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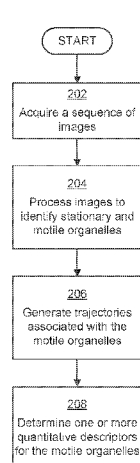
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(54) Title: HIGH-THROUGHPUT SCREENING OF REGULATORS OF AXONAL TRANSPORT



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(57) Abstract: Provided herein are techniques for processing a sequence of images of neurons to identify stationary and motile organelles of the neurons, generating tracking data associated with movement of the one or more motile organelles across the sequence of images, and determining an overall percent motile based on the identified stationary organelles and motile organelles. Also provided herein are compounds identified using the systems described herein, wherein the compounds, and pharmaceutically acceptable salts, and compositions thereof are administered to a subject in need thereof in a sufficient amount to increase or promote organelle (e.g., mitochondrial) motility, or administered in a sufficient amount to treat a disease or disorder associated with decreased organelle motility. The compounds described herein may be used in methods for increasing or promoting organelle (e.g., mitochondrial) motility in a cell, and methods for treating a disease. Also provided in the present disclosure are pharmaceutical compositions, kits, methods, and uses including or using a compound described herein.

FIG. 2

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

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HIGH-THROUGHPUT SCREENING OF REGULATORS OF AXONAL TRANSPORT

RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/792,353, filed January 14, 2019, and entitled “HIGH-THROUGHPUT SCREENING OF REGULATORS OF AXONAL TRANSPORT,” the entire contents of which are incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with government support under Grant Nos. R01GM069808, R35GM122547, and P30 HD018655 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

1. Technical Field

Some embodiments of the present invention relate to processing a sequence of images of neurons to identify stationary and motile organelles of the neurons and to track motile organelles of the neurons. In some embodiments, such techniques may be used in connection with testing of pharmaceuticals or other compounds to identify whether a pharmaceutical affects (e.g., increases) motility of organelles and may be a candidate for treatment of one or more neurodegenerative conditions.

2. Discussion of Related Art

Axonal transport is a cellular process responsible for movement of organelles to and from a neuron's cell body, through the cytoplasm of its axon. Study of axonal transport can provide valuable insight into the functioning and growth of neurons.

SUMMARY

In one embodiment, there is provided a system comprising an imaging platform configured to acquire a sequence of images of neurons that have been transfected. The imaging platform is further configured to process the sequence of images to: identify one or more stationary organelles and one or more motile organelles of the neurons, and generate tracking data associated with movement of the one or more motile organelles

across the sequence of images. The imaging platform is also configured to determine an overall percent motile based on the identified one or more stationary organelles and one or more motile organelles, the overall percent motile indicative of a percentage of the one or more motile organelles over a total number of organelles.

5 In another embodiment, there is provided a method comprising acquiring a sequence of images of neurons that have been transfected. The method further comprises processing the sequence of images to: identify one or more stationary organelles and one or more motile organelles of the neurons, and generate tracking data associated with movement of the one or more motile organelles across the sequence of images. The
10 method also comprises determining an overall percent motile based on the identified one or more stationary organelles and one or more motile organelles, the overall percent motile indicative of a percentage of the one or more motile organelles over a total number of organelles.

 In a further embodiment, there is provided at least one computer-readable storage
15 medium encoded with computer-executable instructions that, when executed by a computer, cause the computer to carry out a method comprising acquiring a sequence of images of neurons that have been transfected. The method further comprises processing the sequence of images to: identify one or more stationary organelles and one or more motile organelles of the neurons, and generate tracking data associated with movement
20 of the one or more motile organelles across the sequence of images. The method also comprises determining an overall percent motile based on the identified one or more stationary organelles and one or more motile organelles, the overall percent motile indicative of a percentage of the one or more motile organelles over a total number of organelles.

25 In one aspect, provided herein are compounds identified using the systems described herein, wherein the compounds (*e.g.*, protease inhibitors, kinase inhibitors) are administered to a subject in need thereof in a sufficient amount to increase or promote organelle (*e.g.*, mitochondrial) motility, or administered in a sufficient amount to treat a disease or disorder associated with decreased organelle (*e.g.*, mitochondrial) motility. In
30 one aspect, provided are pharmaceutical compositions and kits comprising compounds described herein. In another aspect, provided are methods for increasing or promoting organelle (*e.g.*, mitochondrial) motility in a cell, the method comprising contacting the cell with an effective amount of a compound identified using the systems described

herein. In another aspect, provided are methods for treating a disease (*e.g.*, diseases associated with reduced organelle (*e.g.*, mitochondrial) motility, or neurological diseases), the method comprising administering an effective amount of a compound identified using the systems described herein to a subject in need thereof.

5 The foregoing is a non-limiting summary of the invention, which is defined by the attached claims.

BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings are not intended to be drawn to scale. In the
10 drawings, each identical or nearly identical component that is illustrated in various figures is represented by a like numeral. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

FIG. 1 is a schematic diagram of some exemplary components of a system
15 described herein for identifying failure in axonal transport;

FIG. 2 is a flowchart of a process that may be implemented in some embodiments for identifying failure in axonal transport;

FIG. 3 is a flowchart of a process that may be implemented in some embodiments for identifying stationary and motile organelles;

20 FIG. 4 is a flowchart of a process that may be implemented in some embodiments for determining an effectiveness of a pharmaceutical in treating a neurodegenerative disorder;

FIG. 5 shows larger mitochondria and mitochondrial networks being eliminated from images for evaluation of axonal transport;

25 FIG. 6 shows large objects such as somata and cell debris being eliminated from images for evaluation of axonal transport;

FIG. 7 shows identification of stationary mitochondria based on minimum intensity projection;

30 FIG. 8 shows stationary mitochondria being subtracted from images to facilitate tracking of motile mitochondria;

FIG. 9 illustrates a sequence of images where objects that exhibit significant movement are marked with an arrow;

FIG. 10 is a graphical representation of mitochondrial tracks that represent the motile mitochondria identified in FIG. 9;

FIG. 11A is a graphical representation of mitochondrial tracks in neurons for treated cultures;

5 FIGs. 11B and 11C illustrate graphs and heat-maps showing percent motile calculations and integrated distance calculations for treated cultures;

FIG. 12A is a graphical representation of mitochondrial tracks in SNPH^{-/-} and SNPH^{+/+} neurons;

10 FIGs. 12B and 12C illustrate graphs and heat maps showing percent mobile calculations and integrated distance calculations for SNPH^{-/-} and SNPH^{+/+} neurons;

FIG. 13 is a block diagram of a computing device with which some embodiments may operate;

FIG. 14A-14B show TPP1 loss of function experiments in neurons. FIG. 14A shows dose-dependent motility in the presence of TPP1 shRNA. FIG 14B shows
15 Western blots of α -TPP1 and α -tub with either scrambled shRNA, TPP1 shRNA B, or TPP1 shRNA C, showing the depletion of TPP1 in neuronal cultures by shRNA B (shRNA 1) and shRNA C (shRNA 2) .

FIG. 15A shows aurora kinase inhibitors identified using the systems described herein. FIG. 15A is a Venn diagram illustrating the classification of specific aurora
20 kinase inhibitors (Aurora A inhibitors versus Aurora B inhibitors), pan aurora family inhibitors (inhibitors of both Aurora A and Aurora B).

FIG. 16A-16F show that Aurora B is a negative regulator of mitochondrial motility in axons. FIG. 16A shows mobile mitochondria in axon in the presence of the Aurora B inhibitor Hesperadin versus DMSO (control) as shown by MAPS in one frame
25 in a time-lapse sequence. FIG. 16B shows the percentage of mobile mitochondria in axon in the presence of the Aurora B inhibitor Hesperadin versus DMSO (control). FIG. 16C shows the time mitochondria spend in motion in the presence of the Aurora B inhibitor Hesperadin versus DMSO (control). FIG. 16D shows scrambled shRNA and a AurKB knock-down, and their effect on mobile mitochondria in axon, as shown by MAPS in one
30 frame in a time-lapse sequence. FIG. 16E shows the percentage of mobile mitochondria in axon in the presence of AurKB shRNA versus DMSO (control). FIG. 16F shows the time mitochondria spend in motion in the presence of AurKB shRNA versus DMSO (control).

FIG. 17 shows that the Aurora kinase B inhibitor Hesperadin promotes retrograde transport rather than anterograde transport.

FIG. 18 shows that the Aurora kinase B shRNA promotes bidirectional transport with a slight anterograde bias.

5 FIG. 19A shows that the Aurora kinase B inhibitor Hesperadin does not affect the density of mitochondria or stationary organelles, while FIG. 19B shows that Aurora B shRNA reduces the density of stationary organelles.

Figures 20A to 20I show a compound screen and identification of hits. Figures 20A and 20B show 2-D plots where each axis corresponds to one replicate of the library of Z-scores (KS of integrated distance) obtained in the screen. The cut-off levels of Z-score=3 are marked as dotted lines. Compounds that enhanced transport (triangles) or suppressed transport (diamonds) were defined as hits if their KS of integrated distance was equal to or above Z=3. Non-hits are gray. Box and enlargement in Figure 20B hold the location of the hits that increased mitochondrial trafficking. The correlation coefficient for the two replicates was 0.75 (including positive and negative controls and test compounds) or 0.38 (test compounds only). Figure 20C shows Z-scores for all three parameters for each confirmed enhancer of motility. Figures 20D to 20I: For each confirmed hit, the effect on the percent of motile mitochondria is shown together with a dose-response curve, for which the enhancement of motility was expressed as a percent of maximal enhancement. n=3 wells/plate from a minimum of two plates for Figures 20D to 20I.

Figures 21A to 21E show compounds that suppressed mitochondrial motility. Figure 21A shows the effect of suppressors of mitochondrial trafficking on all three descriptors of motility from the screen. Note that only the percent motile descriptor indicates the sign of the effect; the positive KS values for integrated distance and displacement indicate the significance of the change but not its direction. Figure 21 shows the effect of the same compounds on TMRM fluorescence in the soma. TMRM fluorescence could not be determined when the test compounds were fluorescent (#) or greatly altered mitochondrial distribution (##). Figure 21C shows the effect of NMS-873 and NH125, suppressors that did not alter mitochondrial membrane potential, on all three descriptors. Figure 21D shows the effect of NMS-873 and NH125 on TMRM fluorescence. Figure 21E shows that although hits in the original screen, paclitaxel and

24(S) hydroxycholesterol treatment lacked a dose response relationship for upon retesting.

Figures 22A to 27H show TPP1 is the relevant substrate of AAF-CMK. Figure 22A shows known cellular targets of AAF-CMK. Figures 22B and 22C show the Z-score of percent motile (Figure 22B) and KS integrated distance (Figure 22C) in neurons transfected for at least three days with either scrambled or TPP2 shRNA (TPP2-i) and treated with 10 μ M AAF-CMK one hour prior to and during time-lapse imaging. TPP2-i neither mimicked nor occluded the effect of AAF-CMK. Figures 22D and 22E show lack of effect of 2 hour incubation with TPP2 inhibitor butabindide at 10 μ M (Figure 22D) and proteasome inhibitor carfilzomib at 5 μ M (Figure 22E) on percent motile and lack of dose-dependent response. Figures 22F and 22G show Z-scores for percent motile (Figure 22F) and KD Id (Figure 22G) in wells transduced either with scrambled shRNA or with TPP1 shRNAs and treated with either solvent or 10 μ M AAF-CMK for two hours before time-lapse imaging. Two non-overlapping sequences targeting TPP1 were used (TPP1-i 1 and 2) (Z-scores of KD displacement were similar). Figure 22H shows the effect of TPP1 overexpression on the three motility descriptors. At least 4 wells per condition (each well representing the traces collected from more than 10,000 mitochondria) were used for the calculation of Z-scores. Statistical significance between groups of Z-scores was determined using unpaired T-test. **** = $P < 0.0001$

Figures 23A and 23B show validation of TPP1/2 shRNAs. Figure 23A shows a representative western blot showing the depletion of TPP2 protein in neuronal cultures by the TPP2 shRNA but not by the TPP1 shRNA (shRNA 1 is presented). Figure 23B shows depletion of TPP1 in neuronal cultures by shRNA 1 and shRNA 2.

Figures 24A to 24L show Aurora B is the target of the kinase inhibitor class of hits. **= $p < 0.01$. Figure 24A shows the Z-score of percent motile in neurons transfected with either scrambled or AurKB shRNA and treated with 3 μ M Hesperadin one hour prior to and during time-lapse imaging. Figure 24B shows the Z-score of percent motile in neurons transfected with either scrambled or AurKA shRNA and treated with 3 μ M Hesperadin one hour prior to and during time-lapse imaging. Figure 24C shows a representative image of mitochondrial traces in a field of neurons either transfected with scrambled shRNA or with AurKB shRNA. Figure 24D shows the percent of motile mitochondria per well in neurons expressing either scrambled shRNA or shRNA against AurKB. $p = 0.0093$. Figures 24E and 24F show the Z-score of KS integrated distance of

neurons treated as in Figures 24A and 24B. Figure 24G shows the integrated distance of mitochondrial traces in a representative well either transfected with scrambled shRNA or shRNA against AurKB represented as a histogram and as a cumulative distribution. Each dot in the histogram corresponds to a single mitochondrial trajectory.

5 Figure 24H shows the dose-response curve of the effect of Barastertib on percent motility; values were normalized to the maximum % motility at 2 μ M Barastertib. Figure 24I shows the Z-score of percent motile of rat AurKB-i in combination with either WT or a kinase dead form of the RNAi-resistant human AurKB. Figure 24J shows representative images of mitochondrial traces in a field of neurons either transfected with empty plasmid or with a plasmid expressing rat AurKB. Figures 24K and 24L show the

10 effect of AurKB overexpression on percent motility (Figure 24K) and integrated distance travelled (Figure 24L). At least 4 wells per condition (each well containing traces collected from over 10,000 mitochondria on average) were used for the calculation of Z-scores. Statistical significance between groups of Z-scores was determined using

15 unpaired T-test.

Figures 25A to 25C show Aurora family inhibitors and validation of AurKB shRNA. Figure 25A shows the Z-score of KS integrated distance for all Aurora family inhibitors tested in the primary screen. Red bars outline hits that were identified initially, blue bars outline aurora inhibitors that significantly modulate mitochondrial transport but

20 that did not pass the initial cut-off threshold. Figure 25B shows that among the four new compounds, three increase mitochondrial trafficking. Red dotted lines indicates a cut-off of Z-score = 2. Black dotted line indicates Z=0. Figure 25C shows validation of AurKB shRNA in HEK cells expressing rat Myc-AurKB. HEK cells were transfected with the indicated constructs for 5 days prior to lysis and analysis by western blot. Fluorescence

25 images illustrate the transfection efficiency of the shRNA expressing plasmids in HEK cells.

Figures 26A to 26D show the effect of AurKB inhibition on transport of late endosomes. Figure 26A is a representative kymograph of neurons transfected with Rab7-GFP and treated with Hesperadin (as in Figs. 20-23 above). Figure 26B shows the effect

30 of Hesperadin on time Rab7 vesicles spend in motion as well as on the direction of their movement. Figures 26C and 26D show neurons transfected with either scrambled shRNA or shRNA against AurKB and Rab5-mCherry analyzed for percent motile and direction of movement. At least 50 traces from at least 9 axons coming from three

independent transfections were used for all quantifications. D = DMSO; H=Hesperadin; s=scrambled; i= shRNA against AurKB; retro = retrograde transport; antero = anterograde transport.

Figures 27A to 27J show activity of hits in human iPSC-derived neurons. Figure 27A shows representative images of control iPSCs differentiated by neurogenin2-induction into cortical neurons. Nuclei were labeled with Hoechst 33258. GFP expression is driven by viral overexpression alongside the neurogenin2 vector. Neurons were labeled with β 3-tubulin to label all soma and neurites. Figures 27B and 27C show Z-scores of percent motile and KS-values of integrated distance for mitochondrial transport in human cortical neurons transduced with CMV-Mito-DsRed and treated with 3 μ M Hesperadin, 1 μ M Latrunculin A and 10 μ M AAF-CMK for one hour before and during time-lapse imaging. Figures 27D and 27E: Kymographs were used to establish the average time a mitochondrion spends in motion (Figure 27D) as well as its direction (Figure 27E) in human cortical neurons transduced with CMV-Mito-DsRed and treated with either DMSO or Hesperadin. Figures 27F and 27G show Z-scores of percent of motile mitochondria (Figure 27F) and KS value of integrated distance (Figure 27G) in +/-A4V neurons and +/- isogenic control motor neurons. Figure 27H show the effect of Hesperadin, Latrunculin and AAF-CMK on percent of motile mitochondria in +/-A4V motor neurons. Figures 27I and 27J show average time a mitochondrion spends in motion in +/-A4V neurons treated with DMSO or with 3 μ M Hesperadin.

FIG. 28 shows an example interface generated for depicting registration defects; FIG. 29 shows an example interface for registration correction and thresholding; FIG. 30 shows an example interface for single parameter analysis; and FIG. 31 shows an example interface for multi-parametric analysis.

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

DETAILED DESCRIPTION

Described herein are embodiments of an image analysis system to identify stationary and motile organelles within a cell, including stationary and motile organelles within a neuron. In the case of neurons, such stationary and motile organelles may be

identified in axons, and the organelles may be mitochondria. In some embodiments, a sequence of images is analyzed to identify stationary organelles, which are then masked from subsequent analysis of the images in which other organelles (motile organelles) are identified and their movements tracked across the sequence of images. Characteristics of
5 the organelles may be determined, including characteristics of individual stationary and/or motile organelles, and/or characteristics of a group of organelles, such as organelles in a particular cell or in a particular axon of a neuron (or other part of a cell), or organelles in a group of cells. Based on these characteristics, information about a status of a cell, such as a status of axonal transport in a neuron, may be determined.

10 Failures in axonal transport of organelles can be linked to many neurodegenerative conditions/disorders, for example, multiple sclerosis, amyotrophic lateral sclerosis, Charcot-Marie Tooth type 2 disease, and Parkinson's disease. Accordingly, if treatments were developed that increased or improved axonal transport of organelles, these treatments may be candidates for treating or assisting in treating one or
15 more of these neurodegenerative disorders.

Imaging techniques have been proposed for studying organelles in axons, but such prior work focused on studying movement of organelles in individual axons or small populations. Such techniques do not identify modest but potentially significant changes in axonal transport, nor can they assist with evaluating stationary organelles or
20 comparing stationary and motile organelles as part of studying failures or improvements in axonal transport in sufficient quantity to have the statistical power needed for detecting potential pathological or therapeutic conditions. The inventors have recognized and appreciated the advantages that would be associated with techniques identifying potential deficits in axonal transport, including identifying organelles that are not moving
25 and tracking moving organelles.

Moreover, the inventors recognized and appreciated various disadvantages of prior work, including limitations of prior work, that limited efficacy of the prior work in some contexts. For example, prior techniques for studying movement of organelles required capture of a large volume of images at high frequency, to limit time intervals
30 between images. This large volume of images was then analyzed to identify moving organelles by looking for organelles whose position overlaps in successive images, and identifying a moving organelle based on the overlapping positions in a number of successive images. The inventors recognized and appreciated while such a technique

may be useful in some contexts, the high volume of image data and complexity of analysis may lead to long processing times. Such long processing times may limit the amount of times the analysis may be run, and limit throughput of the system.

The inventors have developed an analytic tool that is capable of performing
5 precise automatic tracking of organelles from a sequence of images of neurons to identify a status of (e.g., a failure or deficiency in) axonal transport. In some embodiments, the analytical tool may assist in screening a number of compounds/pharmaceuticals to identify those that impact axonal transport, including that may alleviate the identified axonal transport deficits in neurons.

10 Some embodiments include analyzing a sequence of images of neurons to automatically identify and segregate organelles into stationary and motile organelles, and track movement of the motile organelles across the sequence of images. The inventors have recognized and appreciated various benefits of such an approach. For example, the inventors have appreciated that the presence of stationary organelles across the sequence
15 of images may be one feature indicative of a neurodegenerative condition/disorder, and that determining relative amounts of stationary and motile organelles in a neuron, including by tracking a change in such relative amounts over time, may be another feature that may indicate the presence or progression of such a neurodegenerative condition/disorder. The inventors further recognized and appreciated that, with
20 techniques for identification of stationary organelles in a neuron, including an axon of a neuron, techniques may also be developed for masking stationary organelles from a sequence of images so as to leave in the image motile organelles. Techniques may then additionally be developed for tracking such motile organelles in the images from which stationary organelles have been masked, which in some cases may allow for enhanced
25 tracking of organelles and increased precision of tracking of the motile organelles. Such enhanced tracking may include tracking a movement path of an organelle, and a comparison of an organelle's movement path or characteristics of the movement path to common or ordinary movement paths for organelles. With such techniques, deviations from typical movement of a motile organelles may also be identified and taken as a
30 feature indicative of a potential neurodegenerative disorder. Such enhanced tracking may include tracking of motile organelles with a reduced set of images as compared to techniques that rely on overlapping positions of organelles to perform tracking. With a reduced set of images, potentially captured at a lower frequency, in some cases a

throughput of image analysis, and a throughput of analysis of a status of axonal transport in neurons, may be increased. Such a reduced set of images may include images in which a motile organelle is not represented at an overlapping position in two images of the set, and may have been captured at a frequency that is less than or equal to five images per second, less than or equal to two images per second, or less than or equal to one image per second. In some embodiments, the image capture frequency may be at least one image every five seconds, or at least one image every two seconds, or at least one image every second.

Accordingly, in some embodiments, using analysis techniques described herein, and based on the information gathered by analyzing a sequence of images, one or more quantitative descriptors indicative of the collective behavior of organelles of a neuron or a group of neurons may be determined. For example, an overall percent motile, indicative of a percentage of motile organelles over a total number of organelles (including stationary organelles) identified from the sequence of images, may be determined. The inventors have recognized and appreciated that effectiveness of a pharmaceutical may be assessed based on a comparison between the overall percent motile determined prior to administering the pharmaceutical and after administering the pharmaceutical. In other words, based on the analysis of the overall percent motile both before and after administration of the pharmaceutical, or comparing in parallel samples treated with a pharmaceutical and untreated, a determination can be made regarding whether the pharmaceutical may be effective in treating the disorder, or may be a candidate for further research into whether the pharmaceutical is effective in treating or assisting in treating the disorder.

Described below are examples of systems and methods for evaluating axonal transport, including whether there has been a failure in axonal transport or whether axonal transport has improved (e.g., with administration of a compound). It should be appreciated that embodiments are not limited to operating in accordance with any of the specific examples below, as other examples are possible.

Illustrative Embodiments

FIG. 1 illustrates an example of a system 100 for evaluating axonal transport, in accordance with some embodiments. System 100 may include a high-content or high-throughput screening system. In some embodiments, system 100 includes a culturing

station 110 and an imaging platform 120. The culturing station 110 may be used to culture neurons prior to being imaged by the imaging platform 120. In some embodiments, thirty thousand neurons, or any other suitable number of neurons, may be cultured per well. In some embodiments, the neurons may be derived from any source, including rodent tissue or human iPSC-derived neurons. The culturing station 110 may culture the neurons for a suitable time (e.g., five days) after plating. In some embodiments, multi-well plates may be used, for example, 96-well plates may be used.

In some embodiments, at a time before imaging (e.g., two days before), one or more biomarkers may be introduced into the neurons at the culturing station 110. For example, the neurons may be transfected with a red fluorescent protein targeted specifically to the organelle being studied. In some implementations, the organelle being studied includes mitochondria, and the protein used includes the Mito-DsRed protein, however, other organelles can be studied and other proteins may also be used without departing from the scope of the disclosure.

After the neurons have been cultured and transfected, the well plates may be transferred to the imaging platform 120. The imaging platform 120 may acquire a sequence of images of the transfected neurons (i.e., capture images at a capture rate). In some implementations, the imaging platform 120 may acquire thirty frames at a frequency of approximately two hertz from four fields per well. In some embodiments, four fields may be imaged per well of a 96-well plate containing 30,000 neurons/well at DIV08-09 using a 20X objective lens with 2x2 binning. To minimize well to well differences in exposure to compounds, the fields may be imaged according to the following sequence: field 1 imaged in every well before returning to the start of the plate and imaging field 2 in every well, and similarly for fields 3 and 4. In some embodiments, the imaging may be performed within an environmentally-controlled chamber (e.g., a chamber where temperature and CO₂ are controlled). For example, during imaging, neurons may be maintained in conditioned media at 37°C and 5% CO₂. In some implementations, the imaging platform 120 may acquire the images by detecting light emitted from the neurons transfected with the fluorescent protein within the wells.

In some embodiments only in the primary screen, the plate was incubated with Tetramethylrhodamine, methyl ester (TMRM), and with Hoechst dye upon completion of the time-lapse sequence and then live-imaged again to determine mitochondrial membrane potential.

In some embodiments, TMRM-labeled mitochondria may be analyzed from areas of the wells lacking Mito-DsRed due to low transfection efficiency. Cell bodies may be outlined based on the Hoechst signal, and the intensity of TMRM per cell may be quantified.

5 The imaging platform 120 may analyze the sequence of images to identify a plurality of organelles. In some embodiments, the imaging platform 120 may determine a minimum intensity projection of each organelle across the sequence of images. The inventors have recognized and appreciated that minimum intensity projection can be utilized to differentiate stationary organelles from motile organelles because the
10 minimum intensity projection of a stationary organelle is stronger than the minimum intensity projection of a motile organelle imaged for the same period of time. Such minimum intensity projection may be determined from fluorescence measurements taken from the images captured of cells or organelles subject to the fluorescent projection. By determining the minimum intensity projection of organelles across the sequence of
15 images, the imaging platform 120 may identify stationary organelles and motile organelles. For example, when an organelle appears at approximately the same location across the sequence of images and has a stronger minimum intensity projection than other organelles, the imaging platform 120 may categorize the organelle as a stationary organelle. On the other hand, when an organelle appears to be moving across the
20 sequence of images and has a weaker minimum intensity projection than other organelles, the imaging platform 120 may categorize the organelle as a motile organelle.

 In some embodiments, tracking data associated with the stationary organelles may be generated by the imaging platform 120. The tracking data may include, location information associated with the stationary organelles across the sequence of images,
25 minimum intensity projection information associated with stationary organelles across the sequence of images, a number of stationary organelles identified across the sequence of images, and/or other data.

 In some embodiments, the imaging platform 120 may mask the identified stationary organelles from the sequence of images, which facilitates further analysis of
30 the movement of the motile organelles across the sequence of images. The imaging platform 120 may generate tracking data associated with the movement of the motile organelles. The tracking data associated with the motile organelles can include identification information associated with matching motile organelles across the

sequence of images, tracks or trajectories followed by the matching motile organelles across the sequence of images, minimum intensity projection information associated with motile organelles across the sequence of images, a number of motile organelles identified across the sequence of images, and/or other data. In some embodiments, the sequence of
5 images, the tracking data associated with the stationary and/or motile organelles, and/or other data, may be stored in a data store 125.

In some embodiments, the imaging platform 120 may utilize a linear assignment problem (LAP) framework to match motile organelles between consecutive images, link the matching motile organelles, and generate the trajectories followed by the motile
10 organelles.

In some embodiments, the imaging platform 120 may additionally or alternatively utilize an amount of spatial overlap between motile organelles in consecutive images to match the motile organelles and/or link the matching organelles. For example, an amount of spatial overlap between motile organelles in a previous image
15 and the motile organelles in a current image may be compared. Based on the comparison, a determination may be made regarding whether an amount of spatial overlap that exists between a first motile organelle in the previous image and a second motile organelle in the current image exceeds a predetermined threshold. When the amount of spatial overlap exceeds the predetermined threshold, a determination may be
20 made that the first motile organelle in the previous image matches the second motile organelle in the current image. In this manner, the imaging platform 120 may identify motile organelles having a certain amount of spatial overlap across the sequence of images as matching motile organelles and may assign the same label (or other identifying characteristic) to the matching motile organelles. The label(s) may be included in the
25 tracking data. Once a motile organelle is identified and matched in each image of the sequence of images, the matching motile organelles in the sequence of images may be linked to determine a trajectory followed by the motile organelle across the sequence of images.

In yet other embodiments, the imaging platform 120 additionally or alternatively
30 utilizes a distance between motile organelles in consecutive images to match the motile organelles and/or link the matching organelles. For example, a distance between motile organelles in a previous image and the motile organelles in a current image may be compared. Based on the comparison, a determination may be made regarding whether a

distance between a first motile organelle in the previous image and a second motile organelle in the current image is less than a predetermined value. When the distance is less than the predetermined value, a determination may be made that the first motile organelle in the previous image matches the second motile organelle in the current
5 image. For instance, the imaging platform 120 may identify motile organelles closest to each other across the sequence of images as matching motile organelles and assign the same label (or other identifying characteristic) to the matching motile organelles. The label(s) may be included in the tracking data. Once a motile organelle is identified and matched in each image of the sequence of images, the matching motile organelles in the
10 sequence of images may be linked to determine a trajectory followed by the motile organelle across the sequence of images.

In some embodiments, the tracking data associated with the stationary organelles and/or motile organelles may be analyzed to determine one or more quantitative descriptors indicative of the collective behavior of the organelles across the sequence of
15 images. The one or more quantitative descriptors may include an integrated distance indicative of a total distance travelled by each organelle across the sequence of images or an integrated distance travelled for each motile organelle (length of travel, or the sum of all movements including changes in direction). The one or more quantitative descriptors may include a displacement indicative of a change in position of each organelle across
20 the sequence of images or an absolute distance between point of initiation of travel and the final destination for each motile organelle. The one or more quantitative descriptors may include an overall percent motile indicative of a percentage of motile organelles over a total number of organelles (including stationary organelles) identified from the sequence of images or a proportion of motile organelles per well.

25 In some embodiments, a status of axonal transport (e.g., a failure of axonal transport) may be identified based on the one or more quantitative descriptors.

For example, when one or more stationary organelles are identified, for which an integrated distance is approximately zero, a determination may be made that a failure in axonal transport exists. In another example, when one or more motile organelles are
30 identified, for which the displacement is not within a characteristic range, a determination may be made that a failure in axonal transport exists. Such failure in axonal transport may in turn be indicative of a neurodegenerative disorder.

In some embodiments, the one or more quantitative descriptors may be further utilized to determine an effectiveness of a pharmaceutical in treating the neurodegenerative disorder. For example, the effectiveness of the pharmaceutical may be assessed based on a comparison between the overall percent motile determined prior to administering the pharmaceutical to the neurons and after administering the pharmaceutical to the neurons. In other words, based on the analysis of the overall percent motile both before and after administration of the pharmaceutical, a determination can be made regarding whether the pharmaceutical may be effective in treating the neurodegenerative disorder.

In some embodiments, the imaging platform 120 may generate one or more visual representations of the data (e.g., the tracking data, data associated with the one or more quantitative descriptors, and/or other data) obtained from the analysis of the sequence of images described above. In some implementations, the analysis of the sequence of images may be performed at plate, well, and/or field levels, and visual representations may be generated for the different levels as will be appreciated. In some implementations, the imaging platform 120 may generate visual representations of trajectories followed by motile organelles across the sequence of images. In other implementations, the imaging platform 120 may perform various statistical computations associated with the one or more quantitative descriptors described above. For example, the imaging platform may calculate Z-scores using medians and/or perform Kolmogorov-Smirnov (KS) tests for the one or more quantitative descriptors. The imaging platform 120 may then generate heat maps, traces, and/or other visual representations for each descriptor.

In some embodiments, the generated one or more visual representations may be rendered in a graphical user interface (GUI). In some implementations, the one or more visual representations may be communicated to computing devices 140 (including devices 140A and 140B, referred to generically and collectively herein as device(s) 140). The one or more visual representations may be rendered in a GUI presented on a screen of the devices 140. The devices 140 are illustrated in FIG. 1 as a mobile phone and as a desktop computer, but embodiments are not limited to operating with any form of computing device. The imaging platform 120 may communicate with devices 140 via a network 130, which may be any suitable one or more wired and/or wireless network, including the Internet.

In some embodiments, the imaging platform 120 may generate the one or more visual representations prior to administering the pharmaceutical. The imaging platform 120 may generate one or more second visual representations after administering the pharmaceutical. For example, the one or more visual representations may include a first
5 heat map for the overall percent motile descriptor (prior to administering the pharmaceutical) and the one or more second visual representations may include a second heat map for the overall percent motile descriptor (after administering the pharmaceutical). The inventors have recognized and appreciated that generating such visual representations and allowing users to view the generated visual representations via
10 the GUI (for example, side-by-side via the GUI) may present the users with accurate information needed to confirm the effectiveness of the pharmaceutical in treating a neurodegenerative disorder.

FIG. 2 illustrates an example of a process that may be performed by the imaging platform 120 in some embodiments to evaluate axonal transport. In block 202, the
15 imaging platform 120 acquires a sequence of images of neurons. The neurons may be transfected with a fluorescent protein at the culturing station 110 prior to imaging.

At block 204, the imaging platform 120 may process the sequence of images to identify one or more organelles from the sequence of images. The imaging platform 120 may process the sequence of images to identify one or more stationary organelles and
20 one or more motile organelles. In some embodiments, the imaging platform 120 may utilize the process illustrated in FIG. 3 to identify and distinguish between the stationary and motile organelles. Referring to FIG. 3, the imaging platform 120 may, at block 302, determine a minimum intensity projection of fluorescence for each organelle identified from the sequence of images. At block 304, the imaging platform 120 may, based on the
25 minimum intensity projection values, identify and distinguish between stationary and motile organelles. For example, the imaging platform 120 may identify an organelle with a minimum intensity projection equal to or above a predetermined threshold as a stationary organelle, and an organelle with a minimum intensity projection below the predetermined threshold as a motile organelle.

30 Referring back to FIG. 2, at block 206, the imaging platform 120 may generate one or more trajectories that follow the movement of the one or more motile organelles across the sequence of images. At block 208, the imaging platform 120 may determine one or more quantitative descriptors, e.g., integrated distance, displacement, and/or

overall percent motile, for the stationary and/or motile organelles. The imaging platform 120 may identify a status of axonal transport, including whether there has been a failure of axonal transport or whether axonal transport matches normal or expected performance, based on the one or more quantitative descriptors.

5 FIG. 4 illustrates an example of a process that may be performed by the imaging platform 120 in some embodiments to determine effectiveness of a pharmaceutical in treating a neurodegenerative disorder linked to a failure in axonal transport. As described in detail below, the imaging platform 120 may perform the operations of blocks 402 and 404 both prior to administering a pharmaceutical to the neurons and after
10 administering the pharmaceutical to the neurons in order to determine the effectiveness of the pharmaceutical in treating the neurodegenerative disorder. Alternatively, the operations of blocks 402 and 402 can be used to compare pharmaceutically treated and untreated neurons.

 At block 402, the imaging platform 120 may identify stationary and motile
15 organelles from a sequence of images of neurons. At block 404, the imaging platform 120 may determine an overall percent motile based on the identified stationary and motile organelles. This is indicative of the overall percent motile prior to administering a pharmaceutical to the neurons.

 At a later time, the pharmaceutical may be administered to the neurons, and at
20 block 406, the imaging platform 120 may repeat the operations of blocks 402 and 404. After the operations are repeated, an overall percent motile after administering the pharmaceutical to the neurons is determined.

 At block 408, the effectiveness of the pharmaceutical may be assessed based on a comparison between a first overall percent motile determined prior to administering the
25 pharmaceutical and a second overall percent motile determined after administering the pharmaceutical to the neurons. For example, when the second overall percent motile is greater than the first overall percent motile, a determination can be made that the pharmaceutical is effective.

30 **Example methods for evaluating axonal transport**

 In some aspects, the systems or processes described herein were used to identify stationary and motile mitochondria within E18 rat hippocampal neurons that were cultured on 96-well plates for 6-7 days (days *in vitro* (DIV) 6-7) before transfecting the

cells with Mito-DsRed. After 2 days (DIV 8-9), mitochondrial movement was observable and a sequence of images of the neurons were captured (e.g., by imaging platform 120). Mitochondria were identified in each frame of the sequence of images. A size-based mask was used to identify small (1-10 μ m in size) individual mitochondria that are typically observed in axons and secondary dendrites and to eliminate, from the analysis, larger mitochondria and mitochondrial networks, which correspond to the highly fused mitochondria of somato-dendritic regions, non-neuronal cells, and/or fluorescent debris. A segmentation design/method (as shown in FIG. 5) was used that exploits the fact that somatic mitochondria are densely packed and fill the majority of the somatic cytoplasm. Masking based on the size of objects in the mitochondrial channel resulted in equivalent masking to that obtained by identifying the nucleus and expanding that region to mask the somatic compartment. Masking only on the Mito-DsRed channel avoided the need to use the Hoechst dye which otherwise induced neurotoxicity upon sequential illumination. Usage of the size-based mask allows, for example, analysis to be focused on mitochondria travelling along neurites. FIG. 6 illustrates rat hippocampal neurons transfected with Mito-DsRed to label mitochondria showing the original channel (left) and the masked channel (right) in which large objects such as somata and cell debris have been eliminated.

Because the majority of mitochondria (typically 70-80%) in rat hippocampal cultures remain stationary for long periods of time, it was observed that subtracting the stationary mitochondria from the images substantially improved the accuracy of tracking the moving mitochondria. A minimum intensity projection of fluorescence for each mitochondrion identified from the sequence of images was determined to identify and distinguish between stationary and motile or moving mitochondria. In a minimum intensity projection of the sequence, only stationary mitochondria are visible because the motile mitochondria do not accumulate sufficient fluorescence intensity due to their shifts in location, thereby allowing identification and masking of stationary mitochondria. For example, FIG. 7 shows six frames showing stationary and motile mitochondria and a minimum intensity projection derived from the six frames shows only stationary mitochondria. The identified stationary mitochondria were subtracted or masked from the sequence of images. For example, as shown in FIG. 8, stationary mitochondria (in the middle image) were identified based on minimum intensity projection and the identified mitochondria were subtracted from the image (rightmost

image) to facilitate tracking of the motile mitochondria (outlined in orange in the rightmost image). The motile mitochondria were then tracked using the LAP algorithm.

FIG. 9 illustrates a sequence of images from a small portion of an imaged field, with stationary and motile mitochondria identified based on the minimum intensity projection. Objects that exhibit significant movement are marked with an arrow.

A threshold for a minimum amount of movement was applied to classify “wiggling” mitochondria as stationary, and the “bona-fide” motile mitochondria were tracked using LAP algorithms. FIG. 10 is a graphical representation of mitochondrial tracks, where the arrows and “blue lines” indicate the tracks that represent the motile mitochondria identified in FIG. 9.

Three descriptors of motility were extracted: overall percent motile, integrated distance, and displacement. All the three parameters were evaluated in Z-prime assays to identify a degree of sensitivity of a parameter to changes in mitochondrial motility by known factors that enhance or decrease their axonal transport. To decrease motility, calcimycin, a calcium ionophore that arrests mitochondria was used. 96-well plates were seeded with rat hippocampal neurons and transfected with mito-DSred (at DIV07). 48 of the wells were incubated for a brief period (e.g., fifteen minutes) with 10 μ M calcimycin (at DIV09). In each well, approximately 40,000 mitochondria were imaged. Fewer mitochondrial tracks were detected in calcimycin-treated wells. Also, a reduced percent of motile mitochondria and Z-score of percent motile was observed.

The Komogorov-Smirnov (KS) statistic was used as a descriptor for integrated distance and/or displacement. The KS statistic quantifies a distance between the distribution functions of samples, and is a nonparametric method used for comparing two samples, as it is sensitive to differences in both location and shape of cumulative distribution functions, in this case distances travelled by each mitochondrion in the fields. No difference between distribution leads to KS=0, while significant changes in the curves generates KS>0. Calcimycin, by decreasing the integrated distances and displacement, enhanced KS values and Z-scores for both parameters.

Parameters such as, cell culture conditions, plate type, transfection protocol, control molecules, and assay kinetics were optimized with a target of Z-prime factor Z’>0.5. Z-prime values for percent motile, KS integrated distance, and KS displacement were calculated. Z-prime values for KS of integrated distance and displacement were

higher than 0.5, indicating that the ability of these parameters to distinguish treated and untreated wells was sufficiently robust to power the screen.

In addition, neurons derived from syntrophin knockout mice (SNPH^{-/-}), which show large increases in axonal trafficking of mitochondria relative to control C57B16 mice, were evaluated. 48 wells of a 96-well plate were seeded with hippocampal neurons dissected from each of the genotypes, transfected with Mito-DsRed at DIV06, and images at DIV08. Based on the above-described approach, it was observed that the SNPH^{-/-} neurons showed a significantly higher percentage of motile mitochondria as well as integrated distance and displacement as compared to control SNPH^{+/+} neurons. Thus, it was determined that the above-described approach can identify both enhanced and diminished mitochondrial trafficking along neurites and is robust enough to be used for high content screening.

FIG. 11A is a graphical representation of mitochondrial tracks in neurons either treated with DMSO (mock) or 1 μ M calcimycin. Calcimycin reduced movement (blue lines) and increased the number of stationary objects (red dots). FIG. 11B illustrates a graph (left) showing the percent motile for DMSO-treated and Calcimycin-treated cultures and a heat-map (right) of Z-scores calculated for the DMSO-treated and Calcimycin-treated cultures. FIG. 11C illustrates graphs depicting integrated distance of the tracked objects expressed as cumulative distribution plots to compare either two sets of DMSO-treated cultures (top) or DMSO vs calcimycin cultures (bottom). Heat maps are shown for Kolmogorov-Smirnov (KS) statistical values that compare the distributions of integrated distances. The displacement parameter was similarly altered by calcimycin (KS value $\sim$ 0.3, Z-score $\sim$ 19).

FIGs. 12A is a graphical representation of mitochondrial tracks in murine SNPH^{+/+} and SNPH^{-/-} neurons. FIGs. 12B and 12C illustrate the percent motile and integrated distance parameters in a manner similar to FIGs 11B and 11C.

Example methods and interfaces for detecting and correcting registration defects and hit identification

In some aspects, the systems or processes described herein may be used to perform automated detection and correction of registration defects and run relevant statistical analyses for assay validation and hit identification. FIG. 28 is an example initial loading screen presented by the imaging platform 120. The imaging platform 120

automatically loads all input data and detects the presence of any registration defects. The user may interactively select any well of a plate and the imaging field therein and the imaging platform 120 may display, for the selected well, the stationary and motile organelles (along with the associated tracks) of the selected field. In FIG. 28, the stationary organelles are displayed as dots, while the motile organelles/tracks are represented as lines.

FIG. 29 shows an example user interface with panels for registration correction and thresholding. Panel A of FIG. 29 shows an exploded view of a field of neurons (of FIG. 28) that have registration defects. The registration defects are evident as short movements of most organelles in the field in the same direction. Panel B of FIG. 29 depicts the same exploded view with the defects corrected. The user can choose to delete or ignore the associated wells for further analyses. Panel C depicts the same data thresholded for processive movements. The inset 2902 of Panel B shows a highly non-processive movement (generated by faulty particle tracking), which is thresholded out in the process of generating the data shown in Panel C.

FIG. 30 shows an example user interface with panels for single parameter or descriptor analysis. Panel A of FIG. 30 shows an interface via which the user can perform statistical analysis on a single parameter or descriptor of motility. Panel B depicts a zoomed inset 3002 that shows different statistical tests that can be performed. The results of the analyses can be shown as an interactive heat map of the entire plate.

FIG. 31 shows an example user interface via which a user can perform multi-parametric statistical analysis (i.e., statistical analysis on multiple parameters or descriptors of motility). For example, using the interface of FIG. 31, the user can perform statistical analysis on up to three different parameters/descriptors of motility for each well, assign a combined score to each well and view the plate as a heat map 3102. This allows for a sensitive detection of hits which may display weak changes in a single parameter but a large effect when three parameters are combined. The hits in a screen may be identified by employing the distance of vectors to the origin in a projection. The user can also visualize all the parameters as a three-dimensional plot 3104, from where the user can interactively click on any point. Responsive to the input, a panel 3106 may be generated allowing the user to view the tracks of that corresponding well.

Illustrative Computer Implementations

Techniques operating according to the principles described herein may be implemented in any suitable manner. Included in the discussion above are a series of flow charts showing the steps and acts of various processes that identify failure of axonal transport and/or determine an effectiveness of a pharmaceutical in treating a neurodegenerative disorder linked to the failure of axonal transport. The processing and decision blocks of the flow charts above represent steps and acts that may be included in algorithms that carry out these various processes. Algorithms derived from these processes may be implemented as software integrated with and directing the operation of one or more single- or multi-purpose processors, may be implemented as functionally-equivalent circuits such as a Digital Signal Processing (DSP) circuit or an Application-Specific Integrated Circuit (ASIC), or may be implemented in any other suitable manner. It should be appreciated that the flow charts included herein do not depict the syntax or operation of any particular circuit or of any particular programming language or type of programming language. Rather, the flow charts illustrate the functional information one skilled in the art may use to fabricate circuits or to implement computer software algorithms to perform the processing of a particular apparatus carrying out the types of techniques described herein. It should also be appreciated that, unless otherwise indicated herein, the particular sequence of steps and/or acts described in each flow chart is merely illustrative of the algorithms that may be implemented and can be varied in implementations and embodiments of the principles described herein.

Accordingly, in some embodiments, the techniques described herein may be embodied in computer-executable instructions implemented as software, including as application software, system software, firmware, middleware, embedded code, or any other suitable type of computer code. Such computer-executable instructions may be written using any of a number of suitable programming languages and/or programming or scripting tools, and also may be compiled as executable machine language code or intermediate code that is executed on a framework or virtual machine.

When techniques described herein are embodied as computer-executable instructions, these computer-executable instructions may be implemented in any suitable manner, including as a number of functional facilities, each providing one or more operations to complete execution of algorithms operating according to these techniques. A “functional facility,” however instantiated, is a structural component of a computer system that, when integrated with and executed by one or more computers, causes the

one or more computers to perform a specific operational role. A functional facility may be a portion of or an entire software element. For example, a functional facility may be implemented as a function of a process, or as a discrete process, or as any other suitable unit of processing. If techniques described herein are implemented as multiple functional facilities, each functional facility may be implemented in its own way; all need not be implemented the same way. Additionally, these functional facilities may be executed in parallel and/or serially, as appropriate, and may pass information between one another using a shared memory on the computer(s) on which they are executing, using a message passing protocol, or in any other suitable way.

Generally, functional facilities include routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data types. Typically, the functionality of the functional facilities may be combined or distributed as desired in the systems in which they operate. In some implementations, one or more functional facilities carrying out techniques herein may together form a complete software package. These functional facilities may, in alternative embodiments, be adapted to interact with other, unrelated functional facilities and/or processes, to implement a software program application.

Some exemplary functional facilities have been described herein for carrying out one or more tasks. It should be appreciated, though, that the functional facilities and division of tasks described is merely illustrative of the type of functional facilities that may implement the exemplary techniques described herein, and that embodiments are not limited to being implemented in any specific number, division, or type of functional facilities. In some implementations, all functionality may be implemented in a single functional facility. It should also be appreciated that, in some implementations, some of the functional facilities described herein may be implemented together with or separately from others (i.e., as a single unit or separate units), or some of these functional facilities may not be implemented.

Computer-executable instructions implementing the techniques described herein (when implemented as one or more functional facilities or in any other manner) may, in some embodiments, be encoded on one or more computer-readable media to provide functionality to the media. Computer-readable media include magnetic media such as a hard disk drive, optical media such as a Compact Disk (CD) or a Digital Versatile Disk (DVD), a persistent or non-persistent solid-state memory (e.g., Flash memory, Magnetic

RAM, etc.), or any other suitable storage media. Such a computer-readable medium may be implemented in any suitable manner, including as computer-readable storage media 506 of FIG. 5 described below (i.e., as a portion of a computing device 500) or as a stand-alone, separate storage medium. As used herein, “computer-readable media” (also 5 called “computer-readable storage media”) refers to tangible storage media. Tangible storage media are non-transitory and have at least one physical, structural component. In a “computer-readable medium,” as used herein, at least one physical, structural component has at least one physical property that may be altered in some way during a process of creating the medium with embedded information, a process of recording 10 information thereon, or any other process of encoding the medium with information. For example, a magnetization state of a portion of a physical structure of a computer-readable medium may be altered during a recording process.

In some, but not all, implementations in which the techniques may be embodied as computer-executable instructions, these instructions may be executed on one or more 15 suitable computing device(s) operating in any suitable computer system, including the exemplary computer system of FIG. 1, or one or more computing devices (or one or more processors of one or more platforms or computing devices) may be programmed to execute the computer-executable instructions. A computing device or processor may be programmed to execute instructions when the instructions are stored in a manner 20 accessible to the computing device or processor, such as in a data store (e.g., an on-chip cache or instruction register, a computer-readable storage medium accessible via a bus, a computer-readable storage medium accessible via one or more networks and accessible by the device/processor, etc.). Functional facilities comprising these computer-executable instructions may be integrated with and direct the operation of a single multi- 25 purpose programmable digital computing device, a coordinated system of two or more multi-purpose computing device sharing processing power and jointly carrying out the techniques described herein, a single computing device or coordinated system of computing devices (co-located or geographically distributed) dedicated to executing the techniques described herein, one or more Field-Programmable Gate Arrays (FPGAs) for 30 carrying out the techniques described herein, or any other suitable system.

FIG. 13 illustrates one exemplary implementation of a computing device in the form of a computing device 1300 that may be used in a system implementing techniques described herein, although others are possible. It should be appreciated that FIG. 13 is

intended neither to be a depiction of necessary components for a computing device to operate in accordance with the principles described herein, nor a comprehensive depiction.

Computing device 1300 may comprise at least one processor 1302, a network
5 adapter 1304, and computer-readable storage media 1306. Computing device 1300 may be, for example, an imaging platform, a desktop or laptop personal computer, a personal digital assistant (PDA), a smart mobile phone, a server, or any other suitable computing device. Network adapter 1304 may be any suitable hardware and/or software to enable the computing device 1300 to communicate wired and/or wirelessly with any other
10 suitable computing device over any suitable computing network. The computing network may include wireless access points, switches, routers, gateways, and/or other networking equipment as well as any suitable wired and/or wireless communication medium or media for exchanging data between two or more computers, including the Internet. Computer-readable media 1306 may be adapted to store data to be processed and/or
15 instructions to be executed by processor 1302. Processor 1302 enables processing of data and execution of instructions. The data and instructions may be stored on the computer-readable storage media 1306.

The data and instructions stored on computer-readable storage media 1306 may comprise computer-executable instructions implementing techniques which operate
20 according to the principles described herein. In the example of FIG. 13, computer-readable storage media 1306 stores computer-executable instructions implementing various facilities and storing various information as described above. Computer-readable storage media 1306 may store the sequence of images 1308 acquired by the imaging platform, tracking data 1310 generated based on analysis of the images and/or
25 quantitative descriptors 1310 determined based on the tracking data, or other information determined from analysis of images. Storage media 1306 may also store an analysis facility 1312 that may perform all, some, or any combination of the functionalities described above with respect to image analysis, identification of organelles, determination of a status of axonal transport, and/or determination of effectiveness of a
30 compound/pharmaceutical.

While not illustrated in FIG. 13, a computing device may additionally have one or more components and peripherals, including input and output devices. These devices can be used, among other things, to present a user interface. Examples of output devices

that can be used to provide a user interface include printers or display screens for visual presentation of output and speakers or other sound generating devices for audible presentation of output. Examples of input devices that can be used for a user interface include keyboards, and pointing devices, such as mice, touch pads, and digitizing tablets.

- 5 As another example, a computing device may receive input information through speech recognition or in other audible format.

Illustrative Compounds

- In some aspects, the systems described herein can be employed to identify
- 10 compounds that increase organelle (*e.g.*, mitochondrial) motility. Thus, in some embodiments, provided is a compound identified using the using a system described herein that increases or promotes organelle motility. In some embodiments, the compound increases mitochondrial motility. “Motility” as used herein refers to the movement of organelles (*e.g.*, mitochondria) throughout the cell. “Motility” may also be
- 15 refer to organelle “trafficking” within a cell. In general, motility refers to the ability of organelles (*e.g.*, mitochondria) to move (*e.g.*, redistribute) throughout the cell by interacting with the cellular cytoskeleton. For example, in order to generate the appropriate cellular response to a stimulus or cellular demand, the cytoskeleton employs a complex communication network involving various linker and motor proteins, such as
- 20 tubulin, actin, myosin, dynein, and kinesin. As a specific example, animal cell mitochondria use microtubules to travel long distances and actin filaments for short distances (see, *e.g.*, Wu M., *et al.* (2013) Structural and biomechanical basis of mitochondrial movement in eukaryotic cells. *Int J Nanomed* 8, 4033–4042; which is incorporated by reference herein). Due to their unique cell morphology and function,
- 25 neurons pose an interesting challenge for mitochondrial motility and distribution. Indeed, the regions of neurons that have the highest demand for mitochondrial ATP are the synapses of neurons, which are located at the extremities of neuronal cells, often times millimeters, centimeters, or even meters away from the cell body and nucleus of the neuronal cell (See, *e.g.*, Schwarz T.L. (2013) Mitochondrial Trafficking in Neurons. *Cold Spring Harb Perspec Biol* 5, a011304; which is incorporated herein by reference in
- 30 its entirety).

Disruption of mitochondrial distribution can lead to a variety of neurological diseases, such as, for example, Alzheimer’s, Parkinson’s, and Huntington’s disease (See,

e.g., Woods L.C., *et al.* (2016) Microtubules are essential for Mitochondrial Dynamics-Fission, Fusion, and Motility-in *Dictyostelium discoideum*. *Front Cell Dev Bio* 4, doi: 10.3389/fcell.2016.00019; which is incorporated herein by reference in its entirety).

Thus, in some aspects, provided herein are compounds identified using a system

5 described herein, wherein the compound is administered to a subject in need thereof in a sufficient amount to treat a disease or disorder associated with decreased mitochondrial motility. In some embodiments, the disease is a neurological disease. In some embodiments, the compound crosses the blood brain barrier (BBB). See, *e.g.*, Banks W.A. (2009) Characteristics of compounds that cross the blood brain barrier. *BMC*
10 *Neurol* 9, S3; which is incorporated herein by reference in its entirety. Without wishing to be bound by any particular theory, compounds that cross the blood brain barrier are especially useful in treating diseases localized to the brain and/or central nervous system (CNS) (*e.g.*, a neurological disease).

An “effective amount” of a compound described herein refers to an amount
15 sufficient to elicit the desired biological response. An effective amount of a compound described herein may vary depending on such factors as the desired biological endpoint, the pharmacokinetics of the compound, the condition being treated, the mode of administration, and the age and health of the subject. In certain embodiments, an effective amount is a therapeutically effective amount. In certain embodiments, an
20 effective amount is a prophylactically effective amount. In certain embodiments, an effective amount is the amount of a compound described herein in a single dose. In certain embodiments, an effective amount is the combined amounts of a compound described herein in multiple doses. In certain embodiments, an effective amount is an amount sufficient for increasing or promoting mitochondrial motility (*e.g.*, increasing
25 mitochondrial trafficking). In certain embodiments, an effective amount is an amount sufficient for inhibiting one or more proteases. In certain embodiments, an effective amount is an amount sufficient for inhibiting tripeptidyl-peptidase 1 (TPP1). In certain embodiments, an effective amount is an amount sufficient for inhibiting one or more kinases. In certain embodiments, the kinase is Aurora B kinase. In certain embodiments,
30 the kinase is Aurora B kinase and Aurora A kinase. In certain embodiments, an effective amount is an amount sufficient for treating or preventing a disease. In certain embodiments, an effective amount is an amount sufficient for treating or preventing a neurological disease.

A “therapeutically effective amount” of a compound described herein is an amount sufficient to provide a therapeutic benefit in the treatment of a condition or to delay or minimize one or more symptoms associated with the condition. A therapeutically effective amount of a compound means an amount of therapeutic agent, alone or in combination with other therapies, which provides a therapeutic benefit in the treatment of the condition. The term “therapeutically effective amount” can encompass an amount that improves overall therapy, reduces or avoids symptoms, signs, or causes of the condition, and/or enhances the therapeutic efficacy of another therapeutic agent. In certain embodiments, a therapeutically effective amount is an amount sufficient for treating a disease associated with reduced mitochondrial motility. In certain
10 embodiments, a therapeutically effective amount is an amount sufficient for treating a neurological disease.

A “prophylactically effective amount” of a compound described herein is an amount sufficient to prevent a condition, or one or more symptoms associated with the condition or prevent its recurrence. A prophylactically effective amount of a compound means an amount of a therapeutic agent, alone or in combination with other agents, which provides a prophylactic benefit in the prevention of the condition. The term “prophylactically effective amount” can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent. In
20 certain embodiments, a prophylactically effective amount is an amount sufficient for preventing a disease associated with reduced mitochondrial motility. In certain embodiments, a prophylactically effective amount is an amount sufficient for preventing a neurological disease.

As used herein the term “inhibit” or “inhibition” in the context of enzymes, for example, in the context of a protease and/or a kinase, refers to a reduction in the activity of the protease and/or kinase. In some embodiments, the term refers to a reduction of the level of enzyme activity, *e.g.*, protease activity and/or kinase activity, to a level that is statistically significantly lower than an initial level, which may, for example, be a baseline level of enzyme activity *e.g.*, baseline protease activity and/or baseline kinase
30 activity. In some embodiments, the term refers to a reduction of the level of protease activity to a level that is statistically significantly lower than an initial level, which may, for example, be a baseline level of protease activity. In some embodiments, the term refers to a reduction of the level of protease activity to a level that is less than 75%, less

than 50%, less than 40%, less than 30%, less than 25%, less than 20%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, less than 0.01%, less than 0.001%, or less than 0.0001% of an initial level, which may, for example, be a baseline level of protease activity. In some embodiments, the term refers to a reduction of the level of kinase activity to a level that is statistically significantly lower than an initial level, which may, for example, be a baseline level of kinase activity. In some embodiments, the term refers to a reduction of the level of kinase activity to a level that is less than 75%, less than 50%, less than 40%, less than 30%, less than 25%, less than 20%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, less than 0.01%, less than 0.001%, or less than 0.0001% of an initial level, which may, for example, be a baseline level of kinase activity.

The term “neurological disease” refers to any disease of the nervous system, including diseases that involve the central nervous system (brain, brainstem and cerebellum), the peripheral nervous system (including cranial nerves), and the autonomic nervous system (parts of which are located in both central and peripheral nervous system). Neurodegenerative diseases refer to a type of neurological disease marked by the loss of nerve cells, including, but not limited to, Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, tauopathies (including frontotemporal dementia), and Huntington’s disease. Examples of neurological diseases include, but are not limited to, headache, stupor and coma, dementia, seizure, sleep disorders, trauma, infections, neoplasms, neuro-ophthalmology, movement disorders, demyelinating diseases, spinal cord disorders, and disorders of peripheral nerves, muscle and neuromuscular junctions. Addiction and mental illness, include, but are not limited to, bipolar disorder and schizophrenia, are also included in the definition of neurological diseases. Further examples of neurological diseases include acquired epileptiform aphasia; acute disseminated encephalomyelitis; adrenoleukodystrophy; agenesis of the corpus callosum; agnosia; Aicardi syndrome; Alexander disease; Alpers’ disease; alternating hemiplegia; Alzheimer’s disease; amyotrophic lateral sclerosis; anencephaly; Angelman syndrome; angiomas; anoxia; aphasia; apraxia; arachnoid cysts; arachnoiditis; Arnold-Chiari malformation; arteriovenous malformation; Asperger syndrome; ataxia telangiectasia; attention deficit hyperactivity disorder; autism; autonomic dysfunction; back pain; Batten

disease; Behcet's disease; Bell's palsy; benign essential blepharospasm; benign focal; amyotrophy; benign intracranial hypertension; Binswanger's disease; blepharospasm; Bloch Sulzberger syndrome; brachial plexus injury; brain abscess; brain injury; brain tumors (including glioblastoma multiforme); spinal tumor; Brown-Sequard syndrome;

5 Canavan disease; carpal tunnel syndrome (CTS); causalgia; central pain syndrome; central pontine myelinolysis; cephalic disorder; cerebral aneurysm; cerebral arteriosclerosis; cerebral atrophy; cerebral gigantism; cerebral palsy; Charcot-Marie-Tooth disease; chemotherapy-induced neuropathy and neuropathic pain; chemotherapy-induced peripheral neuropathy (CIPN); Chiari malformation; chorea; chronic

10 inflammatory demyelinating polyneuropathy (CIDP); chronic pain; chronic regional pain syndrome; Coffin Lowry syndrome; coma, including persistent vegetative state; congenital facial diplegia; corticobasal degeneration; cranial arteritis; craniosynostosis; Creutzfeldt-Jakob disease; cumulative trauma disorders; Cushing's syndrome; cytomegalic inclusion body disease (CIBD); cytomegalovirus infection; dancing eyes-

15 dancing feet syndrome; Dandy-Walker syndrome; Dawson disease; De Morsier's syndrome; Dejerine-Klumpke palsy; dementia; dermatomyositis; diabetic neuropathy; diffuse sclerosis; dysautonomia; dysgraphia; dyslexia; dystonias; early infantile epileptic encephalopathy; empty sella syndrome; encephalitis; encephaloceles; encephalotrigeminal angiomas; epilepsy; Erb's palsy; essential tremor; Fabry's

20 disease; Fahr's syndrome; fainting; familial spastic paralysis; febrile seizures; Fisher syndrome; Friedreich's ataxia; frontotemporal dementia and other "tauopathies"; Gaucher's disease; Gerstmann's syndrome; giant cell arteritis; giant cell inclusion disease; globoid cell leukodystrophy; Guillain-Barre syndrome; HTLV-1 associated myelopathy; Hallervorden-Spatz disease; head injury; headache; hemifacial spasm;

25 hereditary spastic paraplegia; hereditary ataxia; hereditary polyneuritis; herpes zoster ophthalmicus; herpes zoster; Hirayama syndrome; HIV-associated dementia and neuropathy (see also neurological manifestations of AIDS); holoprosencephaly; Huntington's disease and other polyglutamine repeat diseases; hydranencephaly; hydrocephalus; hypercortisolism; hypoxia; immune-mediated encephalomyelitis; inclusion body myositis; incontinentia

30 pigmenti; infantile; phytanic acid storage disease; Infantile Refsum disease; infantile spasms; inflammatory myopathy; intracranial cyst; intracranial hypertension; Joubert syndrome; Kearns-Sayre syndrome; Kennedy disease; Kinsbourne syndrome; Klippel Feil syndrome; Krabbe disease; Kugelberg-Welander disease; kuru; Lafora disease;

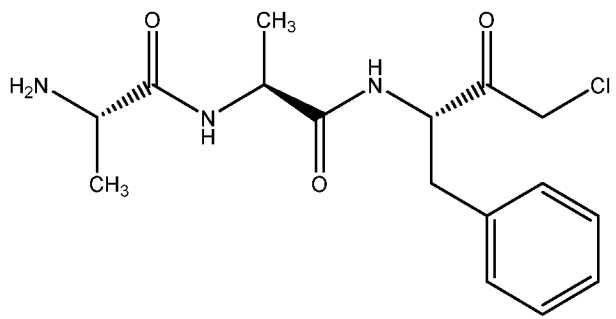
Lambert-Eaton myasthenic syndrome; Landau-Kleffner syndrome; lateral medullary (Wallenberg) syndrome; learning disabilities; Leigh's disease; Lennox-Gastaut syndrome; Lesch-Nyhan syndrome; leukodystrophy; Lewy body dementia; lissencephaly; locked-in syndrome; Lou Gehrig's disease (aka motor neuron disease or amyotrophic lateral sclerosis); lumbar disc disease; Lyme disease-neurological sequelae; Machado-Joseph disease; macrencephaly; megalencephaly; Melkersson-Rosenthal syndrome; Menieres disease; meningitis; Menkes disease; metachromatic leukodystrophy; microcephaly; migraine; Miller Fisher syndrome; mini-strokes; mitochondrial myopathies; Mobius syndrome; monomelic amyotrophy; motor neuron disease; moyamoya disease; mucopolysaccharidoses; multi-infarct dementia; multifocal motor neuropathy; multiple sclerosis and other demyelinating disorders; multiple system atrophy with postural hypotension; muscular dystrophy; myasthenia gravis; myelinoclastic diffuse sclerosis; myoclonic encephalopathy of infants; myoclonus; myopathy; myotonia congenita; narcolepsy; neurofibromatosis; neuroleptic malignant syndrome; neurological manifestations of AIDS; neurological sequelae of lupus; neuromyotonia; neuronal ceroid lipofuscinosis; neuronal migration disorders; Niemann-Pick disease; O'Sullivan-McLeod syndrome; occipital neuralgia; occult spinal dysraphism sequence; Ohtahara syndrome; olivopontocerebellar atrophy; opsoclonus myoclonus; optic neuritis; orthostatic hypotension; overuse syndrome; paresthesia; Parkinson's disease; paramyotonia congenita; paraneoplastic diseases; paroxysmal attacks; Parry Romberg syndrome; Pelizaeus-Merzbacher disease; periodic paralyses; peripheral neuropathy; painful neuropathy and neuropathic pain; persistent vegetative state; pervasive developmental disorders; photic sneeze reflex; phytanic acid storage disease; Pick's disease; pinched nerve; pituitary tumors; polymyositis; porencephaly; Post-Polio syndrome; postherpetic neuralgia (PHN); postinfectious encephalomyelitis; postural hypotension; Prader-Willi syndrome; primary lateral sclerosis; prion diseases; progressive; hemifacial atrophy; progressive multifocal leukoencephalopathy; progressive sclerosing poliodystrophy; progressive supranuclear palsy; pseudotumor cerebri; Ramsay-Hunt syndrome (Type I and Type II); Rasmussen's Encephalitis; reflex sympathetic dystrophy syndrome; Refsum disease; repetitive motion disorders; repetitive stress injuries; restless legs syndrome; retrovirus-associated myelopathy; Rett syndrome; Reye's syndrome; Saint Vitus Dance; Sandhoff disease; Schilder's disease; schizencephaly; septo-optic dysplasia; shaken baby syndrome; shingles; Shy-Drager

syndrome; Sjogren's syndrome; sleep apnea; Soto's syndrome; spasticity; spina bifida; spinal cord injury; spinal cord tumors; spinal muscular atrophy; stiff-person syndrome; stroke; Sturge-Weber syndrome; subacute sclerosing panencephalitis; subarachnoid hemorrhage; subcortical arteriosclerotic encephalopathy; sydenham chorea; syncope; 5 syringomyelia; tardive dyskinesia; Tay-Sachs disease; temporal arteritis; tethered spinal cord syndrome; Thomsen disease; thoracic outlet syndrome; tic douloureux; Todd's paralysis; Tourette syndrome; transient ischemic attack; transmissible spongiform encephalopathies; transverse myelitis; traumatic brain injury; tremor; trigeminal neuralgia; tropical spastic paraparesis; tuberous sclerosis; vascular dementia (multi- 10 infarct dementia); vasculitis including temporal arteritis; Von Hippel-Lindau Disease (VHL); Wallenberg's syndrome; Werdnig-Hoffman disease; West syndrome; whiplash; Williams syndrome; Wilson's disease; and Zellweger syndrome.

In some embodiments, the neurological disease is Alzheimer's disease. In some embodiments, the neurological disease is Parkinson's disease. In some embodiments, the 15 neurological disease is multiple sclerosis. In some embodiments, the neurological disease is amyotrophic lateral sclerosis (ALS). In some embodiments, the neurological disease is chemotherapy-induced peripheral neuropathy (CIPN).

In some embodiments, the compound is a protease inhibitor. The term "protease" refers to any enzyme capable of hydrolyzing a peptide bond. In general, a proteases 20 catalyzes the hydrolysis of peptide bonds (i.e., digests the protein) through a unique mechanism based on the catalytic residue present in the active site of the protease. Exemplary, non-limiting proteases and their catalytic residues are serine proteases, which use a serine alcohol, cysteine proteases, which use a cysteine thiol, threonine proteases, which use a threonine secondary alcohol, aspartic proteases, which use an aspartate 25 carboxylic acid, glutamic proteases, which use a glutamate carboxylic acid, metalloproteases, which use a metal (e.g., zinc), and asparagine peptide lyases, which use an asparagine to perform an elimination reaction and do not require water. In some embodiments, the protease is a serine protease. In some embodiments, the serine protease is tripeptidyl-peptidase 1 (TPP1). TPP1 is a member of the sedolisin family of serine 30 proteases, and functions in the lysosome to cleave N-terminal tripeptides from substrates (e.g., proteins or peptides). Mutations in the *TPP1* gene, which lead to dysfunctional TPP1 proteins, have been implicated in lysosomal storage disorders, such as neuronal ceroid lipofuscinoses (NCLs), which is characterized by accumulation of storage

material and progressive neurodegeneration (see, e.g., Getty AL and Pearce DA (2011) Interactions of the proteins of neuronal ceroid lipofuscinosis: clues to function. *Cell Mol Life Sci* 68, 453-474; which is incorporated by reference herein). Without wishing to be bound by any particular theory, inhibition of TTP1 appears to increase mitochondrial
5 motility in neurons (FIG. 6). In some embodiments, the compound is:

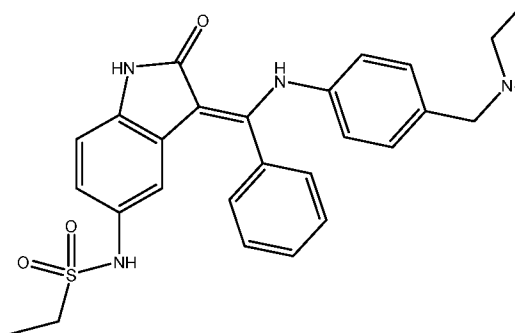


(Ala-Ala-Phe-chloromethylketone)

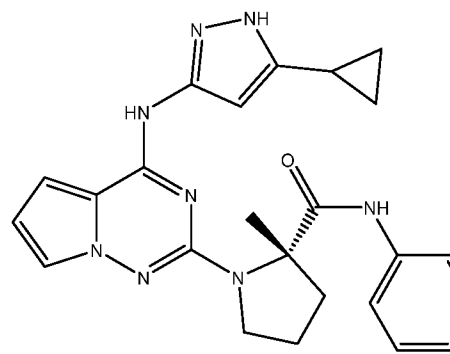
or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof.

In some embodiments, the compound is a kinase inhibitor. The term “kinase”
10 refers to any enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecule(e.g., adenosine triphosphate (ATP)) to specific substrates. Kinases are regulators of a variety of cellular processes, including many signal transduction pathways and coordination of complex functions such as the cell cycle. Kinases are generally classified into groups based on their function, for example, AGC
15 kinases (e.g., protein kinase A (PKA), protein kinase C (PKC), and protein kinase G (PKG)), CaM (calcium/calmodulin dependent) kinases, CK1 (casein kinase 1), CMGC kinases (e.g., CDK, MAPK, GSK3, and CLK kinases), STE kinases (e.g., homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases), TK (tyrosine kinases), TKL (tyrosine-like kinases), and STK (serine/tyrosine kinases). In some embodiments, the kinase is a
20 serine/tyrosine kinase. A “serine/threonine protein kinase” is a kinase enzyme that phosphorylates the OH group of serine or threonine. Serine/tyrosine kinases include, for example, the Aurora kinase family. Without wishing to be bound by any particular theory, serine/tyrosine kinases are essential for cell proliferation, and thus have generated significant interest in the field of cancer research (see, e.g., Giet R and Prigent C (1999)
25 Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *Journal of Cell Science* 112, pp. 3591–3601). Aurora kinases include Aurora A (also known as Aurora 2), Aurora B (also known as Aurora 1), and Aurora C. In some embodiments, the compound is an Aurora B inhibitor. In some embodiments, the

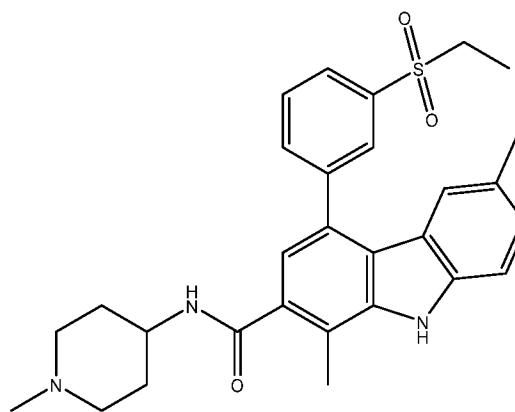
compound is an Aurora B inhibitor, while the compound does not inhibit Aurora A (i.e., a specific Aurora B inhibitor). In some embodiments, the compound inhibits Aurora B and Aurora A. In some embodiments, the compound is a pan-Aurora inhibitor, i.e., the compound inhibits both Aurora A and Aurora B kinase. In some embodiments, the compound is not an Aurora A specific kinase inhibitor. While Aurora kinase inhibitors are known to induce apoptosis via an unspecified “mitochondrial pathway” (Dar A.A., *et al.* (2010) Aurora kinase inhibitors – rising stars in cancer therapeutics? *Mol Cancer Ther* 9, 268), their role as regulators of mitochondrial motility (e.g., trafficking) is provided by the present disclosure. Without wishing to be bound by any particular theory, inhibition of Aurora B appears to increase mitochondrial motility in neurons (FIG. 12A-12F). In some embodiments, the compound is:



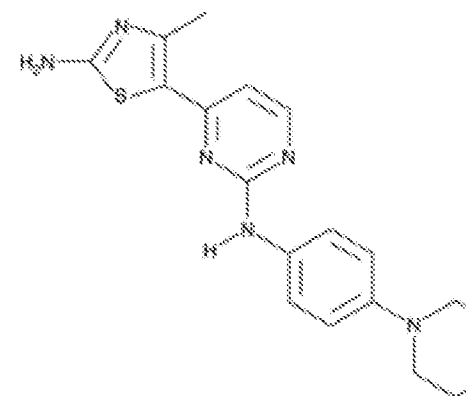
(Hesperadin),



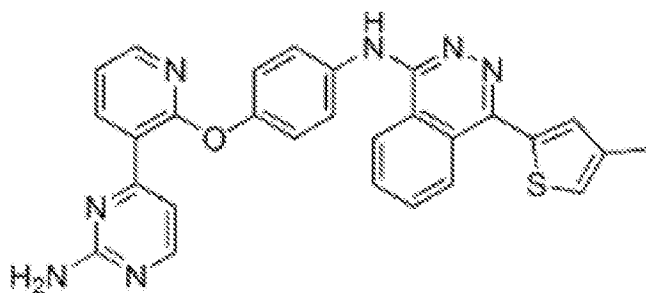
(BMS-754807),



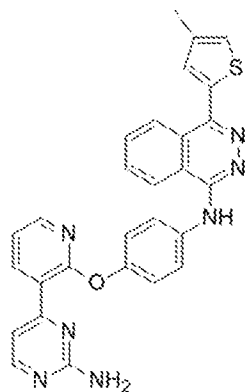
(TAK-901),



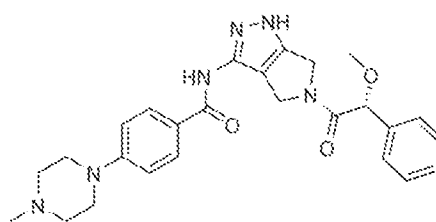
(CYC116),



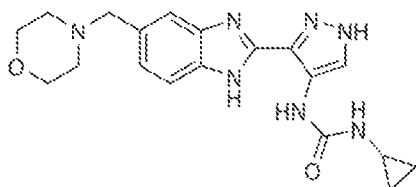
(AMG-900),



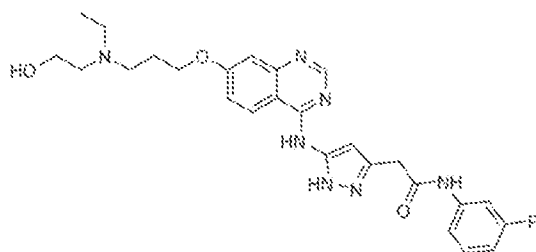
(ESK105),



(ESK106),



(ESK107),



(Barasertib),

or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof.

Example of Enhancers of Mitochondrial Transport; Biological Assays of Exemplary Compounds

In order that the present disclosure may be more fully understood, the following examples are set forth. The synthetic and biological examples described in this

application are offered to illustrate the compounds, pharmaceutical compositions, and methods provided herein and are not to be construed in any way as limiting their scope.

Example 1. Compound Screening Identifies Six Enhancers of Mitochondrial Transport.

5 Two chemical libraries were screened: the Biomol collection (480 compounds) and the Selleck Bioactive collection (2,800 compounds). Both libraries contain well-characterized, potent compounds covering a large portion of what are considered to be druggable targets (see methods). Each chemical library was screened twice, and an average Z-score of 3 or higher in both rounds of screening was taken as the threshold for
10 each descriptor to qualify a compound as a hit. KS of integrated distance was used as the descriptor for Z-score calculations. During the screen, calcimycin was used as a positive control, which consistently yielded an overall Z-prime value of 0.5 or higher.

The distribution of Z-scores, as presented in Figures 20A and 20B, identified 38 suppressors and 8 enhancers of motility. Suppressors covered a variety of chemical
15 classes (Figure 21A), but all but two suppressing compounds either were known to depolymerize microtubules (vincristine and vinblastine) or significantly decreased mitochondrial membrane potential as determined from TMRM intensity (Figure 21B). The latter are likely to suppress motility by depleting axonal ATP or activating the PINK1/Parkin pathway. The compounds that did not affect TMRM or microtubules were
20 NMS-873, a VCP/p97 inhibitor (35) and NH125, a regulator of eEF-2 kinase (36, 37) (Figures 21C and 21D). At present it is unclear how these compounds suppress mitochondrial transport.

The focus was on the compounds that increased motility. Two actin depolymerizers (Latrunculin B and Cytochalasin B), three kinase inhibitors (BMS-
25 754807, Hesperadin and TAK-901) and one peptidase inhibitor (AAF-CMK) were found and subsequently confirmed by retesting (Figure 21C). Two other initial positives failed upon retesting, paclitaxel and the bioactive lipid 24(S)-hydroxycholesterol. Dose response curves with the six active enhancers showed that all compounds acted at sub-micromolar concentrations with the exception of AAF-CMK, which acted at low
30 micromolar concentrations (Figures 21D to 21I). F-actin has been observed previously to inhibit axonal transport of mitochondria (26) and thus identification of Latrunculin B and Cytochalasin B validated the screen.

TPP1 Is a Negative Regulator of Mitochondrial Motility in Hippocampal Axons

AAF-CMK is a potent inhibitor of two tripeptidyl peptidases (Figure 22A), the lysosomal enzyme Tripeptidyl Peptidase 1 (TPP1) (38) and the post-proteasome exopeptidase tripeptidyl peptidase II (TPPII) (39). AAF-CMK also marginally inhibits the chymotrypsin like activity of the proteasome. To distinguish among these potential targets available small molecule inhibitors were used for each and shRNA against TPP1 and TPP2. TPP2 was excluded as the relevant target because an effective TPP2 shRNA (Figure 23A) neither enhanced mitochondrial transport nor occluded the effect of AAF-CMK (Figures 22B and 22C). Furthermore Butabindide, a selective inhibitor of TPP2 (40), failed to mimic the efficacy of AAF-CMK (Figure 22D). The contribution of the proteasome was similarly ruled out because inhibitors of the chymotrypsin-like activity of the proteasome such as carfilzomib (Figure 22E) did not enhance mitochondrial motility. In contrast, two shRNA against TPP1 (Figure 23B) potently enhanced mitochondrial transport and occluded any further effect of AAF-CMK (Figures 22F and 22G). Conversely, overexpression of TPP1 suppressed mitochondrial movement (Figure 22H). Thus, the lysosomal enzyme TPP1 is a novel negative regulator of mitochondrial trafficking, although the mechanism by which it influences mitochondria remains unclear.

Aurora B Regulates Mitochondrial Motility in Hippocampal Axons

The three kinase inhibitors (BMS-754807, Hesperadin and TAK-901) share targets within the Aurora kinase family (Aurora A, B, and C). BMS-754807 was originally developed as an IGFR inhibitor, although it can inhibit Aurora B in low nanomolar range. Two selective inhibitors of IGFR (Picopodophyllin and OSI-906) were included in the screen but did not enhance mitochondrial motility; the Aurora family was therefore focused on as the likely relevant cellular substrate for these hits. To test this hypothesis first the initial screen was re-examined to determine the effects of all the Aurora family inhibitors that had been present in the libraries. Three additional compounds, Danusertib, CYC116 and AMG-900 (Figures 25A and 25B) had enhanced mitochondrial transport in both replicates although they had not met the original selection criteria because in at least one of the replicates the enhancement of motility did not reach the threshold of Z score=3. All 6 positives were either pan-Aurora inhibitors or Aurora B (AurKB) inhibitors. Aurora C (AurKC) is expressed primarily in the germline

and so shRNAs were used against Aurora A (AurKA) and AurKB (Figure 25C) to determine the relevant cellular target. Knock-down of AurKB, but not of AurKA, both mimicked and occluded the effect of AurKB inhibitor Hesperadin on percent motile (Figures 24A to 24B) and integrated distance (Figures 24E to 24G). Furthermore, 5 Barasertib, a selective AurKB inhibitor, also enhanced mitochondrial transport in a dose-dependent manner (Figure 24A), while the more selective AurKA inhibitors in the compound screen did not (Figures 25A and 25B). The effect of AurKB knock-down on mitochondrial transport was reversed by overexpression of an shRNA-resistant AurKB construct, but not by the kinase-dead form of AurKB (Figure 24I). Conversely, 10 AurKB overexpression suppressed mitochondrial transport (Figures 24J to 24L). Thus, AurKB is a novel negative regulator of mitochondrial transport in hippocampal neurons. Neither Hesperadin nor knock-down of AurKB altered the motility of late endosomes as determined by tracking of late endosomes (Figures 26A to 26D).

15 **Exemplary Hit Compounds Enhance Mitochondrial Transport in Human Cortical Neurons and Alleviate Mitochondrial Transport Defects in ALS Motor Neurons**

It was next asked if the screen was able to identify mechanisms relevant to axonal transport in human neurons. It was first tested whether the pathways identified in the 20 screen were conserved in neurons differentiated from iPSCs with a protocol that generates ~90% pure cortical neurons ((41) and Figure 27A). Neurons were transduced with lentivirus encoding Mito-DsRed, and treated with a representative compound of each class of hit (Latrunculin, Hesperadin, and AAF-CMK) before automated imaging and analysis with MAPS. All three compounds significantly enhanced mitochondrial 25 transport (Figures 27B and 27C). Subsequent kymography of axons revealed that inhibition of AurKB increased both anterograde and retrograde mitochondrial motility (Figures 27D and 27E). Thus, the three classes of hits from the screen (inhibitors of F-actin, TPP1, and AurKB) appear to act on mitochondrial motility through mechanisms that are conserved between rat hippocampal and human cortical neurons.

30 Previous studies have demonstrated that the dominant ALS mutation SOD1A4V reduces mitochondrial transport in motoneuron axons (5). To determine whether the hit compounds have the potential to restore defective mitochondrial trafficking in diseased neurons, transport in human motor neurons derived from an iPSC line from a SOD1+/A4V patient was examined (18). Motor neurons were differentiated from the

SOD1+/A4V line as well as from the TALEN-corrected isogenic control, transduced neurons with a lentivirus coding for mito-DSred, and performed imaging on the platform followed by MAPS analysis (Figure 27). The automated analysis confirmed the defect in mitochondrial motility that had previously been described in these cells by kymographic analysis (5) (Figures 25G and 25H). Treatment with latrunculin, Hesperadin, and AAF-CMK each increased mitochondrial motility in the +/A4V neurons (Figure 27H). A more detailed analysis by kymography found that AurKB inhibition with exemplary compound Hesperadin restored mitochondrial transport back to the levels of the TALEN-corrected control (Figures 27I and 27J).

A novel approach has been employed to identify exemplary druggable regulators of axonal transport and thereby uncover cellular targets that influence mitochondrial movement. It is shown that 1) axonal transport can be used as an end-point in an image-based screening campaign; 2) three classes of exemplary compounds and their targets can enhance mitochondrial transport in primary neurons; and 3) the targets of exemplary active compounds are functionally conserved in human neurons and can be targeted to reverse mitochondrial transport defects in ALS motor neurons.

The development of MAPS, which combines a tracking algorithm and data analysis package, enabled exploration of the druggable space for regulators of mitochondrial transport. This method allowed precise quantitative description of motility parameters for tens of thousands individual mitochondria per condition, a scale that is orders of magnitude higher in sampling power than kymography can achieve. However, while these features of MAPS allowed the screening of thousands of actives, they do not offer information on the direction of transport, nor can they unambiguously identify specifically axonal regulators because mitochondria traveling along secondary dendrites are included in the analysis; for these parameters, kymography is necessary.

The results of the present screen validate the approach. As expected, mitochondrial motility was arrested by exemplary compounds that depolarized mitochondria and by vincristine and vinblastine, which disrupt microtubules (42). F-actin is known to inhibit mitochondrial trafficking (24, 25, 43) and two exemplary compounds that destabilize the F-actin cytoskeleton were identified as enhancers of motility: latrunculin, which sequesters globular monomeric actin, and cytochalasin, which blocks the addition of G-actin to the growing end of the microfilament. An attractive hypothesis is that mitochondria use F-actin as a structural scaffold onto which they can anchor in

response to particular stop signals. As appropriate for such a function, F-actin is found in periodic rings along the axonal shaft (44) and is highly enriched at nascent presynaptic sites (45). It is important to note, however, that in the present study, as in previous studies, the effect is subtle – it does not mobilize the majority of the stationary mitochondria and much remains to be learned about the relative importance and interplay of different mechanisms that arrest or anchor mitochondria.

The screening methods described herein can have applications beyond the particulars of the present screen. Although the need for large numbers of neurons of consistent quality was chosen to be met by screening on rodent hippocampal neurons, it was confirmed that the present screen identified hits and druggable targets that are also effective on human neurons (Figures 27A to 27C). The screening method is applicable to genetic screens as well as the acute drug effects assayed. MAPS would, for example, be very suitable for using axonal transport as an endpoint in a CRISPR-based genetic screen in which iPSC-progenitor lines are mutated and then differentiated to neurons prior to screening. In addition, the algorithms are applicable to cargos other than mitochondria, as long as the experiment is designed with attention to object density and speeds so as to allow robust automated tracking (30). The screen is also applicable to libraries larger than those tested. However, because the screen involves taking thousands of short time-lapse images from each well, escalation to a larger plate size would need to take into account the delay between the moment a compound is added and the moment where mitochondria are imaged. Larger compound screens would thus require careful design of drug dosage and duration of treatment.

Although the focus was on enhancers of motility, two compounds arrested mitochondria without affecting TMRM intensity and these may point to novel regulatory pathways of mitochondrial motility: NMS-873 is a VCP/p97 inhibitor (35) and NH-125 is a putative regulator of eEF2 kinase (36, 37). VCP/p97 is implicated in mitochondrial clearance by the PINK1/Parkin pathway and in removal of mitofusin (46), but no mechanistic connection of either NMS-873 or NH125 to mitochondrial transport is known at this time.

TPP1 inhibition by AAF-CMK or knockdown by RNAi enhanced mitochondrial motility. TPP1 is a lysosomal enzyme, and loss of function mutations in the gene are causally linked to a familial lysosomal storage disorder (38). How TPP1 regulates mitochondrial transport remains unclear but lysosomal dysfunction and mitochondrial

transport share linkages to Parkinson's disease (47). The pathological consequences of loss of TPP1 activity suggest that it is not itself an attractive therapeutic target in ALS, but further elucidation of the pathway by which it regulates mitochondrial motility might identify targets downstream that did not cause lysosomal storage defects.

5 By both pharmacologic and knockdown methods, it was found that AurKB is a regulator of mitochondrial transport. AurKB has a well-characterized role in orchestrating cell division and cytokinesis, but the role of AurKB in differentiated cells is largely unexplored. Recently, AurKB has been found to be expressed in post-mitotic neurons of the zebrafish brain and spinal cord, where it regulates axonal outgrowth and
10 regeneration (48). AurKB is significantly upregulated upon axotomy (49) and may mediate regenerative sprouting, and yet paradoxically, axon regeneration involves enhanced mitochondrial trafficking (50-52) and yet AurKB decreased mitochondrial traffic in the study presented herein. An intriguing possibility is that AurKB participates in a pathway that positions stationary mitochondria at critical places such as branch
15 points, nascent synapses, and growth cones (28). As such it could act in regenerating axons to capture the increased axonal flux of mitochondria at necessary junctures. Acute pharmacological inhibition of AurKB preferentially stimulated retrograde transport (Figs. 20-23), while sustained inhibition with shRNA promoted both directions (Figs. 20-23). Therefore it is possible that AurKB has a preferred substrate in the retrograde
20 motor and a less direct, or possibly compensatory effect on anterograde movement. Thus it is plausible that AurKB promotes axon regeneration by biasing transport down the axon by suppressing retrograde movement. AurKA has been shown to disrupt the association of kinesin with mitochondria in dividing cells (53), but the mechanism by which AurKB regulates mitochondrial trafficking is presently unclear.

25 Extensive evidence links neurodegeneration to defects in mitochondrial transport, including the evidence presented here for the SOD1A4V/+ neurons. An appealing hypothesis, therefore, is that improving mitochondrial transport and thereby improving the energy supply and Ca²⁺-buffering capacity of axons may prolong the life and function of affected neurons and ameliorate progression of the disease. The
30 particular targets identified here, however, may not be ideal therapeutics: TPP1 inhibition is likely to cause deleterious side effects (38) and Aurora B inhibitors, though investigated for chemotherapeutic purposes have counterindicating toxicities due to their effects on dividing cells. The success of the screen in identifying novel

pathways and in demonstrating that hit compounds can be effective in improving transport in human neurons and in a pathogenic genetic background, would indicate that a broader application of the screening protocol may be a productive line of investigation. The arrest of mitochondria at important sites, such as synapses, is likely to be as
5 important for neuronal function as sufficient levels of trafficking. A drastic increase in mitochondrial motility, as in the case of syntaphilin knockout mice, might therefore diminish mitochondrial supply at crucial sites and this may explain the failure of syntaphilin knockout to be effective in a mouse model of ALS. The ability of the hits identified here to restore normal levels of transport rather than to mobilize most axonal
10 mitochondria (54) maybe a valuable feature of a pharmacological approach.

In summary, a unique exemplary assay was developed that overcomes the inherent difficulty of tracking motile objects in high throughput. The exemplary assay is sensitive and successfully identified both novel and known regulators of axonal transport of mitochondria. The finding that TPP1 and Aurora B kinase play a previously
15 unsuspected role in neurons by regulating mitochondrial trafficking has created an opportunity to explore the cell biological mechanisms of these pathways. Finally, because defective axonal transport in ALS motor neurons is restorable upon treatment with the exemplary hit compounds here identified, tools are now available to test the potential of modulating axonal transport as a neuroprotective intervention.

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Exemplary Methods for Generating Data on Exemplary Compounds in Assays

Plasmids. Mito-DsRed (pDsRed2-Mito) was a gift of G. Hajnoczky. Rab7-GFP was a gift from Richard Pagano (Addgene plasmid #12605). FLAG-TPP1, human AurKB and rat AurKB were purchased from OriGene (9620 Medical Center Drive,
25 Suite 200, Rockville, MD 20850). Synapsin-Mito-DSred was a gift of Kasper Roet. CMV-Mito-DsRed and synapsin-Mito-DsRed expressing lentivirus were produced at the BCH viral Core. shRNA expressing lentivirus and coding plasmids were obtained from Origene. Rat TPP1, TPP2 and AurKB shRNAs were selected for their efficacy based on Western blots (Figures 23A, 23B, and 25C). Transfection efficiency of shRNAs was
30 verified by fluorescent microscopy, as the plasmids also express eGFP. Co-transfection efficiency with Mito-DsRed was almost 90% in primary neuronal cultures. All plasmids were sequence-verified prior to use.

Western blotting. Cells were harvested in lysis buffer (0.05M Tris pH7.5, 150mM NaCl, 1% Triton, 0.1mM PMSF, 1:500 Calbiochem protease inhibitor cocktail set III). For quantification, membranes were incubated with fluorescent secondary antibodies and images were acquired on Typhoon laser scanner (GE Healthcare, 5 Wilmington, MA) within the linear range. If not indicated, western blots are representative of at least three independent transfections.

Compound screen. E18 rat hippocampal neurons were routinely seeded at 30,000 cells/well on Greiner Cellstar 96 well plates, cat# 655090 (4238 Capital Drive, Monroe, NC 28110) coated with 20 µg/mL poly-L-Lysine (Sigma Aldrich) and 3.5 10 µg/mL laminin (ThermoFisher Scientific), and maintained in Neurobasal medium (ThermoFisher Scientific) supplemented with B27 (ThermoFisher Scientific), L-glutamine, and penicillin/streptomycin. Cells were transfected with Mito-DSred at DIV6-7 using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA 02451) and imaged at DIV8-9. Small molecule libraries were obtained from ICCB-Longwood 15 (iccb.med.harvard.edu/). Compounds were pin-transferred into Greiner 384 well plates (cat#784201) at the ICCB-Longwood facility and immediately added to the cells using Agilent Bravo Liquid Handling Platform (Agilent, 5301 Stevens Creek Blvd, Santa Clara, CA 95051). Cells were incubated with compounds at 3 µM for 1 hour before and during time-lapse imaging.

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Time-lapse high-content imaging of mitochondrial transport. Neurons were imaged on an ArrayScan™ XTI imaging platform (ThermoFisher Scientific, Waltham, MA 02451). 30 frames per field were acquired at the maximum speed of 2 Hz. Four fields were imaged per well of a 96-well plate containing 30,000 rat hippocampal 25 neurons/well at DIV08-09 using a 20X objective with 2x2 binning. To minimize well to well differences in exposure to compounds, the fields were imaged according to this sequence: field 1 was imaged in every well before returning to the start of the plate and imaging field 2 in every well, and similarly for fields 3 and 4. Imaging one field per well for 96 wells took ~30 min when acquiring 30 frames at 2Hz in each well. As a result, 30 collecting a series from each of the 4 fields took a total of 2 hours. This protocol was used for the primary screen, compound retesting and dose response curves in Figures 20A to 20I, as well as for all experiments in Figures 22A to 22H, 24A to 24L, and 27A to 27C. During imaging, neurons were maintained in conditioned media at 37C and

5%CO₂. Only in the primary screen, the plate was incubated with Tetramethylrhodamine, methyl ester (TMRM, ThermoFisher Scientific, cat#T668), and with Hoechst dye upon completion of the time-lapse sequence and then live-imaged again to determine mitochondrial membrane potential.

5 **Image Analysis.** In brief, data is processed by two CellProfiler™ pipelines: the first pipeline creates the minimum intensity projections of the time-lapse sequences, and the second pipeline employs the projections to create a mask of the stationary objects and performs the tracking of the motile objects using the Linear Assignment Problem method. The outcome of the Cell Profiler analysis was processed in MATLAB® (MathWorks, 1 Apple Hill Drive, Natick, Ma 01760) using a custom script with a graphic user interface that represents data at the field and plate levels, creates heat-maps, and performs statistical computations such as Z-scores and KS statistics. TMRM levels were analyzed using Image J. Briefly, TMRM-labeled mitochondria were analyzed from areas of the wells lacking Mito-DsRed due to the low transfection efficiency. Cell bodies were outlined based on the Hoechst signal, and the intensity of TMRM per cell was quantified. 100-150 cells were analyzed per compound.

15 **Statistical Analysis.** Statistical analysis was conducted in GraphPad Prism v6.0 and Matlab. Data were represented either using R (R-project.org) or GraphPad Prism v6.0 (GraphPad Software, 7825 Fay Avenue, Suite 230 La Jolla, CA 02037). If not indicated in the figures, **** = $p < 0.0001$; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = $p > 0.05$. Z-scores were calculated for each parameter using the formula $Z = (x - \mu) / \sigma$ for a minimum of 4 wells per condition.

25 **Kymography.** Rat hippocampal neurons were isolated according to standard procedures and cultured as in (21). Briefly, neurons were obtained from E18 embryos, plated at 150 x 103 on 12-mm glass coverslips (Bellco Glass) or on Greiner Cellstar 96 well plates coated and maintained as above. Neurons were transfected at DIV07-8 and imaged at DIV9-10 at 5% CO₂ and 37C using NikonTiE Eclipse 20X. Time-lapse imaging was performed every 2 s for ~300s. Movies were analyzed using the Kymolyzer macro for ImageJ developed by the laboratory (Pekkurnaz and Basu, unpublished). In all kymographs, anterograde motility is to the right, retrograde motility is to the left. All animal procedures were approved by the Institutional Animal Care Committee at the Boston Children's Hospital.

Immunofluorescence. Neurogenin2-induced cortical neurons were fixed with 4% paraformaldehyde (PFA) by adding 50ul 8% PFA to 50ul media and incubating for 20 minutes at room temperature. After three washes with 1X PBS, the cells were blocked with blocking reagent (2% Bovine Serum Albumin, 5% normal goat serum, 0.1% tritonX100 in 1XPBS) for 1 hour at room temperature and immunostained with primary antibody overnight at 4C. The next day, cells were washed three times with 1X PBS before staining with secondary antibodies. After a final three washes with 1X PBS, cells were stored in 1X PBS + 0.02% sodium azide.

Antibodies. The following primary antibodies were used: mouse anti-b3-tubulin Sigma catalog# T8660 at 1:800, a-TPP1 Abcam rabbit #ab96498 at 1:200, Mouse α -Myc, clone 4A6 (EMD Millipore, Billerica, MA, catalog #05724) at 1:1000. The following secondary antibodies and dyes were used: anti-mouse Alexa-Fluor568 ThermoFisher catalog# A11031 at 1:500, Hoechst 33258 ThermoFisher catalog# H3569 at 1:1000.

Compounds. Libraries were obtained from ICCB-L (iccb.med.harvard.edu/compound-libraries). Compounds that were retested were purchased from Sellechem, except Latrunculin B, A and cytochalasin B, which were purchased from Tocris Biosciences. Unless indicated otherwise, compounds were used at 3 μ M and were added one hour before and during time-lapse imaging.

iPSC Culture and Cortical Neuron Differentiation. The iPSC line, GON0515-03 #5, was derived from peripheral blood mononuclear cells of a normal individual using CytoTune-iPS Sendai Reprogramming Kit (ThermoFisher, #A1378001) at the BCH Human Neuron Core. This iPSC line has a normal karyotype and expresses pluripotency markers. iPSCs were maintained in mTeSR-1 media (STEMCELL Technologies, #85850) on Geltrex (ThermoFisher, #A1413301), and passaged about once a week with Gentle Cell Dissociation Reagent (STEMCELL Technologies, #07174). Cortical neurons were differentiated according to a protocol published by Zhang et al. in 2013 with minor modifications described below. iPSCs were treated with Accutase (Innovative Cell Technologies, #AT104) and plated at 90,000 cells/cm² in mTeSR-1 media supplemented with 10uM ROCK inhibitor, Y-27632 (Cayman Chemical, #10005583) on Geltrex-coated 12-well plates. On day 2, no mouse glia cells were added and Ara-C (Sigma-Aldrich, #C1768) was used at a final concentration of 2uM. To label mitochondria in neurons, a lentivirus carrying CMV-Mito-DsRed was

added to the media on day 5, supplemented with 8ug/mL polybrene (Sigma-Aldrich, #TR-1003-G). Mitochondrial motility was assayed after day 14.

iPSC culture and motor neuron differentiation. All cell cultures were maintained at 37 °C, 5% CO₂. Cells tested negative for mycoplasma contamination.

5 iPSCs were grown on Matrigel (BD Biosciences) with mTeSR1 media (Stem Cell Technologies). Culture Medium was changed every 24 hours and cells were passaged by accutase (Innovative Cell Technologies) as required. Transfection with the HB9::GFP reporter was performed as described in (55). A 1kb HB9 promoter fragment (gift from Hynek Wichterle) controlling the expression of myristoylated GFP was inserted into a
10 donor plasmid specific for the AAVS1 locus (Sigma). Subsequently, 2.5 million iPS cells were single-cell dissociated using accutase and electroporated using the Neon transfection system (100ul tip; 1600V Voltage, 20ms Width, 1 Pulse; Life Technologies) with 2 g of AAVS1 ZFN plasmid and 6 g of modified AAVS1 donor plasmid. After nucleofection cells were plated on matrigel with mTeSR1 in the presence of ROCK
15 inhibitor. After 48hrs, puromycin selection was applied and surviving clonal colonies were individually passaged and gDNA was extracted. PCR was used to confirm proper targeting of the cassette. Primer sequences are available upon request. Faithful expression of the reporter was verified using expression of the motor neuron marker Isl1 (Abcam (Ab109517), 1:1000) and the pan-neuronal marker Map2 (Milipore (MAB378),
20 1:1000). Stem cell cultures were differentiated into motor neurons as previously described in Kiskinis et al., 2014. Briefly, iPSCS were dissociated to single cells and plated in suspension in low-adherence flasks (400k/mL), in mTeSR media with 10uM ROCK inhibitor. Media was gradually diluted (50% on day 3 and 100% on day 4) to KOSR (DMEM/F12, 10% KOSR) between days 1-4 and to a neural induction medium
25 (NIM: DMEM/F12 with L-glutamine, NEAA, Heparin (2 ug/mL), N2 supplement (Invitrogen) for days 5-24. From days 1-6 cells were cultured in the presence of SB431542 (10 uM, Sigma Aldrich) and Dorsomorphin (1 uM, Stemgent), and from days 5-24 with BDNF (10 ng/mL, R&D), ascorbic acid (AA, 0.4 ug/mL, Sigma), Retinoic Acid (RA, 1 uM, Sigma) and Smoothed Agonist 1.3 (SAG 1.3, 1 uM, Calbiochem).
30 On day 24 floating cell aggregates were dissociated into single cells with Papain/DNase (Worthington Bio) and HB9::GFP positive cells isolated using fluorescence activated cell sorting (FACS). Subsequently, 30k motor neurons were plated onto poly-D-lysine/laminin-coated 96 well plates together with 30k mouse primary glial cells (P1).

Cell cultures were fed every 2-3 days with complete Neurobasal media containing L-glutamine, NEAA, glutamax, N2 and B27, with BDNF/CNTF/GDNF (10 ng/mL, R&D) and ascorbic acid (0.2 ug/mL, Sigma). After 14 days, the cultures were transduced with synapsin-MitoDSred lentivirus and used for subsequent mitochondrial movement kinetics experiments.

The term “pharmaceutically acceptable salt” refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, Berge *et al.* describe pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences*, 1977, 66, 1-19, incorporated herein by reference. Pharmaceutically acceptable salts of the compounds of this invention include those derived from suitable inorganic and organic acids and bases. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids, such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid, and perchloric acid or with organic acids, such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid, or malonic acid or by using other methods known in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Salts derived from appropriate bases include alkali metal, alkaline earth metal, ammonium, and $N^+(C_{1-4} \text{ alkyl})_4^-$ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations

formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate, and aryl sulfonate.

The term “solvate” refers to forms of the compound, or a salt thereof, that are associated with a solvent, usually by a solvolysis reaction. This physical association may include hydrogen bonding. Conventional solvents include water, methanol, ethanol, acetic acid, DMSO, THF, diethyl ether, and the like. The compounds described herein may be prepared, e.g., in crystalline form, and may be solvated. Suitable solvates include pharmaceutically acceptable solvates and further include both stoichiometric solvates and non-stoichiometric solvates. In certain instances, the solvate will be capable of isolation, for example, when one or more solvent molecules are incorporated in the crystal lattice of a crystalline solid. “Solvate” encompasses both solution-phase and isolatable solvates. Representative solvates include hydrates, ethanولات, and methanولات.

The term “hydrate” refers to a compound that is associated with water. Typically, the number of the water molecules contained in a hydrate of a compound is in a definite ratio to the number of the compound molecules in the hydrate. Therefore, a hydrate of a compound may be represented, for example, by the general formula $R \cdot x H_2O$, wherein R is the compound, and x is a number greater than 0. A given compound may form more than one type of hydrate, including, e.g., monohydrates (x is 1), lower hydrates (x is a number greater than 0 and smaller than 1, e.g., hemihydrates ($R \cdot 0.5 H_2O$)), and polyhydrates (x is a number greater than 1, e.g., dihydrates ($R \cdot 2 H_2O$) and hexahydrates ($R \cdot 6 H_2O$)).

The term “prodrug” refers to a compound that has one or more cleavable groups and become by solvolysis or under physiological conditions the compounds described herein, which are pharmaceutically active *in vivo*. Such examples include, but are not limited to, choline ester derivatives and the like, N-alkylmorpholine esters and the like. Other derivatives of the compounds described herein have activity in both their acid and acid derivative forms, but in the acid sensitive form often offer advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985). Prodrugs include acid derivatives well known to practitioners of the art, such as, for example, esters prepared by reaction of the parent acid with a suitable alcohol, or amides prepared by reaction of the parent acid compound with a substituted or unsubstituted amine, or acid anhydrides,

or mixed anhydrides. Simple aliphatic or aromatic esters, amides, and anhydrides derived from acidic groups pendant on the compounds described herein are particular prodrugs. In some cases it is desirable to prepare double ester type prodrugs such as (acyloxy)alkyl esters or ((alkoxycarbonyl)oxy)alkylesters. C1-C8 alkyl, C2-C8 alkenyl, C2-C8 alkynyl, 5 aryl, C7-C12 substituted aryl, and C7-C12 arylalkyl esters of the compounds described herein may be preferred.

The terms “composition” and “formulation” are used interchangeably.

Also encompassed by the present disclosure are pharmaceutical compositions comprising any compound provided herein (e.g., a kinase inhibitor or a protease 10 inhibitor), or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof, and optionally a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition comprises more than one of the compounds provided herein (e.g., a kinase inhibitor and a protease inhibitor, more than one kinase inhibitors, or more than one protease inhibitors), or a pharmaceutically acceptable salt, solvate, hydrate, or 15 prodrug thereof, and optionally a pharmaceutically acceptable excipient. In certain embodiments, the compound described herein, or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof, is provided in an effective amount in the pharmaceutical composition. In certain embodiments, the effective amount is a therapeutically effective amount. In certain embodiments, the effective amount is a 20 prophylactically effective amount.

Pharmaceutical compositions described herein can be prepared by any method known in the art of pharmacology. In general, such preparatory methods include bringing the compound described herein, or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof (*i.e.*, the “active ingredient”) into association with a carrier or 25 excipient, and/or one or more other accessory ingredients, and then, if necessary and/or desirable, shaping, and/or packaging the product into a desired single- or multi-dose unit.

Pharmaceutical compositions can be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. A “unit dose” is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the 30 active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage, such as one-half or one-third of such a dosage.

Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition described herein will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered.

- 5 The composition may comprise between 0.1% and 100% (w/w) active ingredient.

Pharmaceutically acceptable excipients used in the manufacture of provided pharmaceutical compositions include inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Excipients such as cocoa
10 butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and perfuming agents may also be present in the composition.

Exemplary diluents include calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol,
15 sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, and mixtures thereof.

Exemplary granulating and/or dispersing agents include potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose, and wood products, natural sponge, cation-exchange resins,
20 calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate
25 (Veegum), sodium lauryl sulfate, quaternary ammonium compounds, and mixtures thereof.

Exemplary surface active agents and/or emulsifiers include natural emulsifiers (*e.g.*, acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin),
30 colloidal clays (*e.g.*, bentonite (aluminum silicate) and Veegum (magnesium aluminum silicate)), long chain amino acid derivatives, high molecular weight alcohols (*e.g.*, stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol),

carbomers (*e.g.*, carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (*e.g.*, carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (*e.g.*,
5 polyoxyethylene sorbitan monolaurate (Tween[®] 20), polyoxyethylene sorbitan (Tween[®] 60), polyoxyethylene sorbitan monooleate (Tween[®] 80), sorbitan monopalmitate (Span[®] 40), sorbitan monostearate (Span[®] 60), sorbitan tristearate (Span[®] 65), glyceryl monooleate, sorbitan monooleate (Span[®] 80), polyoxyethylene esters (*e.g.*, polyoxyethylene monostearate (Myrj[®] 45), polyoxyethylene hydrogenated castor oil,
10 polyethoxylated castor oil, polyoxymethylene stearate, and Solutol[®]), sucrose fatty acid esters, polyethylene glycol fatty acid esters (*e.g.*, Cremophor[®]), polyoxyethylene ethers, (*e.g.*, polyoxyethylene lauryl ether (Brij[®] 30)), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, Pluronic[®] F-68, poloxamer P-188, cetrimonium
15 bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, and/or mixtures thereof.

Exemplary binding agents include starch (*e.g.*, cornstarch and starch paste), gelatin, sugars (*e.g.*, sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol, *etc.*), natural and synthetic gums (*e.g.*, acacia, sodium alginate, extract of Irish
20 moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum[®]), and larch arabogalactan), alginates, polyethylene oxide, polyethylene glycol, inorganic calcium salts, silicic acid,
25 polymethacrylates, waxes, water, alcohol, and/or mixtures thereof.

Exemplary preservatives include antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, antiprotozoan preservatives, alcohol preservatives, acidic preservatives, and other preservatives. In certain embodiments, the preservative is an antioxidant. In other embodiments, the preservative is a chelating
30 agent.

Exemplary antioxidants include alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol,

potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and sodium sulfite.

Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA) and salts and hydrates thereof (*e.g.*, sodium edetate, disodium edetate, trisodium edetate, 5 calcium disodium edetate, dipotassium edetate, and the like), citric acid and salts and hydrates thereof (*e.g.*, citric acid monohydrate), fumaric acid and salts and hydrates thereof, malic acid and salts and hydrates thereof, phosphoric acid and salts and hydrates thereof, and tartaric acid and salts and hydrates thereof. Exemplary antimicrobial preservatives include benzalkonium chloride, benzethonium chloride, benzyl alcohol, 10 bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and thimerosal.

Exemplary antifungal preservatives include butyl paraben, methyl paraben, ethyl 15 paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and sorbic acid.

Exemplary alcohol preservatives include ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and phenylethyl alcohol.

20 Exemplary acidic preservatives include vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and phytic acid.

Other preservatives include tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), 25 ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, Glydant[®] Plus, Phenonip[®], methylparaben, Germall[®] 115, Germaben[®] II, Neolone[®], Kathon[®], and Euxyl[®].

Exemplary buffering agents include citrate buffer solutions, acetate buffer 30 solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium gluconate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium

phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, and mixtures thereof.

Exemplary lubricating agents include magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behenate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, and mixtures thereof.

Exemplary natural oils include almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macadamia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary synthetic oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and mixtures thereof.

Liquid dosage forms for oral and parenteral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredients, the liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (*e.g.*, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can

include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents. In certain embodiments for parenteral administration, the conjugates described herein are mixed with solubilizing agents such as Cremophor[®], alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and mixtures thereof.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can be a sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, (c) humectants such as glycerol, (d) disintegrating agents such as agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, (e) solution retarding agents such as paraffin, (f) absorption accelerators such as quaternary ammonium compounds, (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, (h) absorbents such as kaolin and bentonite clay, and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may include a buffering agent.

Solid compositions of a similar type can be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the art of pharmacology. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of encapsulating compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type can be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The active ingredient can be in a micro-encapsulated form with one or more excipients as noted above. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings, and other coatings well known in the pharmaceutical formulating art. In such solid dosage forms the active ingredient can be admixed with at least one inert diluent such as sucrose, lactose, or starch. Such dosage forms may comprise, as is normal practice, additional substances other than inert diluents, *e.g.*, tableting lubricants and other tableting aids such as magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms may comprise buffering agents. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of encapsulating agents which can be used include polymeric substances and waxes.

In order to prolong the effect of a drug, it is often desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form may be accomplished by dissolving or suspending the drug in an oil vehicle.

Suitable devices for use in delivering intradermal pharmaceutical compositions described herein include short needle devices. Intradermal compositions can be

administered by devices which limit the effective penetration length of a needle into the skin. Alternatively or additionally, conventional syringes can be used in the classical mantoux method of intradermal administration. Jet injection devices which deliver liquid formulations to the dermis *via* a liquid jet injector and/or *via* a needle which pierces the
5 stratum corneum and produces a jet which reaches the dermis are suitable. Ballistic powder/particle delivery devices which use compressed gas to accelerate the compound in powder form through the outer layers of the skin to the dermis are suitable.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration
10 to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification
15 with ordinary experimentation.

Compounds provided herein are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions described herein will be decided by a physician within the scope of sound medical judgment. The specific therapeutically effective dose level
20 for any particular subject or organism will depend upon a variety of factors including the disease being treated and the severity of the disorder; the activity of the specific active ingredient employed; the specific composition employed; the age, body weight, general health, sex, and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific active ingredient employed; the duration of the
25 treatment; drugs used in combination or coincidental with the specific active ingredient employed; and like factors well known in the medical arts.

The compounds and compositions provided herein can be administered by any route, including enteral (*e.g.*, oral), parenteral, intravenous, intramuscular, intramedullary, intrathecal, subcutaneous, intraventricular, transdermal, interdermal,
30 rectal, intravaginal, intraperitoneal, mucosal, nasal, bucal, sublingual; by intratracheal instillation, bronchial instillation, and/or inhalation; and/or as an oral spray, nasal spray, and/or aerosol. Specifically contemplated routes are oral administration, intravenous administration (*e.g.*, systemic intravenous injection), regional administration *via* blood

and/or lymph supply, and/or direct administration to an affected site. In general, the most appropriate route of administration will depend upon a variety of factors including the nature of the agent (*e.g.*, its stability in the environment of the gastrointestinal tract), and/or the condition of the subject (*e.g.*, whether the subject is able to tolerate oral
5 administration). In certain embodiments, the compound or pharmaceutical composition described herein is suitable for oral administration to a subject. In certain embodiments, the compound or pharmaceutical composition described herein is suitable for intravenous administration to a subject.

The exact amount of a compound required to achieve an effective amount will
10 vary from subject to subject, depending, for example, on species, age, and general condition of a subject, severity of the side effects or disorder, identity of the particular compound, mode of administration, and the like. An effective amount may be included in a single dose (*e.g.*, single oral dose) or multiple doses (*e.g.*, multiple oral doses). In certain embodiments, when multiple doses are administered to a subject, any two doses
15 of the multiple doses include different or substantially the same amounts of a compound described herein. In certain embodiments, when multiple doses are administered to a subject, the frequency of administering the multiple doses to the subject is three doses a day, two doses a day, one dose a day, one dose every other day, one dose every third day, one dose every week, one dose every two weeks, one dose every three weeks, or one
20 dose every four weeks. In certain embodiments, the frequency of administering the multiple doses to the subject is one dose per day. In certain embodiments, the frequency of administering the multiple doses to the subject is two doses per day. In certain embodiments, the frequency of administering the multiple doses to the subject is three doses per day. In certain embodiments, when multiple doses are administered to a
25 subject, the duration between the first dose and last dose of the multiple doses is one day, two days, four days, one week, two weeks, three weeks, one month, two months, three months, four months, six months, nine months, one year, two years, three years, four years, five years, seven years, ten years, fifteen years, twenty years, or the lifetime of the subject. In certain embodiments, a dose (*e.g.*, a single dose, or any dose of multiple
30 doses) described herein includes independently between 0.1 μ g and 1 μ g, between 0.001 mg and 0.01 mg, between 0.01 mg and 0.1 mg, between 0.1 mg and 1 mg, between 1 mg and 3 mg, between 3 mg and 10 mg, between 10 mg and 30 mg, between 30 mg and 100 mg, between 100 mg and 300 mg, between 300 mg and 1,000 mg, or

between 1 g and 10 g, inclusive, of a compound described herein. In certain
embodiments, a dose described herein includes independently between 1 mg and 3 mg,
inclusive, of a compound described herein. In certain embodiments, a dose described
herein includes independently between 3 mg and 10 mg, inclusive, of a compound
5 described herein. In certain embodiments, a dose described herein includes
independently between 10 mg and 30 mg, inclusive, of a compound described herein. In
certain embodiments, a dose described herein includes independently between 30 mg and
100 mg, inclusive, of a compound described herein.

Dose ranges as described herein provide guidance for the administration of
10 provided pharmaceutical compositions to an adult. The amount to be administered to, for
example, a child or an adolescent can be determined by a medical practitioner or person
skilled in the art and can be lower or the same as that administered to an adult.

A compound or composition, as described herein, can be administered in
combination with one or more additional pharmaceutical agents (*e.g.*, therapeutically
15 and/or prophylactically active agents). The compounds or compositions can be
administered in combination with additional pharmaceutical agents that improve their
activity (*e.g.*, activity (*e.g.*, potency and/or efficacy) in treating a disease in a subject in
need thereof, in preventing a disease in a subject in need thereof, in reducing the risk to
develop a disease in a subject in need thereof, and/or in inhibiting the activity of a kinase
20 or protease in a subject or cell), improve bioavailability, improve safety, reduce drug
resistance, reduce and/or modify metabolism, inhibit excretion, and/or modify
distribution in a subject or cell. It will also be appreciated that the therapy employed may
achieve a desired effect for the same disorder, and/or it may achieve different effects. In
certain embodiments, a pharmaceutical composition described herein including a
25 compound described herein and an additional pharmaceutical agent shows a synergistic
effect that is absent in a pharmaceutical composition including one of the compound and
the additional pharmaceutical agent, but not both.

The additional pharmaceutical agents include, but are not limited to, anti-
proliferative agents, anti-cancer agents, anti-angiogenesis agents, anti-inflammatory
30 agents, immunosuppressants, anti-bacterial agents, anti-viral agents, cardiovascular
agents, cholesterol-lowering agents, anti-diabetic agents, anti-allergic agents, and pain-
relieving agents.

Also encompassed by the disclosure are kits (*e.g.*, pharmaceutical packs). The kits provided may comprise a pharmaceutical composition or compound described herein and a container (*e.g.*, a vial, ampule, bottle, syringe, and/or dispenser package, or other suitable container). In some embodiments, provided kits may optionally further include a
5 second container comprising a pharmaceutical excipient for dilution or suspension of a pharmaceutical composition or compound described herein. In some embodiments, the pharmaceutical composition or compound described herein provided in the first container and the second container are combined to form one unit dosage form.

Thus, in one aspect, provided are kits including a first container comprising a
10 compound or pharmaceutical composition described herein. In certain embodiments, the kits are useful for treating a disease (*e.g.*, a neurological disease) in a subject in need thereof. In certain embodiments, the kits are useful for preventing a disease (*e.g.*, a neurological disease) in a subject in need thereof. In certain embodiments, the kits are useful for reducing the risk of developing a disease (*e.g.*, a neurological disease) in a
15 subject in need thereof. In certain embodiments, the kits are useful for inhibiting the activity of a kinase in a subject or cell. In certain embodiments, the kits are useful for inhibiting the activity of a protease in a subject or cell. In certain embodiments, the kits are useful for increasing or promoting mitochondrial motility in a subject or cell.

In certain embodiments, a kit described herein further includes instructions for
20 using the kit. A kit described herein may also include information as required by a regulatory agency such as the U.S. Food and Drug Administration (FDA). In certain embodiments, the information included in the kits is prescribing information. In certain embodiments, the kits and instructions provide for treating a disease (*e.g.*, neurological disease) in a subject in need thereof. In certain embodiments, the kits and instructions
25 provide for preventing a disease (*e.g.*, neurological disease) in a subject in need thereof. In certain embodiments, the kits and instructions provide for reducing the risk of developing a disease (*e.g.*, neurological disease) in a subject in need thereof. In certain embodiments, the kits and instructions provide for inhibiting the activity of a kinase in a subject or cell. In certain embodiments, the kits and instructions provide for inhibiting
30 the activity of a protease in a subject or cell. In certain embodiments, the kits and instructions provide for increasing or promoting mitochondrial motility in a subject or cell. A kit described herein may include one or more additional pharmaceutical agents described herein as a separate composition.

As described above, the systems described herein can be employed to identify compounds that increase or promote organelle (*e.g.*, mitochondrial) motility. Thus, in some embodiments, provided here are compounds identified using the using a system described herein that increases or promotes mitochondrial motility for use in treating a
5 disease or disorder in associated with decreases mitochondrial motility.

In one aspect, provided herein is a method for increasing or promoting mitochondrial motility in a cell. In some embodiments, the method comprises contacting the cell with an effective amount of a compound identified using the system of any of the preceding claims. In some embodiments, the effective amount is an amount sufficient to
10 increase or promote mitochondrial motility in the cell. In some embodiments, the cell is a nervous system cell. In some embodiments, the cell is a neuron. In some embodiments, the cell is *in vivo*.

In another aspect, provided herein is a method for treating a disease. In some embodiments, the disease is associated with reduced mitochondrial motility. The term
15 “reduced” refers to an amount of activity that is less than the basal amount of mitochondrial motility (*e.g.*, mitochondrial trafficking) in a subject or a cell (*e.g.*, a normal subject or cell, or a subject or cell that does not display characteristics of the disease). In some embodiments, mitochondrial motility in a subject suffering from the disease or a diseases cell is reduced by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%,
20 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or more compared to a subject that is not suffering from a disease or a normal cell (*e.g.*, a normal or non-diseased cell). In some embodiments, the disease is a neurological disease. In some embodiments, the neurological disease is Alzheimer’s disease, Parkinson’s disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), or chemotherapy-induced
25 peripheral neuropathy (CIPN). In some embodiments, the neurological disease is Alzheimer’s disease. In some embodiments, the neurological disease is Parkinson’s disease. In some embodiments, the neurological disease is multiple sclerosis (MS). In some embodiments, the neurological disease is amyotrophic lateral sclerosis (ALS). In some embodiments, the neurological disease is chemotherapy-induced peripheral
30 neuropathy (CIPN).

Other Potential Embodiments

Embodiments have been described where the techniques are implemented in circuitry and/or computer-executable instructions. It should be appreciated that some embodiments may be in the form of a method, of which at least one example has been provided. The acts performed as part of the method may be ordered in any suitable way. Accordingly, embodiments may be constructed in which acts are performed in an order different than illustrated, which may include performing some acts simultaneously, even though shown as sequential acts in illustrative embodiments.

Various aspects of the embodiments described above may be used alone, in combination, or in a variety of arrangements not specifically discussed in the embodiments described in the foregoing and is therefore not limited in its application to the details and arrangement of components set forth in the foregoing description or illustrated in the drawings. For example, aspects described in one embodiment may be combined in any manner with aspects described in other embodiments.

Use of ordinal terms such as “first,” “second,” “third,” etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of “including,” “comprising,” “having,” “containing,” “involving,” and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

The word “exemplary” is used herein to mean serving as an example, instance, or illustration. Any embodiment, implementation, process, feature, etc. described herein as exemplary should therefore be understood to be an illustrative example and should not be understood to be a preferred or advantageous example unless otherwise indicated.

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In the claims articles such as “a,” “an,” and “the” may mean one or more than one
5 unless indicated to the contrary or otherwise evident from the context. Claims or
descriptions that include “or” between one or more members of a group are considered
satisfied if one, more than one, or all of the group members are present in, employed in,
or otherwise relevant to a given product or process unless indicated to the contrary or
otherwise evident from the context. The invention includes embodiments in which
10 exactly one member of the group is present in, employed in, or otherwise relevant to a
given product or process. The invention includes embodiments in which more than one,
or all of the group members are present in, employed in, or otherwise relevant to a given
product or process.

Furthermore, the invention encompasses all variations, combinations, and
15 permutations in which one or more limitations, elements, clauses, and descriptive terms
from one or more of the listed claims is introduced into another claim. For example, any
claim that is dependent on another claim can be modified to include one or more
limitations found in any other claim that is dependent on the same base claim. Where
elements are presented as lists, e.g., in Markush group format, each subgroup of the
20 elements is also disclosed, and any element(s) can be removed from the group. It should
it be understood that, in general, where the invention, or aspects of the invention, is/are
referred to as comprising particular elements and/or features, certain embodiments of the
invention or aspects of the invention consist, or consist essentially of, such elements
and/or features. For purposes of simplicity, those embodiments have not been
25 specifically set forth *in haec verba* herein. It is also noted that the terms “comprising”
and “containing” are intended to be open and permits the inclusion of additional
elements or steps. Where ranges are given, endpoints are included. Furthermore, unless
otherwise indicated or otherwise evident from the context and understanding of one of
ordinary skill in the art, values that are expressed as ranges can assume any specific
30 value or sub-range within the stated ranges in different embodiments of the invention, to
the tenth of the unit of the lower limit of the range, unless the context clearly dictates
otherwise.

Where ranges are given herein, embodiments are provided in which the endpoints are included, embodiments in which both endpoints are excluded, and embodiments in which one endpoint is included and the other is excluded. It should be assumed that both endpoints are included unless indicated otherwise. Furthermore, it is to be understood

5 that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also understood that where a series of numerical values is

10 stated herein, embodiments that relate analogously to any intervening value or range defined by any two values in the series are provided, and that the lowest value may be taken as a minimum and the greatest value may be taken as a maximum. Where a phrase such as “at least”, “up to”, “no more than”, or similar phrases, precedes a series of numbers herein, it is to be understood that the phrase applies to each number in the list in

15 various embodiments (it being understood that, depending on the context, 100% of a value, e.g., a value expressed as a percentage, may be an upper limit), unless the context clearly dictates otherwise. For example, “at least 1, 2, or 3” should be understood to mean “at least 1, at least 2, or at least 3” in various embodiments. It will also be understood that any and all reasonable lower limits and upper limits are expressly

20 contemplated where applicable. A reasonable lower or upper limit may be selected or determined by one of ordinary skill in the art based, e.g., on factors such as convenience, cost, time, effort, availability (e.g., of samples, agents, or reagents), statistical considerations, etc. In some embodiments an upper or lower limit differs by a factor of 2, 3, 5, or 10, from a particular value. Numerical values, as used herein, include values

25 expressed as percentages. For each embodiment in which a numerical value is prefaced by “about” or “approximately”, embodiments in which the exact value is recited are provided. For each embodiment in which a numerical value is not prefaced by “about” or “approximately”, embodiments in which the value is prefaced by “about” or “approximately” are provided. “Approximately” or “about” generally includes numbers

30 that fall within a range of 1% or in some embodiments within a range of 5% of a number or in some embodiments within a range of 10% of a number in either direction (greater than or less than the number) unless otherwise stated or otherwise evident from the context (except where such number would impermissibly exceed 100% of a possible

value). It should be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one act, the order of the acts of the method is not necessarily limited to the order in which the acts of the method are recited, but the invention includes embodiments in which the order is so limited. In some
5 embodiments a method may be performed by an individual or entity. In some
embodiments steps of a method may be performed by two or more individuals or entities such that a method is collectively performed. In some embodiments a method may be performed at least in part by requesting or authorizing another individual or entity to perform one, more than one, or all steps of a method. In some embodiments a method
10 comprises requesting two or more entities or individuals to each perform at least one step of a method. In some embodiments performance of two or more steps is coordinated so that a method is collectively performed. Individuals or entities performing different step(s) may or may not interact.

 The recitation of a listing of chemical groups in any definition of a variable
15 herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

20 This application refers to various issued patents, published patent applications, journal articles, and other publications, all of which are incorporated herein by reference. If there is a conflict between any of the incorporated references and the instant specification, the specification shall control. In addition, any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any
25 one or more of the claims. Because such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the invention can be excluded from any claim, for any reason, whether or not related to the existence of prior art.

 Having thus described several aspects of at least one embodiment, it is to be
30 appreciated that various alterations, modifications, and improvements will readily occur to those skilled in the art. Such alterations, modifications, and improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of

the principles described herein. Accordingly, the foregoing description and drawings are by way of example only.

CLAIMS

What is claimed is:

- 5 1. An system comprising:
an imaging platform configured to:
acquire a sequence of images of neurons that have been transfected;
process the sequence of images to:
identify one or more stationary organelles and one or more motile
10 organelles of the neurons, and
generate tracking data associated with movement of the one or
more motile organelles across the sequence of images; and
determine an overall percent motile based on the identified one or more
stationary organelles and one or more motile organelles, the overall percent
15 motile indicative of a percentage of the one or more motile organelles over a
total number of organelles.
2. The system of claim 1, wherein processing the sequences of images comprises:
analyzing a minimum intensity projection of fluorescence of a plurality of
20 organelles of the neurons; and
identifying the one or more stationary organelles and the one or more motile
organelles based on the analysis.
3. The system of claim 2, wherein analyzing a minimum intensity projection further
25 comprises:
determining whether the minimum intensity projection of an organelle exceeds a
predetermined threshold; and
when the minimum intensity projection of the organelle exceeds the
predetermined threshold, identifying the organelle as a stationary organelle.

30

4. The system of claim 2, wherein analyzing a minimum intensity projection further comprises:

determining whether the minimum intensity projection of an organelle is below a predetermined threshold; and

5 when the minimum intensity projection of the organelle is below the predetermined threshold, identifying the organelle as a motile organelle.

5. The system of claim 1, wherein the imaging platform is further configured to: generate one or more visual representations associated with the overall percent

10 motile.

6. The system of claim 1, wherein the imaging platform is configured to perform the acquiring, the processing, and the determining prior to administering a pharmaceutical to the neurons.

15

7. The system of claim 6, wherein the imaging platform is configured to repeat the acquiring, the processing, and the determining after administering the pharmaceutical to the neurons.

20 8. The system of claim 7, wherein the imaging platform is further configured to: compare a first overall percent motile determined prior to administering the pharmaceutical with a second overall percent motile after administering the pharmaceutical; and

determine an effectiveness of the pharmaceutical based on the comparison.

25

9. The system of claim 1, wherein processing the sequence of images to generate the tracking data comprises:

comparing an amount of spatial overlap between a first motile organelle of the one or more motile organelles in a previous image and a second motile organelle of the one or more motile organelles in a current image.

30

10. The system of claim 9, wherein the processing further comprises:

determining, based on the comparison, whether an amount of spatial overlap that exists between the first motile organelle in the previous image and the second motile organelle in the current image exceeds a predetermined threshold; and

5 in response to a determination that the amount of spatial overlap that exists between the first motile organelle in the previous image and the second motile organelle in the current image exceeds the predetermined threshold:

determining that the first motile organelle in the previous image matches the second motile organelle in the current image.

10

11. The system of claim 1, wherein processing the sequence of images to generate the tracking data comprises:

comparing a distance between a first motile organelle of the one or more motile organelles in a previous image and a second motile organelle of the one or more motile
15 organelles in a current image.

12. The system of claim 10, wherein the processing further comprises:

determining, based on the comparison, whether a distance between the first motile organelle in the previous image and the second motile organelle in the current
20 image is less a predetermined value; and

in response to a determination that the distance between the first motile organelle in the previous image and the second motile organelle in the current image is less the predetermined value:

determining that the first motile organelle in the previous image matches the
25 second motile organelle in the current image.

13. The system of claim 1, wherein processing the sequence of images to generate the tracking data comprises:

identifying the one or more motile organelles in each image of the sequence of
30 images; and

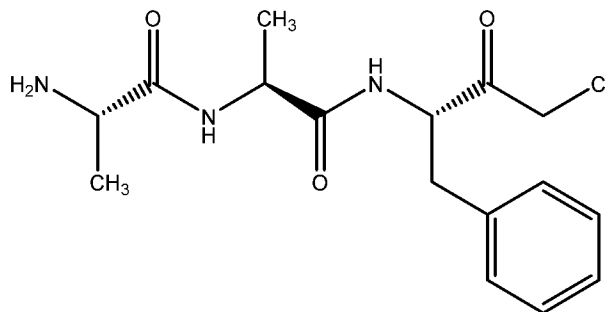
linking the one or more motile organelles identified in each image of the sequence of images to determine one or more trajectories followed by the one or more motile organelles across the sequence of images.

14. A method comprising:
acquiring a sequence of images of neurons that have been transfected;
processing the sequence of images to:
- 5 identify one or more stationary organelles and one or more motile
organelles of the neurons, and
 generate tracking data associated with movement of the one or more
motile organelles across the sequence of images; and
 determining an overall percent motile based on the identified one or more
- 10 stationary organelles and one or more motile organelles, the overall percent motile
indicative of a percentage of the one or more motile organelles over a total number of
organelles.
15. The method of claim 14, wherein processing the sequences of images comprises:
- 15 analyzing a minimum intensity projection of fluorescence of a plurality of
organelles of the neurons; and
 identifying the one or more stationary organelles and the one or more motile
organelles based on the analysis
- 20 16. The method of claim 15, wherein analyzing a minimum intensity projection
further comprises:
 determining whether the minimum intensity projection of an organelle exceeds a
predetermined threshold; and
 when the minimum intensity projection of the organelle exceeds the
- 25 predetermined threshold, identifying the organelle as a stationary organelle
17. The method of claim 15, wherein analyzing a minimum intensity projection
further comprises:
 determining whether the minimum intensity projection of an organelle is below a
- 30 predetermined threshold; and
 when the minimum intensity projection of the organelle is below the
predetermined threshold, identifying the organelle as a motile organelle

18. The method of claim 14, further comprising:
generating one or more visual representations associated with the overall percent motile.
- 5 19. The method of claim 14, wherein the one or more stationary organelles include one or more stationary mitochondria and the one or more motile organelles include one or more motile mitochondria.
20. At least one computer-readable storage medium encoded with computer-
10 executable instructions that, when executed by a computer, cause the computer to carry out a method comprising:
acquiring a sequence of images of neurons that have been transfected;
processing the sequence of images to:
identify one or more stationary organelles and one or more motile
15 organelles of the neurons, and
generate tracking data associated with movement of the one or more motile organelles across the sequence of images; and
determining an overall percent motile based on the identified one or more stationary organelles and one or more motile organelles, the overall percent motile
20 indicative of a percentage of the one or more motile organelles over a total number of organelles.
21. A compound identified using the system of any of claims 1-13, wherein the compound is administered to a subject in need thereof in a sufficient amount to increase
25 mitochondrial motility.
22. A compound identified using the system of any of any of claims 1-13, wherein the compound is administered to a subject in need thereof in a sufficient amount to treat a disease or disorder associated with decreased mitochondrial motility.
- 30 23. The compound of claim 21 or 22, wherein the compound is a protease inhibitor.

24. The compound of claim 23, wherein the protease is tripeptidyl-peptidase 1 (TPP1).

25. The compound of claim 23 or 24, wherein the compound is:



5

(Ala-Ala-Phe-chloromethylketone)

or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof.

26. The compound of claim 21 or 22, wherein the compound is a kinase inhibitor.

10

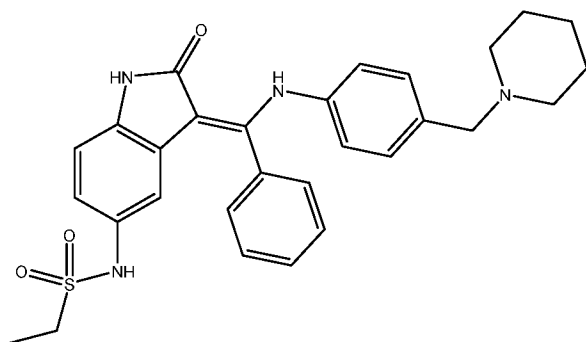
27. The compound of claim 26, wherein the kinase is Aurora B kinase.

28. The compound of claim 26 or 27, wherein the compound is an Aurora B kinase inhibitor, and wherein the compound does not inhibit Aurora A kinase.

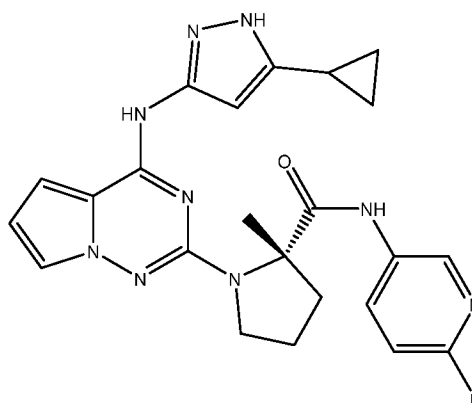
15

29. The compound of claim 26 or 27, wherein the compound is an Aurora A kinase inhibitor and an Aurora B kinase inhibitor.

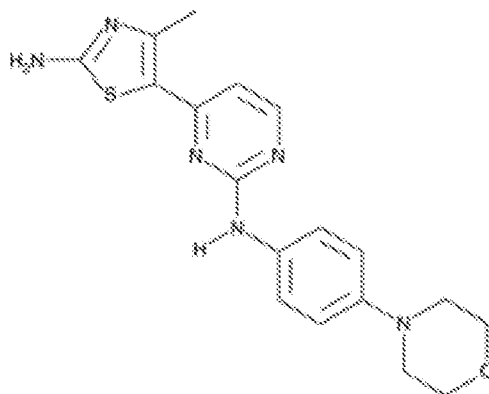
30. The compound of any one of claims 26-29, wherein the compound is:



(Hesperadin),

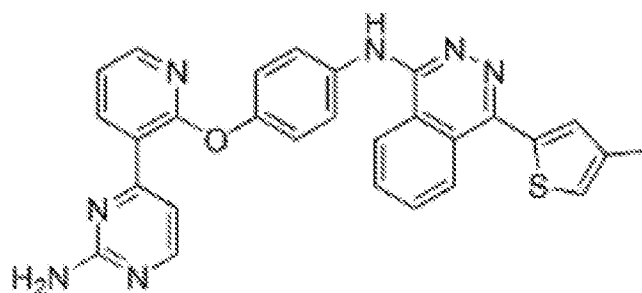


(BMS-754807),

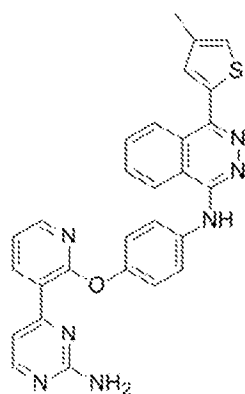


(TAK-901),

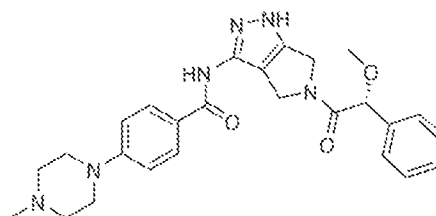
(CYC116),



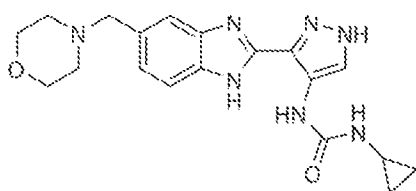
(AMG-900),



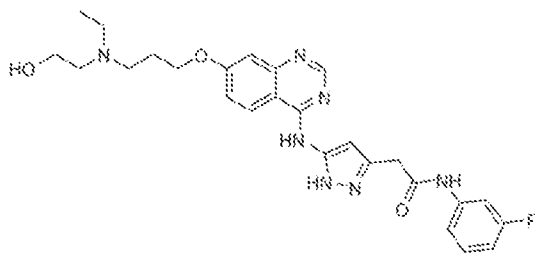
(ESK105),



(ESK106),



(ESK107),



(Barasertib),

or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof.

31. The compound of any one of claims 21-30, wherein the compound crosses the
5 blood brain barrier.
32. A pharmaceutical composition comprising a compound of any one of claims 21-
31, or a pharmaceutically acceptable salt, solvate, hydrate, or prodrug thereof, and a
pharmaceutically acceptable excipient.
- 10 33. A method for increasing mitochondrial motility in a cell, the method comprising
contacting the cell with an effective amount of a compound identified using the system
of any of the preceding claims 1-13.
- 15 34. The method of claim 33, wherein the effective amount is an amount sufficient to
increase mitochondrial motility in the cell.
35. The method of claim 33 or 34, wherein the cell is a nervous system cell.
- 20 36. The method of any one of claims 33-35, wherein the cell is a neuron.
37. The method of any one of claims 33-36, wherein the cell is *in vitro*.
38. The method of any one of claims 33-36, wherein the cell is *in vivo*.

39. A method for treating a disease, the method comprising administering an effective amount of a compound identified using the system of any of the preceding claims to a subject in need thereof.

5 40. The method of claim 39, wherein the disease is associated with reduced mitochondrial motility.

41. The method of claim 39 or 40, wherein the disease is a neurological disease.

10 42. The method of claim 31, wherein the neurological disease is Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), or chemotherapy-induced peripheral neuropathy (CIPN).

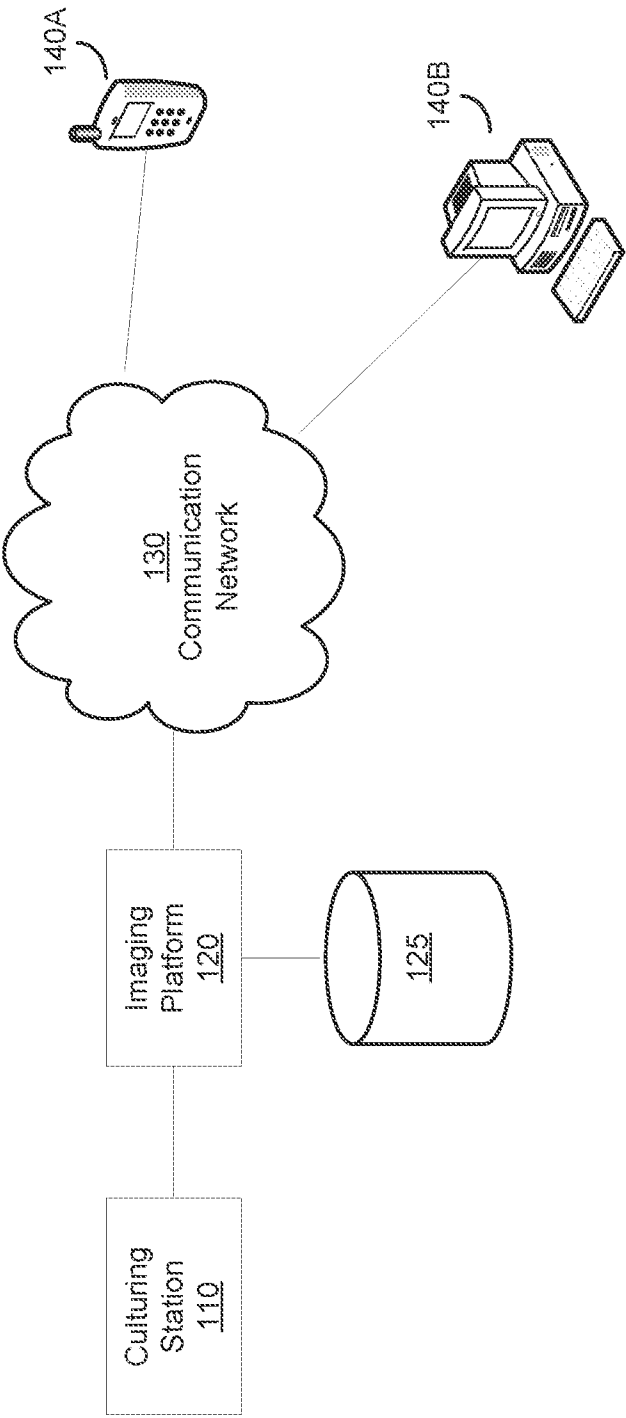
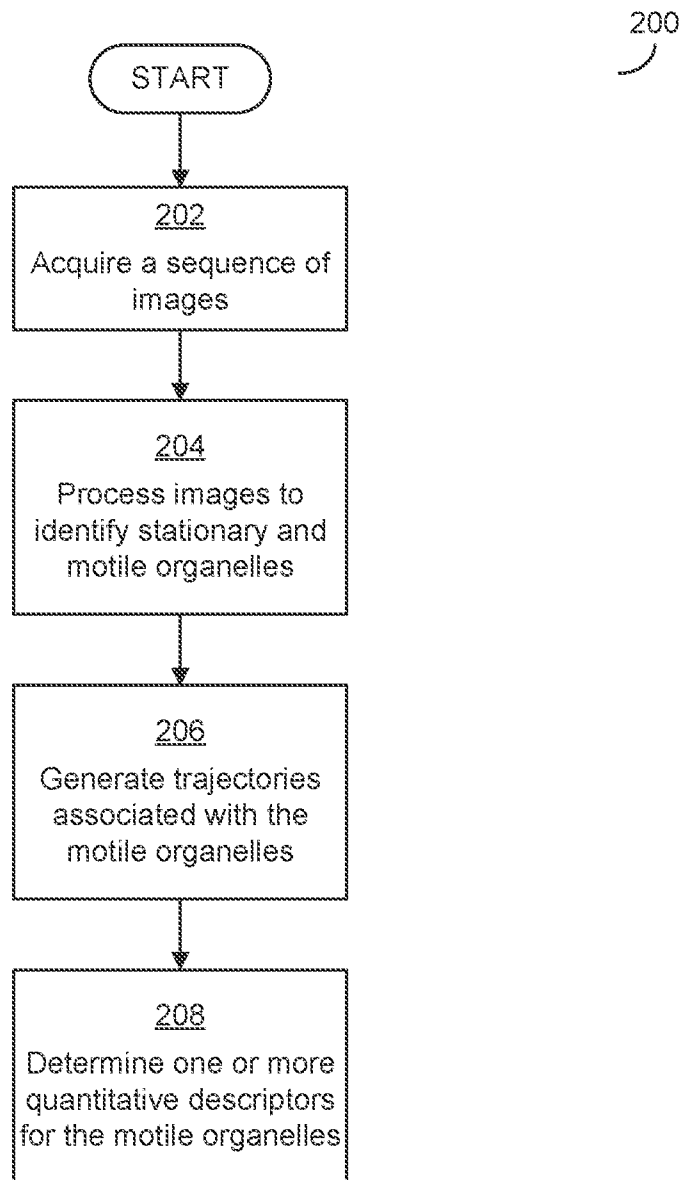
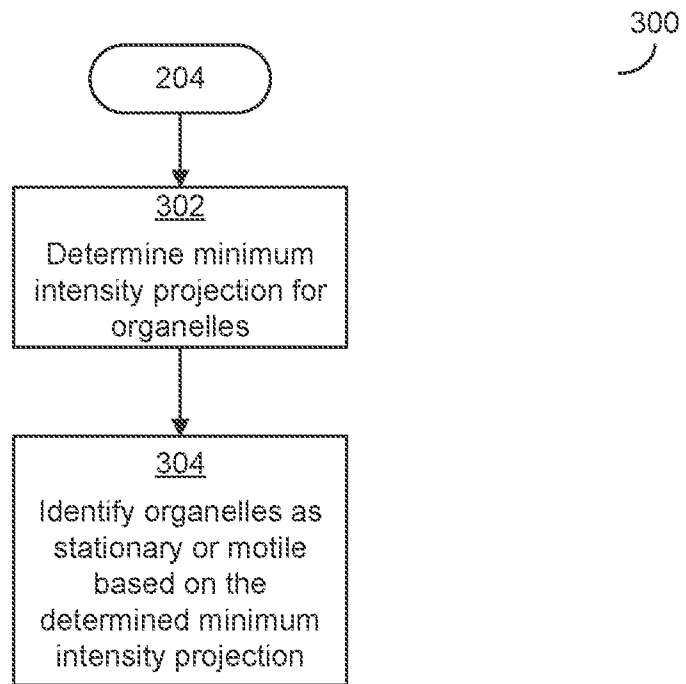


FIG. 1

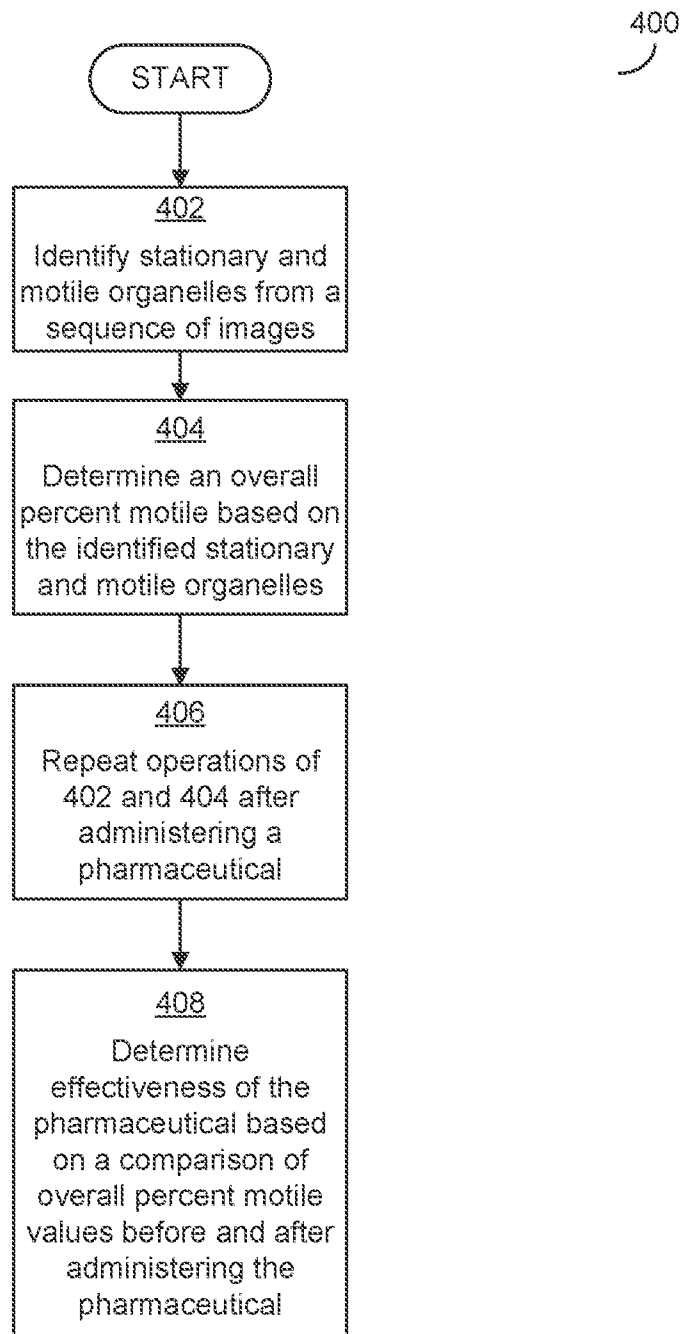
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**FIG. 2**

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**FIG. 3**

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**FIG. 4**

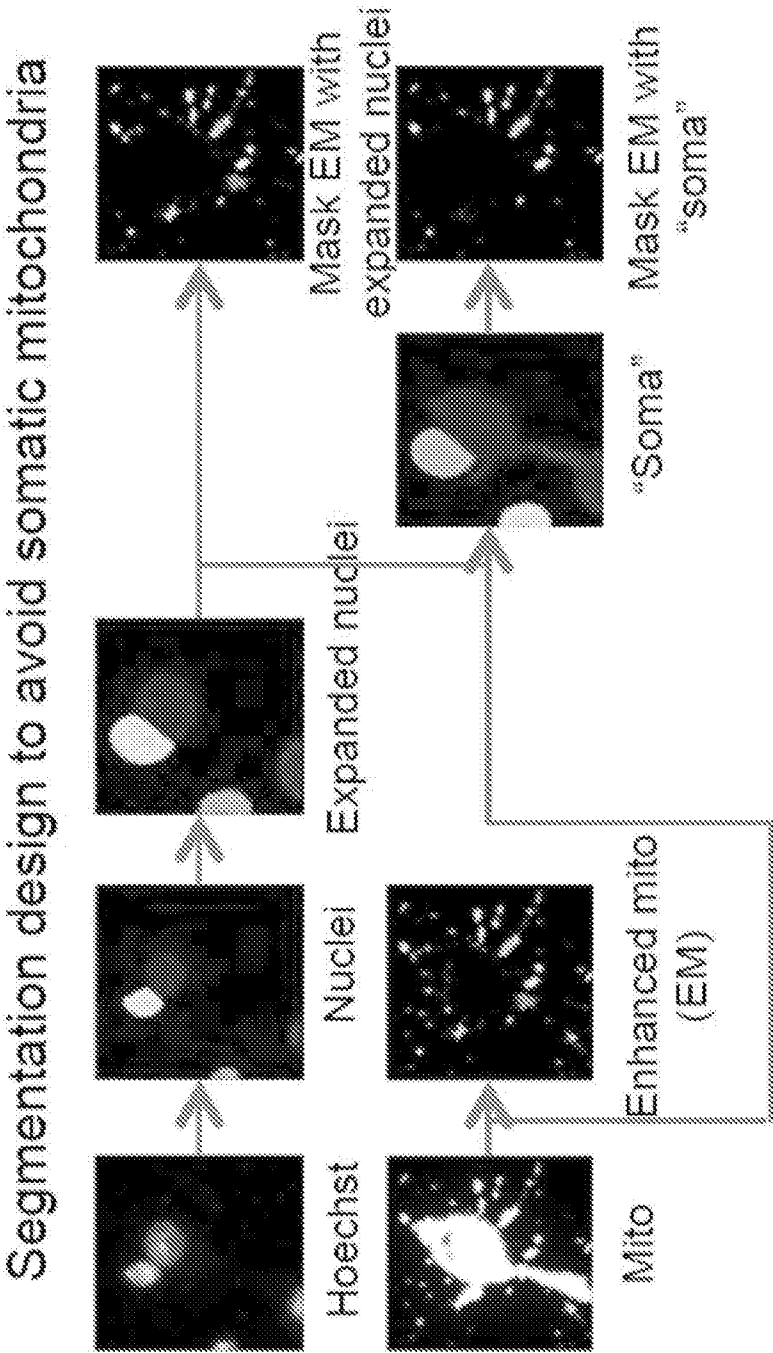


FIG. 5

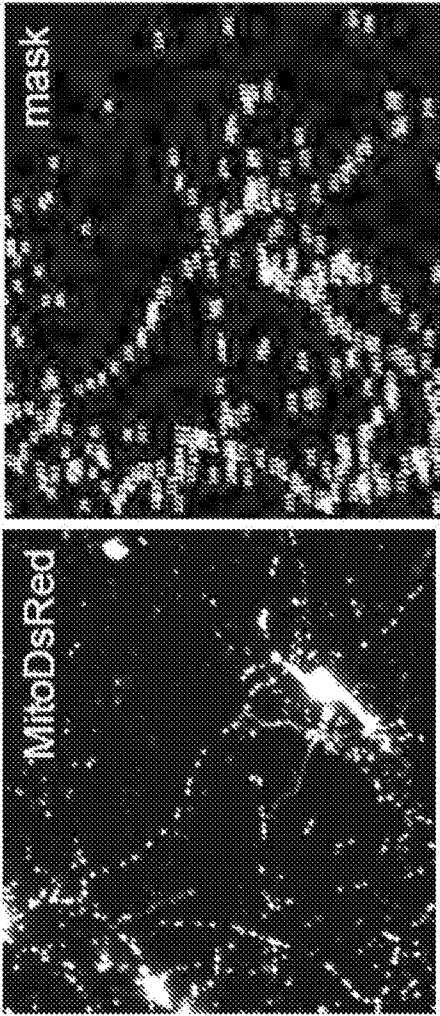


FIG. 6

Minimum intensity projection approach

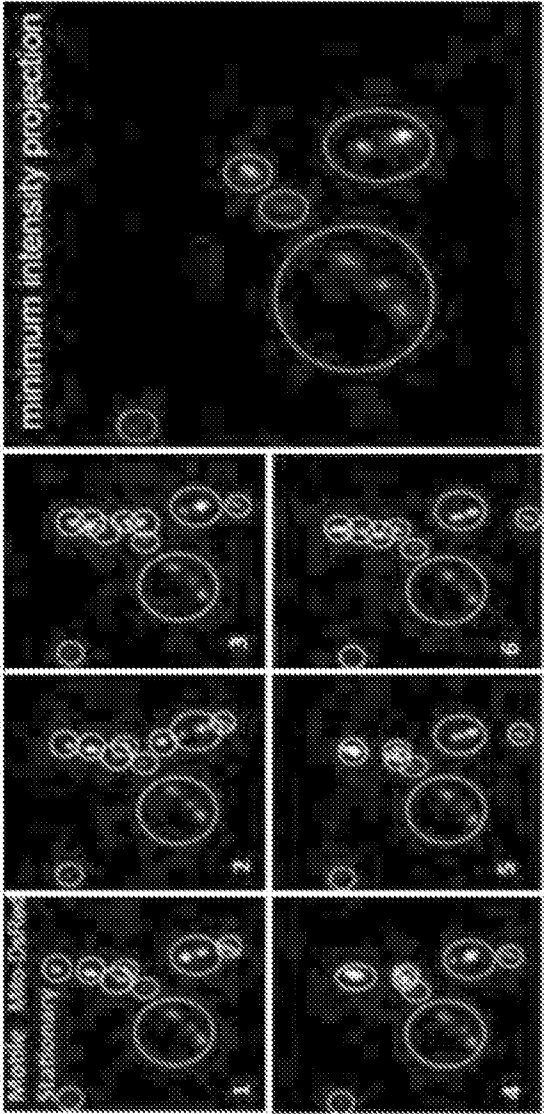


FIG. 7

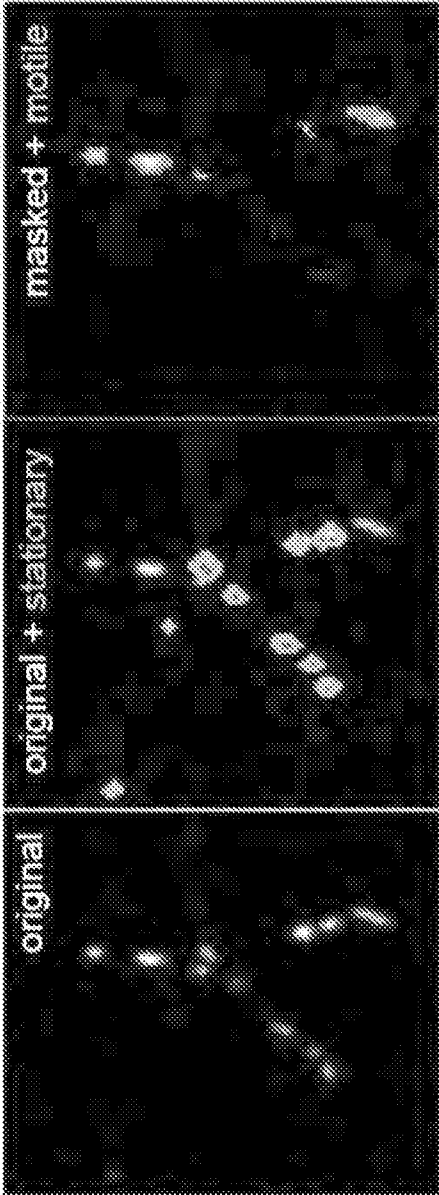


FIG. 8

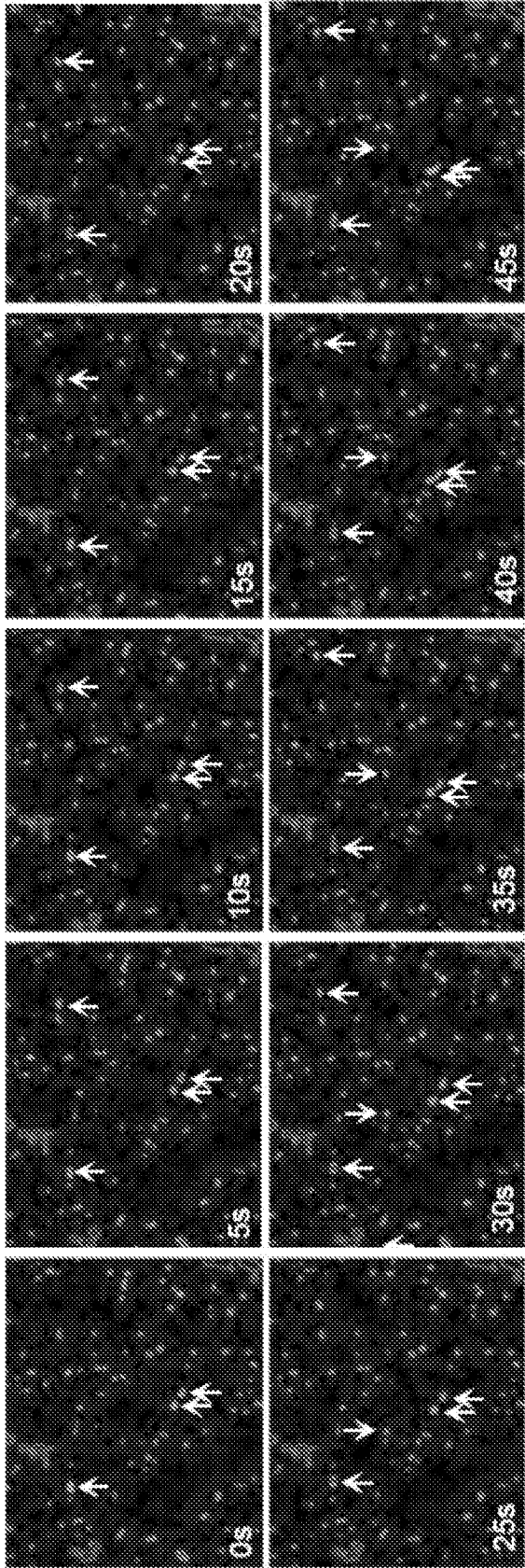


FIG. 9

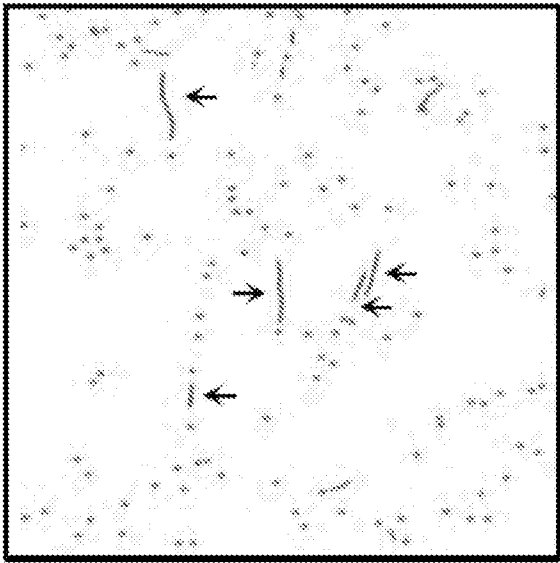


FIG. 10

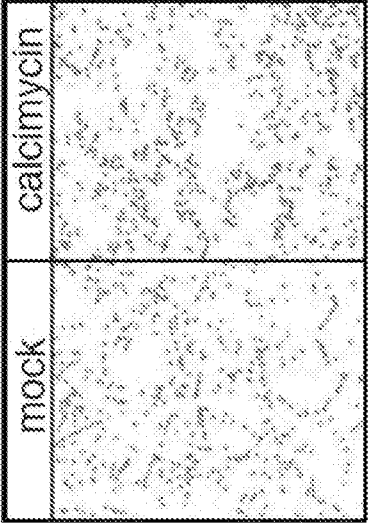


FIG. 11A

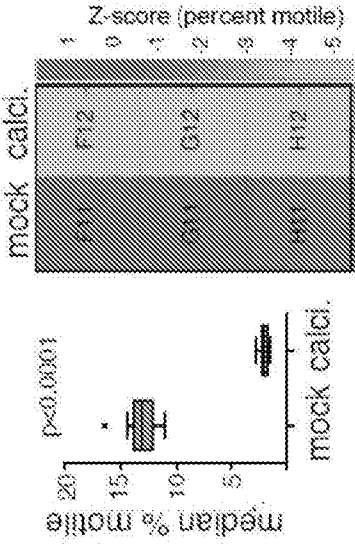


FIG. 11B

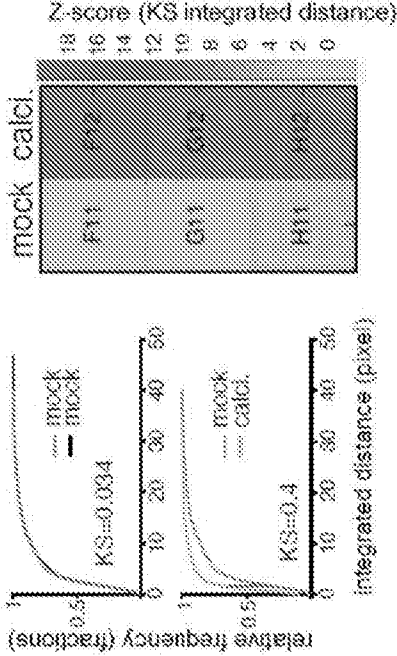


FIG. 11C

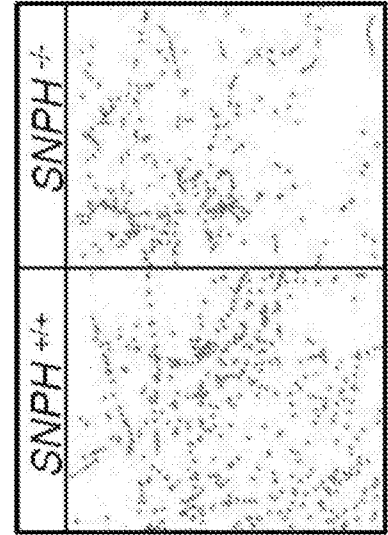


FIG. 12A

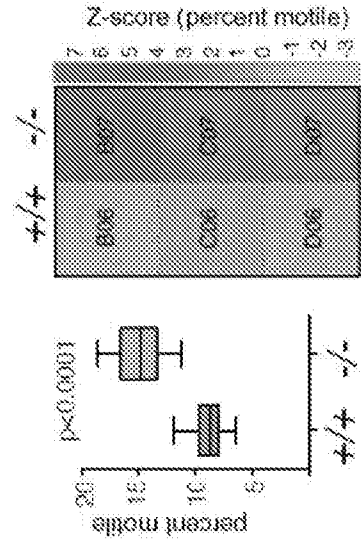


FIG. 12B

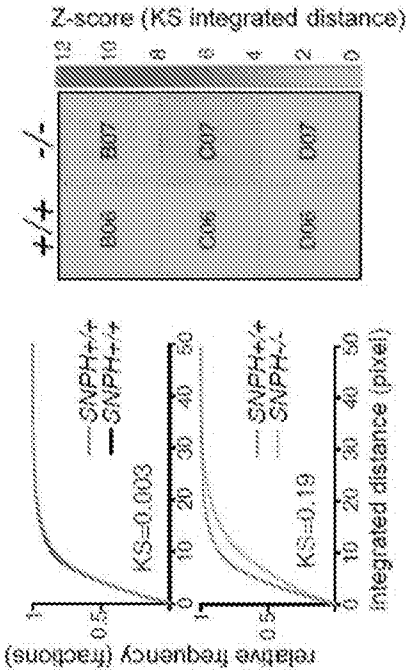
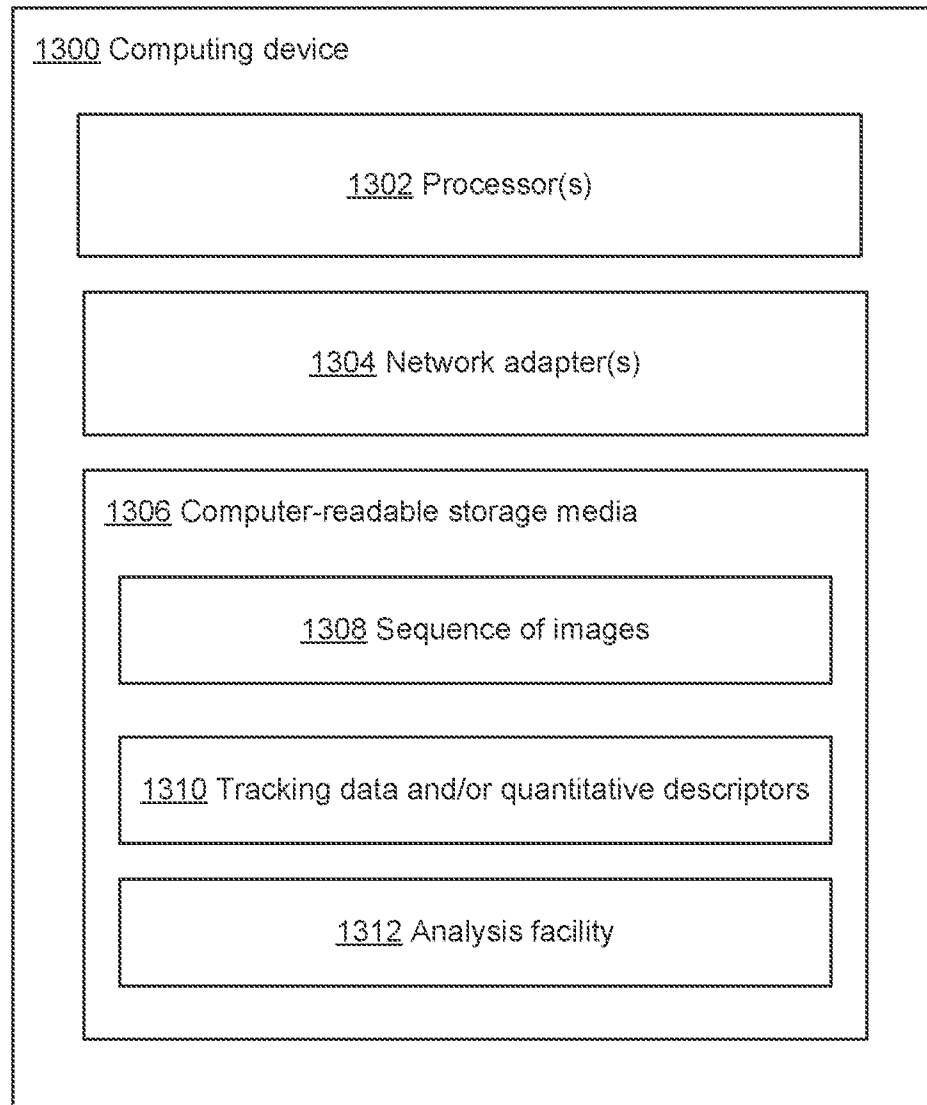


FIG. 12C

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**FIG. 13**

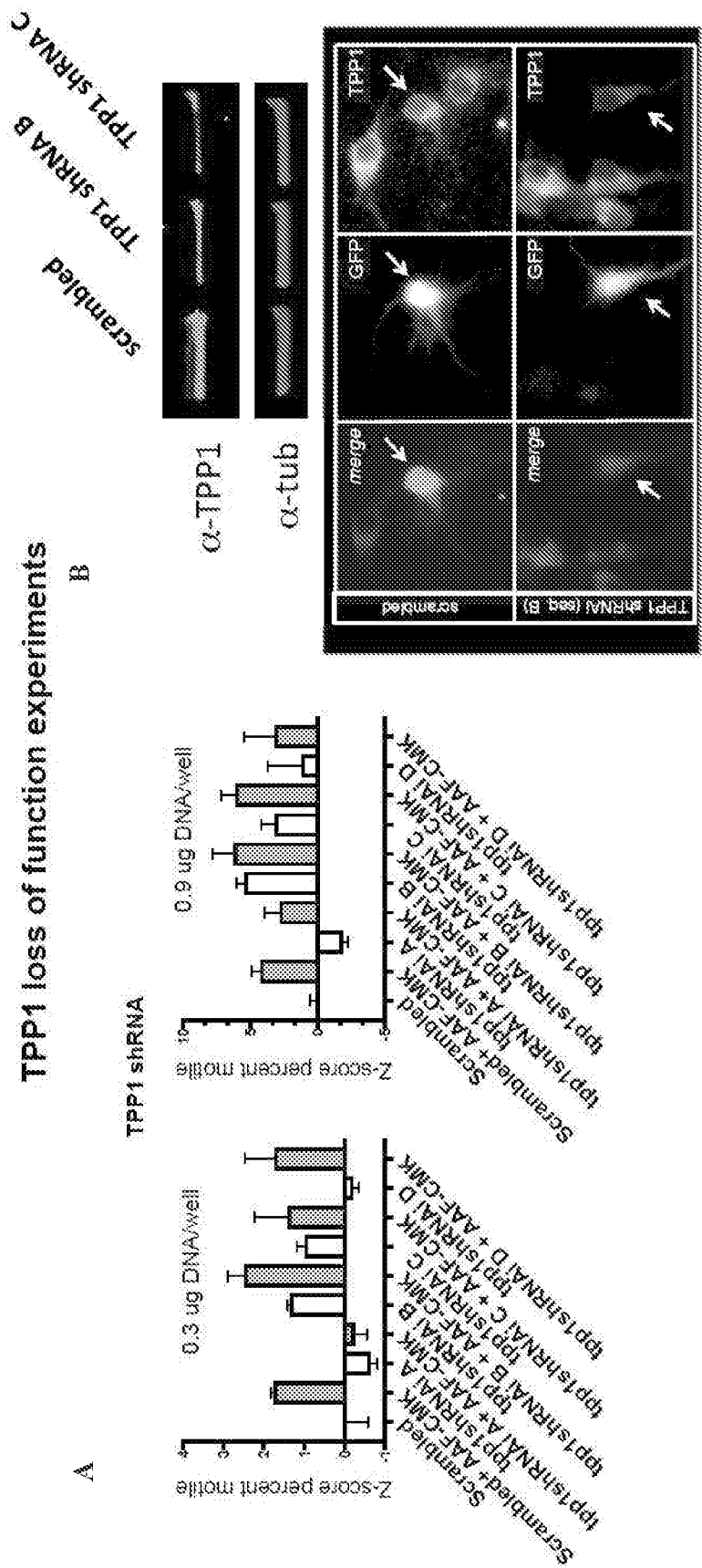


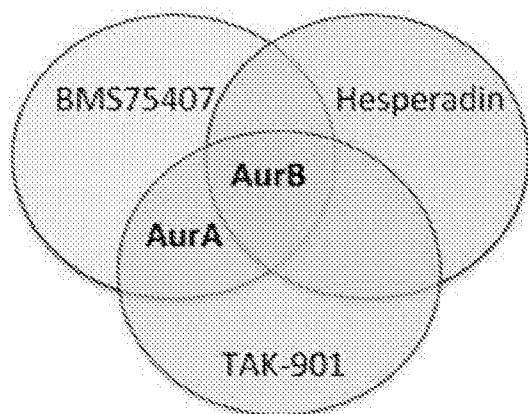
FIG. 14

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All specific inhibitors of AurA are non-hits

A

**Aurora family is a shared
target**



BMS75407 is not considered an inhibitor of this family but can inhibit *kinase1* and *kinase2* with IC₅₀~10nM *in vitro*

FIG. 15A

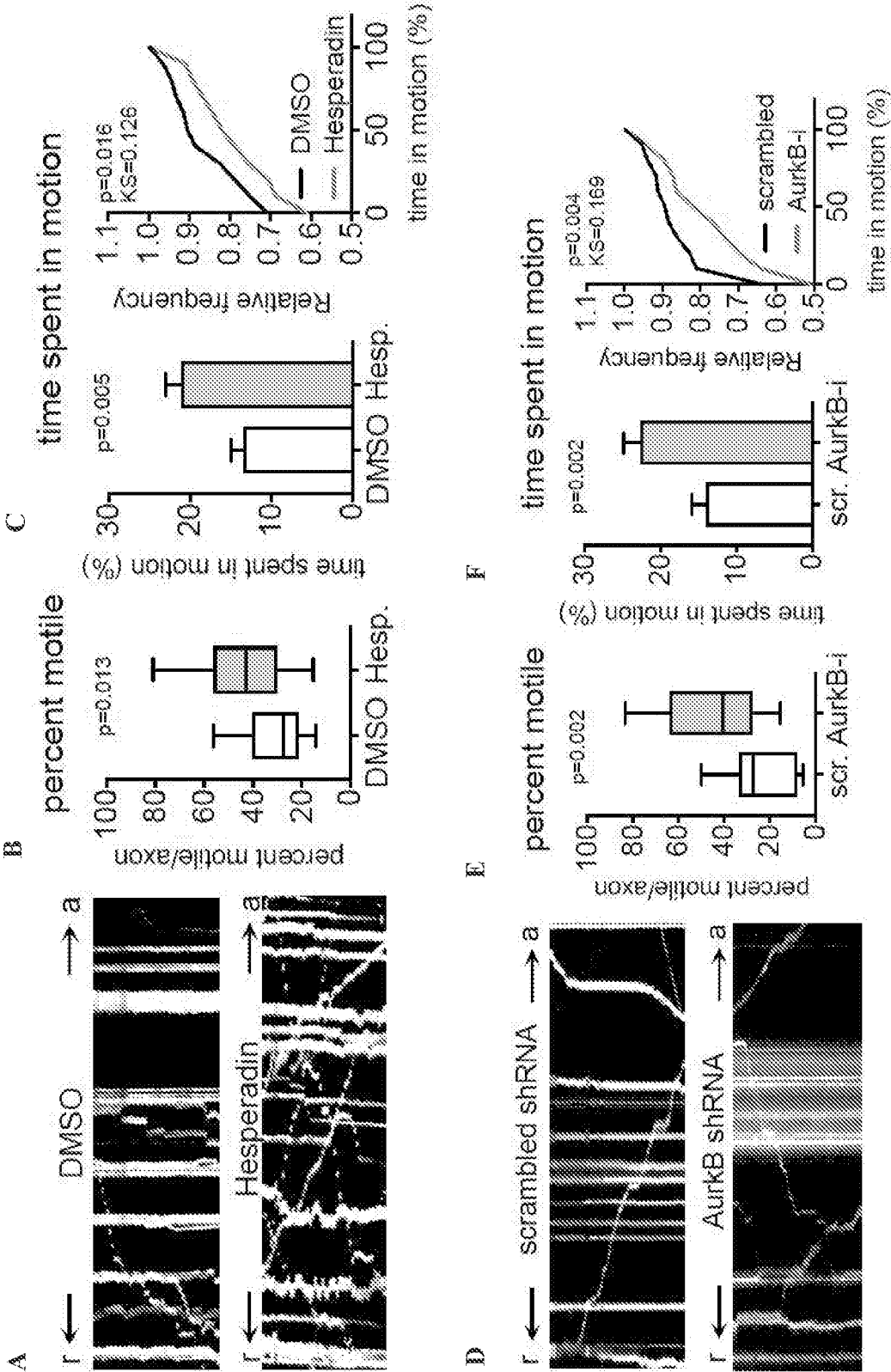


FIG. 16

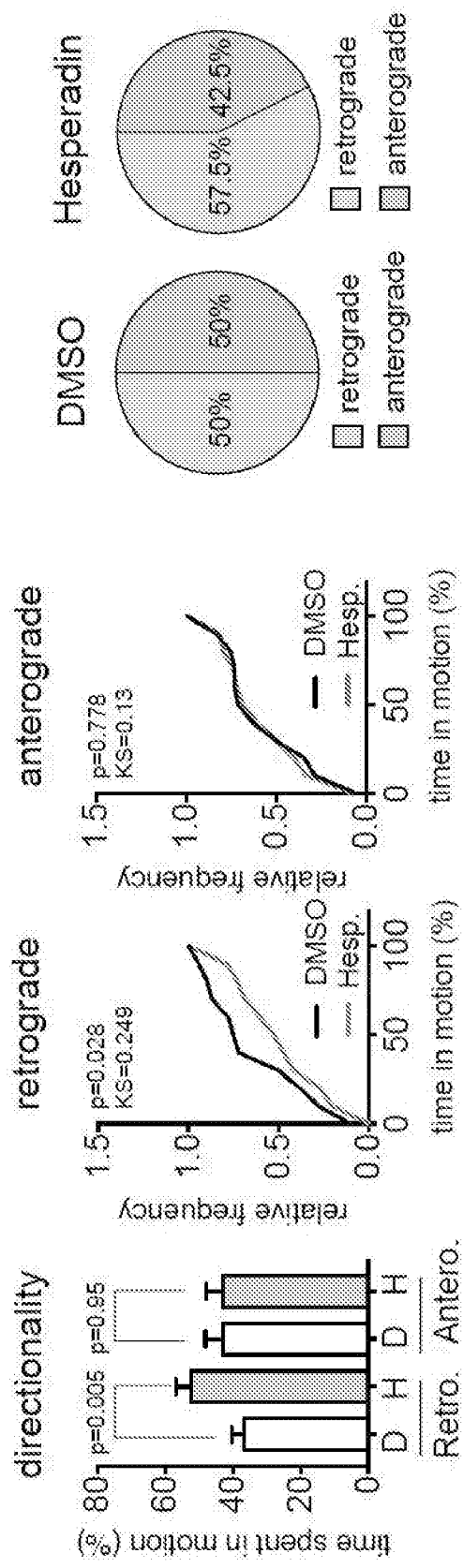


FIG. 17

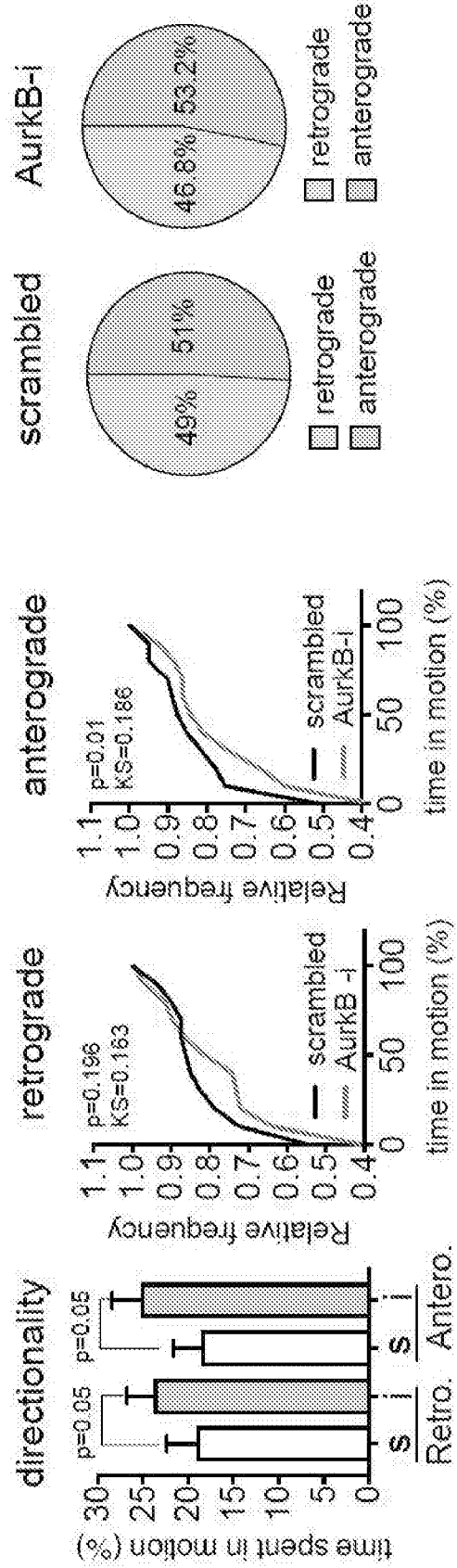


FIG. 18

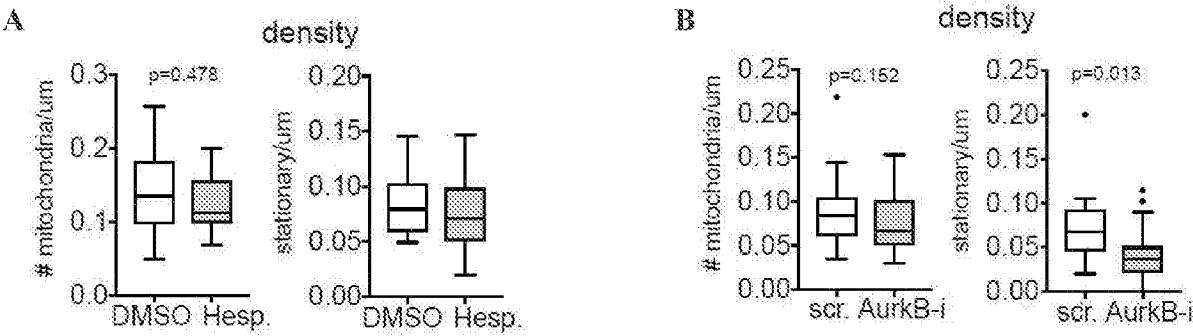


FIG. 19

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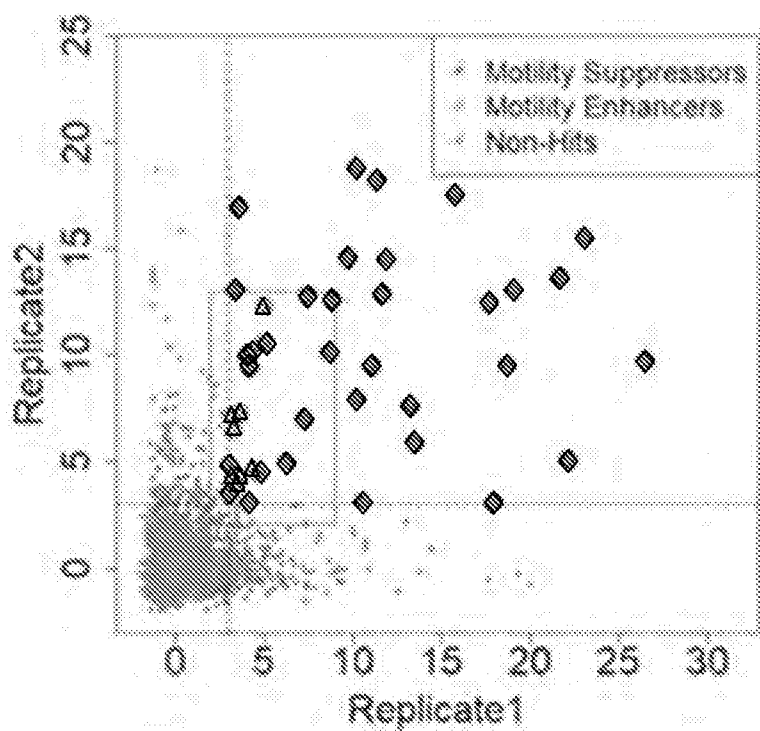


FIG. 20A

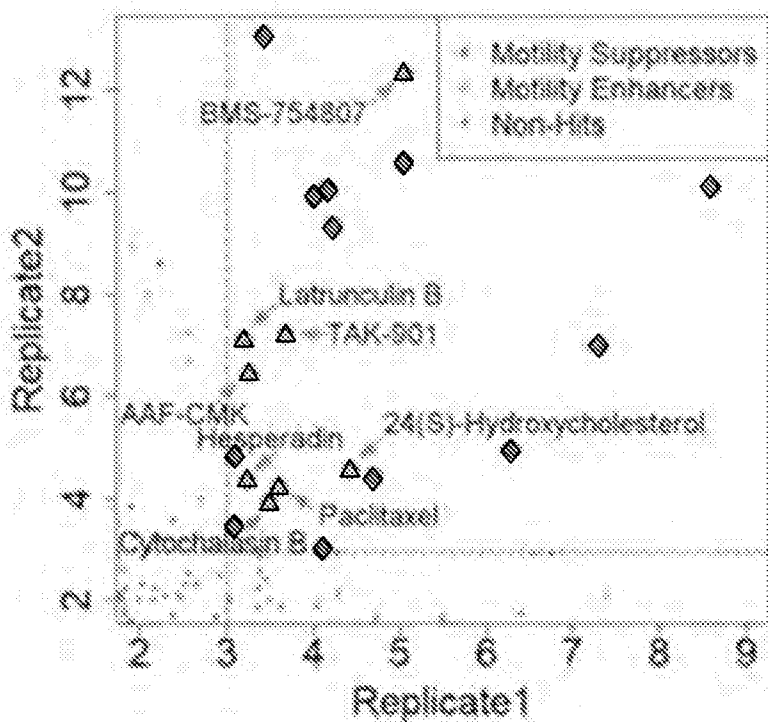


FIG. 20B

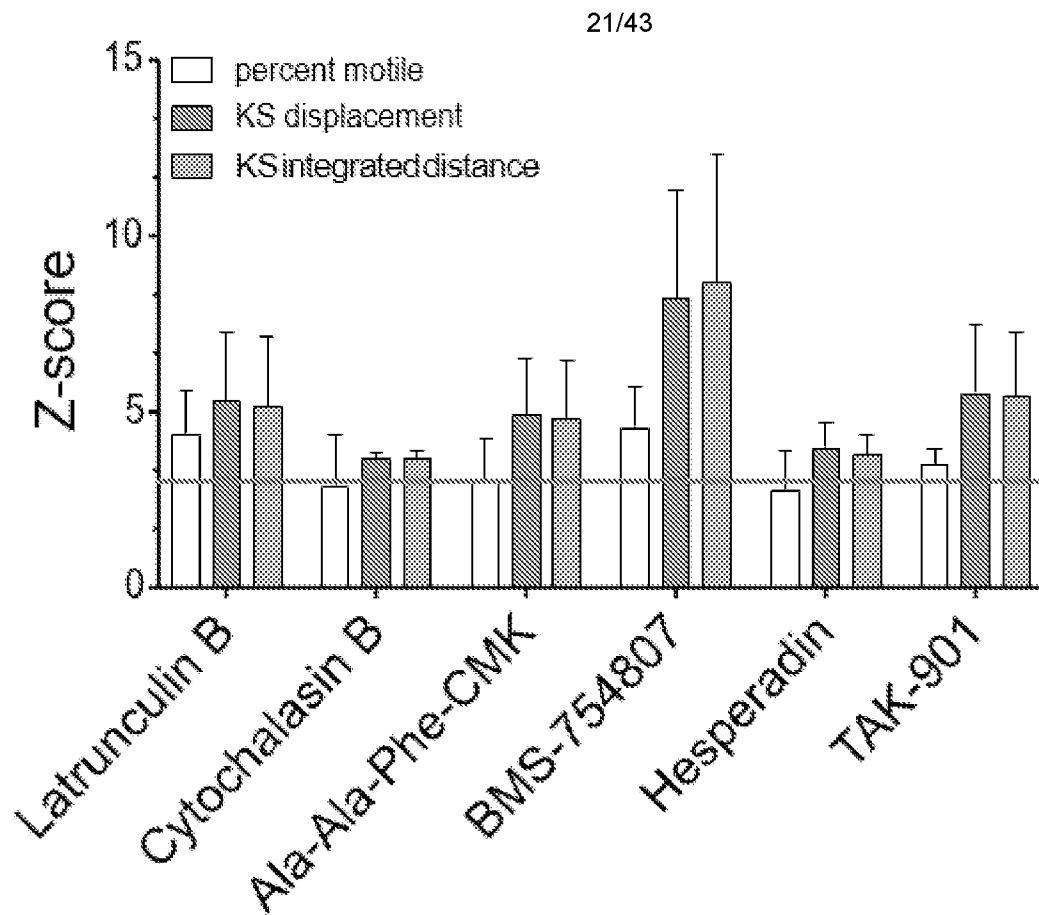


FIG. 20C

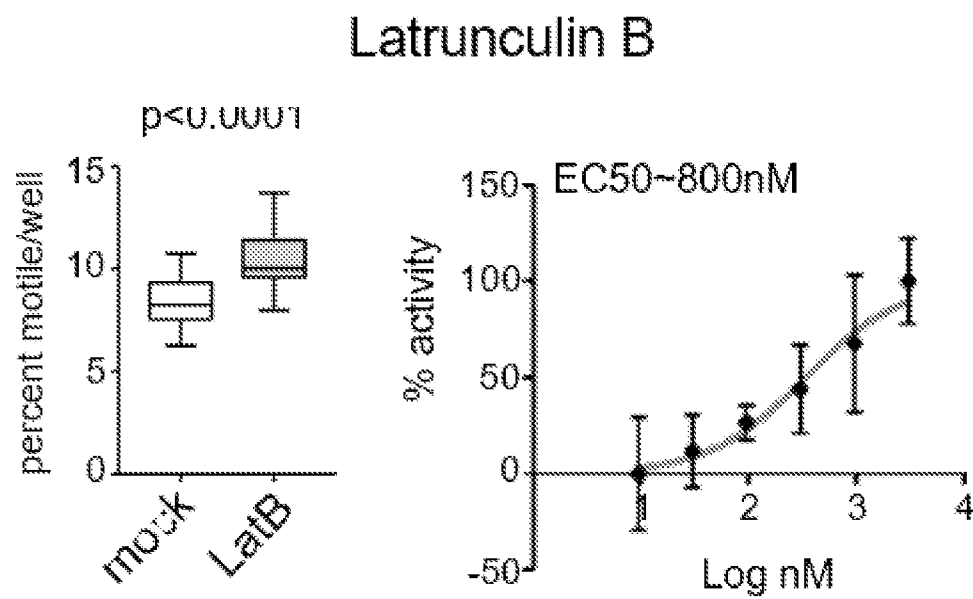


FIG. 20D

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Cytochalasin B

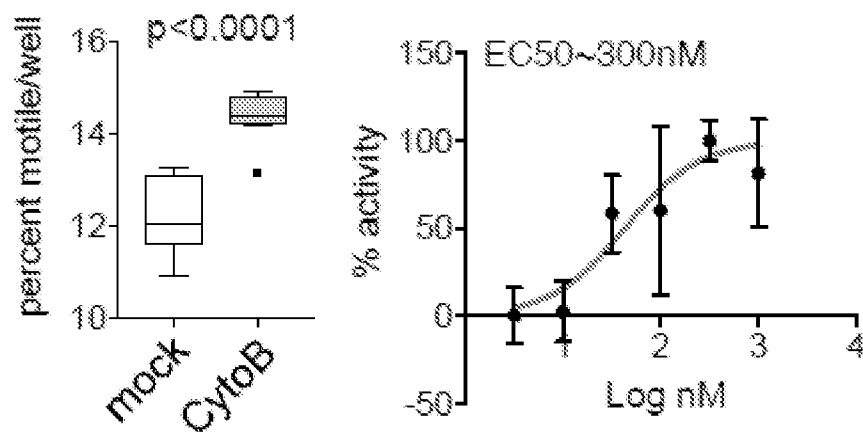


FIG. 20E

Ala-Ala-Phe-CMK

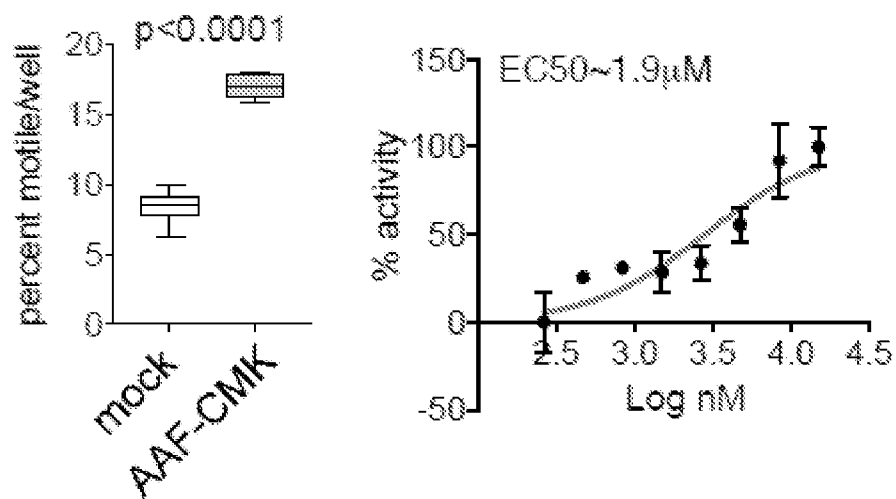


FIG. 20F

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BMS-754807

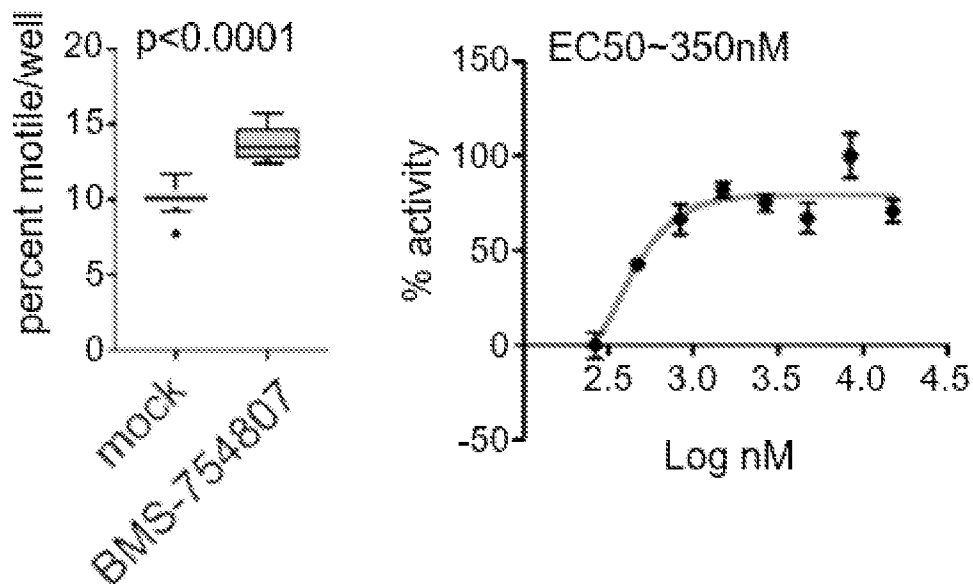


FIG. 20G

Hesperadin

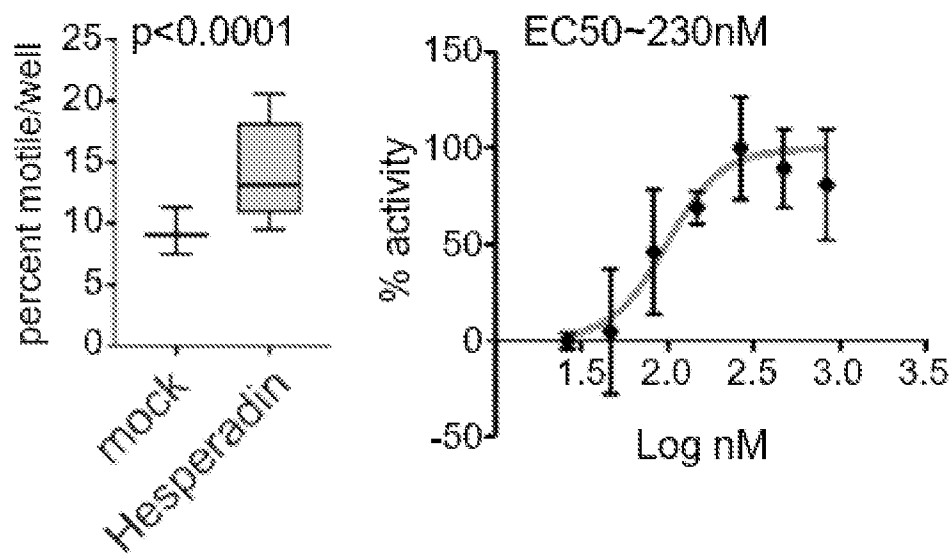


FIG. 20H

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TAK-901

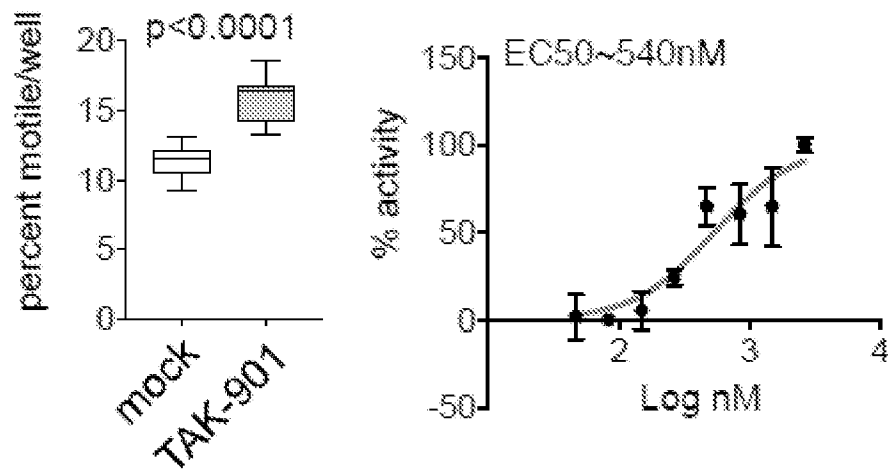


FIG. 20I

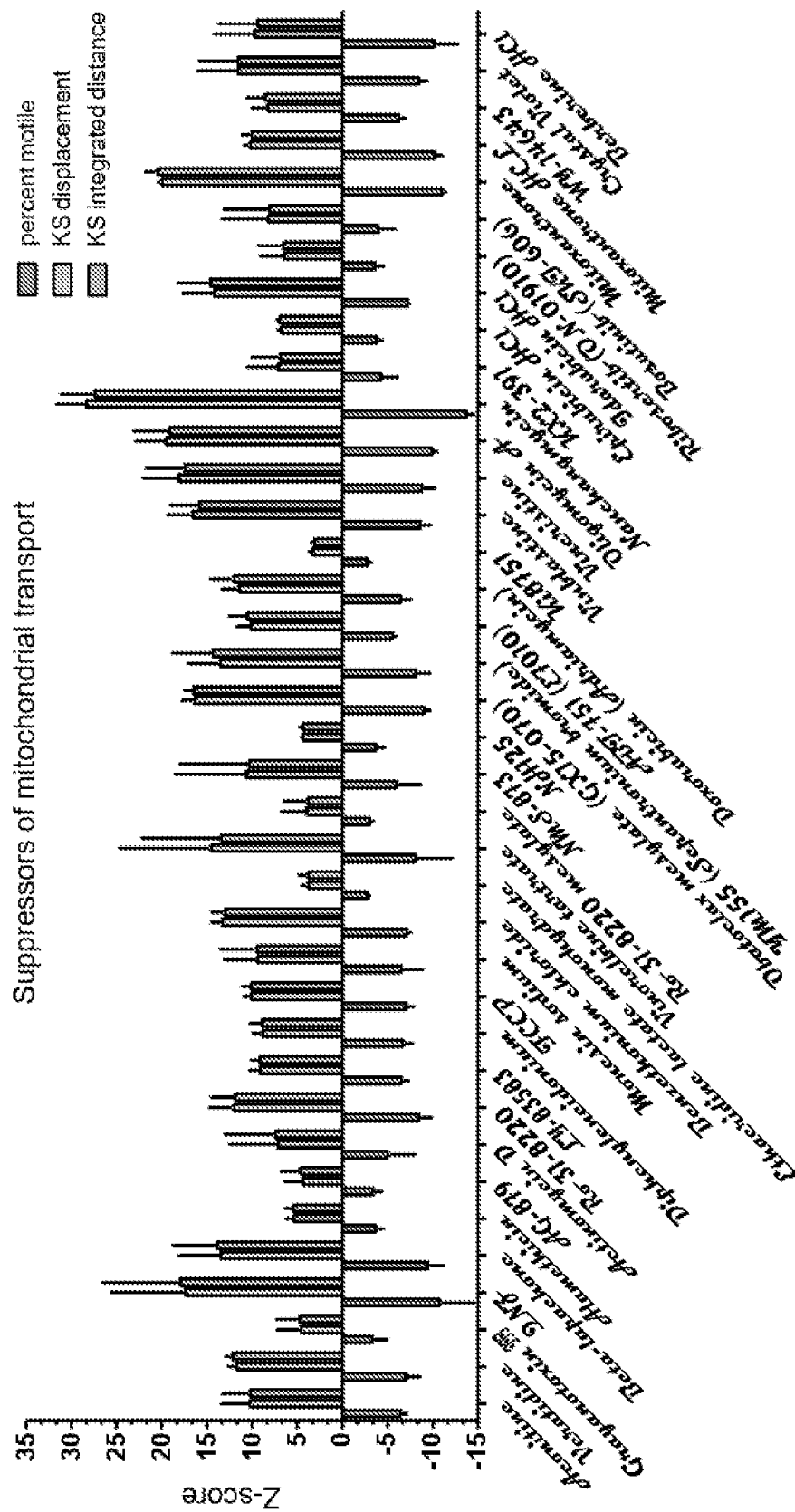


FIG. 21A

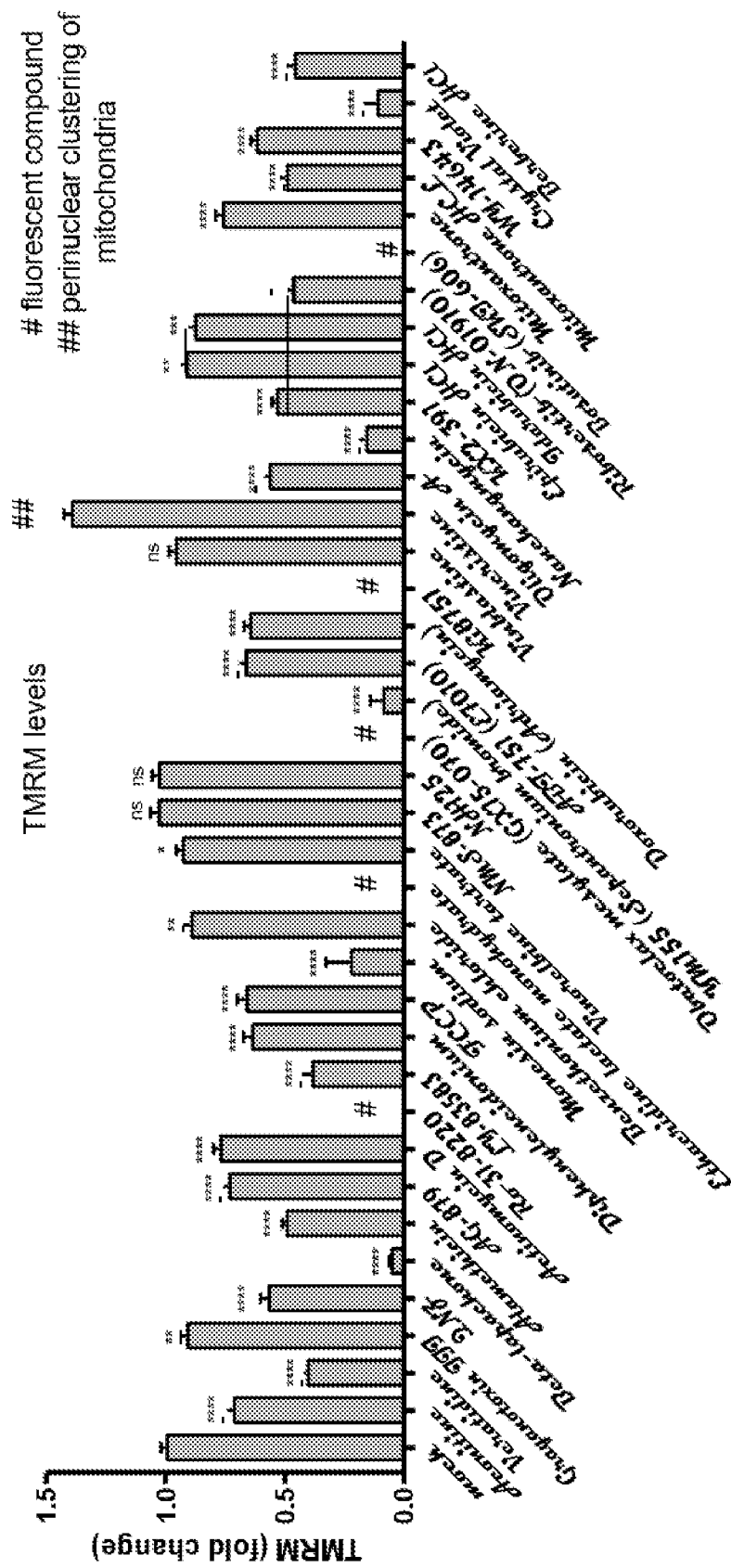


FIG. 21B

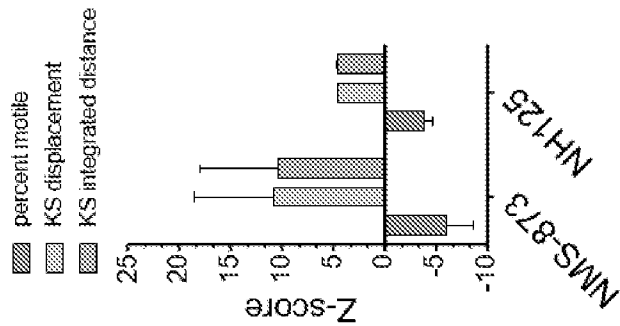


FIG. 21C

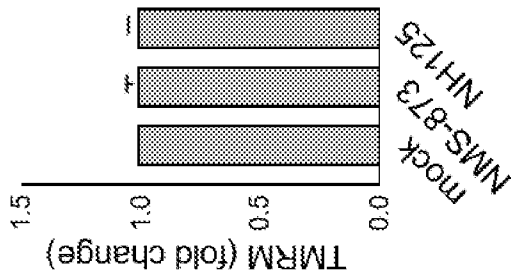


FIG. 21D

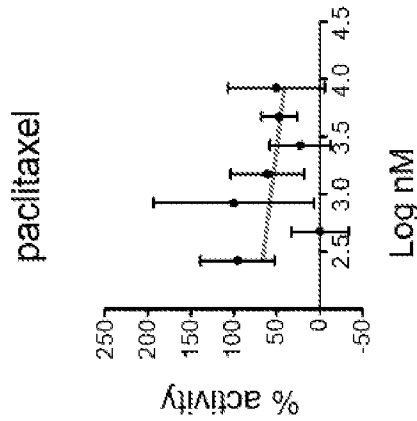
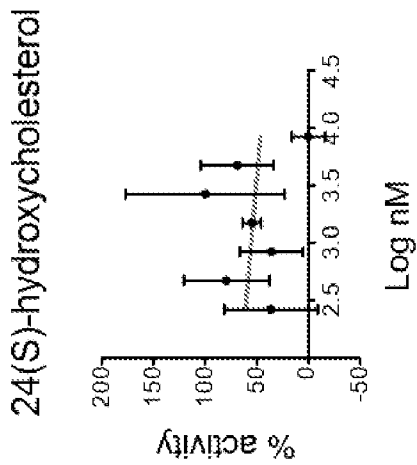


FIG. 21E



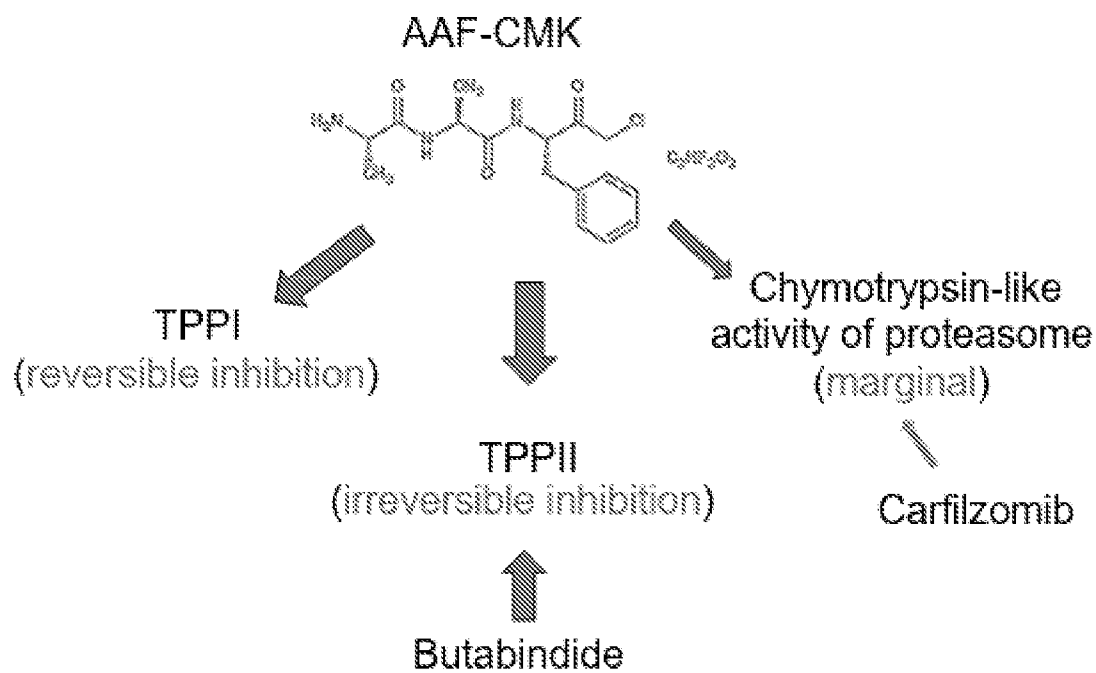


FIG. 22A

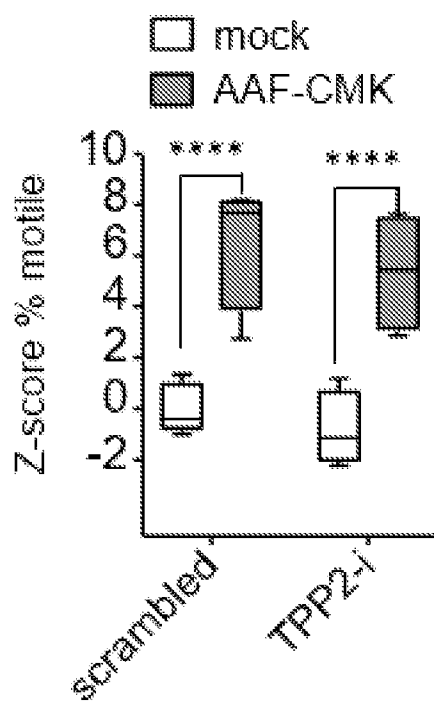


FIG. 22B

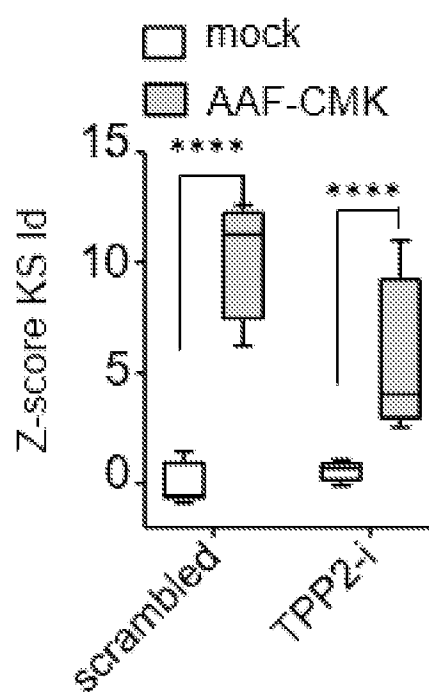


FIG. 22C

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Butabindide

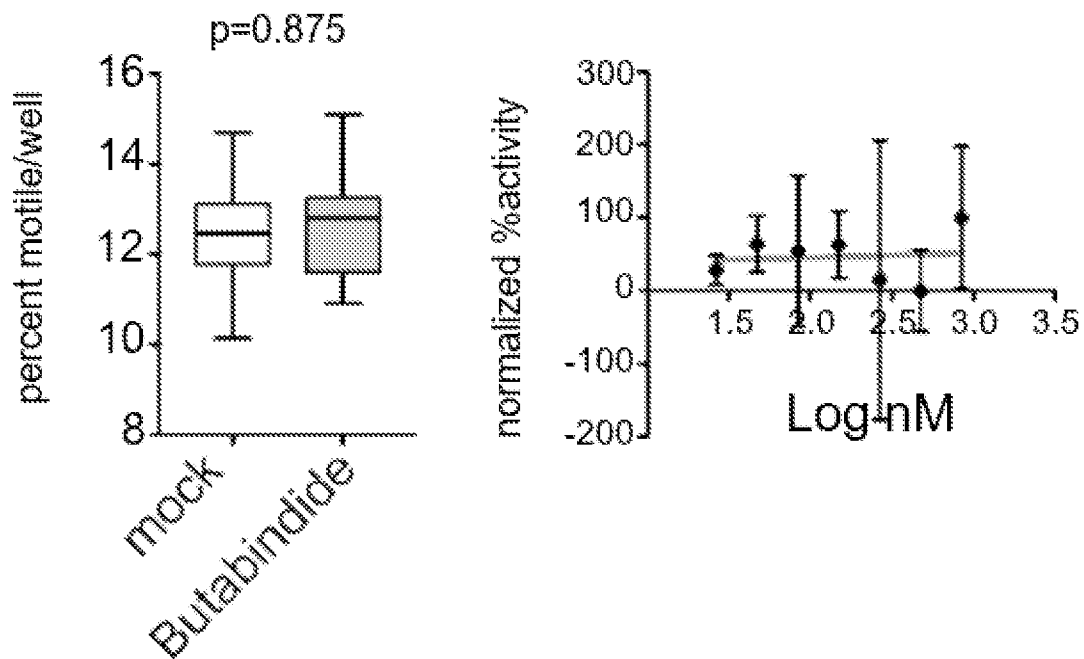


FIG. 22D

Carfilzomib

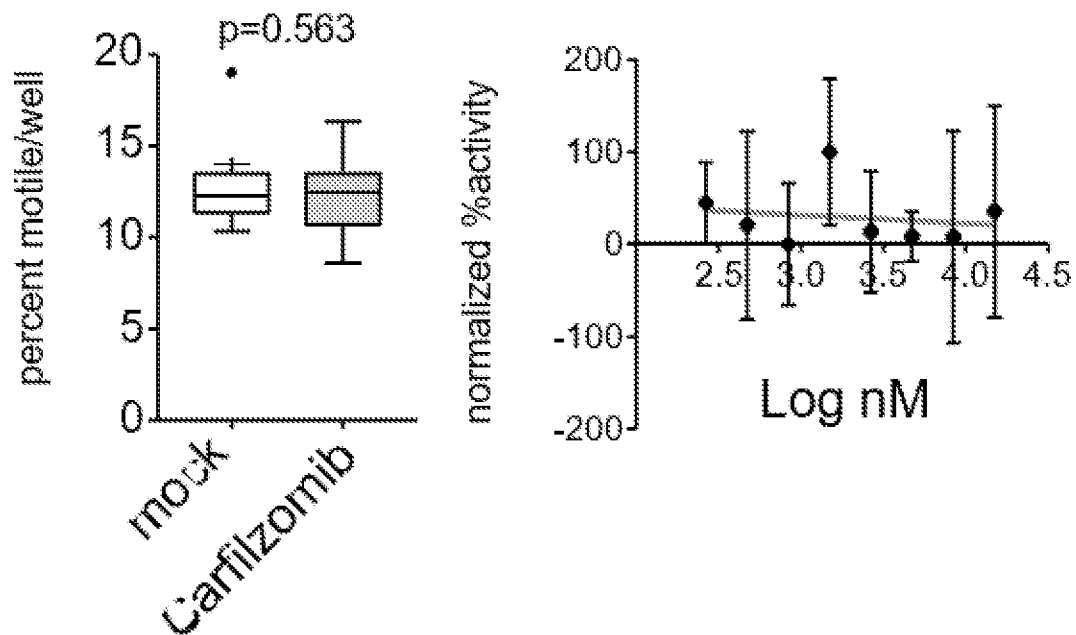


FIG. 22E

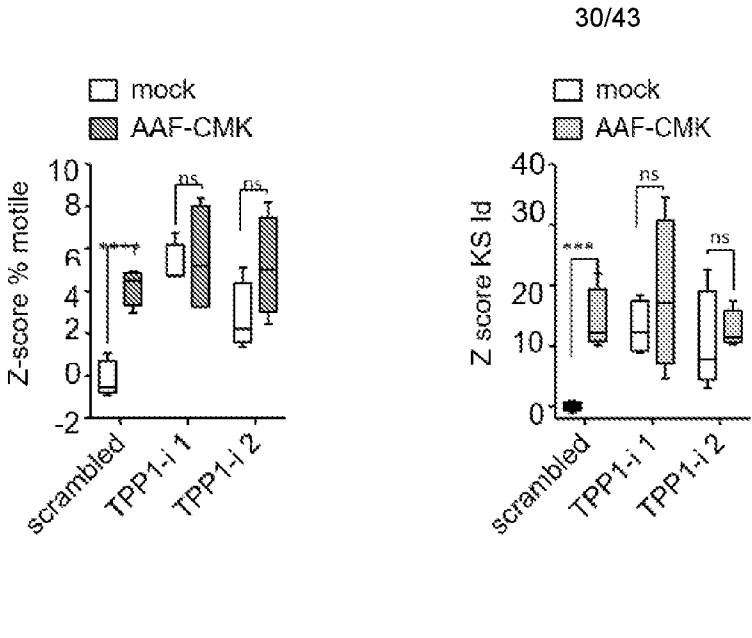


FIG. 22F

FIG. 22G

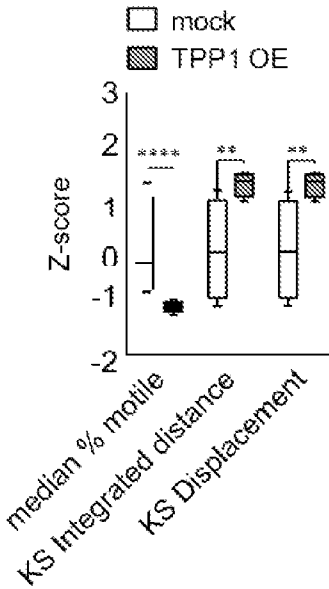


FIG. 22H

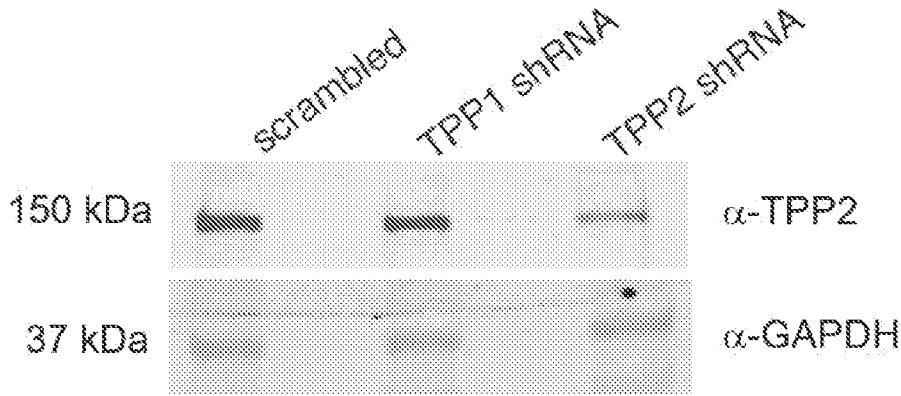


FIG. 23A

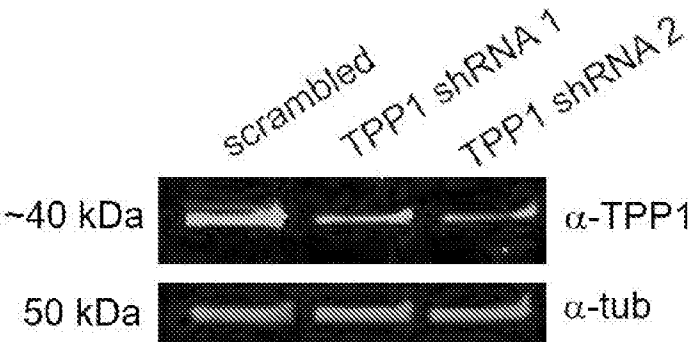


FIG. 23B

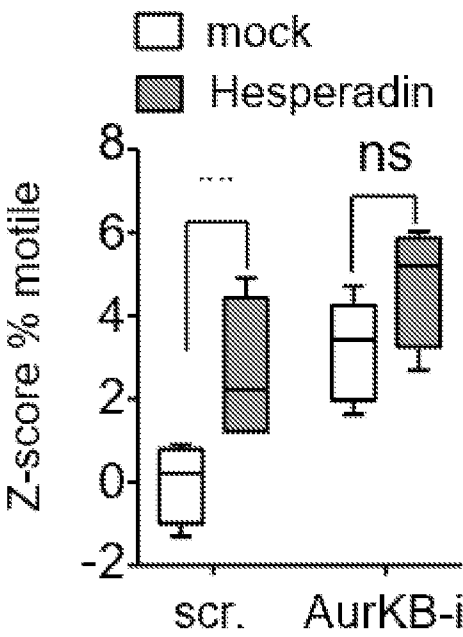


FIG. 24A

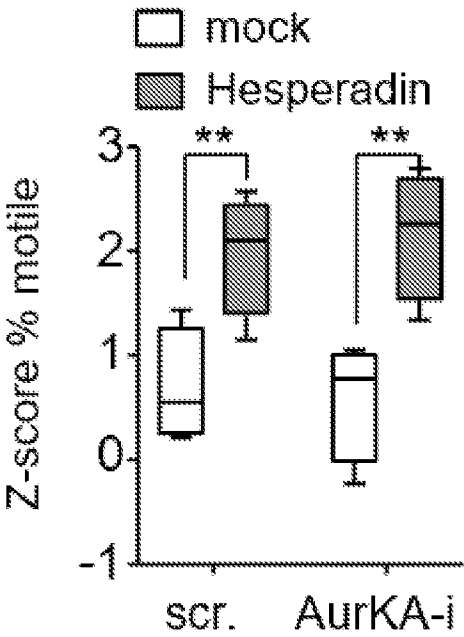


FIG. 24B

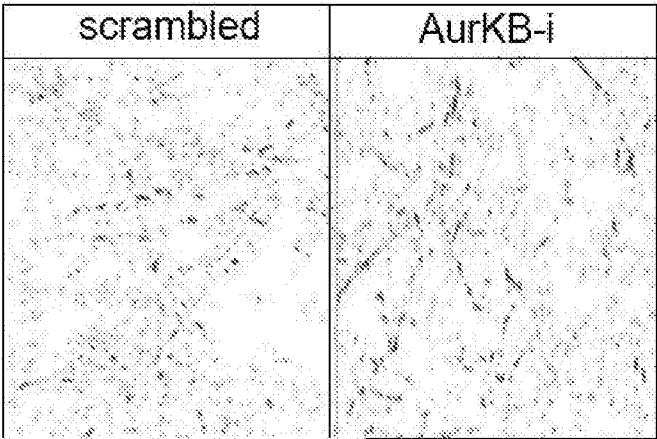


FIG. 24C

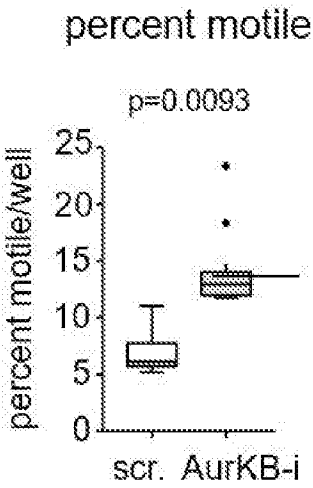


FIG. 24D

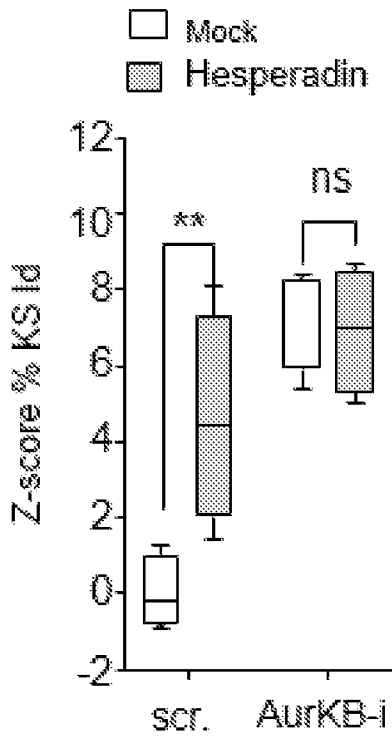


FIG. 24E

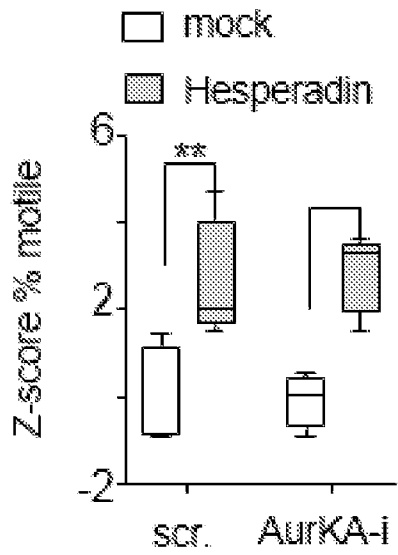


FIG. 24F

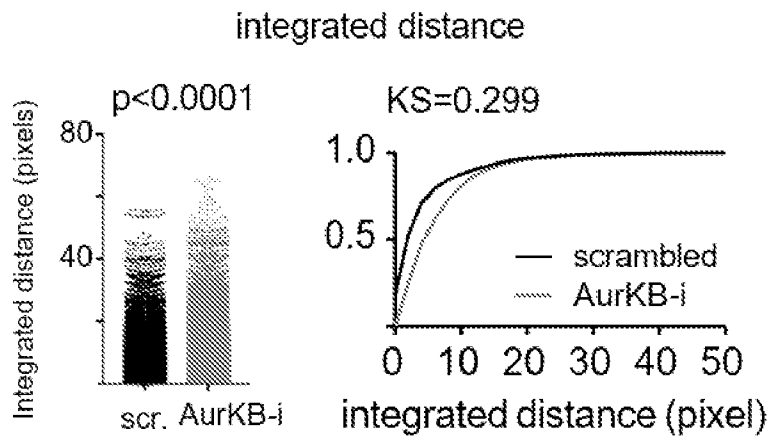


FIG. 24G

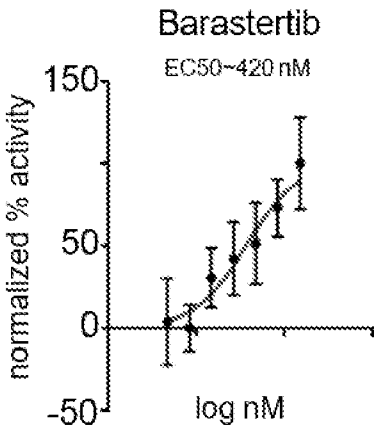


FIG. 24H

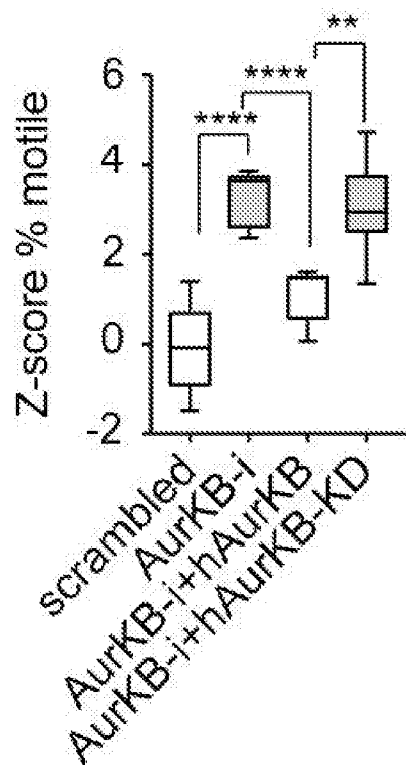


FIG. 24I

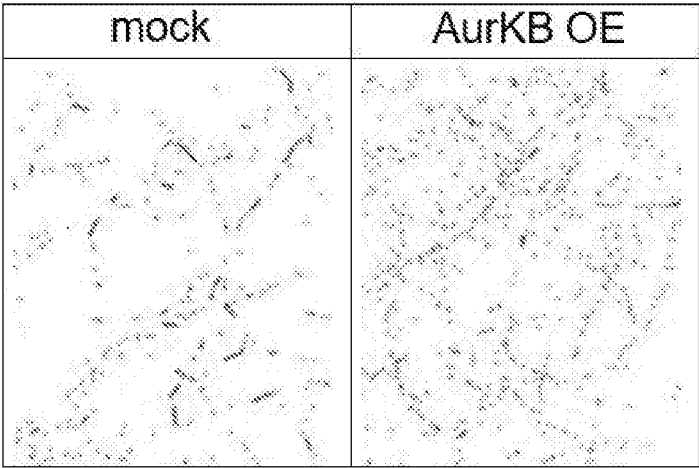


FIG. 24J

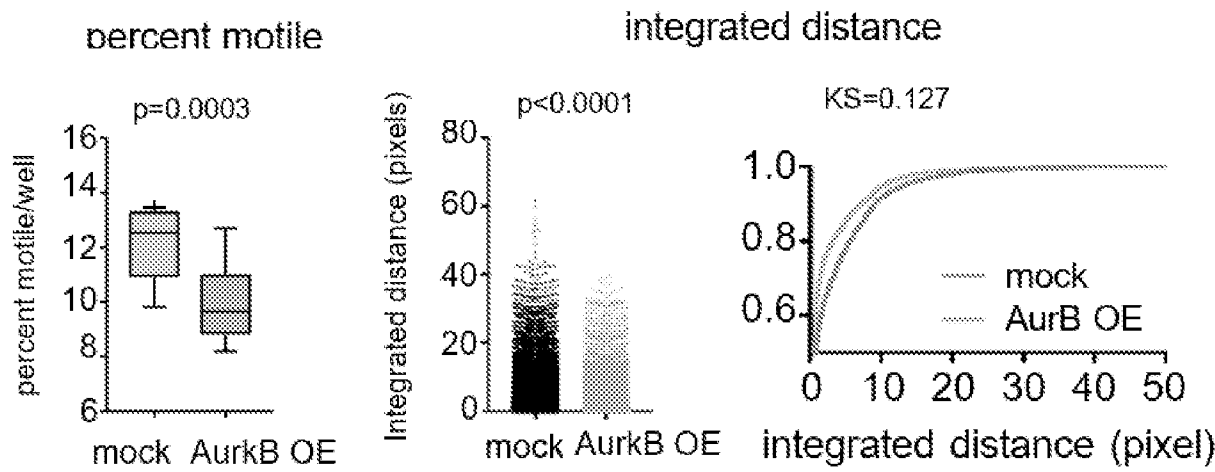


FIG. 24K

FIG. 24L

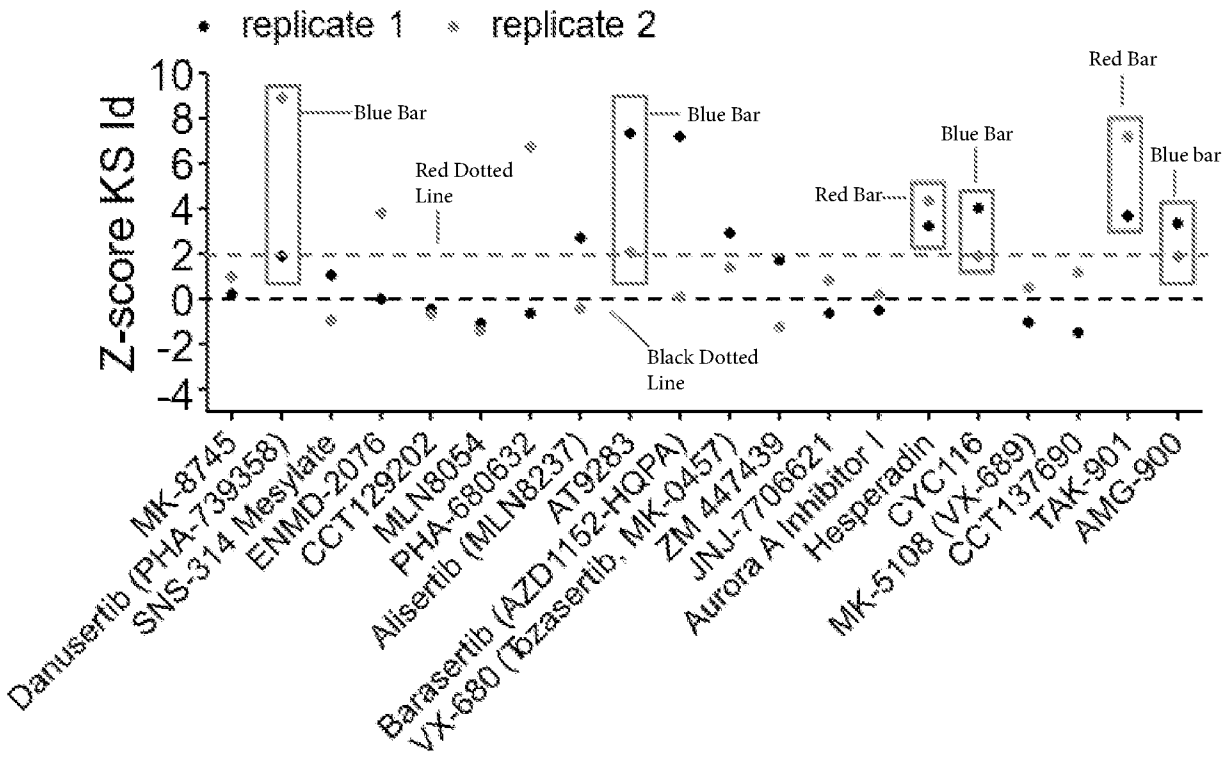


FIG. 25A

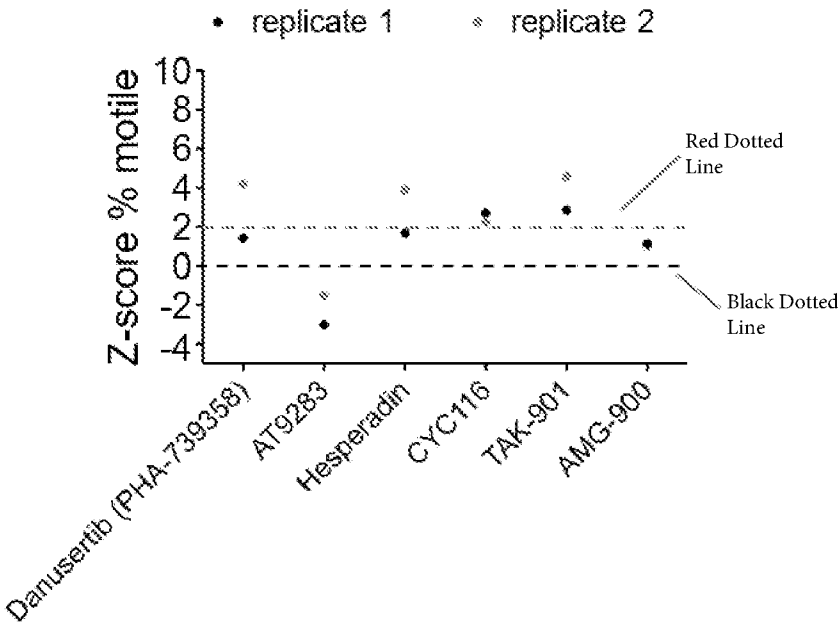


FIG. 25B

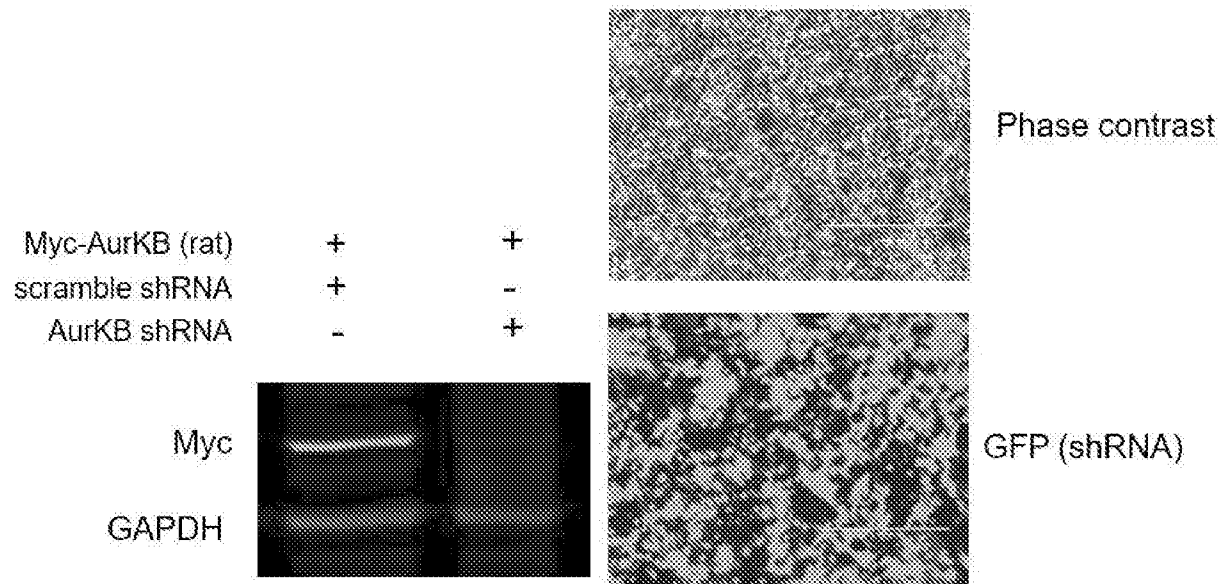


FIG. 25C

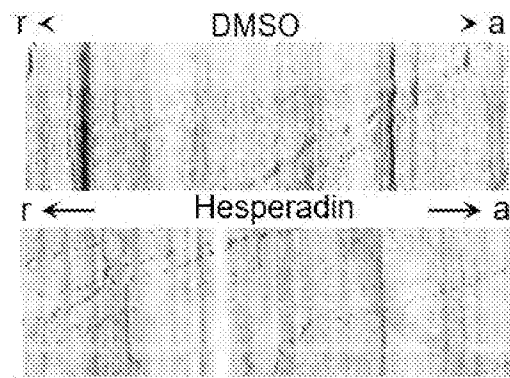


FIG. 26A

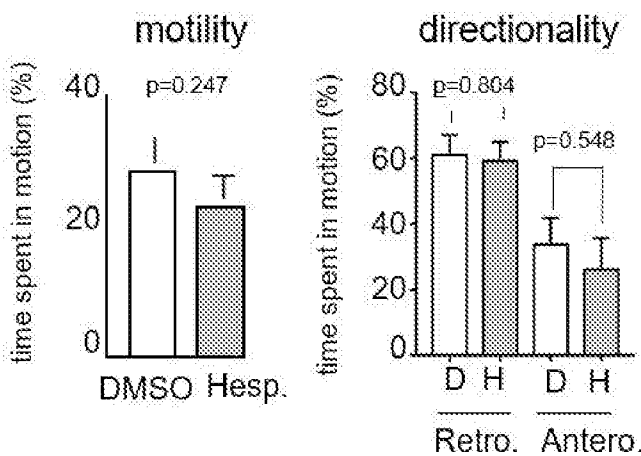


FIG. 26B

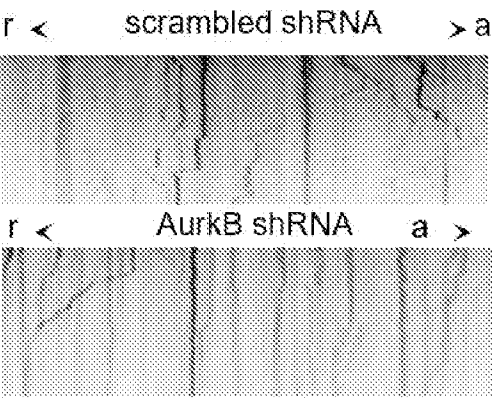


FIG. 26C

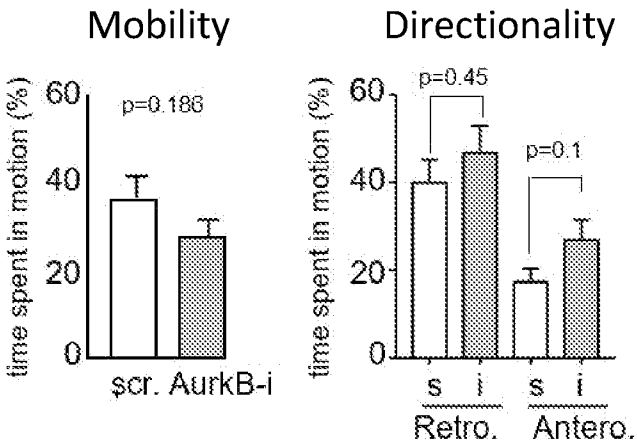


FIG. 26D

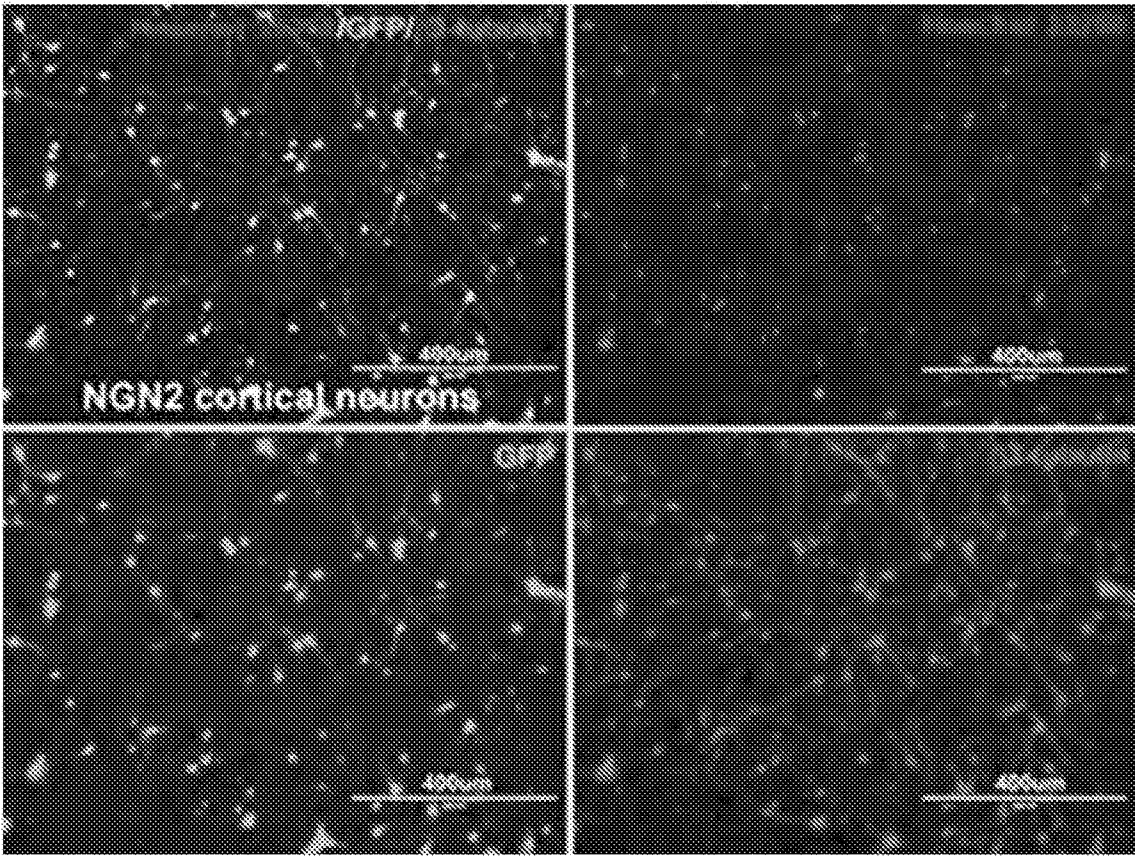


FIG. 27A

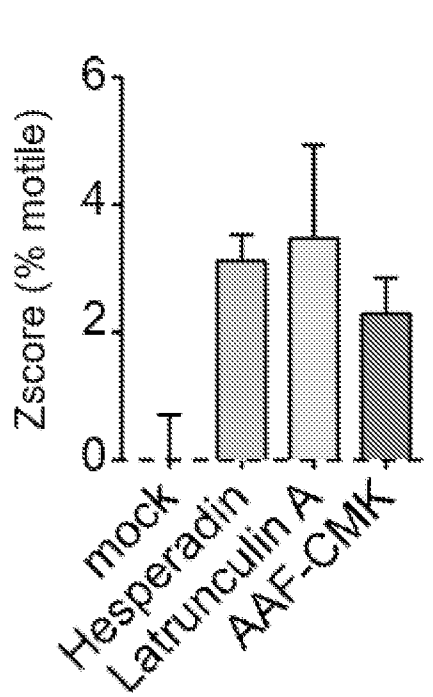


FIG. 27B

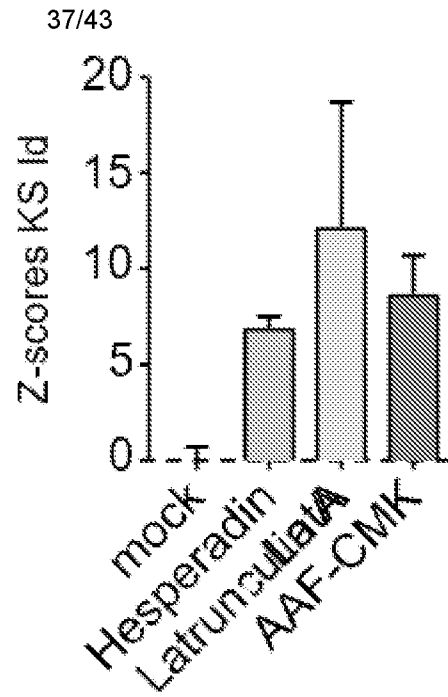


FIG. 27C

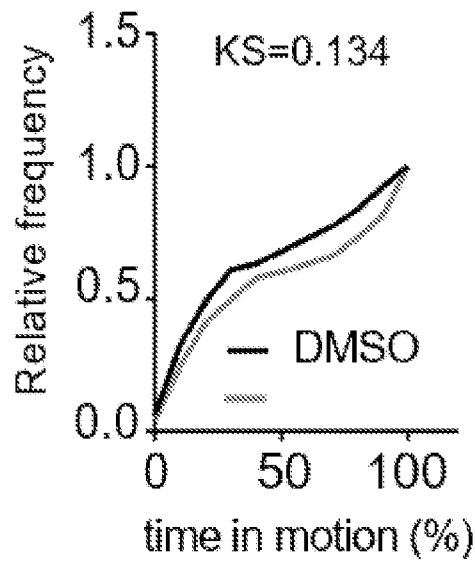
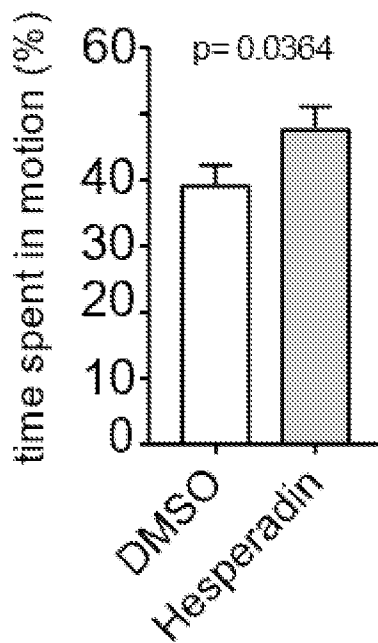


FIG. 27D

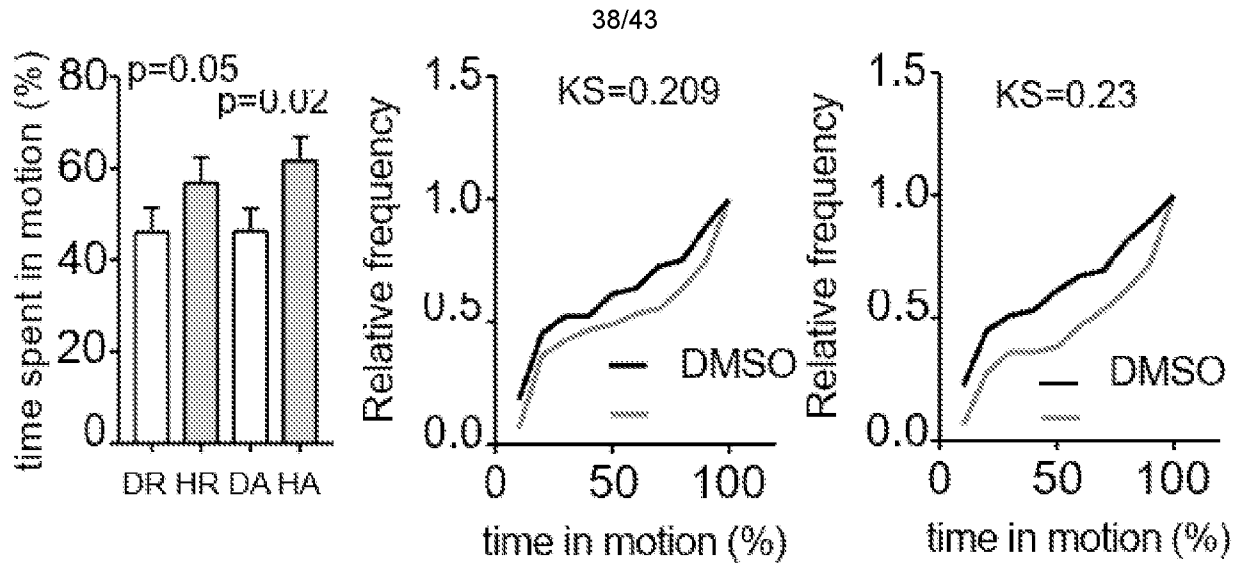


FIG. 27E

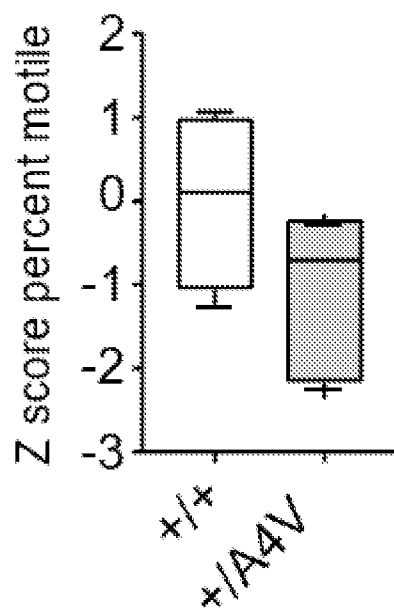


FIG. 27F

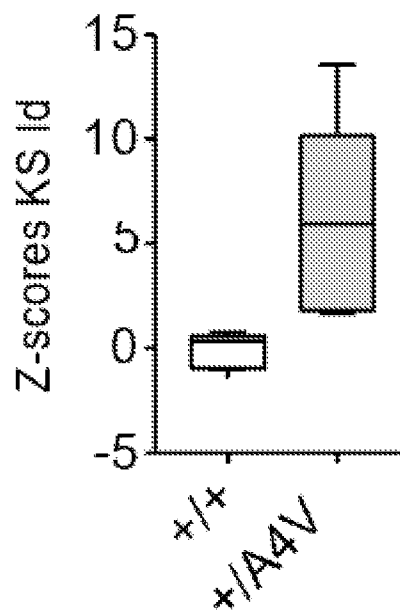


FIG. 27G

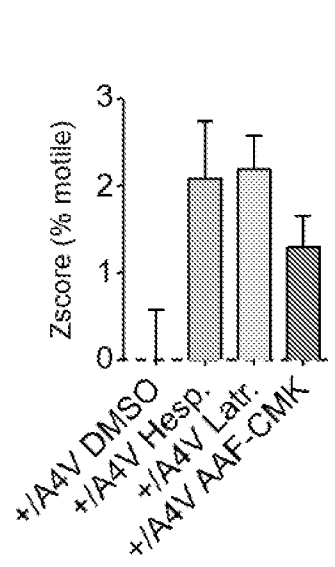


FIG. 27H

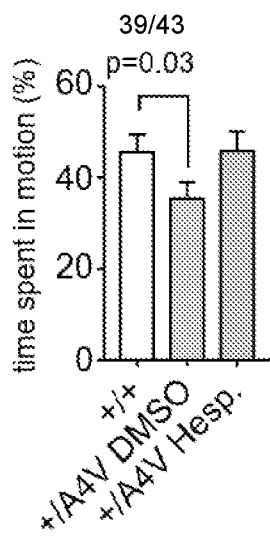


FIG. 27I

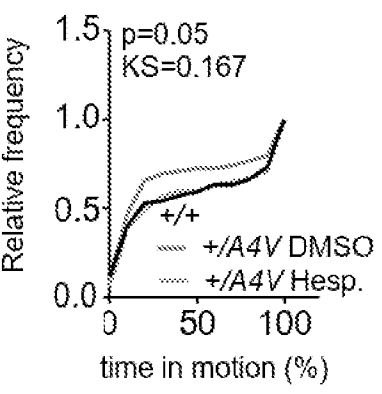
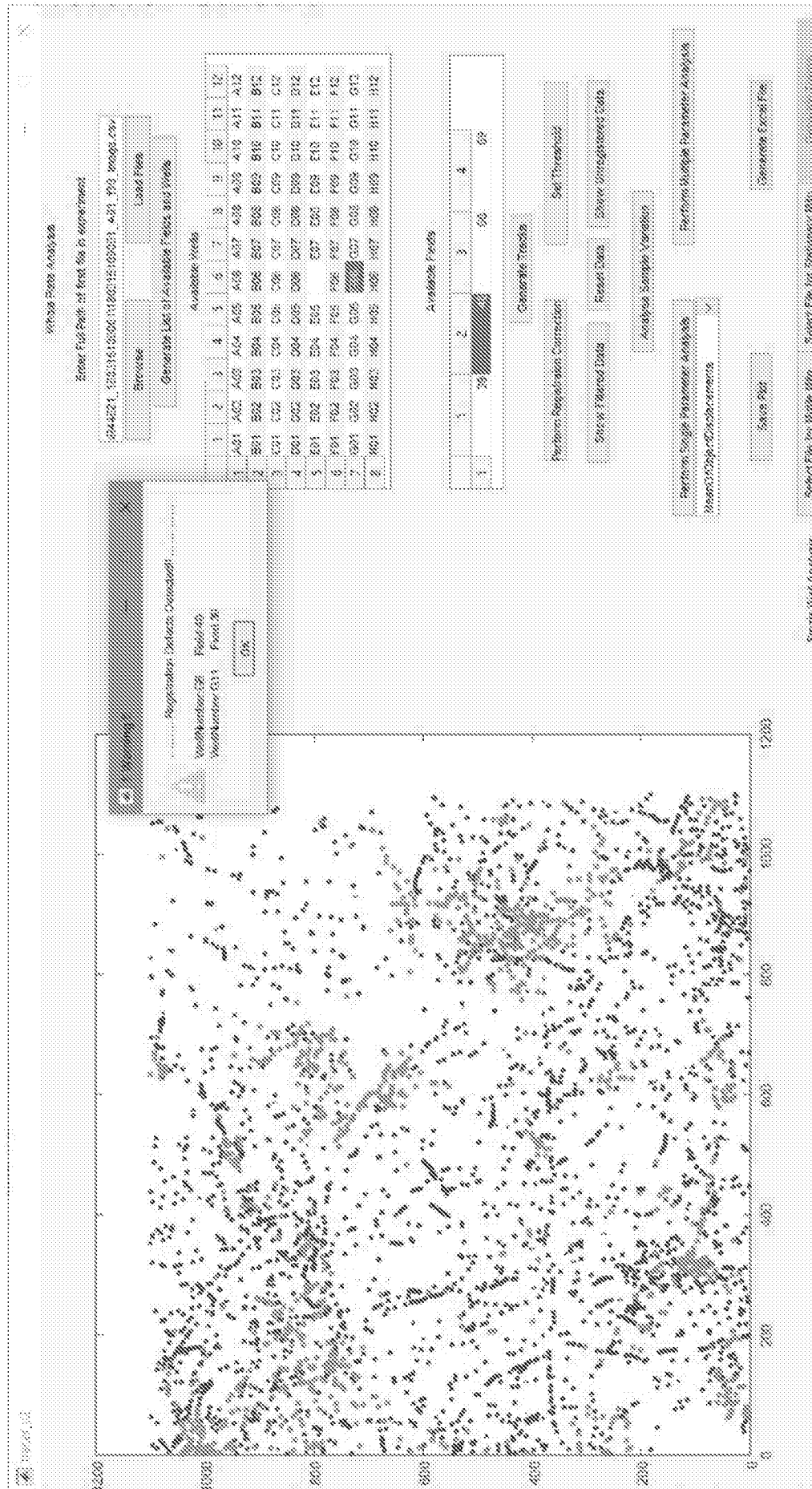
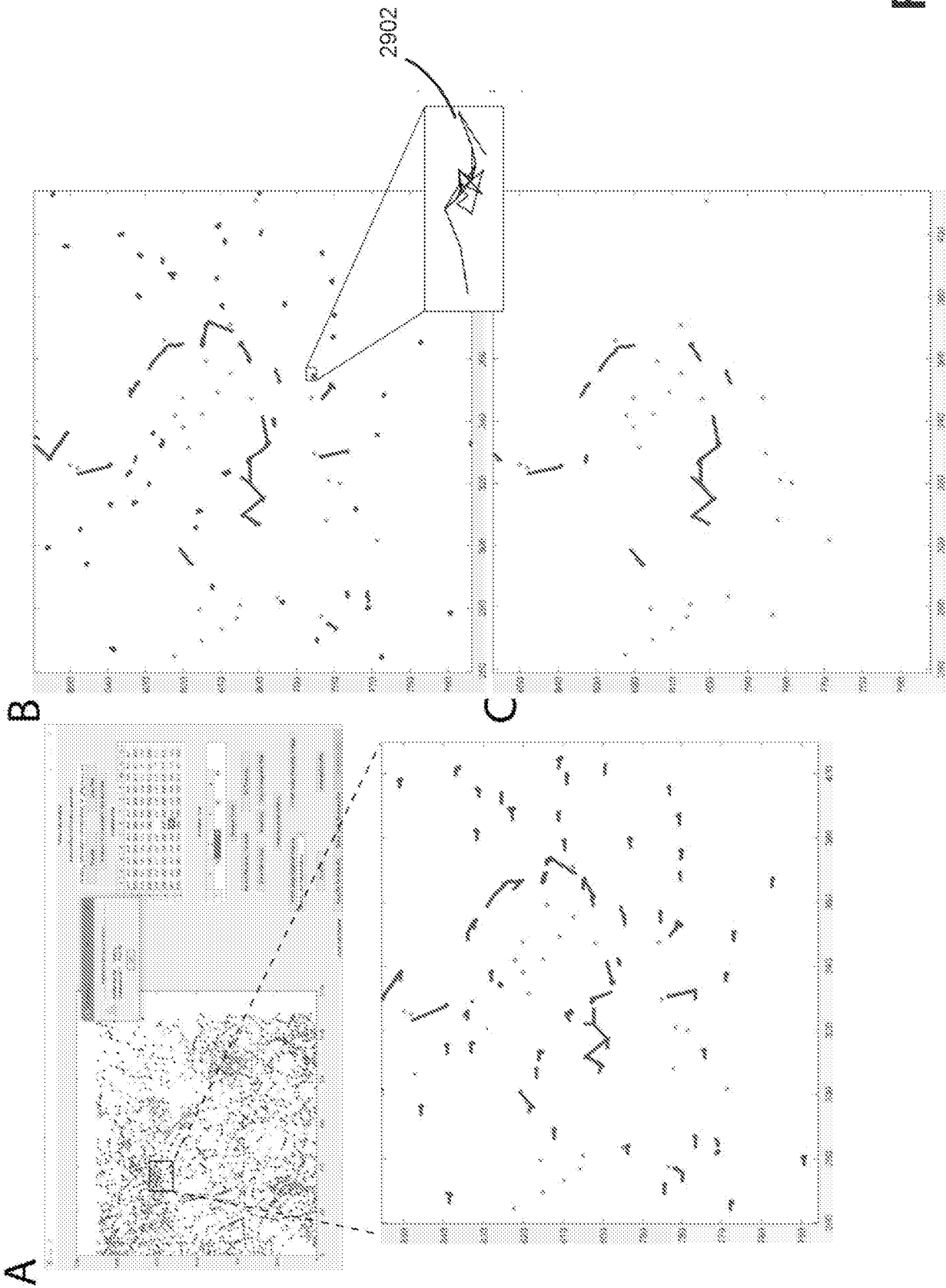


FIG. 27J



826

FIG. 29



3002

B

Perform Single Parameter Analysis

ZScoresOfMedianOfPercentMotility

MeanOfObjectDisplacements

NumberOfObjects

MedianOfObjectDisplacements

KS2StatOfObjectDisplacements

MeanOfObjectIntDistance

MedianOfObjectIntDistance

KS2StatOfObjectIntDistance

MeanOfPercentMotility

MedianOfPercentMotility

ZScoresOfMeanOfObjectDisplacement

ZScoresOfMedianOfObjectDisplacement

ZScoresOfKS2StatOfObjectDisplacement

ZScoresOfMeanOfObjectIntDistance

ZScoresOfMedianOfObjectIntDistance

ZScoresOfKS2StatOfObjectIntDistance

ZScoresOfMeanOfPercentMotility

ZScoresOfMedianOfPercentMotility

A

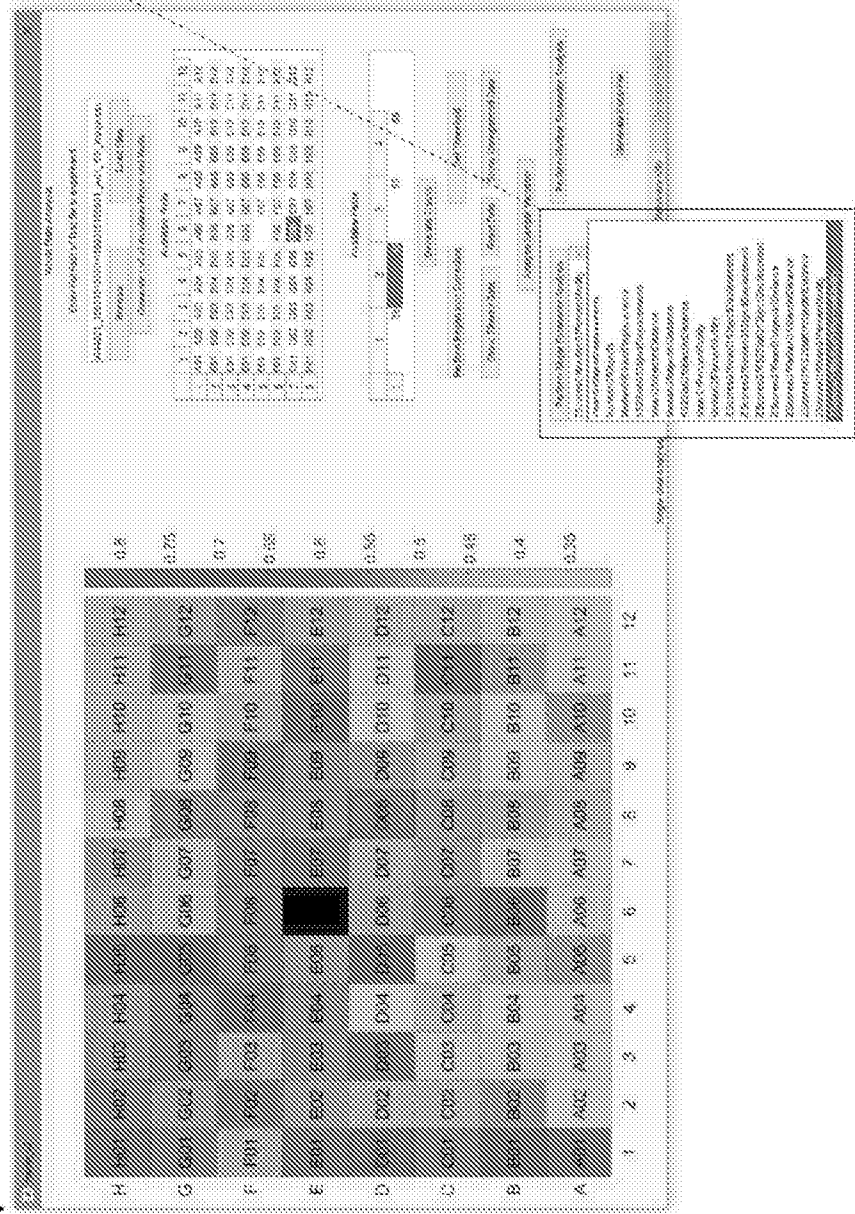
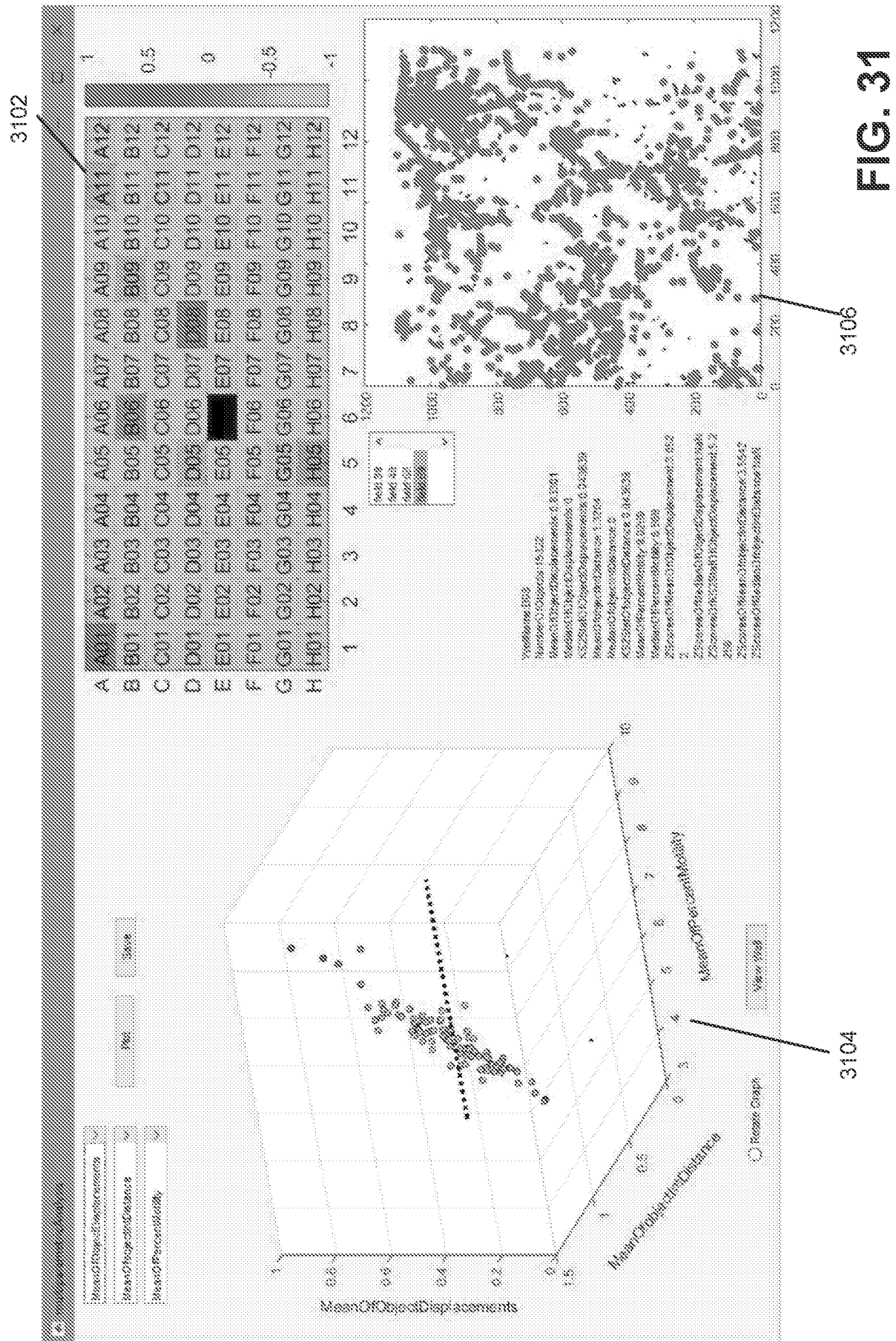


FIG. 30



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/061557

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/11; A61K 31/351; A61K 31/665; A61P 25/28; G01N 33/50; G01N 33/68 (2020.01)

CPC - A61K 31/665; A61K 31/708; A61K 38/1709; A61P 25/28; G01N 33/5029; G01N 33/5058; G01N 33/5079; G01N 33/6896; G01N 33/92; G01N 2333/775 (2020.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2018/106918 A1 (THE CHILDREN'S MEDICAL CENTER CORPORATION) 14 June 2018 (14.06.2018) entire document	1, 5-14, 18-22, 33, 34 ----- 2-4, 15-17
Y	GERENCSEI et al. "Measurement of Instantaneous Velocity Vectors of Organelle Transport: Mitochondrial Transport and Bioenergetics in Hippocampal Neurons," Biophysical Journal, 15 September 2008 (15.09.2008), Vol. 95, No. 6, Pgs. 3079-3099. entire document	2-4, 15-17
A	US 2016/0272618 A1 (CHILDREN'S MEDICAL CENTER CORPORATION et al) 22 September 2016 (22.09.2016) entire document	1-22, 33, 34
A	HOLLENBECK et al. "The Axonal Transport of Mitochondria," Journal of Cell Science, 01 December 2005 (01.12.2005), Vol. 118, Iss. 23, Pgs. 5411-5419. entire document	1-22, 33, 34
A	BROUGH et al. "Agonist-Induced Regulation of Mitochondrial and Endoplasmic Reticulum Motility," Biochem J, 28 June 2005 (28.06.2005), Vol. 392, Pt 2, Pgs. 291-297. entire document	1-22, 33, 34
A	US 2007/0178535 A1 (HUANG et al) 02 August 2007 (02.08.2007) entire document	1-22, 33, 34
A	WANG et al. "Imaging Axonal Transport of Mitochondria," Methods in Enzymology, 01 January 2009 (01.01.2009), Vol. 457, Pgs. 319-333. entire document	1-22, 33, 34

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 January 2020

Date of mailing of the international search report

10 FEB 2020

Name and mailing address of the ISA/US

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Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/061557

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 23-32, 35-42
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.