

QuoVadoPro, an autonomous tool for measuring intracellular dynamics using temporal variance.

AUTHOR(S) AND CONTACT INFORMATION:

Himanish Basu^{1, 2} (himanishbasu@fas.harvard.edu), Thomas L Schwarz^{1, 3 *}
(thomas.schwarz@childrens.harvard.edu)

1. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA 02115, USA.
2. Division of Medical Sciences, Harvard Medical School, Boston, MA 02115, USA.
3. Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

* Corresponding author: thomas.schwarz@childrens.harvard.edu

ABSTRACT:

Trafficking of intracellular cargo is essential to cellular function and can be defective in pathological states including cancer and neurodegeneration. Tools to quantify intracellular traffic are thus necessary for understanding this fundamental cellular process, studying disease mechanisms and testing the effects of therapeutic pharmaceuticals. In this article we introduce an algorithm called “QuoVadoPro”, that autonomously quantifies the movement of fluorescently tagged intracellular cargo. QuoVadoPro infers the extent of intracellular motility based on the variance of pixel illumination in a series of time-lapse images. The algorithm is an unconventional approach to the automatic measurement of intracellular traffic and is suitable for quantifying movements of intracellular cargo under diverse experimental paradigms. QuoVadoPro is particularly useful to measure intracellular cargo movement in non-neuronal cells, where cargo trafficking occurs as short movements in mixed directions. The algorithm can be applied to images with low temporal or spatial resolutions and to intracellular cargo with varying shapes or sizes, like mitochondria or endoplasmic reticulum: situations in which conventional methods such as kymography and particle tracking cannot be applied. In this article we present a stepwise protocol for using the QuoVadoPro software, illustrate its methodology with common examples, discuss critical parameters for reliable data analysis and demonstrate its use with a previously published example.

Basic Protocol 1: QuoVadoPro, an autonomous tool for measuring intracellular dynamics using temporal variance.

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INTRODUCTION

Intracellular transport of proteins and organelles is vital for maintaining cellular organization and physiology. Live cell imaging of intracellular structures has revealed an astonishing level of dynamic movements and reorganization. As local metabolic needs transiently arise within a cell or as environmental cues change, the rapid trafficking of cellular cargoes enables a swift reorganization of the cell's proteins and organelles to adapt the cell to the new conditions. In eukaryotes, the rapid movement and reorganization of intracellular cargoes is powered by molecular motors like Kinesin, Dynein and Myosin, which carry the cargo on a network of microtubules and actin filaments spanning the entire cytosol (Hartman & Spudich, 2012; Hirokawa, 1998; Miki, Okada, & Hirokawa, 2005; Pilling, Horiuchi, Lively, & Saxton, 2006; Reck-Peterson, Redwine, Vale, & Carter, 2018; Schnapp & Reese, 1989; Titus, 2018; Verhey, Kaul, & Soppina, 2011).

Many factors influence the rates and directions of intracellular traffic. The motors involved and the principles that govern their movement differ from cargo to cargo and are further shaped by cellular health, intracellular location, and signaling mechanisms. Changes in intracellular trafficking can influence pathologies like cancer (Caino et al., 2016; Goldenring, 2013; Parachoniak & Park, 2012) and neurodegeneration (Baloh, Schmidt, Pestronk, & Milbrandt, 2007; Collard, Cote, & Julien, 1995; Gunawardena & Goldstein, 2001; Hafezparast et al., 2003; Williamson & Cleveland, 1999). Studying the rates of intracellular traffic both in normal and disease states is thus vital to understand an essential cell biological process. Assays of intracellular motility may also shed insight into disease mechanisms or reveal potential points of therapeutic intervention (Misgeld & Schwarz, 2017; Schwarz, 2013; Shlevkov et al., 2019; Vale, 2003).

We have developed two algorithms, called “Kymolyzer” and “QuoVadoPro”, to quantify intracellular movement. Kymolyzer is semi-autonomous and utilizes kymography to analyze intracellular trafficking. Kymolyzer is useful to measure the speeds, directionality and flux of intracellular cargo in cells where intracellular movement occurs along a specified path, like that along the aligned microtubules found in neuronal axons and dendrites. These conditions, however, are not met in most non-neuronal cell lines and in neuronal cell bodies. In those places, the intracellular cargoes move in short processive runs on microtubules and actin filaments that have mixed orientations. To analyze such movements characterized

by short runs in heterogeneous directions, we have developed QuoVadoPro. QuoVadoPro is fully autonomous and uses the variance in pixel occupancy in a series of time lapse images as an indicator of the extent of movement of a fluorescently tagged intracellular cargo.

In this article, we describe the algorithm and software of QuoVadoPro. QuoVadoPro is useful to measure intracellular traffic in most cell types and is especially effective in quantifying movement in cells whose microtubules are not in parallel arrays and thus are not readily analyzable by kymography. Traditionally, automated object detection and tracking is used to quantify intracellular trafficking in such cell lines (Chenouard et al., 2014; Jaqaman et al., 2008; Lee et al., 2019; Wasim & Treanor, 2018). Although able to descriptively quantify motility, object tracking algorithms are often computationally intensive, are not easily adaptable to tracking different organelles, and require images of high temporal and spatial resolutions. Most significantly, object tracking is not reliable in tracking the movement of reticular or

partially reticular structures such as mitochondria or endoplasmic reticulum (ER). Manually tracking the movement of individual mitochondria in every frame of a movie (Caino et al., 2016; Caino et al., 2017) has overcome some of the challenges of automated detection and tracking. These approaches however are extremely low throughput and may ignore mitochondria in a fused reticulum.

QuoVadoPro takes a non-traditional approach that does not employ conventional object detection and tracking. Through QuoVadoPro, we infer the motility along an X-Y plane by analyzing the variance in pixel illumination over time. QuoVadoPro has several advantages over conventional object tracking: it is not influenced by the heterogeneity of sizes, shapes, or reticular structure of objects to be tracked; it can infer motility from images with low temporal and spatial resolutions and from images with high object densities; it is computationally light; and it can be used to monitor the movement of different types of cargo with minimal user driven changes to input parameters. QuoVadoPro is packaged as an ImageJ based macro set that can be installed and used through a graphical user interface. It can accept most microscopy file formats and outputs motility scores and 2D motility heatmaps for visual representation.

BASIC PROTOCOL 1

QuoVadoPro, an autonomous tool to measure intracellular dynamics using temporal variance.

QuoVadoPro is a new method to quantify intracellular movement. In this algorithm, we use the temporal variance in pixel illumination by fluorescently tagged intracellular structures to infer on motility within a cell. It provides a quick, fully autonomous and computationally light way to measure intracellular movements.

Time-lapse images of fluorescently tagged intracellular objects with high motility display a high pixel variance over time. This is because the pixels have a high probability of being transiently occupied by moving objects. On the other hand, time-lapse images of less motile intracellular objects have pixels that are either constantly occupied by the objects or are empty; and thereby have low variance in intensity over time. Thus, although not a direct measure of motility, temporal variance in pixel illumination serves as a proxy for motility. Based on this principle, QuoVadoPro can quantify intracellular traffic under diverse experimental and imaging conditions, even in samples that are otherwise poorly analyzed by conventional methods like kymography or automated object tracking. Further, by normalizing the variance in pixel illumination with the sum of pixel illumination during a time-lapse, QuoVadoPro distinguishes the processive movements from short discontinuous back and forth movements (also referred to as jitters or jiggle) and weighs the former more heavily.

The algorithm works as a set of three macros that can be installed on Fiji (a distribution of ImageJ that is bundled with commonly used plugins) and can be run sequentially through a user-friendly graphical interface. In the first step, the user selects an image series to be analyzed (representing a time-lapse of fluorescently tagged intracellular objects). During analysis, the user can choose to have parts of the image analyzed separately (for example, if a single image has two cells that need to be quantified separately) or analyze the image as a whole. QuoVadoPro then extracts the part of the image (if indicated) or uses the whole image to create a binary mask of the fluorescently tagged objects in all the frames of the time-lapse. In the second step, the algorithm analyzes the variance in the binary image stack created in step 1 and creates a 2-dimensional heatmap of movement. A motility value is then derived from such a heatmap for quantification. The optional third step can be used to automatically collate data from multiple such quantifications through a re-iterative loop.

QuoVadoPro uses the contrast of the fluorescently tagged objects over their local backgrounds to convert them into binary masks. The algorithm can thus work robustly irrespective of object shape, size and absolute fluorescence. In addition, QuoVadoPro does not rely on tracking movements of individual objects from one frame to another and can thus work on images irrespective of frame-to-frame object displacements (governed by temporal and spatial resolutions and movement speeds of the intracellular objects). These features render QuoVadoPro as a versatile software to analyze intracellular traffic with minimal usersupervision.

Necessary Resources

Hardware:

Workstation with internet access

Software:

Please read ***Instructions_Readme*** file and download folder ***“QuoVadoPro_<date>.zip”*** from <https://github.com/ThomasSchwarzLab/QuoVadoPro>

Sample File:

<https://drive.google.com/file/d/1yJZzhlsAGEFrIBoIS0iB6nUDzguzjQcu/view?usp=sharing>

The sample file contains a time lapse image stack of a cos-7 cell expressing a fluorescent tag that marks the mitochondria (mito-DsRed, addgene:55838) and a cytosolic GFP (meGFP-N1, addgene:54767) that helps visualize the cell boundary. To use this sample file, the users should download it into a separate folder on their computer before processing the file through QuoVadoPro.

The cell depicted in the sample file has been used as an example to illustrate the steps and features of QuoVadoPro in figures 1, 2 and 4.

Movie S1 is a video made from the cell shown in the sample file. The video depicts moving mitochondria in the cos-7 cell along with its motility heatmap.

Protocol steps—Step annotations

1. Installation instructions:

- a. Download and install FIJI from: <https://imagej.net/Fiji/Downloads>.
- b. Download the zipped folder named **“QuoVadoPro_<date>.zip”** from <https://git>
- c. From FIJI, go to the following tabs: Plugins > Macros > run. In the file selection window that opens up, select the file named **installer_QuoVadoPro_.ijm** within the downloaded and unzipped folder and run it.
- d. Restart FIJI to complete macro installation. Following the restart, a new tab should appear as follows: Plugins > Macros > QuoVadoPro.
- e. If images have drift, the optional install of the Fiji registration plugin, StackReg (<https://imagej.net/StackReg>) may be needed.

2. **Step 1: Segmentation of objects by local intensity-based thresholding.** As the first step in QuoVadoPro, the user selects a file containing images representing a time-lapse of fluorescently tagged intracellular objects. The user then has the choice to outline a specific region of interest (ROI) in the image or use the whole image for analysis. All fluorescently tagged objects in the whole image or defined ROI are converted to binary masks. This conversion of objects to binary masks (thresholding or segmenting) ensures that the quantification done by QuoVadoPro is independent of absolute intensities. To create the binary masks, the fluorescent objects are thresholded based on their signal over the local background (figure 1). At this step, the user has the option of using multiple tools to remove the background from the images while creating the binary masks of the objects.

Instructions for running Step 1:

- a. To run Step 1 of QuoVadoPro go to the following tabs in FIJI: Plugins>Macros>QuoVadoPro>Step1_preprocess_Cells_IntensitybasedSegmentation. A file selection window will open to select the file containing the movie (time-lapse) to be analyzed.

Note: QuoVadoPro uses the bioformats reader in Fiji to open image files. Most microscopy image file formats are supported by this reader. In case of an unsupported file format, the user should save the file as a multipage tiff stack before opening the file in step 1.

Note: The user can choose to run step 1 on an already opened file. However, we recommend that the user opens the file using the file selection window that is triggered at step 1a. This ensures that QuoVadoPro can read the metadata and the file location correctly.

- b. After selecting the movie file, the user is asked to draw a coarse outline marking the cell periphery (ROI). The outline should include all the organelles to be tracked (figure 1A-B). If the movie has multiple channels or Z-planes, the macro will ask the user to select one channel and one Z-plane. If no selection or ROI is indicated, the macro will proceed to analyze the full frame of the currently active Z-plane and channel. If the user wants to analyze multiple Z-planes, they can choose to either run QuoVadoPro on each individual Z-plane or use a single projection of all the Z-planes.

Note: QuoVadoPro analyzes the movement in one 2D plane (Z-plane). Given the thickness of the cell imaged and the mode of imaging, the user may choose to supply one Z-plane for analysis or a projection of all the Z-planes. To analyze a projection of all the Z-slices, the user must perform the Z-projection and save the time-lapse as a multi-image tiff stack prior to running step 1 of QuoVadoPro. Typically, most cell lines that are used to monitor intracellular trafficking (such as Cos-7 or U2OS) have a relatively thin cytoplasm, most of which can be captured in one optical section using an epifluorescence microscope, thereby not requiring a Z-projection. However,

certain cell lines have thicker cytoplasm (for example, dividing HELA cells) where multiple confocal optical sections are needed to capture the cytoplasm, and thus may need to be projected post-acquisition and before analysis.

- c. The image or the ROI (if selected in step 1b) is then subjected to a local background subtraction to correct for local variations in background and illumination. The resulting image is then adjusted to a standardized intensity scale (16-bit). This processing standardizes images of different bit depths (8-bit, 16-bit and 32-bit). The user is then asked to supply a threshold intensity value for

segmenting the objects in the movie, along with additional optional inputs to ensure that the binary masks do not incorporate noise artifacts. The following values are asked for:

- I. Gaussian smoothing radius for background removal: Local background correction for each frame is done by subtracting a background image from the original image. The background image is generated by smoothing the original image through a gaussian disk of a defined radius. When the smoothed background image is subtracted from the original image, diffuse background fluorescence is eliminated while retaining the sharp signals in the original image. Each pixel in the resulting image is also normalized for its local background.

The “Gaussian smoothing radius” thus dictates the strength of the background removal. A lower gaussian smoothing radius results in a strong background correction, while a higher radius reduces the strength of the background correction. By default, the radius is set to 5 pixels.

The gaussian smoothing radius necessary for optimal background correction depends on the signal to noise ratio of the image. The user should decide on a value empirically. A value that is set too high (low background correction), will cause large diffuse background objects to be included in the binary mask. Conversely, a gaussian radius value that is set too low (high background correction) will cause over amplification of object edges and thus may result in artifacts in the binary mask. To prevent the over-amplification of object edges, the “Gaussian smoothing radius” should be set to a value that is at minimum equal to the average cross-sectional distance of the fluorescently tagged objects.

- II. Threshold level: This value is the threshold intensity used for segmenting the fluorescent objects after local background subtraction and contrast stretching. As the contrast of the image is stretched to a 16-bit range, the user can decide on a threshold anywhere from 0 to 65535 (max intensity in a 16-bit image). The default threshold is set to 5000 and the working value that needs to be set is dictated by the signal to noise ratio (SNR) of the image.

- For images with high SNR, a strong background subtraction can be applied. In such cases, the background intensities are greatly reduced (brought close to zero) and the threshold for segmentation can thus be set to a low value (just above the background).
- If the image is highly noisy (or grainy), the gaussian radius for background removal should be set to a high value (i.e. weak background subtraction) to prevent the amplification of

noise pixels. In this case, the background intensities will be equalized throughout the image, but low levels of noise may remain. The threshold value should thus be set relatively high, enough to remove any remaining background while retaining the fluorescent objects.

- III. Time Smoothing: In images with a high degree of noise, choosing a threshold that completely removes all background pixels may not be possible even after local background subtraction. In such cases, the user can choose to select a threshold that captures most of the fluorescent objects, even if it retains some of the background pixels. To further remove the background, the user can then utilize the time-smoothing feature in QuoVadoPro. This feature removes any pixels that were not constantly illuminated in the defined set of frames. The time-smoothing feature works on the principle that noise pixels will not be detected at the same position in consecutive frames, whereas the position of fluorescent objects will not change as fast.

This value can be kept as 1 (no time smoothing) for images with high SNR and increased if noise is detected after segmentation. To use the time smoothing feature (i.e. for any value more than 1), the images must have a high temporal resolution (i.e. the frame to frame displacement of each object should be much lower than the size of the object). This ensures the fluorescently tagged objects illuminate the same pixels in consecutive frames, whereas noise pixels do not.

Note: It is important to note that both local background subtraction and time smoothing in QuoVadoPro correct for noise pixels that appear randomly due to camera noise or indistinct background fluorescence. These features however will not eliminate background caused by unintended fluorescent objects (for example auto-fluorescent cytosolic granules), if visible.

- IV. Minimum Particle Size: In addition to time smoothing, QuoVadoPro gives the user another option to remove noise pixels by filtering the images for a minimum particle size. The minimum particle size allows the user to define a lower area limit for all objects detected in the binary mask. Any object occupying an area less than the minimum particle size is removed. By default, this value is set to 1 pixel. The user can choose to increase it if small clusters of pixels are seen to be illuminated in the binary mask due to noise. The minimum size filter should be used in conditions where the objects to be masked have a substantially larger area than noise clusters.

Note: As discussed later, it is important that the binary masks created have minimal noise (pixels that do not represent fluorescent objects). To ensure optimal noise removal, the user should run step 1 multiple times and identify the optimal parameters for background removal and thresholding with every new imaging condition. Once the parameters have been decided, the user should not change them in between samples of the same experiment.

- d. Post segmentation, the macro will ask the user to fine tune the ROI to be analyzed (made in step 1b). If no specific ROI was selected in step 1b (i.e. the whole frame was considered), the user should click "OK" and move onto the next step. However, if the user had selected an ROI in step 1b, the edge of the selection may have been erroneously amplified during background subtraction and segmented within the binary mask. This is especially the case if there is high background fluorescence (for example autofluorescence from growth media) and can introduce artifacts in the quantifications. In this case, the user should amend the selection (ROI) at this step to remove any edge artifacts, if present.
 - e. At the end of step 1, the macro will save the binarized image (figure 1C) and the thresholding information in a folder created at the same location as the image, having the same name as the image along with a time stamp.
3. **Step 2: Construction of motility heatmap and quantification.** In step 2 of QuoVadoPro, the binarized video frames (from step 1) are used to generate a motility heatmap, depicting the movement of fluorescent objects. During this step, the variance in illumination over time and the total time of illumination (obtained by summation of intensities over time) is calculated for each binary pixel. The final motility heatmap is constructed as the ratio of an image representing the variance in pixel illumination (figure 2A) to an image representing the total time (sum) of pixel illumination (figure 2B). Parts of the image containing moving objects display high variance and low sum and thereby appear as hot spots on the heatmap. Conversely, parts of the image containing stationary objects have less variation in pixel residence and thereby have low variance and appear as cold spots on the heat map (figure 2C). Pixels that were never occupied by an object have a sum of zero and are excluded from the analysis. They are assigned a NAN (not a number) value. For representation purposes, they appear as cold pixels on the heatmap.

Note: Normalizing the variance in pixel illumination to the total time of pixel illumination (sum) also allows QuoVadoPro to give more weight to processive runs than to back and forth jitters (see guidelines for understanding results for more details).

The motility heatmap is used to derive a motility score for the whole image. To calculate the motility score, the intensities of the heatmap pixels are summed and normalized to the object area (derived from the number of pixels occupied by the binary mask in the first frame). The normalization with the total object area factors out differences in number or size of objects between images.

Note: The users should note that although the motility score is normalized for the number and area of objects, QuoVadoPro should not be utilized to analyze samples with highly varying object numbers (see commentary for more details).

Instructions for running Step 2:

- a. To run Step 2 of QuoVadoPro go to the following tabs in FIJI: Plugins>Macros>QuoVadoPro>Step2_variance_Calculation. A folder selection window will appear in which the user can select the folder containing the binarized image stack created in step 1. This step can also be run directly on the binarized image that is already open at the end of step 1.
 - b. After the user selects the folder, the macro will automatically find and open the binary image stack and ask the user about the number of sub-stacks to break the image into. By default, this value is kept as 1, where in the macro will process all the images in the movie as a single stack. In the case of long movies or movies in which the experiment may have caused motility to change over time, the user may want to break up the movie into sub-stacks to gain information on how movement changed during the course of the movie.
 - c. During processing, the macro will create the variance image and divide it by the sum image as described before. The final image is represented as a 32-bit heatmap (figure 2C). As this step is finished, the quantification is shown in a text window and is automatically saved as a text file in the same folder as the binary image. The macro then asks the user to set a display range that is used to convert the 32-bit heatmap into RGB images. It is important that all the images are set to the same display range within the same experiment. The original variance image and the RGB heatmap are stored in the same image folder.
4. **Step 3: Data Collation.** At this step, the user can interactively consolidate the quantifications generated for different cells into one table.

Instructions for running Step 3:

To run Step 3 of QuoVadoPro go to the following tabs in FIJI: Plugins>Macros>QuoVadoPro>Step3_ CollateDataFiles. A folder selection window will appear. At the folder selection window, the user should select the parent folder containing the subfolders with the quantification text files. The macro will automatically go through the sub-folders and re- iteratively assemble all the data into one table, which the user can then save through a dialogue box.

GUIDELINES FOR UNDERSTANDING RESULTS

Explanation of motility scores quantified by QuoVadoPro

QuoVadoPro infers the motility of fluorescent objects based on the variation in pixel illumination. To quantify movement from a time-lapse of fluorescently tagged objects, as the first step, QuoVadoPro converts the fluorescence signal into binary masks (figure 1). At the second step, the algorithm uses the binarized images to generate a motility heatmap. Each pixel in the heatmap corresponds to the ratio of the temporal variance to the temporal sum of that pixel across all the binarized images of the time-lapse (figure 2). To generate a motility score, the pixel intensities of the heatmap are summed and normalized to the area of the masked objects.

The final motility scores as calculated from binary time-lapse images can also be represented by the following formula:

$$\text{motility score} = \left(\sum \left(\frac{\text{variance}_{\text{time}}}{\text{sum}_{\text{time}}} \right) \right) / (n\text{Pixels}_{\text{segmentedfirstframe}})$$

$\text{variance}_{\text{time}}$ represents the variance of pixel intensities over time. sum_{time} represents the sum of pixel intensities over time.

$n\text{Pixels}_{\text{segmentedfirstframe}}$ represents the number of pixels that were segmented in the first frame (indicating the total area occupied by the fluorescent objects)

Pixels having $\text{sum}_{\text{time}}=0$ are assigned NaN (not a number) values and are not considered in the calculations. For representation, these are shown as cold spots on the heatmap.

The motility scores generated by QuoVadoPro are saved as a text file in a folder created in step 1 of the algorithm. The folder is created at the same location as the image and has the same name as the image in addition to a time stamp. In the text file the following quantification parameters are reported:

- I) MotilityScore: This value represents the final quantified motility score as detailed above. The reported motility score is on an arbitrary scale (i.e. does not have a unit).
- II) Number of Slices: This is the number of time points that were present in the time lapse.
- III) Number of substacks considered: During the analysis of an image stack representing a time lapse, QuoVadoPro can break the image stack into multiple sub-stacks (if requested by the user during step 2). The “Number of substacks considered” value represents the number of sub-stacks the original image stack was broken into. If the user has chosen not to break the image stack into smaller subsets, this value will be stated as 1. Breaking the time lapse into smaller segments is often useful (especially for long movies), to get information on how movement may have changed during the image acquisition. Once the number of sub-stacks to be considered is set at Step 2, it should not be changed in between samples of the same experiment.
- IV) Thresholding parameters: The values represented under this tab are the values that the user inputs in step 1 of QuoVadoPro - the strength of background subtraction, the threshold value used for image segmentation and the noise removal parameters.
- V) Total pixel intensity and area occupied in heatmaps of each sub-stack: The sum of intensities of all pixels from the heatmaps constructed from each sub-stack and the area occupied by the objects in each sub-stack are shown under this tab. To calculate the motility score for each sub-stack, the sum of intensities of the heatmap pixels is normalized to the object area.

QuoVadoPro gives more weight to processive movements than to back and forth jitters

Normalizing the temporal variance to the temporal sum of each binary pixel to construct the motility heatmaps, allows QuoVadoPro to give more weight to processive movements than to short back and forth jitters. With processive movement, an object transiently illuminates several sets of pixels, each of which thus have a high variance and low sum and appear as hot spots on the image (figure 3A-B, left panels). When an object moves back and forth (i.e. displays non-processive movement), it illuminates the same set of pixels repeatedly (figure 3A-B, right panels). Each pixel illuminated by an object moving back and forth shows a high sum and a lower variance than pixels depicting processive movements and thus creates a less intense spot on the heatmap.

COMMENTARY

QuoVadoPro was designed to measure intracellular movement in systems where kymography cannot be applied. Kymography depends on the ability to identify discrete intracellular objects and track them along a well-defined path. As such, it works well to follow organelles or protein complexes in axons, dendrites, filopodia, and fungal hyphae (Alieva et al., 2019; Bielska et al., 2014; Kerber et al., 2009; Klinman & Holzbaur, 2016; Maday, Twelvetrees, Moughamian, & Holzbaur, 2014). In more complex systems, such as non-neuronal cells where microtubules and actin filaments (the paths of intracellular traffic) have mixed orientations, alternative manual or automated solutions have been employed to quantify intracellular traffic (Chenouard et al., 2014; Dokka, Park, Jansen, DeAngelis, & Angelaki, 2019; Jaqaman et al., 2008; Wasim & Treanor, 2018). QuoVadoPro takes a unique approach to automatically measure intracellular movements. The algorithm uses the variation in pixel illumination in time lapse images of fluorescently tagged intracellular objects, as a proxy parameter that can reveal the extent of intracellular movements. This unique approach to measuring intracellular movements makes QuoVadoPro suitable for analyzing intracellular traffic in most experimental systems.

QuoVadoPro can measure intracellular movements in an XY plane irrespective of the directions of movements and can thus be used to analyze samples not addressable by kymography. QuoVadoPro can also be applied to images where conventional object tracking is not applicable, for example in quantifying the movement of complex reticular structures like mitochondria and the ER and where individual organelles may vary from sub-micron to more than 100µm lengths (Bosch & Calvo, 2019; Costantini & Snapp, 2013; Friedman, Webster, Mastronarde, Verhey, & Voeltz, 2010; Koopman, Visch, Smeitink, & Willems, 2006; Leonard et al., 2015). This heterogeneity can prevent object tracking algorithms from faithfully detecting and therefore tracking the movements of the network. QuoVadoPro does not depend on object detection; it can infer the movement of cellular structures irrespective of size and shape. In addition, high temporal and spatial resolutions and low object densities are prerequisites for object tracking algorithms. These requirements ensure that individual cellular objects can be reliably assigned in every frame of a movie, thus allowing the changes in their positions to be tracked. QuoVadoPro may be the method of choice when these conditions do not prevail because it does not need to track the objects from frame to frame. Thus, QuoVadoPro can operate with data sets with low temporal and spatial resolutions and high object densities.

Unlike kymography or object tracking algorithms, however, QuoVadoPro is not intended to extract in-depth parameters describing different features of motility of individual intracellular objects. For that analysis, the previous methods are preferable if they can be accurately applied. Instead, the QuoVadoPro algorithm is designed to be a fast and automatic method to provide a proxy readout for

total motility within a cell. The algorithm is versatile, is computationally light and requires minimal user input and supervision. It should be stressed, that the variance algorithm is only a proxy for motility. Drastic differences in the shapes of organelles or cells in between experimental samples can also alter the output, even if these events may not involve motors acting on the cargo.

Critical Parameters

To ensure that a change in the variance of pixel occupancy faithfully reflects a change in intracellular motility, the following parameters should be carefully noted:

- **Rate of image acquisition:** As QuoVadoPro analyzes the variance of pixel illumination over the entire span of a time lapse, the total number of frames in the time lapse (as governed by the image acquisition rate) has a significant impact on the final motility score. Thus, when comparing two samples, variation in frame rates should be kept to a minimum.
- **Noise removal from images:** In step 1, QuoVadoPro generates a stack of binary masks from a time lapse movie of fluorescent objects. The binarization of fluorescence allows the algorithm to omit differences in absolute fluorescence during quantification. To make the binary masks, QuoVadoPro uses local contrast to segment the fluorescently tagged objects. Accurate quantifications depend on reliably eliminating noise pixels. Thus, images with high signal to noise ratios are preferred. The erroneous detection of noise pixels in certain frames can influence the variance (figure 4A). The user can choose from multiple tools described in step 1, such as smoothing in time and defining a lower limit of object area to eliminate noise pixels (figure 4B).
- **Number of objects:** The edges of objects may be scored slightly differently from frame to frame, even for stationary objects. This results in an increased variance at object edges. Though the algorithm weighs processive movement more heavily than this edge-jitter and normalizes for total object area, the signal due to edge-jitter can still contribute to the calculated variance. Since edge-jitter scales with the number of discrete objects in an image, the total number of discrete objects has an impact on the variance. Thus, QuoVadoPro should be used to analyze experimental samples with similar numbers of discrete objects. For example, QuoVadoPro can be used to analyze mitochondrial movement only in cases where the samples do not have large differences in mitochondrial fission or fusion.
- **Cell shape:** Quantification through QuoVadoPro is done on a single plane or on 2-dimensional projections of 3-dimensional images. Thus, the algorithm is best used in samples that do not exhibit large changes in cell shape as that may influence the distribution of objects in the plane or projection analyzed.

Example of data analysis using QuoVadoPro

As a demonstration of QuoVadoPro, we used the algorithm to analyze previously published data of mitochondrial movement in LN229 cells (Caino et al., 2016). The authors in this study show that mitochondrial movement in tumor cells is essential to promote cell invasion and membrane dynamics. They study mitochondrial movement in the presence and absence of an anchoring protein called syntaphilin (SNPH). The authors manually track individual mitochondria in these cells to demonstrate that knocking down SNPH increases mitochondrial motility. We have re-analyzed the published video of the LN229 cells using QuoVadoPro (figure 5). Our observations indicate a 20% increase of mitochondrial motility upon knockdown of SNPH, similar to the authors' observation.

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FIGURE LEGENDS:

Figure 1. Example of a Cos-7 cell having fluorescently tagged mitochondria illustrating object segmentation carried out during step 1 of QuoVadoPro.

Cos-7 transfected with GFP to facilitate determining the outline of the cell (A) and with a red fluorescent marker of the mitochondrial matrix, mitoDsRed, is shown (B). The cell was imaged at 3.3Hz for 3 minutes. Mitochondria at each time frame are segmented into binary masks by local intensity-based thresholding. During this step, the user has the option to select a region of interest (ROI), like the cell outline (dotted line). All objects outside the ROI are removed. The images in the time-lapse are then corrected for local background. Following this, the mitochondria are segmented using a predefined intensity threshold (C). Due to local background correction, all mitochondria are faithfully segmented as binary objects, irrespective of their individual intensities.

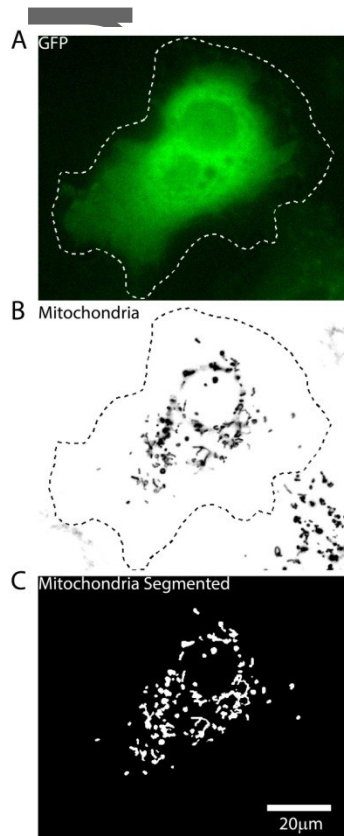


Figure 2. Demonstration of step 2 of QuoVadoPro for the Cos-7 cell whose segmentation was illustrated in Figure 1.

Following the binarization of images, in step 2, QuoVadoPro calculates the variance (A) and the sum (B) of each binary pixel over all time frames. The final motility heatmap (C) is represented as the ratio of variance over sum of intensity of each pixel. Pixels that contain a moving object, for example the mitochondrion that has moved towards the edge of the cell (arrow), display low sum and high variance. These pixels will therefore be represented as hot points on the heatmap. Pixels that contain a stationary object, will have a low variance but a high sum, and will therefore appear as cold points on the heatmap (arrowhead).

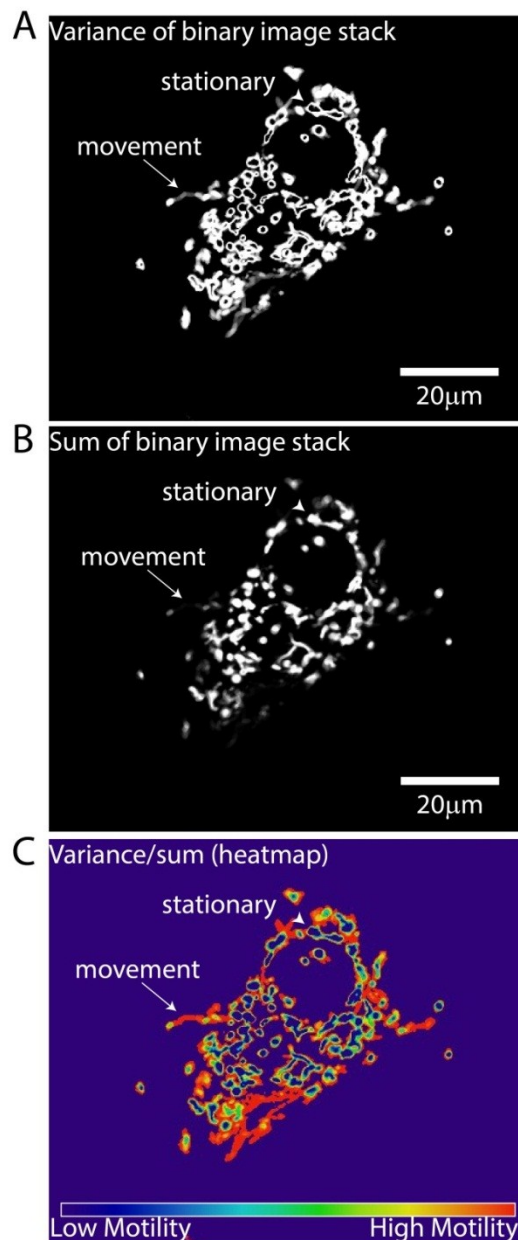


Figure 3. Example of neuronal axons with fluorescently tagged mitochondria analyzed by QuoVadoPro, demonstrating that processive movements are weighed more than back and forth movements.

Two axon segments expressing fluorescent tags on mitochondria (mitoDsRed) are shown (A, top panels) along with their kymographs (A, bottom panels). These segments are analyzed by QuoVadoPro, and their respective variance images, sum images and heatmaps along with final motility scores are depicted (B). One of the axons has processively moving mitochondria (an example of which has been marked by the arrow), while the other one contains mitochondria that are largely jiggling back and forth (an example of which is marked by the arrowhead). When analyzed over time, pixels containing processive movements show high variance and low sum (arrow, panel B). Binarized pixels depicting back and forth movements have a high variance and a high sum (arrowhead, panel B). The motility heatmaps are constructed as a ratio of variance over sum. Thus, processive movements create more hot points on the motility heatmaps compared to non-processive movements. Heatmaps that contain processively moving objects thus have a higher motility score than heatmaps of non-processive movements. Horizontal scalebars depict 15 μm and vertical scalebars indicate 30s.

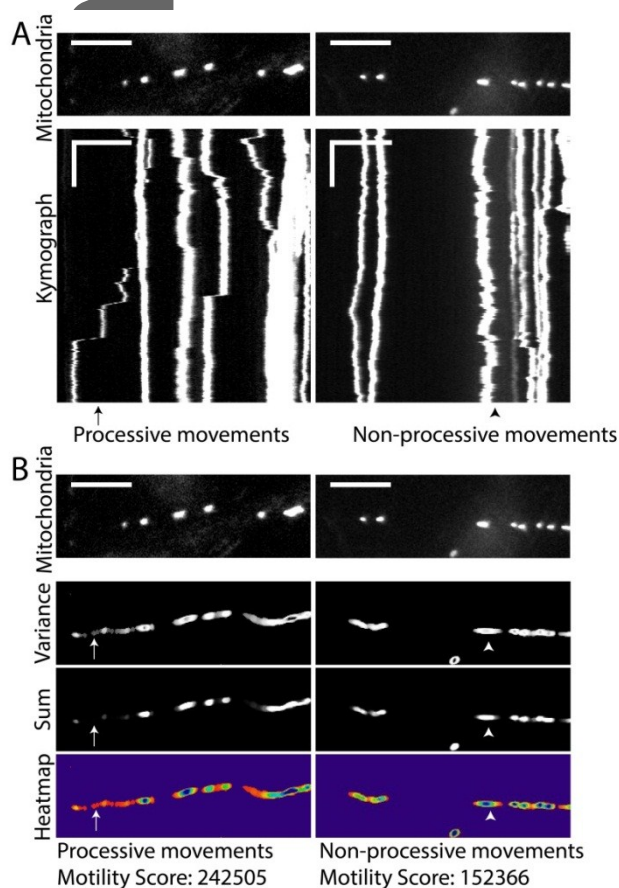


Figure 4. Impact of pixel noise and demonstration of noise removal during step 1 of QuoVadoPro.

To demonstrate the effect of pixel noise on quantification by QuoVadoPro, artificial noise is introduced in the sample cell with fluorescent mitochondria (previously shown in figures 1 and 2). When the noise in the images is not filtered out, the noise pixels are retained in the 2D motility heatmap and significantly affect the motility score (A). However, when the noise pixels are filtered out, the impact of noise is greatly reduced or eliminated (B). QuoVadoPro gives the user multiple tools to remove image noise during its operation in step 1. In this example one such tool is used, wherein a minimum size threshold can be declared for all discrete objects in the image. Any object below the minimum size threshold is eliminated. Since most image noise is in the form of isolated pixels, the user can choose a minimum size threshold of a few pixels (typically less than 3 pixels), to remove most of the noise.

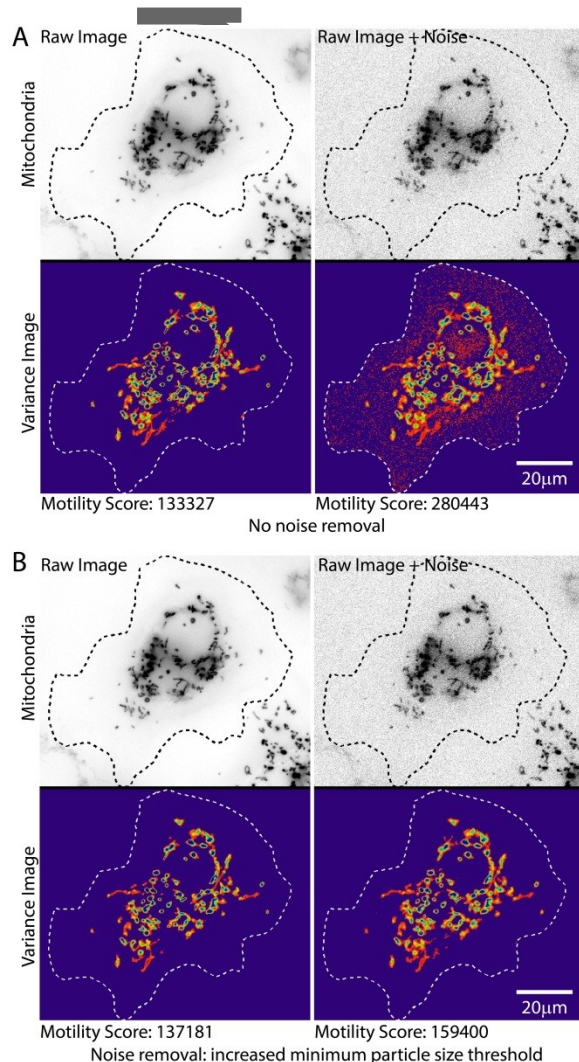
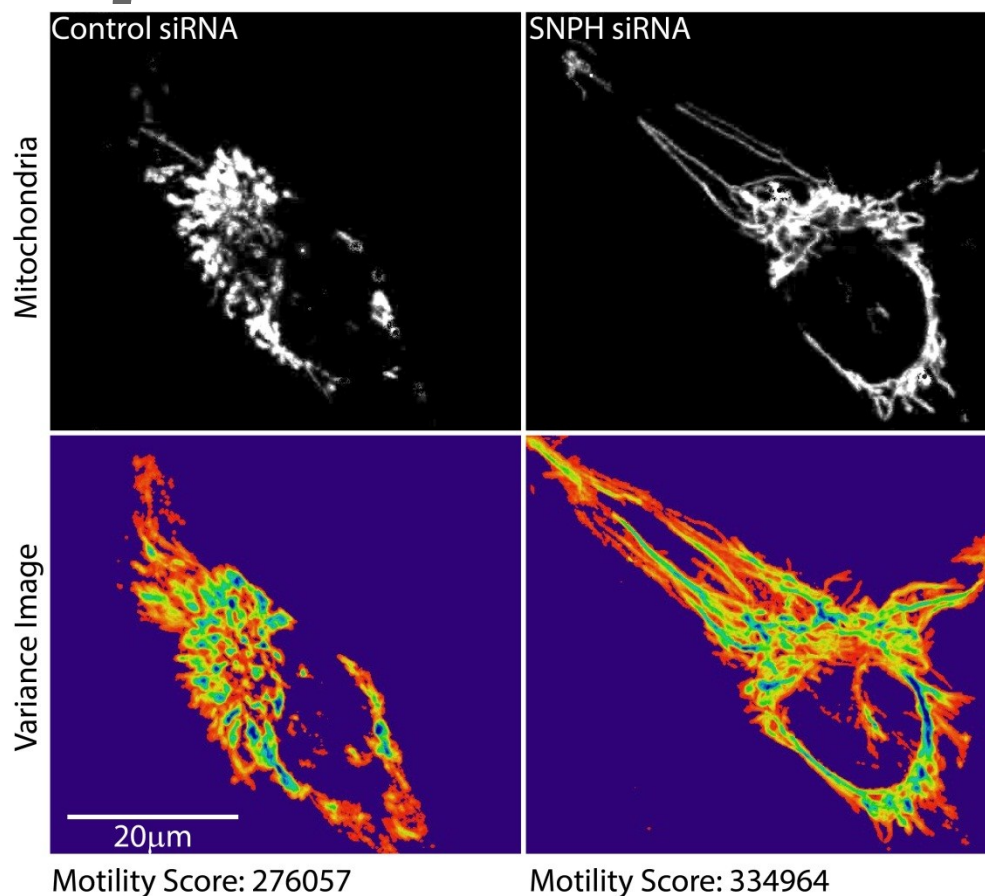


Figure 5. Demonstration of mitochondrial motility analysis by QuoVadoPro on previously published example.

To demonstrate the utility and robustness of QuoVadoPro, the algorithm is used to analyze a previously published video (Caino et al., 2016) of mitochondrial movement in LN229 cells. The mitochondria are shown (top panels) along with their heatmaps (bottom panels). In concordance with the data previously obtained by manual tracking (Caino et al., 2016), the analysis by QuoVadoPro demonstrates a 20% increase in mitochondrial motility upon knockdown of an anchoring protein syntaphilin (SNPH).



Movie S1. Example of mitochondrial movement in a Cos-7 cell.

A video of a cos-7 cell expressing GFP to mark the cytosol (left panel) along with mitoDsRed to mark the mitochondria (middle panel) is shown. The time lapse image series is analyzed by QuoVadoPro to generate a motility heatmap (right panel).

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