AN *IN VIVO* ASSAY FOR QUANTITATIVE ANALYSIS OF ARP2/3 COMPLEX INHIBITORS

by

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A THESIS

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Branched networks in the actin cytoskeleton are critical for a variety of cellular processes such as motility and endocytosis. New branched actin filaments are nucleated by Arp2/3 complex, and the deregulation of this protein assembly is linked to a variety of diseases including cancer. Several classes of small molecule inhibitors of Arp2/3 complex have been discovered and characterized in previous research. These molecules are useful tools because they allow researchers to turn off the activity of the complex in different processes, and they have potential as drugs due to the increased activity of Arp2/3 complex in some diseases. These inhibitors have been characterized *in vitro* and in some cell-based systems, but there has been limited *in vivo* quantitative analysis. My project developed an *in vivo* assay for quantitatively measuring the effects of Arp2/3 complex inhibitors on cytoskeleton dynamics. The assay uses *Drosophila* S2 cells expressing a low level of GFP-tagged actin and total internal reflection fluorescence (TIRF) microscopy to extract velocity data from the cell's actin cytoskeleton following treatment with inhibitors. Unlike previous experiments, this assay uses live cell imaging to directly measure actin dynamics in eukaryotic cells, and retains much of the complexity of biological systems including the presence of cell membranes and many different proteins. The previously used methods can be implemented in concert with this one to provide a more comprehensive characterization of these inhibitors.

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Introduction

Every organism on earth is made up of life's building block: the cell. These highly complex machines are run mostly through the diverse functions of a category of biomolecules called proteins. Proteins fulfill many of the functions required for life to exist: some proteins called **enzymes** oversee chemical reactions, some allow things to pass in and out of cells through the membrane, and some proteins serve as the structural scaffolding of the mostly water-filled cells. In eukaryotes—the domain of life which includes (among many others) humans, plants, and bugs—this scaffolding system is known as the cytoskeleton.

The cytoskeleton consists of many different structural and regulatory proteins, and has many different functions, but the part most relevant to my research is the branched actin cytoskeleton, which is able to produce forces that reshape the cell and even allow it to move (Figure 1).[1] With the power of the actin cytoskeleton, cells can bring outside substances in by pulling the membrane into the cell and pinching it off to form a membrane enclosed sphere inside the cell, a process called endocytosis. Endocytosis is the process by which many viruses enter host cells, but it is also important for cell signaling and nutrient uptake. Cancer cells can create structures called invadopodia by disruption of the regulation of the actin cytoskeleton to degrade surrounding tissue, aiding in **metastasis**.[2] Lamellipodia are dynamic protrusions that allow cells to move, a function that immune cells use in wound healing.[3] Because of the actin cytoskeleton's crucial role in cellular function, many researchers are interested in understanding its complicated function.

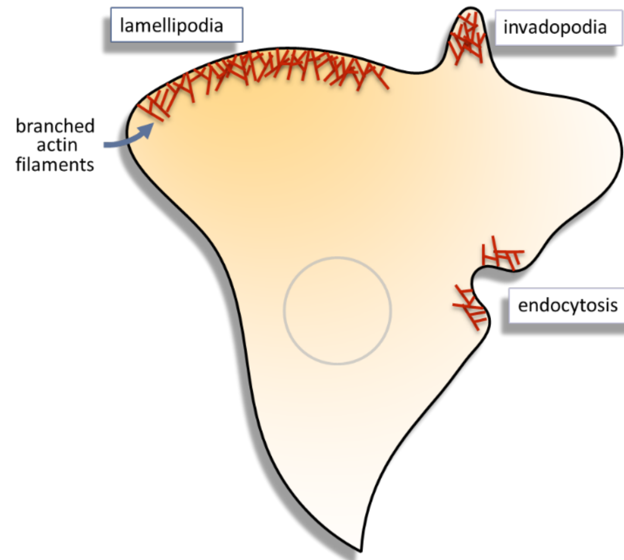

Figure 1: Some functions of the branched actin cytoskeleton in a eukaryotic cell

Branched actin filaments are indispensable for the creation of different structural features including lamellipodia, invadopodia, and endocytic sites.

The actin cytoskeleton consists of many actin filaments: long strands of actin subunits that are linked together in a process called polymerization. These filaments are dynamic, with monomers being added and removed constantly, but the initiation of a new filament, called nucleation, is highly regulated. In branched networks, there is only one protein that is able to nucleate a new branched filament, a seven-subunit protein called Arp2/3 complex (Arp = actin-related protein) (Figure 2).[1]

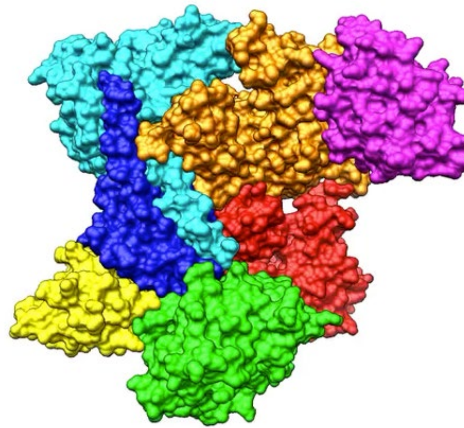

Figure 2: A molecular model of Arp2/3 complex

The seven subunits of Arp2/3 complex are Arp2 (red), Arp3 (orange), ARPC1 (green), ARPC2 (cyan), ARPC3 (pink), ARPC4 (blue), and ARPC5 (yellow)

Arp2/3 complex is inherently inactive, so the nucleation rate is slow in the absence of one of several classes of nucleation promoting factors (NPFs), the largest family of which are Wiskott–Aldrich syndrome proteins (WASP).[4,5] Activation by WASP family proteins leads to exclusively branched filaments, where Arp2/3 complex binds to the side of an existing (mother) filament, and the daughter filament is extended out at a ~70° angle from the first (Figure 3).[6,7]

In addition to activating proteins, researchers have discovered many proteins in cells that regulate Arp2/3 complex by inhibiting its activity, as well as small organic molecules that have the same effect.[8,9] The study of biological processes is made difficult by the high degree of complexity in these systems. Small molecule inhibitors of proteins are useful research tools because they allow a researcher to specifically eliminate the activity of a desired protein in a manner that is fast and reversible, leading to a better isolation of the specific effects of that protein. These inhibitors have been

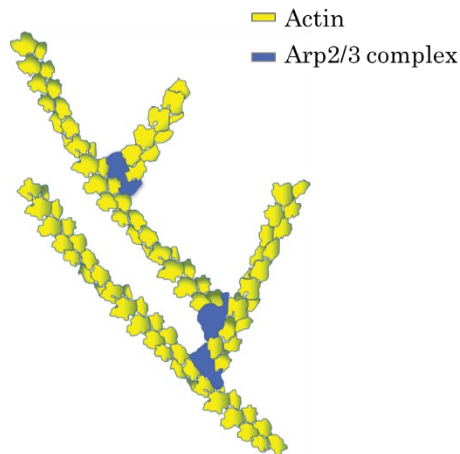

Figure 3: A few branched actin filaments

The actin monomers (yellow) are linked together consecutively to form a filament. Branched filaments are created when Arp2/3 complex (blue) binds to a pre-existing filament and serves as the nucleation point for a new filament.

used to elucidate the importance of **Arp2/3 complex** in many different cellular processes, including the membrane trafficking of aquaporin, the protein that allows water to move in and out of cells,[10] the formation of new neural connections in our brains via **axonal** remodeling,[11] and the maintenance of long-term memory.[12] Small-molecule inhibitors are also highly researched as drugs because of their ability to inhibit disease-causing activities.[13]

Inhibitors have great potential, but their effects need to be carefully analyzed to ensure there are no off-target effects. The interdependence of biological systems can make determining the exact effects of a molecule difficult, so it is important to fully understand the impact of these substances before they are introduced into biological research systems. There are some tools currently used to characterize Arp2/3 complex; the original papers to characterize the inhibitors measured their effects on ***in vitro*** actin **polymerization** at different concentrations, as well as some ***in vivo*** experiments that measured the total actin present and the fraction of **motile** cells that were active with

and without inhibitor, but there was no *in vivo* analysis of the inhibitors' direct effect on actin dynamics.[9,13,14] Research that uses inhibitors now typically involves adding a high concentration of the molecule to completely stop Arp2/3 complex activity rather than looking at a range of concentration effects.

A simple, direct, *in vivo* assay will be an important tool for characterizing new inhibitors that continue to be identified in the years to come, or for working to improve the efficiency of previously discovered inhibitors. There are current efforts to improve the potency of a commonly used inhibitor, CK-666, using mostly *in vitro* methods, but having the feedback from an *in vivo* assay would help to judge the improvement of these new versions in the cellular context.

It is important to choose the right system within which to model these inhibitory effects, and for this sort of analysis, ***Drosophila*** S2 cells are a good candidate. The result of day-old fly embryos isolated and induced to grow in culture medium, cultured S2 cells are inexpensive and easy to maintain, extensively researched and well understood, and ideal for **fluorescence microscopy**.[15]

When S2 cells are plated on the **lectin** concanavalin A, they spread to approximately 20 μm—twice their normal diameter—and actin-rich, dynamic lamellipodial structures form around the perimeter of the cell.[16] One powerful method for visualizing actin dynamics in live cells is fluorescent speckle microscopy (FSM). This technique requires a small percentage (0.5-2%) of a protein to be fluorescently labeled. The result is cells that are "speckled" with fluorescence, with each speckle made up of a small region with significantly higher labeling density than the surrounding area (Figure 4).

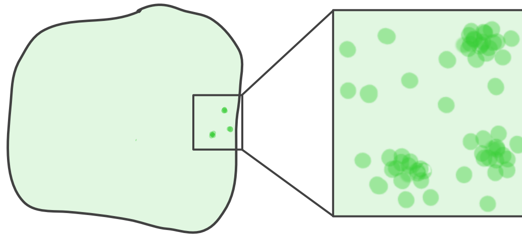

Figure 4: Appearance of “speckles” in speckle microscopy

The cell has an overall slightly green appearance due to the labeling of actin. Each individual dot in the zoomed-in region represents a single labeled actin monomer, and the areas where they form clusters create dots that are visible with the microscope and shown on the left, in the un-zoomed cell. There are many more unlabeled actin monomers in the cell that are not visible with fluorescence microscopy.

As actin filaments are assembled at the edge of the cell and disassembled further from the membrane, the actin network treadmills and the speckles can be seen to move inward (Figure 5).[17]

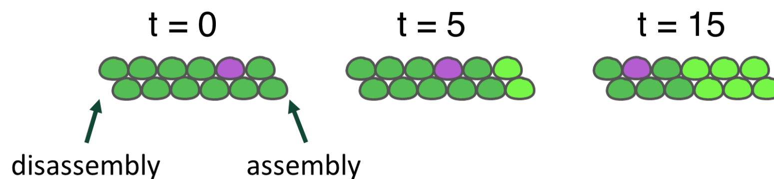

Figure 5: How actin assembly dynamics cause speckles to move

Actin filaments are polarized with nearly all new actin monomers (light green ovals) added to one end and removed from the other. At the edge of the cell membrane, this dynamic means that an individual actin monomer (in purple) moves inward over time.

This movement is directly related to the kinetics of the system and is one of the best representations of actin network dynamics in live cells.

Individual speckles can be identified and analyzed in several ways. The simplest of these is the kymograph, a graphical representation of position over time. These data can be analyzed manually, or with the help of software programs that seek to expedite the process and eliminate human bias.[18] The data can be compiled and compared for different concentrations of the inhibitor. One commonly calculated parameter is the half-maximal inhibitory concentration (IC50), which describes the concentration at which the inhibitor has half of its maximum effect.

Results

To gauge the feasibility of the assay, an early trial was performed with a high concentration of the inhibitor CK-666, as well as untreated control cells and cells treated with an equal amount of DMSO as contained in the CK-666 condition (Figure 6). DMSO is a commonly used solvent in biology, but it has wide-ranging effects on different cell types, including cell death.[19] The concentration of DMSO used in this experiment was below 1%, which is relatively low, but because the effects of DMSO on *Drosophila* S2 cells are not well characterized, it was necessary to assess the effect of the DMSO alone on actin dynamics. An inhibitor concentration of 200 µM was chosen because in most studies using CK-666 that is the concentration used to entirely eliminate Arp2/3 complex activity. In this experiment, the cells were incubated for only a few minutes after addition of the inhibitor before imaging.

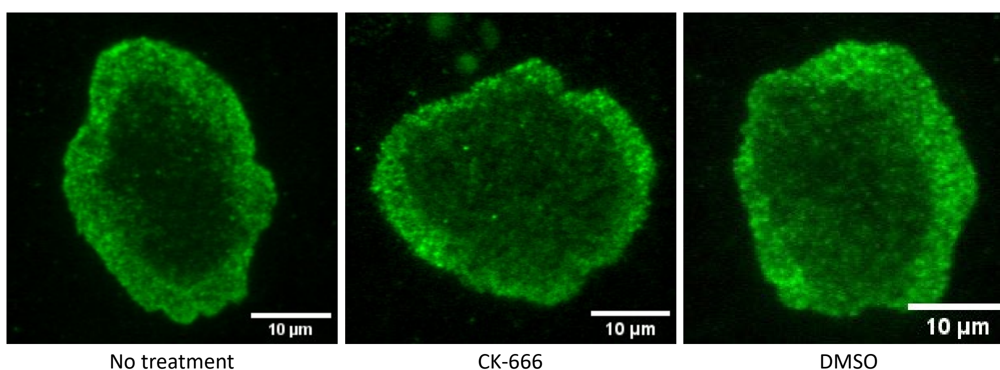

Figure 6: Representative frames from videos

Movies were taken under three conditions: untreated (containing complete media), treated with 200 µM CK-666 (containing complete media and CK-666 dissolved in DMSO), and treated with 0.04% DMSO (containing complete media and DMSO). Each frame is one of 76 in each video.

Figure 6 shows representative frames from a video for each condition. Under all

conditions, the speckles are clear and more highly concentrated in a ring around the edge of the cell where the lamellipodia form. Cell size and shape varied slightly but with no discernible trend between conditions. The speckles are transient and do not always appear at the precise edge of the cell and travel the entire width of the actin network, but their lifetimes were long enough to analyze. For each video, 6 to 8 kymographs were created (Figure 7a) (later experiments used 4-6 kymographs per video). Kymographs plot the pixels of a chosen line at a series of timepoints, so that the movement of a particle along that line appears as a diagonal trace. The slopes of the traces in these kymographs can be extracted and converted to velocity. These velocities

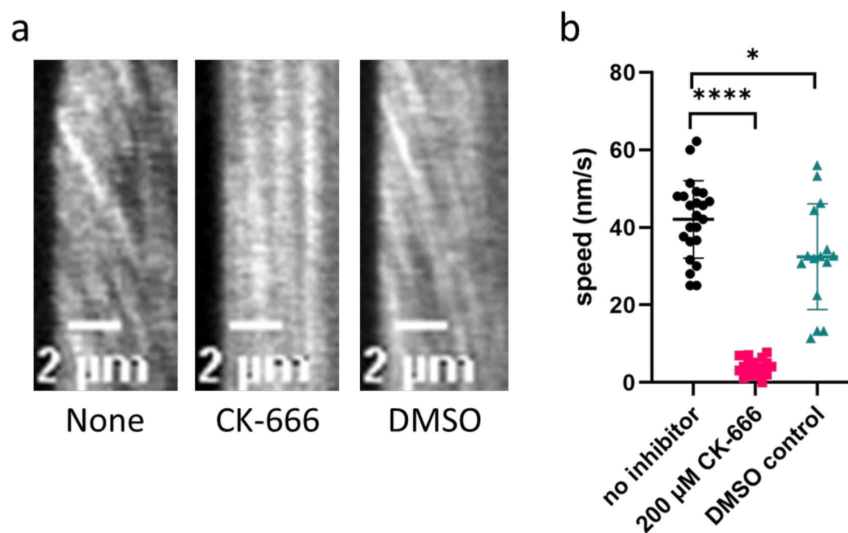

Figure 7: The effect of a high concentration of inhibitor and its corresponding DMSO concentration on actin dynamics

A) An example kymograph from every condition: no treatment, 200 µM CK-666, and 0.4% DMSO. B) Treadmilling speed was extracted from kymographs and plotted for all points. The DMSO control was on average significantly slower than untreated cells. The symbol * indicates $p < 0.05$, and **** indicates $p < 0.0001$.

for each condition are plotted in Figure 7b. In this experiment, the untreated cells had an average velocity of around 40 nm/s, and after treatment with 200 µM CK-666, their

velocity was around 5 nm/s. This result was promising because the inhibitor had a clear and drastic effect on the network treadmilling. Interestingly, the dose of inhibitor, which has been shown previously to eradicate all nucleation activity of Arp2/3 complex, did not stop actin treadmilling here. Some areas of some cells had zero velocity, but others were just extremely slow. This variation in velocity between different areas in the same cell and between cells extended to all experimental conditions.

The DMSO control cells had a very wide range of treadmilling speeds with a few being nearly as slow as the inhibited cells and a few being nearly as fast as the fastest untreated cells. The average velocity was around 30 nm/s. This condition was significantly slower ($p<0.05$) than the untreated control cells, which was surprising since 0.4% DMSO was lower than the expected damaging dose. Before further experimentation with CK-666, an appropriate concentration of DMSO to use in experiments needed to be determined.

DMSO is the solvent used to create stock solutions of CK-666. Previous stocks of CK-666 were made at a concentration of 50 mM in DMSO. These stocks, when added to the cell medium to create a final concentration of CK-666 of 200 μM, led to a final concentration of DMSO of 0.4%. The maximum solubility of CK-666 in DMSO is 100 mM. Using stocks at this concentration allows final concentrations of DMSO to be as low as 0.2% when adding 200 μM CK-666. Concentrations in a range from 0-0.5% were tested (Figure 8).

The ranges of velocities were large again, even more so than the previous experiment. There were more data points collected this time, indicating that this experiment may be capturing the true spread of the data whereas before there were not

enough measurements to see it all. DMSO concentrations of 0%, 0.2%, and 0.3% all had average velocities around 40 nm/s and were not found to be significantly different from each other. As predicted, 0.5%, like 0.4%, was significantly slower ($p<0.05$)

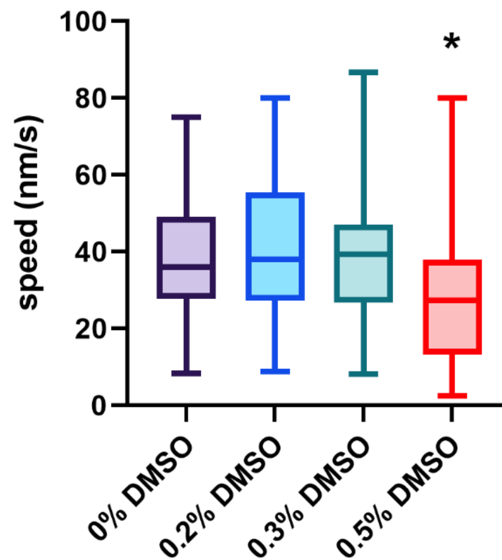

Figure 8: The effect of DMSO concentration on actin treadmilling velocity

DMSO concentration of 0, 0.2, 0.3, and 0.5% were tested to determine the concentration at which it would not affect actin dynamics. Concentrations of 0.2 and 0.3% were found to be not significantly different from 0% DMSO.

than 0% DMSO. From this data it was determined that either 0.2% or 0.3% DMSO could be used.

While 200 µM CK-666 completely stops Arp2/3 complex activity *in vitro*, in the previous experiment, the speckles had a small but non-zero velocity in the presence of this concentration of CK-666. In order to attempt to capture the full range of effects of CK-666, the next round of experiments was planned with concentrations of 0, 50, 100, 200, and 300 µM CK-666. With CK-666 stocks at 100 mM, this necessitated a DMSO concentration of 0.3% in the 300 µM condition. DMSO was thus added to all solutions

to give every condition a final concentration of 0.3% DMSO. Incubation times for CK-666 vary in the literature from just a few minutes to several hours, so one hour was chosen as an intermediate time.[10,20]

Representative kymographs from each condition are shown in Figure 9a. Velocities were extracted and are plotted in Figure 9b. The average speed of the uninhibited cells was around 40 nm/s, which corresponds well to the data from previous

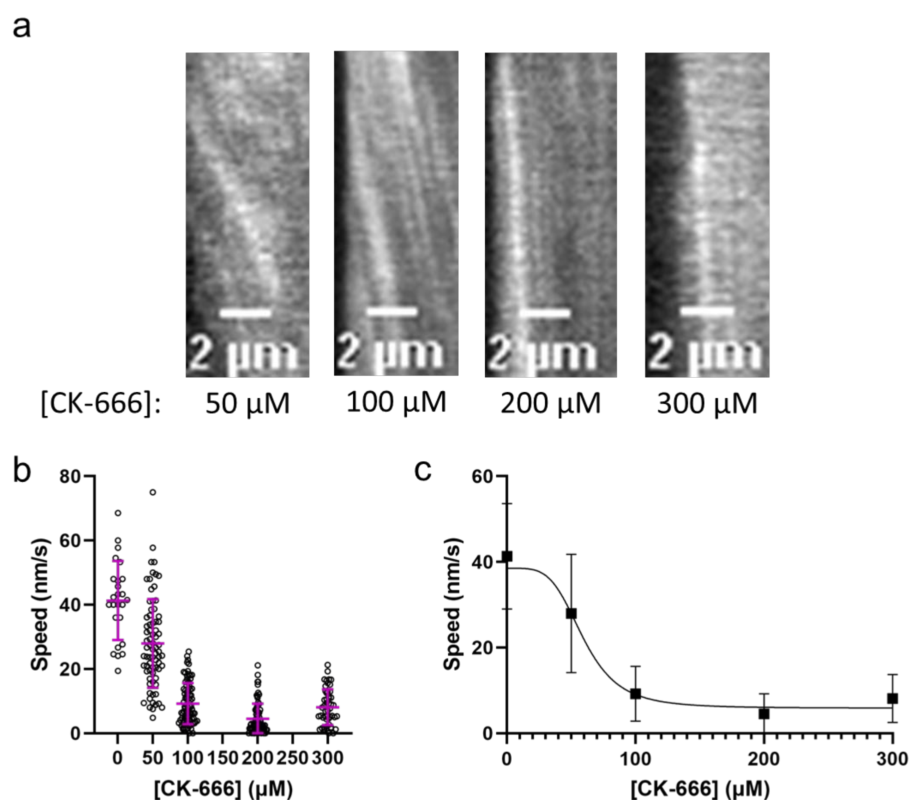

Figure 9: The concentration-dependent effect of CK-666 on actin network treadmilling velocity.

A) Representative kymographs from all conditions except 0 μM CK-666. B) Velocity data calculated and plotted for every kymograph. C) Mean value and standard deviation plotted, fit to a dose-response curve. IC50 = 59 μM.

experiments. When 50 μM CK-666 is added, this speed drops to just under 30 nm/s. Between 100 and 200 μM inhibitor, the treadmilling speed levels out around 5 nm/s. To

calculate the IC50 value, the data were fit to a dose-response curve (Figure 9c). This value was found to be 59 μM.

Discussion

This work has developed an assay that can be used to characterize the effects of Arp2/3 complex inhibitors in living systems. The assay detects changes in the activity of Arp2/3 complex by measuring the treadmilling rate of actin networks, and can differentiate sufficiently between activities of the complex to fit a dose-response curve and calculate IC50 values for a given inhibitor. Comparing these parameters between inhibitors will give researchers an additional method to understand the safety and efficacy of new Arp2/3 complex inhibitors in their experiments.

This assay is easy to learn and teach to others, doesn't require the purification of any proteins, and can be carried out relatively quickly with less than an hour of work required for most days of the experiment set up. The effects of CK-666 on the treadmilling speed were similar following incubations of just two minutes or one hour, so lengthy incubations are not necessary in the future. The concentration of DMSO in the final medium should be kept below 0.4%, if possible, to prevent inhibition of the network due to DMSO rather than the target molecule. If not possible, a control experiment should be sufficient to measure and account for the effect of DMSO.

In S2 cells at high concentrations of CK-666, average network velocity was as low as 5 nm/s but didn't further approach zero with increasing inhibitor concentration. This suggests that in this system, no amount of CK-666 will ever completely stop Arp2/3 complex activity. This may indicate that the *Drosophila* Arp2/3 complex retains some degree of activity when bound to CK-666, or the continued activity could be caused by Arp2/3 complex-independent nucleation activity. Testing other inhibitors with the assay in the future could help illuminate the possible causes of this non-zero

velocity. If the velocity at saturation changes with different inhibitors it would favor the first possibility, and if doesn't it would favor the second.

The IC50 for CK-666 with this assay was 59 μM, significantly higher than previously reported IC50s of 16 μM with BtArp2/3 complex (cow), and 5 μM with SpArp2/3 complex (yeast),[9] meaning that more inhibitor must be used to reach the same level of inhibition in this system than in the previous work. This difference could be due to a number of things. The *drosophila* Arp2/3 complex could have structural differences that decrease the binding strength of CK-666 leading to a higher IC50 value. This number also reflects the permeability of the cell membrane, which is not accounted for in *in vitro* experiments. Impermeability could increase the IC50, since higher concentrations of inhibitor in the medium would be needed to achieve a given concentration inside cells.

These experiments show the promise of using speckle fluorescence microscopy to analyze the effects of Arp2/3 complex inhibitors, but they are somewhat preliminary. The effects of CK-666 should be reversible, and confirming that this reversibility in present with this assay will confirm that the system behaves as expected. An experiment where CK-666 is washed out by replacing the media with fresh, inhibitor-free media and the cells are imaged again would be sufficient to show reversibility.

Additionally, the video analysis, including the generation and quantification of kymographs can be time intensive. In an effort to automate speckle analysis, several groups have developed software that detects and tracks the positions of speckles during their lifetime.[21,22] These programs exist independently and as add-ons to applications like ImageJ. There are also more general ImageJ plugins such as TrackMate[23] that are

designed to track a broad range of particles in images and can be harnessed for speckle analysis. These programs could be helpful if the assay ever needed to be greatly scaled up by eliminating a significant amount of time researchers would otherwise need to spend doing manual analysis. These programs could also help reduce potential human error or bias.

If this assay is to be used for characterizing newly discovered inhibitors, it will be important to show that it works for many different inhibitors. Testing it on another well-characterized inhibitor such as CK-869 would be the next natural step. However, the early successes already suggest the assay will be effective and can be used to test new inhibitors. Researchers have developed a version of CK-666[14] that was found to be a better inhibitor of Arp2/3 complex *in vitro*, but it would be interesting to test how it performs *in vivo*. This assay will provide researchers with precise measurements of the effects of this and other new compounds inside living cells, providing complementary information to experiments conducted with purified proteins *in vitro*.

Methods

Cell Strain Preparation

Drosophila S2 cells can be purchased, but in this case were donated by the Prehoda lab. The cells are stored in liquid nitrogen at a density of 1.1×10^7 cells/mL in 45% conditioned complete Schneider's *Drosophila* Medium (complete refers to media that cells have been growing in) containing 10% **heat-inactivated fetal bovine serum** (FBS), 45% fresh complete Schneider's *Drosophila* Medium containing 10% heat-inactivated FBS, and 10% dimethyl sulfoxide (DMSO). Cell **aliquots** were thawed quickly in a 37°C water bath and cultured in Schneider's *Drosophila* medium with 10% FBS in T25 flasks at 28°C. To maintain densities between 1×10^6 and 2×10^7 cells/mL, cells were diluted 1:10 twice a week.

To prepare the DNA for labeling actin with GFP, **pMT-GFP-actin** DNA was transformed into DH5α *E. coli* cells and grown overnight in LB media containing 100 μg/mL **ampicillin**. The **plasmid** DNA was purified from cells using the Thermo Scientific GeneJet Plasmid miniprep kit following included protocols. The DNA was **eluted** in 50 μL of warm (~50°C) water instead of the included elution buffer at concentrations of 659 and 636 ng/μL.

One day before transfection, cells were diluted in Schneider's *Drosophila* medium with 10% FBS to a total volume of 2 mL at a density of 0.5×10^6 cells/mL in 6 well plates and returned to the incubator. The next day, for each transfection, 1 μg of DNA and 4 μL of FuGENE HD transfection reagent were added to 100 μL of *Drosophila* medium without FBS and incubated at room temperature for 20 minutes.

This mixture was added to the cells plated the day before, and the cells were returned to the incubator. Protocol from Kögler et al. 2021.[24]

Inhibitor Preparation

CK-666 powder was purchased from Sigma-Aldrich and stored at 4°C until solutions were prepared. The powder was dissolved in DMSO to a concentration of 100 mM. Aliquots of 10 µL were prepared and stored at -20°C. For an experiment, one aliquot was diluted with fresh complete media to desired concentration before being added to the cells.

Imaging and Analysis

Nunc Lab-Tek II 8-Chambered Coverglasses were purchased from Thermo Fisher Scientific. For handling, the coverglasses were stored on a kimwipe in a petri dish to protect the surface from scratches. To prepare the slides, 250 µL of 5 µM Concanavalin A (ConA) dissolved in phosphate-buffered saline (pH 7.4) was added to each well needed for the experiment and incubated at room temperature for 30-60 minutes. The ConA solution was removed, and the wells were rinsed with 200 µL of water. The water was removed, and the slide was allowed to fully dry before being stored at 4°C until needed.

Two days after transfection, 50 µL of transfected culture was transferred to each well of the slide along with 200 µL fresh complete media. The slide was moved to the incubator and the cells allowed to spread and attach for one hour. The desired amount of inhibitor solution was added to a well after removing an equal volume of media

carefully from the well. The cells were incubated with inhibitor for an hour before imaging.

Cells were imaged on a Nikon inverted microscope with a 100X TIRF objective. Movies were collected for 2.5 minutes with the 488 nm laser at 3 mW power, with 100 msec exposure times at 2 second intervals.

Movies were cropped and background fluorescence was subtracted in ImageJ in preparation for analysis. Four to ten kymographs were generated for each cell by drawing lines perpendicular to the cell membrane and processing with KymographBuilder, an ImageJ plugin.[25] Kymographs were analyzed using the kymograph line selection tool, an ImageJ macro which extracts dx and dy for a drawn line segment. Change in y was converted to time by multiplying by two, since images were taken every two seconds. The calculated velocities for each condition were compiled in Excel and graphed in GraphPad Prism. T-tests and curve fitting were also conducted in Prism.

Glossary

Aliquot: a portion of a larger whole

Ampicillin: a type of antibiotic, commonly used to prevent the growth of unwanted bacteria

Arp2/3 complex: a protein complex that is responsible for the nucleation of actin filaments

Axon: the part of a neuron that transmits electrical impulses

Drosophila melanogaster: the scientific name for fruit flies

Elute: to remove material by washing with a solvent

Enzyme: a protein that catalyzes (speeds up) a chemical reaction

Fluorescence microscopy: a form of microscopy that uses lasers and fluorescence instead of white light to view specimens

Heat-inactivated fetal bovine serum: a substance derived from the blood of a cow fetus which is commonly used in eukaryotic cell culture because of its high levels of growth factors. Heat-inactivated refers to the heating of the serum to inactivate some compounds which may inhibit cell growth

In vitro: experiment takes place outside of a cellular context, with different necessary components being mixed together in a test tube or other receptacle

In vivo: experiment takes place in a living organism or cell

Lectin: a group of proteins with the ability to bind to carbohydrates (sugar molecules)

Metastasis: the process by which cancer spreads to another part of the body from the initial tumor

Motile: capable of motion

Plasmid: a small circular strand of DNA

pMT-GFP-actin: a plasmid containing the gene for actin and GFP under the control of a promoter that turns on gene expression in the presence of certain metals

Polymerization: the process in which smaller molecules, called monomers, combine chemically to produce a large chainlike or network molecule, called a polymer

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