measured. Total fluorescence intensity is the sum of the intensity of every pixel measured (Fig. 1) and thus has no relationship to concentration because it does not have an area or volume component to it. Simply put, the same intensity from a large area would represent a lower concentration than that same intensity in a small area. Likewise, the percent of a given protein inside the nucleus has no area or volume component, and so it conveys less information than a concentration measurement. The image measurement most similar to concentration is the mean fluorescence intensity per pixel (Fig. 1, often referred to as mean fluorescence), as it is the total fluorescence intensity divided by the number of pixels measured (Eq. 1). Thus, it is proportional to the concentration. In many cases, just reporting the mean fluorescence intensity in the compartment of choice may be sufficient.

$$\text{Mean Fluorescence Intensity} = \frac{\text{Total Fluorescence Intensity Measured}}{\text{Number of pixels Measured}} \quad \text{Eq. 1}$$

When calculating nuclear localization, one is often concerned with whether the macromolecule in question is more concentrated in the nucleus, or in the cytoplasm. In this case, a mean nuclear fluorescence value would have to be accompanied by a mean cytoplasmic value for comparison. For this reason, a commonly used metric for the nucleocytoplasmic distribution of a molecule is the ratio of the nuclear mean fluorescence to the cytoplasmic mean fluorescence. When this is less than 1, the molecule is predominantly cytoplasmic (by concentration), when greater than one, it is predominantly nuclear, and at 1, it has an equal concentration in both compartments. It is worth noting that the relative measure of protein localization to the nucleus or the cytoplasm is not necessarily indicative of activity. Small amounts of protein in the cytoplasm, for example, may still have significant activity there despite the concentration being higher in the nucleus. For example, the yeast polarity factor Cdc24 is sequestered to the nucleus during vegetative growth and is released to the cytoplasm during chemotropic growth (Nern and Arkowitz, 2000). Overall, the predominant localization of Cdc24 is still nuclear, but the small increase in the cytoplasmic concentration is capable of facilitating gradient tracking.

A commonly used measurement for nuclear localization is % Nuclear (Eq. 2). As mentioned above, there is no area or volume information in a percentage value, but it does work well in situations where a protein is very strongly in one compartment rather than another. For example, % Nuclear works well for quantifying a protein that was 95% nuclear, and has now become 94% nuclear. It works less well for cases where nuclear and cytoplasmic concentrations are closer to each other. Interpreting the distribution of protein in a cell based on % Nuclear is very difficult, because it is not always intuitive how the percent in the nucleus is related to the concentrations of the protein in the nucleus and cytoplasm. In Figure 2, we show how two cells with different sizes can have the same N/C ratio, but very different % Nuclear values. The small size of the nucleus compared to the cytoplasm means that a molecule becomes more concentrated just by moving into the nucleus. In Figure 2, the small cell has an area equal to 20% of the area of the cytoplasm, while the large cell has a nuclear area equal to 5% of the area of the cytoplasm. This leads to confusing situations where one might expect a cell that has 28% nuclear localization to have more than twice as much nuclear concentration than a cell with 10% nuclear localization, but in fact, both have an N/C ratio of 2 and very similar mean fluorescence in the nucleus (Figure 2). The disconnect between %Nuclear and actual concentrations is evident throughout Figure 2, where large changes in nuclear concentration correspond with relatively minor changes in cytoplasmic intensity (i.e. N/C of 0.5 to 3). The relative sizes of the nucleus and cytoplasm mean that only a small proportion of the total protein in the cell needs to localize to the nucleus to achieve a higher concentration in the nucleus than in the cytoplasm.

$$\% \text{Nuclear} = \frac{\text{Total Nuclear Intensity}}{\text{Total Cytoplasmic Intensity} + \text{Total Nuclear Intensity}} \times 100 \quad \text{Eq. 2}$$

3.2 Background subtraction

While there are many methods to performing background subtraction, here we will discuss three approaches, in order of increasing difficulty. The first two approaches should cover most applications.

3.2.1 Use the [Process: Subtract Background] command in ImageJ.—The ImageJ Subtract Background command is the simplest process here, requiring only one step (after determining the appropriate parameters), and it will work for most situations. This uses a rolling ball method and requires that the user choose an appropriate “rolling ball radius.” The radius should be larger than the features in your image. An appropriate size can be calculated by measuring sizes of the cells or it can be determined operationally by experimenting with different radius sizes while having the “Preview” and “Create background (don’t subtract)” options checked in the subtract background window. The goal is to choose a radius size that is large enough that cellular features are not appearing in the background. Once an appropriate radius size has been chosen, “Create background (don’t subtract)” can be deselected and “OK” can be pressed. The effects of the “sliding paraboloid” and “disable smoothing” options can be examined to determine if they enhance the background subtraction (Figure 3A). This method of background subtraction is sufficient for the vast majority of situations where the background is smooth and continuous.

3.2.2 Perform a pseudo-flat-field correction: The rolling ball method described above sometimes results in artifacts (Figure 3B), specifically if the background fluorescence is not smooth and continuous. The pseudo-flat-field correction is less prone to artifacts caused by imaging problems, such as dead pixels (figure 3B). It is good at maintaining small features and eliminating large, field wide features (like background fluorescence). This method involves creating a duplicate of the image that is blurred to the extent that it has lost cell detail, and only maintains the general background fluorescence intensity and then subtracting that blurred image from the first (Figure 3C). This is achieved by duplicating the image of interest [Image: Duplicate], followed by a Gaussian blur with a large radius. Use [Process: Filters: Gaussian Blur] and try starting with a radius of 150. The goal is to blur the image to the point that the cells (or other relevant features) are no longer discernible. The blurred image should just have the characteristics of the background fluorescence. Once a blurred image has been obtained, use [Process: Image Calculator] to subtract the blurred image from the original image. The resultant image should have more even illumination and diminished background fluorescence.

3.2.3 Perform a flat-field correction: If the above methods are not sufficient to even the illumination and remove background signal without causing artifacts, the more thorough method is to experimentally determine the background characteristics of the microscope and imaging conditions to determine what the background fluorescence looks like. This may be necessary when using data with a very low signal to noise ratio. This approach involves acquiring dark images and fluorescent images with the microscope and camera, and performing a flat-field correction based specifically on the system being used. A detailed method has been described previously (Model, 2006; Model and Burkhardt, 2001).

3.3 Calculation of N/C

The N/C calculation required is simply the mean nuclear intensity divided by the mean cytoplasmic intensity (Eq. 3). When measuring a region of interest (ROI), one might record both a total intensity and mean intensity. The mean intensity is the total intensity in the ROI divided by the number of pixels in the ROI, and is therefore analogous to concentration. It is important to subtract the background fluorescence intensity (section 3.2 above) from the data prior to calculating the N/C ratio, as the background can drastically change the calculated N/C.

$$N/C = \frac{\text{Mean Nuclear Intensity}}{\text{Mean Cytoplasmic Intensity}} \quad \text{Eq. 3}$$

4 Manual ROI approach

Selecting an ROI (Figure 1, 3) can be accomplished with any of the selection tools in ImageJ/FIJI. To make keeping track of the ROIs easier while generating them, they can be added to the ROI manager using [Analyze: Tools: ROI Manager], or the short cut key 't'. This allows review of the ROIs, and they can be saved to maintain a record of where each measurement came from.

Generating ROIs is relatively simple, but the question often arises, what should be selected? In order to generate a nuclear ROI, one could select the entire nucleus, or just a subset of the nucleus. The key consideration for ROI selection is whether the protein is uniformly distributed. If the protein has a homogenous distribution, then an ROI of any size should accurately capture the concentration of the protein. If, however, the protein exhibits a heterogeneous distribution, choices for selecting a subset of the compartment or the whole compartment must be made. If, for example, the protein is excluded from nucleoli, you may want to exclude nucleoli from the ROI. If the protein has a heterogeneous distribution, selecting the entire compartment may be the best course of action. These considerations of protein uniformity are the same for both the nucleus and the cytoplasm. Selecting the entire nucleus and the entire cytoplasm is a generally justifiable approach, but ROI generation will take more effort than, for example, selecting similar sized circles in the nucleus and cytoplasm.

5. Image Segmentation and Mask based calculations

Rather than hand-drawing regions of interest to measure mean nuclear and mean cytoplasmic fluorescence, it is possible to use ImageJ to select the nucleus and cytoplasm of all cells in a process called image segmentation. We will divide the image into segments containing the features in which we are interested. A mask is a binary image, where the regions of interest are equal to 1, and the background regions that are not interesting are 0. In ImageJ, binary images are not made up of 1's and 0's. Instead, they are represented as 8-bit grayscale images where the region of interest is 255 (white in an 8 bit grayscale image), and the background is equal to 0 (black in an 8 bit grayscale image). This means that normal math that would work with a mask does not work in ImageJ, although ImageJ functions that work with binary images will treat the 8 bit image properly as a mask. Another important

fact to remember while working with masks in ImageJ is that ImageJ also inverts the look-up table for masks, such that 255 is black, and 0 is white; the opposite of how images are usually represented.

Segmentation of an image is easiest when the feature of interest is bright and the rest of the image is dark, as this allows segmentation through thresholding of intensities. Nuclei are readily stained with a variety of fluorescence dyes, including DAPI and Hoechst, making generation of nuclear masks relatively easy. This process has significantly higher processing overhead than the approach above, but scales to large numbers of cells well. For a small dataset, drawing ROIs by hand may be the most expedient approach, but for the quantification of hundreds to thousands of cells, image segmentation could save significant amounts of time.

5.1 Generate a nuclear mask:

Using an image of a nuclear marker, DAPI for example, to generate a mask that segments nuclei (Figure 4). Use [Image: Adjust: Threshold] to set the threshold, and then, without hitting the Apply button on the threshold dialog, perform [Analyze: Analyze Particles]. This will generate ROI's and a mask if that option is selected. Save these. If the image is noisy and the mask is not very smooth, the image can be blurred. Try a 1 or 2 pixel radius Gaussian blur [Process: Filters: Gaussian Blur] on the image that is being thresholded. Do not use this image for measurements, just for mask production.

5.2 Measure the Nuclei:

Select the fluorescence image you wish to measure. Go to [Analyze: Set Measurements] and make sure that you have selected "Area" and "Mean gray value." Selecting "Integrated Density" will save some calculations later in the process, but is not required (Integrated Density is the sum of all pixels measured). Then select the ROI manager and press "Measure." A new results window will open. Save this information

5.3 Generate a mask that contains the cytoplasm:

There are two basic approaches that could be used for this, either selecting the entire cell by thresholding, or increasing the size of the nuclear mask such that it expands into the cytoplasm. *In both cases, these selections will still contain the nucleus.*

5.4.1 Generate a whole cell mask: Using an image that has fluorescence marking the whole cell, generate a threshold that segments the cells. (Figure 6). As above, generate a mask. If some cells are not successfully segmented through the initial threshold step, they can be separated using the pencil tool. Drawing a white line between two cts.

OR

5.4.2 Generate a larger nuclear mask: If the nuclear mask is enlarged, it will cover the cytoplasm proximal to the nucleus. When measuring a homogeneously distributed protein, this should work well. Duplicate the nuclear mask (ctrl+shift+d) and then use [Process: Binary: Dilate] to make the new mask larger. (Figure 7). The amount you should

increase the size will depend on your specific data, but try two to three iterations of dilation to start. Once you have enlarged the mask as much as you want, run [Analyze: Analyze Particles] on the larger mask, which will add the new ROI's to the ROI manager. An advantage to this method over generating a whole cell mask, is that it is more likely to have a 1 to 1 correspondence between the nuclear mask and the cytoplasm containing mask. In Figure 7, you can see that there are 5 nuclei, but 6 cells are detected in Figure 6. Because this method merely increases the size of the nuclei, the number of segmented objects should not change.

5.4.3 Automated Segmentation using Machine Learning.—The previous sections on mask generation are an attempt at high throughput segmentation, rather than hand drawn ROIs. They are based on simple thresholding (with some user input) to segments the cells, and there are many experimental systems where thresholding alone will not work and the number of images makes hand drawn ROIs impractical. In these situations, machine learning based segmentation may be called for. Machine learning is a broad term encompassing computational approaches to image classification and segmentation that involve the algorithm becoming more optimal as it ‘learns’ more about the problem (Kan, 2017). In traditional machine learning the user chooses the criteria that images will be scored upon, while the newer approach of deep learning uses algorithms that apply a series of convolutions to the image and finds those that work best as criteria. There are multiple approaches to machine learning-based segmentation of cells available ranging from FIJI plugins to software that will require programming experience to use (Arganda-Carreras et al., 2017; Janowczyk and Madabhushi, 2016; Litjens et al., 2017; Van Valen et al., 2016). The use of these algorithms will require varying degrees of facility with programming, a set of properly segmented images for training, and access to computational resources with high end Graphics Processing Units (GPUs). A detailed discussion of their use is beyond the scope of this paper.

5.5 Measure the larger mask:

Select the same fluorescence image that the nuclear measurements came from, and measure the larger ROIs for that image. Save this data. **Problem solving:** Sometimes the nucleus is very close to the edge of the cell, and an enlarged nuclear mask will extend outside of the cell. If this happens, set a threshold that excludes the outside of cells, and make sure to select “Limit to threshold” in the [Analyze; Set Measurements...] dialog. If the larger mask happens to be impinging upon another cell (that is, two nuclear masks have merged into one mask), this can be fixed manually by separating the masks with the pencil tool set to the background color. A high throughput way to solve this problem is to separate the fused masks using a mask generated from the Voronoi diagram of the original nuclear masks. This will generate lines that are equidistant from their two closest nuclei. Using a duplicate of the nuclear masks, run [Process: Binary: Voronoi] to generate the Voronoi lines, threshold them to create a mask, and then subtract that mask from the larger mask.

5.6 Measure the background:

Even if you have performed background subtraction/flat field correction, there may still be some level of background signal. Measure an area outside of the cells, and record the background for that image.

5.7 Generate a count mask:

This step exists to verify that you are measuring the same cells in both sets of ROIs. On a small data set or a single image, this may not be necessary, but in larger, more complex data sets, this provides the ability to verify that you are measuring what you think you are measuring, and the information necessary to fix the data if you find that there is a problem. Select the larger mask image, and run [Analyze: Analyze Particles] again selecting “Show: Count Masks”. This will generate a new mask where each separate object will have the value of the number object that it is, e.g. object number one will consist entirely of 1s, object number two will consist entirely of 2s, etc. Measure this count mask with both the nuclear ROIs from part A and the larger ROIs from C. If the two masks are measuring the same cell, they will return the same Mean gray value from this process. This is easily checked by placing the two sets of data next to each other in Excel, Google Sheets, or other spreadsheet software, and using an IF formula. The general form of this is “=IF([Logical Test], [Value if True], [Value if False])”. If the cell count measurements for the nucleus mask is in cell A1, and the cell count measurement for the whole cell mask is in B1, then the formula “=IF(A1=B1, 1,0)” would return 1 if they were equal, and 0 if they were not equal. If the values are not equal, the mean gray value measured will indicate where the problem lies.

5.8 Calculate N/C:

If none of the count mask measurements have come back as 0, or mismatches have been resolved, then the N/C ratios can be calculated. The two sets of data are the nuclear measurements and the larger measurements that contain the cytoplasm. To calculate the cytoplasmic mean from this data, calculate the cytoplasmic intensity (Eq. 4) and the cytoplasmic area (Eq. 5). Now calculate the mean cytoplasmic intensity (Eq. 6). Finally, the N/C ratio can be calculated as before (Eq. 3).

$$\text{Cytoplasmic Intensity} = \{(Large\ Mask\ mean) \times (Large\ Mask\ Area)\} - \{(Nuclear\ Mean) \times (Nuclear\ Area)\} \quad \text{Eq. 4}$$

$$\text{Cytoplasmic Area} = Large\ Mask\ Area - Nuclear\ Area \quad \text{Eq. 5}$$

$$\text{Cytoplasmic Mean} = \frac{\text{Cytoplasmic Intensity}}{\text{Cytoplasmic Area}} \quad \text{Eq. 6}$$

5.9 Calculating N/C from 3D data:

If you have 3D data, you may want to calculate the N/C ratio using the entire 3D data set. This is generally not necessary, as it gives very similar results to the whole cell mask approach described above (Supplementary Figure S1), but increases the complexity of the

analysis. It may prove useful in situations with very heterogeneous distribution of the protein of interest, or if the shape of the cell is in some way confounding to a 2D analysis. You would do this in the same basic manner as the whole cell mask approach above, using sections 5.1 and 5.4.1 to generate a nuclear mask and a whole cell mask, respectively. In this case, however, a mask would be made for each Z position, so there would be a stack of nuclear masks, and a stack of whole cell masks. These masks must each be associated with the correct cell, a difficult process in ImageJ (although there may be plugins that would help), but easily undertaken using MATLAB. We have included a MATLAB script as supplementary information that will take as input a fluorescence stack, a 3D nuclear mask, and a 3D whole cell mask and calculate whole cell N/C ratios.

6. Consistency is Key:

It is possible to generate a fluorescence standard curve to calculate concentrations in microscopy (Riddick and Macara, 2005). However, the methods we have discussed here are all relative, rather than absolute. As such, it is the trends in measured values that have the most meaning. In examining the results of the quantitation methods discussed above (Figure 7), we find that the relative N/C ratios of the cells are remarkably consistent from one method to another, only the absolute values differ. Different approaches to measuring the N/C ratio will achieve slightly different results, but if the measurements are performed in a consistent manner, the trends in the data should be the same, regardless of the approach. The most important thing is that you not compare data generated by two different approaches, as you may be measuring the differences in the approaches rather than the differences in the data.

7. Double Check the Results:

When choosing an image analysis approach, consider the following questions:

1. **Do the results of the quantitation numbers qualitatively match the visual inspection of the images?** If the data looks one way to your brain, and another way when quantified, it is possible that a mistake was made or that the method is not appropriate. Quantification of images is an attempt to put numbers to something our brains are remarkably good at doing, detecting patterns in images. There is the danger of confirmation bias in getting quantitation that matches the expected outcome, however. In order to avoid confirmation bias, positive and negative controls are important, as well as considering points 2 and 3 below.
2. **Does the outcome of this approach match the outcome of an alternative approach?** Compare alternative methods, and assess whether they change the outcome. The results in Figure 7 is an example of the type of examination one could apply to validate a quantitative approach.
3. **Does this approach have a false positive problem?** Compare quantitation between different trials of negative controls to ensure that the chosen approach does not find differences between data sets that are not different.

Conclusion

Measuring nucleocytoplasmic localization by fluorescence microscopy has multiple advantages over biochemical fractionation. We have focused on measuring the mean fluorescence per pixel in the compartment of interest, which can be reported directly, or the N/C ratio of the concentrations can be reported in situations where the protein is changing the compartment in which it is predominantly localized. Because concentration of reactants drives biochemical reactions in the forward direction, we encourage the use of fluorescence measurements that are related to concentration. Given the growing emphasis placed upon rigor and reproducibility in science, quantitation of microscopy results has become the standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Adam SA, Sterne-Marr R, and Gerace L (1992). Nuclear protein import using digitonin-permeabilized cells. *Methods Enzymol* 219, 97–110. [PubMed: 1488017]
2. Arganda-Carreras I, Kaynig V, Rueden C, Eliceiri KW, Schindelin J, Cardona A, and Sebastian Seung H (2017). Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification. *Bioinformatics* 33, 2424–2426. [PubMed: 28369169]
3. Chatterjee M, and Paschal BM (2015). Disruption of the ran system by cysteine oxidation of the nucleotide exchange factor RCC1. *Mol Cell Biol* 35, 566–581. [PubMed: 25452301]
4. Chou CC, Zhang Y, Umoh ME, Vaughan SW, Lorenzini I, Liu F, Sayegh M, Donlin-Asp PG, Chen YH, Duong DM, et al. (2018). TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. *Nat Neurosci* 21, 228–239. [PubMed: 29311743]
5. Cole RW, Jinadasa T, and Brown CM (2011). Measuring and interpreting point spread functions to determine confocal microscope resolution and ensure quality control. *Nat Protoc* 6, 1929–1941. [PubMed: 22082987]
6. Czubryt MP, Austria JA, and Pierce GN (2000). Hydrogen peroxide inhibition of nuclear protein import is mediated by the mitogen-activated protein kinase, ERK2. *J Cell Biol* 148, 7–16. [PubMed: 10629214]
7. Datta S, Snow CJ, and Paschal BM (2014). A pathway linking oxidative stress and the Ran GTPase system in progeria. *Mol Biol Cell* 25, 1202–1215. [PubMed: 24523287]
8. Dimauro I, Pearson T, Caporossi D, and Jackson MJ (2012). A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC Res Notes* 5, 513. [PubMed: 22994964]
9. Dormann D, Rodde R, Edbauer D, Bentmann E, Fischer I, Hruscha A, Than ME, Mackenzie IR, Capell A, Schmid B, et al. (2010). ALS-associated fused in sarcoma (FUS) mutations disrupt Transportin-mediated nuclear import. *EMBO J* 29, 2841–2857. [PubMed: 20606625]
10. Gasset-Rosa F, Chillon-Marin C, Goginashvili A, Atwal RS, Artates JW, Tabet R, Wheeler VC, Bang AG, Cleveland DW, and Lagier-Tourenne C (2017). Polyglutamine-Expanded Huntingtin Exacerbates Age-Related Disruption of Nuclear Integrity and Nucleocytoplasmic Transport. *Neuron* 94, 48–57 e44. [PubMed: 28384474]
11. Hamilton N (2009). Quantification and its applications in fluorescent microscopy imaging. *Traffic* 10, 951–961. [PubMed: 19500318]

12. Hao N, and O'Shea EK (2011). Signal-dependent dynamics of transcription factor translocation controls gene expression. *Nat Struct Mol Biol* 19, 31–39. [PubMed: 22179789]
13. Hymer WC, and Kuff EL (1964). Isolation of Nuclei from Mammalian Tissues through the Use of Triton X-100. *J Histochem Cytochem* 12, 359–363. [PubMed: 14193857]
14. Janowczyk A, and Madabhushi A (2016). Deep learning for digital pathology image analysis: A comprehensive tutorial with selected use cases. *J Pathol Inform* 7, 29. [PubMed: 27563488]
15. Kan A (2017). Machine learning applications in cell image analysis. *Immunol Cell Biol* 95, 525–530. [PubMed: 28294138]
16. Kau TR, Way JC, and Silver PA (2004). Nuclear transport and cancer: from mechanism to intervention. *Nat Rev Cancer* 4, 106–117. [PubMed: 14732865]
17. Kelley JB, Datta S, Snow CJ, Chatterjee M, Ni L, Spencer A, Yang CS, Cubenas-Potts C, Matunis MJ, and Paschal BM (2011). The defective nuclear lamina in Hutchinson-gilford progeria syndrome disrupts the nucleocytoplasmic Ran gradient and inhibits nuclear localization of Ubc9. *Mol Cell Biol* 31, 3378–3395. [PubMed: 21670151]
18. Kelley JB, and Paschal BM (2007). Hyperosmotic stress signaling to the nucleus disrupts the Ran gradient and the production of RanGTP. *Mol Biol Cell* 18, 4365–4376. [PubMed: 17761537]
19. Komeili A, and O'Shea EK (2000). Nuclear transport and transcription. *Curr Opin Cell Biol* 12, 355–360. [PubMed: 10801461]
20. Li Y, Roberts J, AkhavanAghdam Z, and Hao N (2017). Mitogen-activated protein kinase (MAPK) dynamics determine cell fate in the yeast mating response. *J Biol Chem* 292, 20354–20361. [PubMed: 29123025]
21. Litjens G, Kooi T, Bejnordi BE, Setio AAA, Ciompi F, Ghafoorian M, van der Laak J, van Ginneken B, and Sanchez CI (2017). A survey on deep learning in medical image analysis. *Med Image Anal* 42, 60–88. [PubMed: 28778026]
22. Model MA (2006). Intensity calibration and shading correction for fluorescence microscopes. *Curr Protoc Cytom Chapter 10, Unit10* 14. [PubMed: 18770832]
23. Model MA, and Burkhardt JK (2001). A standard for calibration and shading correction of a fluorescence microscope. *Cytometry* 44, 309–316. [PubMed: 11500847]
24. Nagara Y, Tateishi T, Yamasaki R, Hayashi S, Kawamura M, Kikuchi H, Iinuma KM, Tanaka M, Iwaki T, Matsushita T, et al. (2013). Impaired cytoplasmic-nuclear transport of hypoxia-inducible factor-1alpha in amyotrophic lateral sclerosis. *Brain Pathol* 23, 534–546. [PubMed: 23368766]
25. Nern A, and Arkowitz RA (2000). Nucleocytoplasmic shuttling of the Cdc42p exchange factor Cdc24p. *J Cell Biol* 148, 1115–1122. [PubMed: 10725324]
26. Newmeyer DD, Finlay DR, and Forbes DJ (1986). In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J Cell Biol* 103, 2091–2102. [PubMed: 3097026]
27. Paine PL, Moore LC, and Horowitz SB (1975). Nuclear envelope permeability. *Nature* 254, 109–114. [PubMed: 1117994]
28. Pemberton LF, and Paschal BM (2005). Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* 6, 187–198. [PubMed: 15702987]
29. Poon IK, and Jans DA (2005). Regulation of nuclear transport: central role in development and transformation? *Traffic* 6, 173–186. [PubMed: 15702986]
30. Riddick G, and Macara IG (2005). A systems analysis of importin- α - β mediated nuclear protein import. *J Cell Biol* 168, 1027–1038. [PubMed: 15795315]
31. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, and Eliceiri KW (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* 18, 529. [PubMed: 29187165]
32. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676–682. [PubMed: 22743772]
33. Schneider CA, Rasband WS, and Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671–675. [PubMed: 22930834]

34. Snow CJ, Dar A, Dutta A, Kehlenbach RH, and Paschal BM (2013). Defective nuclear import of Tpr in Progeria reflects the Ran sensitivity of large cargo transport. *J Cell Biol* 201, 541–557. [PubMed: 23649804]
35. Van Valen DA, Kudo T, Lane KM, Macklin DN, Quach NT, DeFelice MM, Maayan I, Tanouchi Y, Ashley EA, and Covert MW (2016). Deep Learning Automates the Quantitative Analysis of Individual Cells in Live-Cell Imaging Experiments. *PLoS Comput Biol* 12, e1005177. [PubMed: 27814364]
36. Wentz SR, and Rout MP (2010). The nuclear pore complex and nuclear transport. *Cold Spring Harb Perspect Biol* 2, a000562. [PubMed: 20630994]

Figure 1: Digital Images are a Matrix of Fluorescence Intensities.
A) A grayscale fluorescence image magnified such that the individual pixels are readily visible. Each pixel is colored in grayscale based on its numerical value. B) Grayscale values overlaid on the image show that a digital image is a matrix of numbers. C) A region of interest is selected with the magenta line. The 3 × 3 selection of pixels defines the numbers to be used for quantitation D) The 9 individual values are used to calculate the total intensity and the area measured. Mean intensity is calculated from the total intensity and the area using Eq. 1.

Figure 2: Relationship between N/C and % Nuclear.

Shown are outlines of two cells, divided into nuclear and cytoplasmic compartments. Fluorescence intensities were simulated for the nucleus and cytoplasm at different N/C ratios (shown in white at the top), while maintaining the same total fluorescence intensity between each image. The percentage of the signal that is nuclear is shown at each N/C ratio in the color of the cell it represents. Nuclear and cytoplasmic size have a large effect on % nuclear, but not on N/C ratio. Also note that an increase in nuclear concentration does not have an equivalent decrease in cytoplasmic concentration because of the smaller size of the nucleus. The brightness and contrast of the images of 0.5 N/C up to 3 N/C are identical, while 10 N/C and 50 N/C were rescaled to avoid saturating the images. The simulated images were blurred and had noise added to more closely resemble actual fluorescence images.

Figure 3: Background Subtraction.

A) Background subtraction of an image of yeast expressing a GFP tagged protein. The ImageJ rolling ball function [Process: Subtract Background] was run on the starting image with a radius of 50 and no options selected (middle), or with “sliding paraboloid” selected (right). B) An example of the type of problem that can lead to artifacts with a rolling ball approach. The starting image had several pixels set to 0 to simulate a camera with dead pixels or some other obstruction in the light path. As in (A), the ImageJ [Process: Subtract Background] was used with a radius of 50 and no options selected (middle) or with “sliding paraboloid” selected (right). C) Example of a pseudo flat field correction using the same starting image as (B). A [Process: Filter: Gaussian blur] with a radius of 50 pixels was applied to the starting image (middle). This blurred image was then subtracted from the starting image using [Process: Image Calculator] to yield the difference image (right) which does not suffer from the same artifacts as the rolling ball method produces in (B).

Figure 4: Quantitation with Hand Drawn Regions of Interest.

Shown are DIC, DAPI, and Ran-GFP (*S. cerevisiae* Gsp1-GFP). ROIs (yellow) were drawn by hand in the nucleus and cytoplasm. The vacuole was avoided in the cytoplasm, as it excludes Ran.

Figure 5: Generation of Nuclear Masks.

Using the same images as Figure 3, the DAPI channel was thresholded to generate a nuclear mask and associated ROIs (yellow).

Figure 6: Generating a Whole Cell Mask.

The Ran channel from Figure 3 was thresholded to generate whole cell masks. If the nuclear mask is subtracted from the whole cell mask, a cytoplasmic mask is generated. The method described here does not generate the cytoplasmic mask directly, but calculates the fluorescence and area from the nuclear mask and the whole cell mask. The selected area includes a daughter cell with no nucleus, which will slightly complicate the data analysis, as its measurements will have to be removed. Mammalian cells will likely display similar problems in generation of whole cell masks, although not for the same reason. Rather than daughter cells, it is more likely to be caused by variations in cytoplasm thickness, such that parts of the cell will appear to be separate objects.

Figure 7: Dilating Nuclear Masks:

Instead of selecting cytoplasmic area by selecting the whole cell, here the nuclear mask is enlarged several times to capture cytoplasmic signal just outside the nucleus. This has the advantage of preserving a one to one relationship between the nuclear masks and the cytoplasmic masks under most circumstances. The new area selected will not be as large as in the whole cell method (Figure 4) but in cases where the cytoplasmic signal is very homogenous, it should suffice.

Figure 8. Different methods yield the same relative measurements.

Graphs of the Ran N/C results of each cell, using the different selection methods discussed above. While the absolute values differ (most notably between Nuclear Dilation and the other methods), the relative values of the N/C ratios are well maintained through all three methods.