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Fluorescence Microscopy for ATP Internalization Mediated by Macropinocytosis in Human Tumor Cells and Tumor-xenografted Mice

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TITLE:

Fluorescence Microscopy for ATP Internalization Mediated by Macropinocytosis in Human Tumor Cells and Tumor-xenografted Mice

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SUMMARY:

We developed a reproducible method to visualize the internalization of nonhydrolyzable fluorescent adenosine triphosphate (ATP), an ATP surrogate, with high cellular resolution. We validated our method using independent *in vitro* and *in vivo* assays—human tumor cell lines and immunodeficient mice xenografted with human tumor tissue.

ABSTRACT:

Adenosine triphosphate (ATP), including extracellular ATP (eATP), has been shown to play significant roles in various aspects of tumorigenesis, such as drug resistance, epithelial-mesenchymal transition (EMT), and metastasis. Intratumoral eATP is 10^3 to 10^4 times higher in concentration than in normal tissues. While eATP functions as a messenger to activate purinergic signaling for EMT induction, it is also internalized by cancer cells through upregulated macropinocytosis, a specific type of endocytosis, to perform a wide variety of biological

functions. These functions include providing energy to ATP-requiring biochemical reactions, donating phosphate groups during signal transduction, and facilitating or accelerating gene expression as a transcriptional cofactor. ATP is readily available, and its study in cancer and other fields will undoubtedly increase. However, eATP study remains at an early stage, and unresolved questions remain unanswered before the important and versatile activities played by eATP and internalized intracellular ATP can be fully unraveled.

These authors' laboratories' contributions to these early eATP studies include microscopic imaging of non-hydrolysable fluorescent ATP, coupled with high- and low-molecular weight fluorescent dextrans, which serve as macropinocytosis and endocytosis tracers, as well as various endocytosis inhibitors, to monitor and characterize the eATP internalization process. This imaging modality was applied to tumor cell lines and to immunodeficient mice, xenografted with human cancer tumors, to study eATP internalization *in vitro* and *in vivo*. This paper describes these *in vitro* and in *vivo* protocols, with an emphasis on modifying and finetuning assay conditions so that the macropinocytosis-/endocytosis-mediated eATP internalization assays can be successfully performed in different systems.

INTRODUCTION:

The opportunistic uptake of intratumoral extracellular (ie) nutrients has recently been named a key hallmark for cancer metabolism[1]. One of these important nutrients is ATP, as the concentration of ieATP is 10^3 and 10^4 times higher than that found in normal tissues, in the range of several hundred µM to low mM[2-5]. As a key energy and signaling molecule, ATP plays a central role in cellular metabolism in cancerous and healthy cells[6-8]. Extracellular ATP is not only involved in cancer cell growth, but it also promotes drug resistance[9]. Previously unrecognized functions of ATP, such as hydrotropic activity, have recently been identified, thus implicating ATP involvement in diseases such as Alzheimer's[10]. Indeed, it seems our understanding of ATP and its functions in cancer cells, healthy cells, and other diseased cells is far from complete. However, due to ATP's instability and high turnover rates in cells, it is technically challenging to monitor ATP's movement across the cell membrane and into the cell.

To address this problem and fill the need of this research area, a method was developed in which nonhydrolyzable fluorescent ATP (NHF-ATP) (**Figure 1**) was used as a surrogate to visualize the internalization of ATP and observe the intracellular spatial localization of internalized ATP, both *in vitro* and *in vivo*[11,12]. NHF-ATP has been demonstrated to substitute for endogenous ATP to investigate ATP movement across animal cell membranes, both in cancer cell lines and in human tumor tissue xenografted on immunodeficient mice[11,12]. Moreover, administering macropinocytosis inhibitors to cells blocked eATP internalization, suggesting that intracellular uptake of eATP involves a macropinocytotic mechanism[9,11,12]. This protocol permits immunobased colabeling against cell-specific proteins and thus identification of which cell type internalizes NHF-ATP. Using *in vivo* tumor xenografts and high-resolution microscopy, NHF-ATP can be visualized spatially across the tissue sample and even within a single cell. These methods also permit quantitative analysis, such as the percentage of cellular uptake, number of macropinocytotic vesicles, and internalization kinetics. This paper describes in detail how NHF-ATP, working alone or together with endocytosis-tracer fluorescent dextrans[13-16], can be used in

different experimental settings to study ATP's internalization and intracellular localization, following internalization in cells.
[Place **Figure 1** here]

PROTOCOL:

All procedures reported herein were performed in accordance with Ohio University's IACUC and with the NIH.

1. **Selection of nonhydrolyzable fluorescent ATP (NHF-ATP) and dextrans**

1.1. Select a fluorophore-conjugated NHF-ATP (**Figure 1A**) and endocytosis tracers, high and low molecular weight fluorescent dextrans (TMR-HMWFD and TMR-LMWFD) (**Figure 1B**), based on the preferred emission wavelengths (e.g., imaging system equipped with appropriate filters) and the specific endocytosis process to be studied.

2. ATP localization studies, *in vitro* (Figure 2)
[Place **Figure 2** here]

2.1. Cell culture and preparation of cells

NOTE: Perform cell culture under sterile conditions in a tissue culture hood.

2.1.1. Prepare Dulbecco's Modified Eagle Medium (DMEM), containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (hereafter called DMEM/FBS), sterile phosphate-buffered saline (PBS), and 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), in a 37 °C water bath.

2.1.2. Culture human cancer cells in DMEM/FBS in a 100 mm tissue culture dish. Maintain the cells in an incubator set to 37 °C with a 5% CO_2 atmosphere.

2.1.3. When the cells reach confluence, passage the cells by first removing the culture medium. Next, rinse the dish with 5 mL of sterile PBS, remove the PBS, and add 3 mL of 0.25% trypsin. Incubate at 37 °C in a 5% CO_2 atmosphere for 5 min.

2.1.4. Retrieve the dish, then add 6 mL of DMEM/FBS to stop the trypsinization. Transfer the cells in suspension to a 15 mL conical tube and centrifuge at 800 × *g* for 5 min to pellet the cells.

2.1.5. After centrifugation, aspirate the supernatant and use 10 mL of DMEM/FBS to resuspend the cell pellet by pipetting.

2.1.6. Count the cell density and viability using a hemocytometer. Use DMEM/FBS to dilute the cell suspension to a density of ~7.5×10^4 cells/mL.

2.2. Preparation of coverslips and seeding cells

2.2.1. Wash 12 mm coverslips with 70% ethanol and wipe them carefully with delicate task wipes. Sterilize the coverslips and one pair of forceps via autoclaving.

2.2.2. In a tissue culture hood, use forceps to place one coverslip into each well of a 24-well tissue culture plate.

NOTE: Later, the coverslip, with cells, will be mounted directly onto a microscope slide for imaging.

2.2.3. Dispense 300 µL of the cell suspension (cells in DMEM/FBS), at a seeding density of ~2.5 × 10^4 cells per well, into the 24-well plate containing the sterilized coverslips. Incubate in sterile conditions at 37 °C with 5% CO_2 flow.

2.3. Starvation of cells

2.3.1. Twenty-four hours after seeding, remove the DMEM/FBS from each well. Immediately add 300 µL of prewarmed serum-free DMEM into each well to serum-starve the cells for 15–18 h to induce uptake of extracellular nutrients.

NOTE: The 15–18 h starvation period is a critical parameter.

2.4. Preparation of NHF-ATP and HMWFD/LMWFD solutions

2.4.1. Use an analytical balance to weigh high-molecular-weight (70 kDa) fluorescent TMR-dextran (TMR-HMWFD, 1 mg/mL), a tracer for visualizing macropinosomes, or NHF-ATP (10 µmol/L) in serum-free DMEM in a 1.5 mL microcentrifuge tube. Place the tubes, protected from light, in a 37 ˚C water bath for 15 min.

2.4.2. Centrifuge at 12,000 × *g* for 5 min at room temperature. Carefully transfer the clear supernatant to a new 1.5 mL microcentrifuge tube, leaving any pellet or debris intact to remove indissoluble crystals.

2.4.3. Add the solutions from step 2.4.1 to the cells in each well, and incubate the cells for 30 min at 37 °C.

NOTE: If HMWFD and NHF-ATP solutions are to be mixed for co-incubation with the cells, prepare both solutions at 2x the final concentrations. The solutions will be mixed later at a 1:1 ratio to achieve the final accurate working concentrations. Avoid light as the reagents are light-sensitive.

2.5. Treatment of cells and fixation

2.5.1. In a fresh 24-well plate, dispense 500 µL of prewarmed PBS into each of five wells.

2.5.2. After cell incubation, carefully pick up each coverslip using forceps. Rinse each coverslip by dipping it in 500 µL of prewarmed PBS. Repeat five times using the five PBS-filled wells.

NOTE: Gentle washing of cells-on-coverslips is critical for the success of this experiment.

2.5.3. After the final PBS wash, tap the coverslip on a delicate task wipe to absorb extra PBS and transfer the coverslip immediately to cold (4 ˚C) 3.7% formaldehyde, preloaded in a 24-well plate. Fix the cells for 15 min at room temperature.

2.5.4. While the cells are being fixed, pre-clean microscope slides with 70% ethanol. Remove the coverslips from the wells and mount them onto the slides, using 5 µL of aqueous mounting medium containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI), per coverslip. Gently blot excess PBS with a paper towel or a delicate task wipe.

2.6. Fluorescence microscopy and image acquisition

2.6.1. Two to 24 hours after the steps above, capture images of cells and internalized HMWFD and/or NHF-ATP using an epifluorescence imaging system and data acquisition software.

NOTE: This sub-section describes steps to acquire images using a Nikon NiU microscope, equipped with epifluorescence imaging capability, and Nikon NIS Elements software. However, other comparable imaging systems and acquisition software may be used. Follow the operating instructions from the manufacturer.

2.6.1.1. Place the slide on the stage of an upright epifluorescence microscope in binocular mode. Access the imaging program.

2.6.1.2. Select the 10x objective, adjust the stage to define focus, and scan the slide from left to right in a serpentine manner to identify the regions of interest.

NOTE: Identifying regions of interest will vary between cell types, with some cell lines/cancer types exhibiting diverse and distinct degrees of TMR-HMWFD and/or NHF-ATP uptake.

2.6.1.3. Select the 40x objective, and switch from binocular mode to image capture mode, using the toggle on the microscope.

2.6.1.4. Click on the **Live Quality** icon on the imaging program to view and subsequently acquire images.

2.6.1.5. Using the **OC Panel** on the **Acquisition** toolbar, define the exposure parameters for each filter cube or fluorescent channel.

NOTE: Select the appropriate exposure time for each channel, as signal intensities are different. For example, select an exposure time of 200 ms for DAPI, 2 s for HMWFD, and 4 s for NHF-ATP. Once the exposure time is determined per channel, use this setting for all images, per channel, with different treatment or conditions.

2.6.1.6. Once the exposure settings have been set for each channel, use the multichannel acquisition toolbar to acquire a 3-channel image with the defined exposure settings.

NOTE: Image acquisition through the multichannel ND acquisition mode enables automatic image capture for each channel of the same field of view. The shutter is closed automatically between turret changes.

2.6.1.7. Alternatively, acquire multichannel images manually by toggling between filter cubes, setting the exposure time, closing/opening shutter in between image acquisition for each channel, and overlaying each image taken for individual channels.

NOTE: The ND acquisition mode automates this process and provides merged images.

2.6.1.8. Save the image as .nd2 file (Nikon Elements format saves metadata). Save TIF files, including the merged channel image and individual channel images.

NOTE: TIF files can be used with a broader selection of software applications.

2.6.1.9. Use the **Object Count** feature on the **Analysis** toolbar to count the number of NHF-ATP-, TMR-HMWFD-, and/or TMR-LMWFD-positive cells on a saved .nd2 image file.

2.6.1.10. Export the data to a spreadsheet through the analysis program.

2.7. Data quantification and analysis

2.7.1. For each condition assayed, image 50 to 100 cells for quantification. Using the data analysis software (software included in the epifluorescence imaging system or other software), count and calculate the mean number of fluorescent vesicles per cell.

2.7.2. Use appropriate statistical methods to analyze the quantified results.

3. ATP internalization in tumors, *ex vivo* (Figure 3)

[Place **Figure 3** here]

3.1. Preparation of cell cultures for implantation

3.1.1. Grow cancer cells to 80% confluence at 37 °C in a 225 cm^2 flask, using DMEM supplemented with FBS, to a final concentration of 10% (v/v) and penicillin/streptomycin at 1% (v/v).

3.1.2. Wash the cells two times with 10 mL of PBS. Pre-warm 0.25% trypsin/EDTA to 37 °C. Add 8 mL of trypsin/EDTA and incubate at 37 °C for 2 min.

3.1.3. Once the cells start detaching from the bottom of the flask, use a 10 mL sterile serological pipette to add 8 mL of DMEM/FBS. Aspirate two times to dislodge any adherent cells. Use the pipette to transfer the detached cells from the flask into a 50 mL conical tube.

3.1.4. Add 10 mL of DMEM/FBS using a 10 mL pipette, and collect all remaining floating cells in the same 50 mL conical tube.

3.1.5. Centrifuge the cell suspension at 600 × *g*, 4 °C for 4 min. Remove the supernatant and resuspend the cells in 1 mL of ice-cold PBS.

3.1.6. Count the cell density using a hemocytometer. Keep the cell suspension on ice while counting.

3.1.7. Centrifuge the cell suspension at 600 × *g*, 4 °C for 4 min. Remove the supernatant and suspend the cells in ice-cold PBS such that the cell density becomes 5×10^6 cells per 100 µL of PBS. Transfer the cell suspension to a 1.5 mL microcentrifuge tube.

3.2. Subcutaneous injection of cancer cells for xenograft tumor development

3.2.1. Use a latex-free syringe (1 mL) with a precision glide needle (27 G needle) for cancer cell injection.

3.2.2. Transfer the cell suspension (5×10^6 in 100 µL of PBS) to a 1.5mL microcentrifuge tube. Draw the cells into the syringe.

3.2.3. Select an injection site on the flank of an immunodeficient (nude) mouse, and gently clean the skin using 75% ethanol. Wipe off excess ethanol with a delicate task wipe.

3.2.4. For subcutaneous injection, hold the needle at an approximately 10° angle to the skin. Insert the needle tip, bevel side up, just underneath the skin, so that only 1–2 mm of the needle is visible outside the skin. Dispense the cells from the syringe slowly over approximately 10 s.

3.2.5. After injecting the entire volume, continue to hold the needle in place for 3–5 s, then withdraw the needle and use a finger to apply gentle but firm pressure to the injection site for 3–5 more seconds to prevent leaking of the injected content.

3.2.6. Monitor and measure tumor growth using vernier calipers until the tumors reach a volume of 200–500 mm^3.

3.3. Preparation of HMWFD and NHF-ATP solutions to be used post-tumor resection

3.3.1. Dissolve 300 µL of 16 mg/mL HMWFD in serum-free DMEM (culture medium), incubate in a 37 °C water bath for 30 min, and centrifuge at 12,000 × *g* for 5 min as described above. Transfer the solution to a 1.5 mL microcentrifuge tube.

3.3.2. Add 40 µL of NHF-ATP analog stock (1 mM) to 160 µL of serum-free DMEM to prepare a 0.2 mM NHF-ATP solution.

3.4. Preparation of experimental wells

NOTE: This experimental design will assay the intracellular internalization of HMWFD + NHF-ATP, indicative of uptake by macropinosomes.

3.4.1. Prepare the wells as follows: Well #1, Control: 200 µL of serum-free DMEM; Well #2, Control: 100 µL of 16 mg/mL LMWFD + 100 µL of serum-free DMEM = 200 µL of 8 mg/mL LMWFD; Well #3, Control: 100 µL of 0.2 mM NHF-ATP + 100 µL of serum-free DMEM = 200 µL of 0.1 mM NHF-ATP; Well #4; Experimental: 100 µL of 16 mg/mL HMWFD + 100 µL of 0.2mM NHF-ATP = 200 µL of 0.1 mM NHF-ATP and 8 mg/mL HMWFD.

3.5. Preparation of tumor tissues

3.5.1. Euthanize the mouse by cervical dislocation or according to the IACUC-approved protocol.

3.5.2. Use a size 10 scalpel to slice the isolated tumors at a thickness of ~500–1,000 µm.

3.5.3. Incubate the tumor slices in serum-free DMEM supplemented with 100 µM NHF-ATP and/or 8 mg/mL H/LMWFD in microcentrifuge tubes for 40 min at 37 °C with 5% CO_2 flow.

NOTE: After incubation, tumor tissue metabolism causes the color of the medium to change.

3.5.4. Rinse the tissues in 37 °C prewarmed PBS (2 mL for each rinse in a 24-well plate).

3.5.5. Transfer the tissue to a new 24-well plate with prewarmed fresh PBS, rinse and repeat four times with gentle shaking.

3.6. Cryo-embedding (preparing frozen tissue blocks)

3.6.1. Prepare identification labels for each tumor to be harvested. Cut a 2 cm piece of laboratory tape and fold in half, adhesive sides together, lengthwise. Use a marking pen to label the tag, e.g., with a mouse/tumor identification number.

3.6.2. Prepare embedding molds by placing stainless steel tissue molds directly on dry ice.

NOTE: Dry ice may cause frostbite, burns, and asphyxiation. Wear insulated gloves when handling dry ice. Use dry ice in a well-ventilated area. Do not store dry ice in a tightly sealed container. Instead, store in a container (such as a Styrofoam cooler) that allows gas to escape.

3.6.3. While the mold chills, place a small pool of tissue-freezing medium into a 10 mm tissue culture plate. Ensure that the volume is enough to submerge the tumor tissue that will be harvested.

3.6.4. Use a perforated spoon to scoop up the resected tumor tissue and immediately place the tissue into a freezing medium, ensuring that the tissue is submerged. Using the perforated spoon, gently roll the tissue in the freezing medium, ensuring that the medium is bathing all the tissue surfaces.

3.6.5. Carefully move the tissue into the embedding mold containing the freezing medium. Place the corresponding label tag vertically into the freezing medium/mold to freeze in place. Ensure the written label is visible outside of the medium.

3.6.6. When the freezing is complete (freezing medium turns opaque-white), remove the tissue block from the mold, place it on dry ice, and repeat for each tumor. Store the tissue blocks at -80 °C for several months before the cryosectioning procedure.

3.7. Preparation of slides of tissues samples

3.7.1. To maximize the chance of finding internalization-positive cells and having more representative tissue regions, collect serial cryosections at -18 to -20 °C using a cryostat.

3.7.1.1. Prechill cryostat tools (blade, razor blade, anti-roll plate, tissue chuck holder, paintbrush) and equilibrate the tumor tissue blocks by placing them in a cryostat chamber at -18 to -20 °C. Set the blade holder angle to 5–10˚. Carefully trim the tissue block, as needed, with a razor blade, and mount it onto the chuck holder using tissue freezing medium as “glue.”

3.7.1.2. Lock the chuck holder into the vertical position on the microtome unit, which advances to the set distance (e.g., 10 µm) with each turn of the hand crank. Position the anti-roll plate to rest just above the height of the blade. To prevent tissue curling before advancing the microtome, carefully slide the thumb over the bottom edge of the tissue block.

3.7.1.3. As the microtome advances and the tissue section falls onto the metal plate, use a paintbrush to guide the tissue section and unroll the tissue, if necessary.

3.7.1.4. Hover the microscope slide over the tissue section without touching so that the section will be attracted to the slide.

NOTE: Cryostat blades (high-profile, disposable) are extremely sharp and can cause serious injury. Use care when handling blades and operating the cryostat. Use a blade protector, if available. Proper training is required.

3.7.1.5. Slice the tumor into sections of 10 µm thickness. Immediately transfer the sliced sections onto a positively charged glass microscope slide.

NOTE: For serial sections, first collect a 10 µm-thick section onto the upper left corner of each of eight positively charged slides. Advance the cryostat through subsequent 100–200 µm of tissue, and discard the tissue. Immediately transfer all sliced sections onto glass microscope slides.

3.7.1.6. Next, collect another 10 µm-thick section, next to the previously placed tissue section, for each of the eight slides. Repeat this serial collection process until each of the eight slides contains eight tissue sections, each 100–200 µm apart. Keep the tissue sections in the dark to preserve fluorescence.

NOTE: Tissue sections on slides can be stored in a slide box at -80 °C for several months.

3.8. Fixation of tissue slides

3.8.1. CRITICAL STEP: Fix the tissue sections in 95% ethanol at -18 °C for 5 min.

3.8.2. Wash the fixed section for 5 min with room temperature PBS, and then mount the fixed tumor sections under a glass coverslip using 10 µL of an aqueous mounting medium with DAPI.

3.8.3. Twelve to 24 h after mounting, examine the fixed tumor sections by fluorescence microscopy and acquire images, as described for the cultured cells above.

3.9. Fluorescence microscopy and image acquisition

3.9.1. Identify regions of interest and acquire images, as described in section 2.6.

3.10. Data quantification and analysis

3.10.1. Quantify the cells and apply appropriate statistical analyses, as in section 2.7.

4. ATP internalization in tumors, *in vivo*

4.1. Prepare cell cultures for implantation as described in section 3.1.

4.2. Subcutaneous injection of cancer cells for xenograft tumor development

4.2.1. Generate xenografted tumors as described in section 3.2.

4.3. ATP and/or dextran injection into xenograft tumors

4.3.1. Prepare the treatment solutions of DMEM (vehicle) or 8 mg/mL HMWFD or LMWFD, with or without NHF-ATP (100 µM) in DMEM, as described above.

4.3.2. Use a 1 mL syringe to collect 50 µL of one treatment solution and inject the solution directly into each xenograft tumor. Repeat the procedure for four biological replicates of each treatment.

4.4. Tissue harvest and cryo-embedding

4.4.1. Prepare identification labels for each tumor to be harvested. Cut a 2 cm piece of laboratory tape and fold in half, adhesive sides together, lengthwise. Use a marking pen to label the tag, e.g., with a mouse/tumor identification number.

4.4.2. Approximately 5 min post-injection, euthanize the mouse by cervical dislocation or according to the IACUC approved protocol.

4.4.3. Using a size 10 scalpel, make an incision adjacent to the tumor and approximately perpendicular to the direction of the needle injection. Use forceps and surgical scissors to resect the tumor tissue from the surrounding tissue.

4.4.4. Divide the tumor into two to four 1 cm^2 pieces, depending on the total tumor size.

4.4.5. Prepare the embedding molds and embed the tissue, as described above in section 3.6. Ensure that harvest time, from intratumoral dextran injection to cryo-embedding, is no more than 7–8 min.

4.5. Preparation of slides of tissues samples

4.5.1. Collect serial tumor sections, as described in section 3.7.

4.6. Fixation of tissue slides

4.6.1. Fix the tissue, as described in section 3.8.

4.7. Fluorescence microscopy and image acquisition

4.7.1. Identify the regions of interest and acquire images, as described in section 2.6.

4.8. Data quantification and analysis

4.8.1. Quantify the cells and apply appropriate statistical analyses, as described in section 2.7.

REPRESENTATIVE RESULTS:

***In vitro* study**

Intracellular internalization of NHF-ATP was demonstrated by co-localization of NHF-ATP with HMWFD or LMWFD (**Figure 4**). The success of this procedure primarily relies on the use of appropriate concentrations of NHF-ATP and dextrans and on determining the appropriate type(s) of dextrans (poly-lysine vs. neutral). For example, to investigate macropinocytosis, HMWFD was chosen as it is internalized only by macropinosomes[13-16]. Alternatively, if clathrin- and/or caveolae-mediated endocytoses are studied, then LMWFD is to be selected because the smaller sizes of these endocytosis-associated endosomes only allow them to engulf LMWFD[14-16]. Fluorescent dextrans are also available in two different forms: poly-lysine dextran and neutral dextran. These dextrans generate different fluorescence intensity and background staining; thus, their recommended applications vary. For example, poly-lysine dextran produces higher fluorescence intensity but also higher background. Neutral dextrans generate sufficient fluorescence intensity with relatively low background signal and are preferred for experiments using cancer cell lines. Using this cost-efficient assay, we generated high-intensity and high signal-to-noise NHF-ATP fluorescent labeling, which matched the fluorescence intensity of dextrans without confounding background fluorescence.

As the selection of the fixation agent and the fixation procedure significantly influenced the outcome of the assay, fixation conditions must be experimentally determined and selected for each specific experiment (i.e., specific cell line or tissue type). Extracellular ATP is not needed in the assay; in fact, high concentrations of eATP in the assay solution may lead to increased background. Importantly, after cell seeding, the optimal time frame for serum starvation is ~15–18 h. If serum starvation is too long, the lack of nutrients will affect cell attachment and lead to the loss of cells in the following steps. If serum starvation is too short, the cell cycle will not be properly arrested, and nuclear staining will not be uniform across the slide. Rinsing the coverslip-bound tissue in warm PBS five times is adequate to remove background staining. It is important to be very gentle with tissue washes. Avoid excessive rinsing, cold rinsing, or forceful washing, as these may damage the morphology of the cells.

While any cancer cell lines can be used in this assay, different cell lines may show different degrees of internalization. We have shown that cancer cell lines with *KRAS* proto-oncogene mutations are advantageous for studying NHF-ATP internalization. While *KRAS* mutations are not required for the internalization of eATP, *KRAS* mutations are associated with increased macropinocytosis *in vitro*[13]. For these experiments, we selected A549 lung cancer cells, which harbor a *KRAS* mutation. Indeed, macropinocytosis of eATP in A549 cells has been previously shown using an *in vitro* ATP internalization assay[13].

***Ex vivo* study**

Figure 5 shows fluorescence microscopic images of NHF-ATP internalization in tumor tissue sections. The success of the *ex vivo* study relies heavily on the proper incubation of NHF-ATP with the tumor tissue and thorough post-collection washing of the tumor sections. A shorter or longer incubation time may disrupt internalization. Determine incubation times, experimentally and in advance, for different tumors. Inadequate rinsing of tumor tissue permits high background

staining and thus a low signal-to-noise ratio.

Selection of the fixation agent is also critical. Fixation in methanol, formaldehyde, acetone, and ethanol can be tested individually and compared to identify the best fixation agent for the specific study system. It is noteworthy that the mounted slides need to be photographed within 12–24 h, but not longer, to prevent the internalized fluorescent molecules from being released from cells and contributing to high background. Finally, to avoid the edge-effect phenomenon—intense staining at the edges of tumor tissue sections—it is critical to collect uniform tissue sections before *ex vivo* incubation.

Given the heterogeneity of tumor tissue, it is important to obtain tissue sections throughout the tumor. The described method of collecting serial tumor sections, taken every 100–200 µm apart, ensures that representative data from different regions of the tumor can be acquired and analyzed. For example, just as different tumor types might generate different ATP internalization data, select intratumoral regions may also vary in ATP internalization kinetics and mechanism.

***In vivo* study**

Figure 6 shows NHF-ATP internalization in injected (xenografted) tumors. An important parameter for a successful and positive *in vivo* study was the NHF-ATP injection and the time between injection and animal euthanasia. During the injection procedure, position the injection needle to reach as much tumor area as possible, and keep the needle intact within the tumor for as long as 1 min to ensure that NHF-ATP remains inside the tumor and does not leak out. The short time interval between the injection and euthanasia ensures that injected NHF-ATP is transported into the tumor cells, but that transport is not too long, so that recipient cells will not significantly metabolize and degrade NHF-ATP, resulting in intracellular smears. After euthanasia and tumor removal, document the injection procedure for each tumor, including the injection site and injection direction. In this way, tumors can be sectioned in specific orientations and along specific anatomical planes so that tumor sections run parallel to the injection direction, leading to the identification of more NHF-ATP-positive cells.

FIGURE LEGENDS:

Figure 1: Structures of nonhydrolyzable fluorescent ATP and tetramethylrhodamine labeled high molecular weight fluorescent dextran. (**A**) Structure of NHF-ATP. (**B**) Schematic representation of HMWFD. Abbreviations: ATP = adenosine triphosphate; NHF-ATP = nonhydrolyzable fluorescent ATP; TMR = tetramethylrhodamine; HMWFD = high molecular weight fluorescent dextran.

Figure 2: *In vitro* procedure to examine ATP internalization. Schematic representation of the protocol to visualize the internalization of extracellular ATP in cultured cancer cells using fluorescence microscopy.

Figure 3: *In vivo* procedure to examine ATP internalization. Schematic representation of the protocol to visualize the internalization of extracellular ATP in tumor xenografts using cryosectioning and fluorescence microscopy.

Figure 4: A549 cells internalize NHF-ATP, which co-localizes with HMWFD *in vitro*. Fluorescence microscopy of A549 cells incubated with both 1 mg/mL HMWFD (left panel, red) and 10 µM NHF-ATP (middle panel, green) for 30 min. HMWFD and NHF-ATP co-localize in macropinosomes (right panel, merged yellow). Insets show high magnification of boxed regions. Scale bars = 20 µm. Abbreviations: NHF-ATP = nonhydrolyzable fluorescent ATP; HMWFD = high molecular weight fluorescent dextran.

Figure 5: A549 cells internalize NHF-ATP along with HMWFD *ex vivo*. Fluorescence microscopy of tumorigenic A549 cells xenografted into immunodeficient (*Nu/J*) mice; NHF-ATP internalization was performed *ex vivo*. Surgically removed tumors were incubated (*ex vivo*) with 8 mg/mL HMWFD (left panel, red) and 100 µM NHF-ATP (middle panel, green). Co-localization of cellular HMWFD and NHF-ATP is shown as a merged image (right panel, yellow). Scale bars = 20 µm. Abbreviations: NHF-ATP = nonhydrolyzable fluorescent ATP; HMWFD = high molecular weight fluorescent dextran.

Figure 6: A549 cells internalize NHF-ATP along with HMWFD *in vivo*. Fluorescence microscopy of tumorigenic A549 cells, xenografted into immunodeficient (*Nu/J*) mice; NHF-ATP internalization was performed *in vivo* with 8 mg/mL HMWFD and 100 µM NHF-ATP being directly injected into tumors of living mice. Co-localization of cellular HMWFD and NHF-ATP is shown as merged images in **A** and **B** (right panels, yellow). (**A**) High-magnification images highlight cellular internalization of NHF-ATP and HMWFD in tumors *in vivo*. Scale bars = 20 µm. (**B**) Low-magnification images depict regional internalization within a tumor tissue section. Scale bars = 100 µm.

DISCUSSION:

A method was developed for spatial, temporal, and quantitative analysis of the cellular internalization of nonhydrolyzable ATP. This method is broadly applicable for use in diverse biological systems, including various tumorigenic models, for which we provide technical instruction and representative data. To acquire interpretable data during *in vivo* ATP internalization studies (section 4 of the protocol), it is critical to limit the experimental time elapsed from intratumoral dextran injection to cryo-embedding. Fixing tissue slides—post-tumor sectioning—is a necessary step before fluorescence microscopic imaging. Together, these two critical steps ensure that tumor cells retain the internalized ATP during the imaging process. Another important consideration during the analysis of ATP internalization is to account for the heterogeneity of xenograft tumors. As tumorigenesis proceeds differently among cancer cell types, it may be necessary to troubleshoot aspects of this method to determine ideal experimental conditions for different cancer cells. To ensure a comprehensive assessment of macropinocytosis of ATP in tumors, it is critical to image tissue sections throughout the tumor as there may be regional variation in terms of resident cells and their ability to internalize eATP. Indeed, this method may uncover different mechanisms of eATP internalization among cancer/tumor cells in future studies.

It is important to note that NHF-ATP can only replace unconjugated ATP or radioactive ATP for

the internalization study. It cannot be used for other studies, such as metabolic studies, as NHF-ATP will behave and be metabolized differently once it is released from macropinosomes and/or endosomes inside cells. Traditionally, ATP internalization was investigated using radioactive ATP[17-19]. However, given the instability of radioactive ATPs, measurable radioactivity inside target cells is not necessarily intact ATP. Because NHF-ATP is nonhydrolyzable and can be visualized microscopically, its use is recommended over radioactive ATP.

The procedure described herein is simple to perform, quick, and cost-effective. If an imaging system is equipped with video capability in future applications, then the dynamic process of ATP internalization can be visualized in real time, revealing information about macropinocytotic kinetics and trafficking in specific tissues. This procedure has been successfully used in other cancer cell lines such as H1299 cells[12], noncancerous cells such as NL-20 cells[12], and neuronal cells, with no or minor modifications (data not shown). As ATP is intimately involved in the Warburg effect in cancer metabolism[6-8,20-22], in diabetes[23-25], and other diseases involving energy metabolism, and as eATP may play important roles in those diseases, the described procedure is likely to have broad applications.

ACKNOWLEDGMENTS:
Cryosectioning was performed on-site at the Ohio University Histopathology Core. This work was supported partly by start-up funds (Ohio University College of Arts & Sciences) to C Nielsen; NIH grant R15 CA242177-01 and RSAC award to X Chen.

DISCLOSURES:
The authors declare no competing interests.

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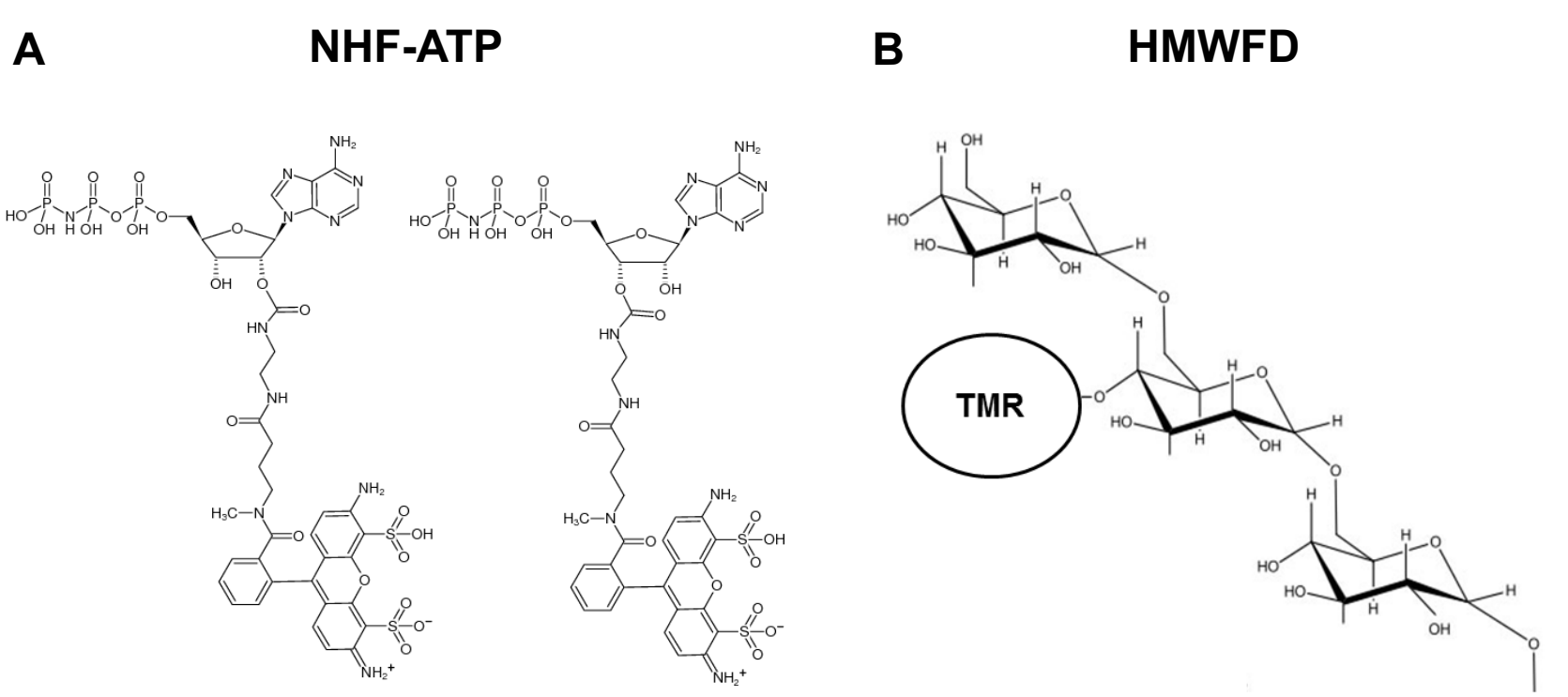
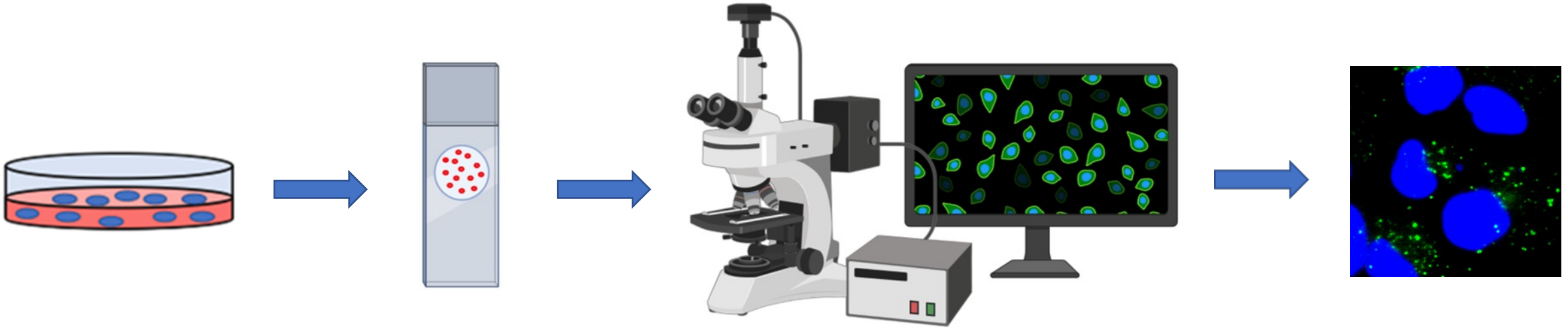


In vitro procedure to examine ATP internalization



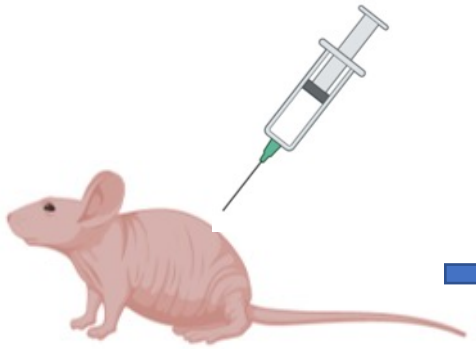
Culture cancer cells atop glass coverslips *in vitro*

Mount cell/coverslips on glass slides

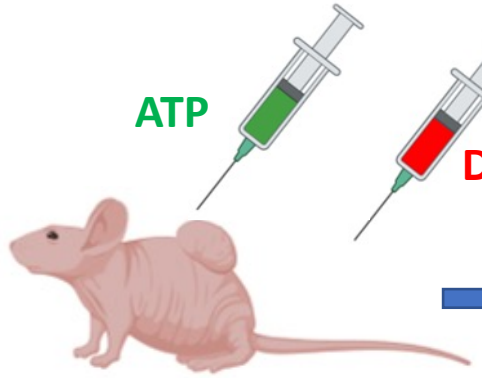
Use fluorescence microscope to visualize cells and acquire images

Representative image of ATP internalization in cancer cells

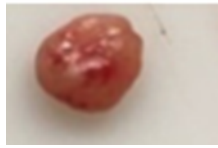
In vivo procedure to examine ATP internalization



Inject cancer cells into nude mice to generate tumor



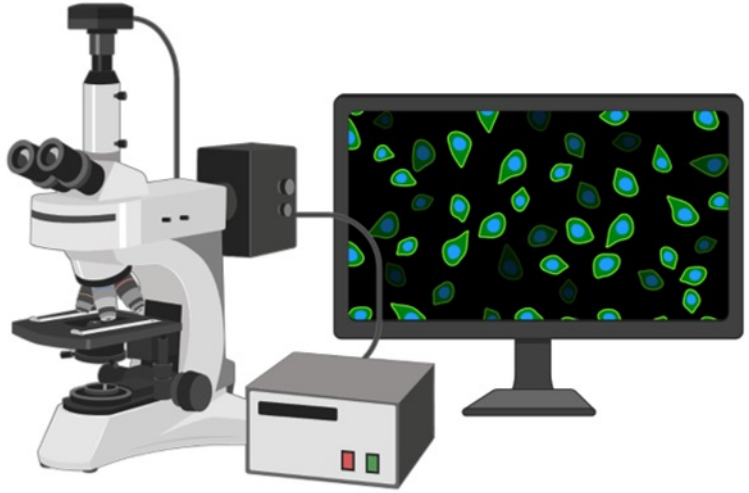
Inject fluorescent ATP or dextran into tumor grown on mice



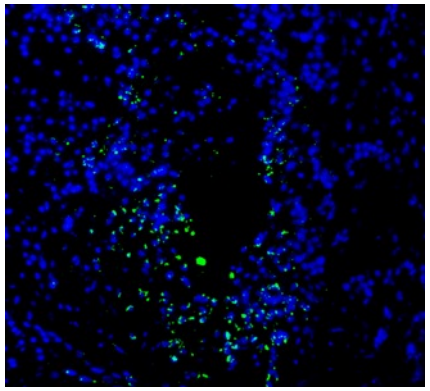
Surgically remove tumor and prepare tissue for cryosectioning

Use cryostat to collect sections through tumor tissue

Mount representative tumor sections onto microscope slide



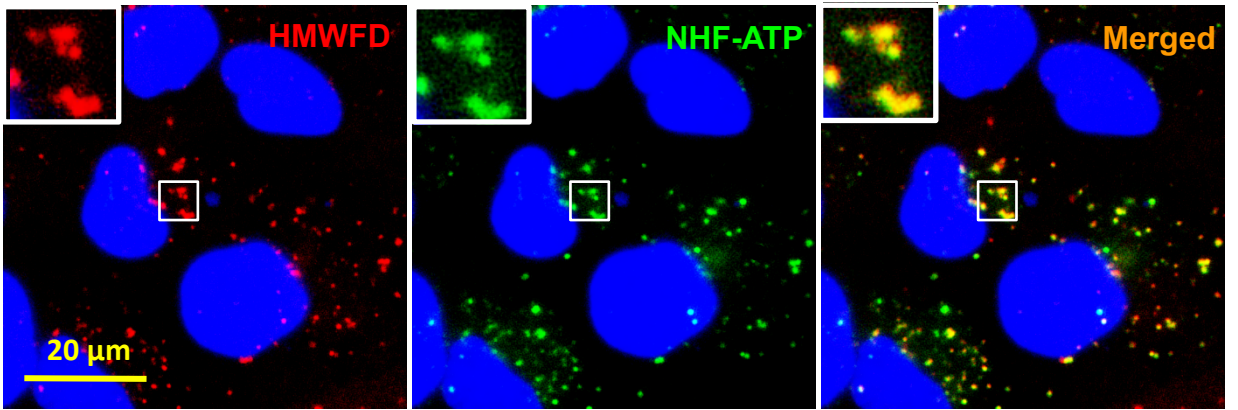
Use fluorescence microscope to visualize tumor tissue and acquire images



Representative image of ATP internalization in tumor tissue

Figure

Click here to access/download;Figure;Nielsen JoVE Figure 4.pdf



Figure

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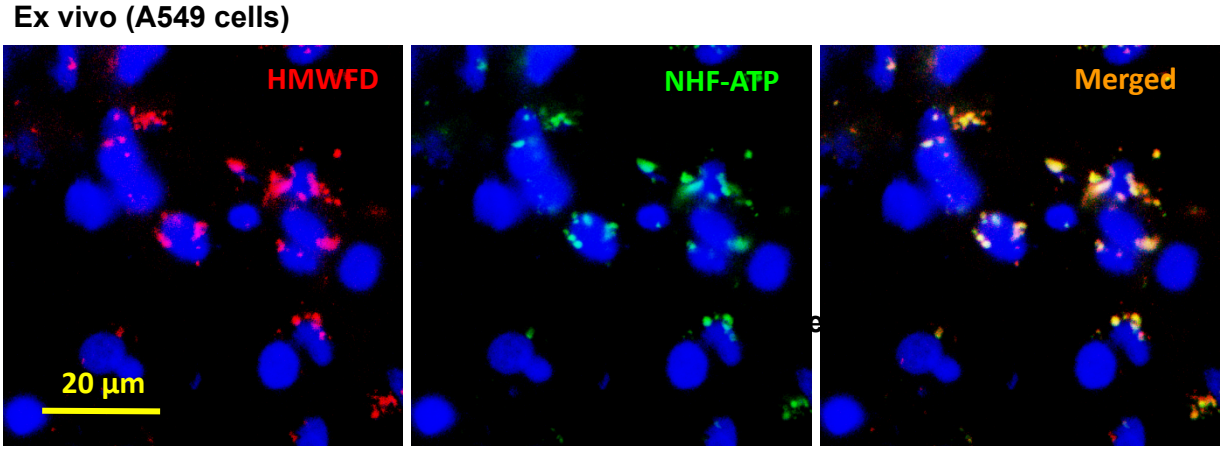

Figure

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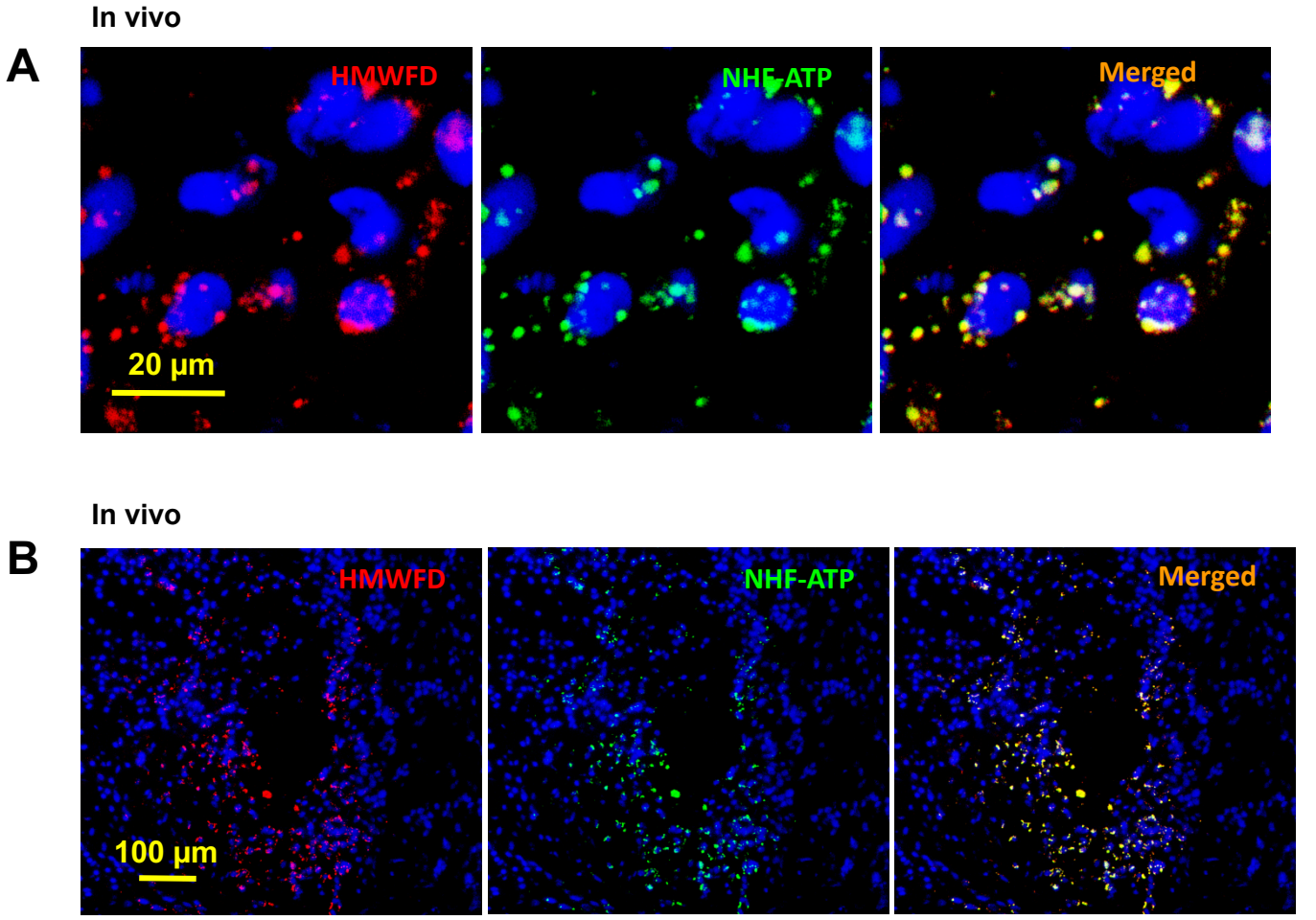

Vineeta Bajaj, Ph.D.
Review Editor, *JoVE*
vineeta.bajaj@jove.com
617.674.1888

2 June 2021

Re: Revisions for JoVE submission JoVE62768 – [EMID:b416054c1feabb36]

Dear Dr. Bajaj,

Thank you for the opportunity to revise and resubmit our manuscript, JoVE62768 **"**ATP internalization, mediated by macropinocytosis and examined by fluorescence microscopy, in human tumor cells and in tumor-xenografted mice." We have addressed all Editorial and Reviewer Comments/Concerns, listed below, and **our responses are bolded.** We have tracked all corresponding changes, within our resubmitted manuscript. We hope that you find our improved manuscript appropriate for print and video publication in *JoVE*.

There are two additional points we would like to make. As the first two authors contributed equally to this manuscript, we changed them to co-first authors, as shown in the revised manuscript beneath author names and affiliations.
The future video part of the protocol requires tumor generation and tumor injection, which requires a significant amount of preparation time (they require approximately 40 days). Thus, we need some advanced notice for the video production. This way, we can purchase nude mice, generate tumors, and prepare them for video shooting.

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have reviewed the manuscript and corrected any spelling/grammar errors.

2. Please provide an email address for each author.

Corinne M. Nielsen	**nielsenc@ohio.edu**
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3. Please define all abbreviations during the first-time use.

We have defined all abbreviations during first-time use and tracked those changes.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets.

We have reformatted all in-text references as numbered superscripts.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Jena Bioscience, Germany, Invitrogen, CA, USA, Invitrogen, TMRHMWFD, Invitrogen, Kim-wipe, ECLIPSE E600 or NiU, Nikon, Nikon Elements format, Eppendorf, Leica Cryostat CM1950, SuperFrost Plus, Fisher Scientific, etc.

We have removed all commercial language from the manuscript and have included appropriate references to commercial products in the Table of Materials and Reagents (Table 1). Within the table, items that have been added or edited appear in bold type.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The text now is written in the imperative tense, throughout the manuscript.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added many detailed steps to our Protocol (tracked edits in the manuscript), in a manner that allows viewers/readers to replicate our Protocol. We also added "CAUTION" and "NOTE" to several steps, throughout the Protocol. Please note that with the additional steps, we have edited our numbering system with intent to make the Protocol easier to read and follow.

8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. Please use complete sentences throughout.

We have edited the Protocol accordingly.

9. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

All centrifuge speeds are now listed as centrifugal force (x g). Please note one instance in which we still report rpm – the speed, at which we gently shake tissue, on an orbital shaker (not a rotor/centrifuge).

10. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

In its current format, our Protocol is 8.5 pages. We have highlighted portions of the Protocol, totaling fewer than three pages (including headings and spacings), that we recommend for video content and production.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All images in figures are original, unpublished data, which have not been used in previous publications; thus, no copyright permission is required.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

We have added new text to our Discussion to address the recommended discussion points. Below, we list where these points can be found, within the Discussion section of our manuscript.

a) Critical steps within the protocol

Discussed in Paragraph 1

b) Any modifications and troubleshooting of the technique

Discussed in final sentences of Paragraph 1

c) Any limitations of the technique

Discussed in Paragraph 2

d) The significance with respect to existing methods

Discussed in Paragraphs 2 and 3

e) Any future applications of the technique

Discussed in Paragraph 3

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a protocol and rationale behind the protocol for visualization of ATP uptake using a fluorescent surrogate in adherent cancer cell lines and xenografts. The steps of the protocol are easy to follow and

Major Concerns:

No major concerns

Minor Concerns:
Please define all the abbreviations (eg, OCT).

We have defined all abbreviations during first-time use and tracked those changes.

In step 2.2, also indicate the volume of cells suspension seed in each well of 24-well plate. The volume determines the time taken for the cells to settle to the bottom of the well and attach.

We have updated the protocol to state that 300 µL of cell suspension (containing 2.5 x 10^4 cells) was plated per well, into a 24-well tissue culture plate.

In step 3.2, indicate the volume of cell suspension that was subcutaneously injected in mice.

The text now states that 100 µL of cell suspension (containing 5 x 10^6 cells) was subcutaneously injected into mice.

In step 3.2.1, line# 137, check the units of centrifugation. It is better to indicate centrifugation as Relative Centrifugal Force (RCF) or g force as it is more precise than revolutions per minute (rpm).

All centrifuge speeds are now listed as centrifugal force (x g). Please note one instance in which we still report rpm – the speed, at which we gently shake tissue, on an orbital shaker (not a rotor/centrifuge).

Reviewer #2:
Manuscript Summary:
This protocol by Nielsen et al is centered on evaluating ATP internalization in cancer cell lines and xenografts. The senior author, Dr. Xiaozhuo Chen, is the world's foremost expert in the field of macropinocytosis-mediated uptake of ATP. The approaches described in this protocol should allow other cancer researchers to easily enter the field, which is impactful because eATP is much higher in tumors compared to normal tissues. Macropinocytosis of ATP can provide energy for ATP-requiring biochemical reactions, contribute phosphate groups for signaling, and control gene expression. The approaches use microscopic imaging of non-hydrolysable fluorescent ATP, coupled with dextrans as endocytic markers. They have applied their imaging modality to tumor cell lines and to xenograft tumors to study eATP internalization in vitro and in vivo, respectively. They have also provided new modifications and optimizations of the assay conditions. This is a very clearly written protocol and an excellent use of figures that are easy to follow.

Major Concerns:
None

Minor Concerns:
Some of the degree signs in the temperature labels may need to be corrected to be superscript.

We have reviewed the text to ensure that all temperature labels are written as ^oC.

Reviewer #3:

Manuscript Summary:

In this study Chen and co-workers describe a fluorescence-based method to monitor cellular uptake of extracellular ATP in vitro, ex vivo and in vivo.

Major Concerns:

I don't have major concerns.

Minor Concerns:

It would be helpful if the Authors could show that in the presence of inhibitors of macropinocytosis ATP uptake is blocked.

In our previous studies (references 9,11,12), we have shown that internalization of ATP is blocked, following administration of macropinocytosis inhibitors. We have added the following text to the Introduction: "Moreover, by administering macropinocytosis inhibitors to cells, we showed that eATP internalization was blocked, suggesting that intracellular uptake of eATP uses a macropinocytotic mechanism[9,11,12]."

Furthermore, the Authors mention that the method also allowed to monitor intracellular trafficking. In fact, intracellular trafficking was not investigated. Mere pinocytosis is shown.

We agree that our representative data demonstrate macropinocytosis of eATP, rather than trafficking. We have deleted the term "trafficking" from the Introduction, and we have edited the introductory text to state that our method may be used to visualize the internalization of ATP and to observe the intracellular spatial localization of internalized ATP, both *in vitro* and *in vivo.* We believe future iterations of our method could involve real-time visualization of the eATP internalization process, revealing information about macropinocytotic kinetics and trafficking in specific tissues. We have added a brief statement, regarding this potential application of our method, to the Discussion section.

Maybe, in the Protocol section, point 2.1.1 should precede point 2.1.

While the numbering in the Protocol section has changed, we have edited the ordering of steps so that instructions for *washing and preparing coverslips* precedes instructions for *placing coverslips into wells*.

Thank you again for the opportunity to re-submit our manuscript. We look forward to hearing from you.

Sincerely,

Xiaozhuo Chen, PhD
Associate Investigator, Edison Biotechnology Institute
Associate Professor, Microbiology
Heritage College of Osteopathic Medicine, Biomedical Sciences