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TITLE:

Imaging of Intracellular ATP in Organotypic Tissue Slices of the Mouse Brain using the FRET-Based Sensor ATeam1.03^{YEMK}

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SUMMARY:

We describe a protocol for cell-type specific expression of the genetically encoded FRET-based sensor ATeam1.03^{YEMK} in organotypic slice cultures of the mouse forebrain. Furthermore, we show how to use this sensor for dynamic imaging of cellular ATP levels in neurons and astrocytes.

ABSTRACT:
Neuronal activity in the central nervous system (CNS) involves a high demand on cellular energy in the form of adenosine triphosphate (ATP). A large share of ATP is needed to re-install ion gradients across plasma membranes degraded by electrical signaling of neurons. There is evidence that astrocytes – while not generating fast electrical signals themselves – undergo increased production of ATP in response to neuronal activity and support active neurons by providing energy metabolites to them. The recent development of genetically encoded sensors for different metabolites now enables the study of such metabolic interactions between neurons and astrocytes. Here, we describe a protocol for cell-type specific expression of the ATP-sensitive Fluorescence Resonance Energy Transfer- (FRET-) sensor ATeam1.03^{YEMK} in organotypic tissue slice cultures of the mouse hippocampus and cortex using adeno-associated viral vectors (AAV). Furthermore, we demonstrate how this sensor can be employed for dynamic measurement of changes in cellular ATP levels in neurons and astrocytes upon increases in extracellular potassium and following induction of chemical ischemia (i.e., an inhibition of cellular energy metabolism).

INTRODUCTION:
Excitatory electrical activity of neurons is largely based on the flux of cations such as sodium (Na^+) and potassium (K^+) across their plasma membranes. Maintenance of the electrochemical gradients of these two ions is thus required for signaling. This is accomplished by the cellular Na^+/K^+-ATPase (NKA), an ubiquitously expressed electrogenic transmembrane pump, which extrudes 3 Na^+ out of the cell in exchange for 2 K^+ from the extracellular space, requiring the consumption of one molecule of ATP per transport cycle[1]. In addition to the NKA, there are several other ATP-consuming ion transporters including the plasma membrane Ca^{2+}-ATPase, which is vital for intracellular Ca^{2+} homeostasis and its export following activity-induced influx[2]. In presynaptic vesicles, a vacuolar-type H^+-ATPase (v-ATPase) creates the proton gradient necessary for neurotransmitter uptake into this compartment[3].

While activity of neurons thus requires a substantial amount of ATP[4], they do not exhibit a significant capacity for storage of energy. Instead, they seem to rely on metabolic interactions with neighboring astrocytes, the major glycogen stores in the brain[5]. It has been suggested that astrocytic glycogen indeed plays an important role in supporting neuronal energy needs; and a key phenomenon in this proposed neuro-metabolic coupling between the two cell types is the capacity of astrocytes to increase their ATP production in response to neuronal activity[6,7,8]. This hypothesis known as astrocyte-neuron lactate shuttle (ANLS) is still under debate, because other work has provided evidence that neurons may also increase their own rate of glycolysis in response to stimulation[9,10], reflecting the necessity of further methods and approaches to study neuro-glia interaction.

Investigation of cellular energy metabolism and ATP levels in neurons and astrocytes to elucidate neuro-glia metabolic interactions has long been hampered by the lack of suitable probes for the detection of changes in metabolite concentrations in living cells in brain tissue. The last decade, however, has provided a surge in the development of new tools and new genetically encoded fluorescent probes for different metabolites, including sensors for ATP, lactate, pyruvate and others[11,12]. Using these tools, it is now possible to directly address questions related to cellular

ATP consumption and changes in cellular energy levels at the single cell level and in a cell-type specific manner in intact brain tissue[13].

In the present work, we describe a procedure to visualize cytosolic ATP dynamics on neurons and astrocytes of cultured organotypic brain slices. We show how to employ adeno-associated viral vectors (AAV) for cell-type specific expression of the genetically encoded ATP-nanosensor ATeam1.03^{YEMK} ([14]) in neurons and astrocytes of slices of mouse brain that can be maintained in cell culture for several weeks[15]. A procedure of how to remove the glial scar that covers cultured tissue slices is described, which improves optical accessibility and imaging of cells in the organotypic tissue layers underneath. Finally, we show how ATeam1.03^{YEMK} can be used to perform FRET-based imaging of changes in cellular ATP levels in this preparation. This method hosts the major advantages that it does not require surgical brain procedures, provides high levels of expression of the sensor and cell type specificity in cultured brain slices, reducing invasiveness or stress in the cells as compared with other methods, like transfection by electroporation or transduction with other viral vectors[10,16,17]. In addition, this protocol can be applied to other FRET based nanosensors, among them other variants of ATeam1.03 that provide lower binding affinity for ATP[14].

PROTOCOL:

The present study was carried out in strict accordance with the institutional guidelines of the Heinrich Heine University Düsseldorf as well as the European Community Council Directive (2010/63/EU). All experiments using organotypic brain slice cultures were communicated to and approved by the Animal Welfare Office at the Animal Care and Use Facility of the Heinrich Heine University Düsseldorf (institutional act number: O50/05). In accordance with the recommendations of the European Commission [18], animals up to 10 days old were killed by decapitation.

1. Preparation of organotypic brain slice cultures (OTCs)

1.1. The day before or at least 30 min prior to the procedure

1.1.1. Prepare the culture plate (under sterile conditions). Remove the lid of the 6 well plate and place 800-850 µL of OTC medium into each well. Keep the plate in the incubator (37 °C, 5% CO_2/95% O_2) until required.

1.1.2. Prepare the washing Petri dishes (under sterile conditions). Add 3 mL of HBSS to each 30 mm Petri dish. A total of 5 dishes per procedure is required. Place them in the incubator (37 °C, 5% CO_2/95% O_2) for at least 30 min – overnight until required.

1.1.3. Prepare the ACSF (**Table 1**). Keep the saline without glucose at 4 °C until the next day.

1.2. On the day of preparation, add the glucose to the ACSF, place it on ice and start bubbling

with 95% O_2 and 5% CO_2 for at least 30 min to result in a pH of 7.4.

1.3. Dissection and slicing

1.3.1. Sacrifice the mouse (BalbC, both sexes) at postnatal days 6 to 8 by rapid decapitation and place the head in a glass Petri dish containing ice-cold ACSF.

1.3.2. Expose the skull by cutting the skin from the back until the posterior tip of the nasal bone. Then, carefully cut the skull from the foramen magnum using a surgical scissor and expose the brain.

NOTE: Verify that the procedure is in accordance with the guidelines of the institution.

1.3.3. Remove the brain and place it on a filter membrane in an ice-cold Petri dish filled with ACSF.

1.3.4. Separate the hemispheres and perform a parasagittal cut at an angle of 45°. Fix one hemisphere at the vibratome tissue stage with superglue. Immediately transfer the tissue block to the vibratome bath containing ice-cold ACSF (bubbled with 5% CO_2/95% O_2). Finally align the tissue. Keep the second hemisphere in ice-cold ACSF until slicing.

1.3.5. Adjust the vibratome to cut slices at 250-400 µm. Slicing at 250 µm will yield approximately 12 slices per animal (400 µm: ~7 slices).

1.3.6. After cutting the slice (**Figure 1A**), identify the hippocampal formation based on its typical morphological appearance (**Figure 1**) and isolate it using hypodermic needles (23 gauge, 1”), keeping the part of the cerebral cortex adjacent to the hippocampus.

NOTE: Preserving the neocortex while culturing helps to preserve the integrity of the hippocampus. However, the hippocampus can be isolated and cultured without cortex if required.

1.3.7. Place the slice on a mesh in warmed, ACSF (34 °C, bubbled with 5% CO_2/95% O_2) until all the slices are collected.

1.3.8. Transfer the slices to the laminar flow cabinet to continue under sterile conditions.

2. Culturing the slices

2.1. Gently transfer the slices from the ACSF into one of the pre-warmed Petri dishes filled with sterile Hank’s salt solution using an inverted sterile glass Pasteur pipette.

NOTE: Sterilization of the tissue is achieved by dilution (step 3.2). Transfer as little ACSF as possible to the culture plate.

2.2. Change the pipette and transfer the slices to the second culture plate. Repeat the process 5 times overall. Transfer as little HBSS as possible to the following culture plates.

2.3. Gently place one slice at a time on the top of the culture insert. Repeat the process for each slice. Avoid turbulences in the pipette and wait until the slice descents to the tip of the Pasteur pipette. One may place up to 4 slices onto a single membrane.

2.4. Carefully remove any excess Hank's solution from the top of the insert by using a fine tip.

2.5. Keep the cultures (**Figure 2B**) in an incubator at the interface between gas (carbogen, 95% O_2 /5% CO_2) and the liquid at 37 °C until the day of experiment. Replace the medium every 2-3 days.

3. Expression of ATP sensors with an adeno-associated viral vector (Figure 2)

NOTE: Make sure to meet all requirements for handling of genetically modified organisms!

3.1. To handle the viral vector, aliquot adeno-associated viral vectors (AAV2/5) at 1-2 µL to avoid repeated freezing and thawing. Store the aliquots at -80 °C.

3.2. Place a flask containing 10% bleach onto the sterile bench to discard all used residual material that was in contact with the vector.

3.3. For transduction, prepare a dilution of 1 µL of the vector with 2-3 µL of DPBS. Stock solutions normally exhibit a physical titer in the magnitude of 10^{12} viral genomes per mL (vg/mL).

3.4. Transfer an insert containing a cultured slice into the sterile hood.

3.5. Without touching the tissue, apply 0.5 µL of the diluted vector directly to the top of each slice.

NOTE: Better expression in the deeper layers of the tissue is obtained by transducing cultured slices at 1-3 days in vitro (DIV). Transduction of older cultures might result in a predominant expression of cells in the surrounding glial scar or a low expression in neurons, respectively.

3.6. Finally, place the slices back into the incubator and maintain them for at least 6 more days. Do not change the medium on the day of transduction.

4. Removal of the glial scar (Figure 3)

4.1. Just before starting an experiment, transfer an insert containing cultured slices into the sterile hood and place it into a 30 mm dish, containing 1 mL of OCT medium or MEM.

4.2. Place the dish under the stereoscope and focus onto the surface of the slice.

4.3. Use two sterile hypodermic needles (23 gauge, 1") to make a short-crosscut right on the narrow edges of a chosen slice (**Figure 3**). This procedure will release the tension in the surface created by the glial scar causing it to retract, thereby exposing the underlying layers (see **Figure 5**).

NOTE: The first tissue layer (glial scar) is mainly formed by reactive astrocytes. In wide-field imaging, this dense tissue layer will result in additional scattering of the light, resulting in blurry images. Removing the scar is thus advantageous to obtain better visibility of the deeper layers, which contain the proper organotypic tissue. Thus, be careful to perform this cut exclusively at the edge and in the upper layer of the slice preparation only and not to damage the tissue underneath. We did not observe differences between data obtained in OTCs with glial scar with those from scar-free OTCs (data not shown).

4.4. Remove the prepared slice from the insert. To this end, use a sterile scalpel and excise it by making straight parallel cuts to the membrane, forming a square or a triangle with the slice in the center, while holding the edges of the membrane with tweezers. If the insert hosts additional slices, transfer it back to the original plate and into the incubator. The surface tension of the medium will prevent its leakage onto the surface of the membrane.

5. FRET-based ATP imaging (Figure 4)

5.1. Before the experiment, prepare E-ACSF and bubble it with 95% O_2 and 5% CO_2 for at least 30 minutes to obtain a pH of 7.4. Switch on the fluorescent light source (Xenon lamp) of the monochromator (**Figure 4**). Start the perfusion just before taking out the slice from the incubator.

NOTE: Keep the saline bubbled with 95% O_2 and 5% CO_2 during the entire experiment.

5.2. Transfer the slice into an experimental chamber that is constantly perfused with freshly carbogenated E-ACSF using a peristaltic pump (**Figure 4**). Then, fix the slice with a grid. Place the chamber onto the microscope stage and connect the perfusion system. Gas-proof laboratory tubing is recommended for perfusion.

NOTE: Experiments can be performed at room temperature or near physiological temperature, depending on the experimental design. Check the stability and reliability of the perfusion flow to avoid changes of focus induced by movement of the tissue and/or changes in shear stress. A standard perfusion velocity for slice work, used by us and many other laboratories, is 1.5 - 2.5 mL/minute.

5.3. Bring the cultured slice into focus using transmission light. Identify the area where experiments shall be performed (example: CA1 region of the hippocampus). Before starting imaging experiments, wait at least 15 min to allow slices to adapt to the saline conditions. For the configuration of the experimental setup, see **Figure 4**.

5.4. Switch on the camera and the imaging software. Then, select the proper filter cube.

5.5. Excite the donor fluorescent protein (eCFP) at 435/17 nm (~435 nm). Set the exposure time between 40 to 90 ms.

NOTE: Overexposure of slices to the fluorescent light may result in phototoxic effects.

5.6. Excitation at 435 nm results in emission at both 475 nm (eCFP; donor) and 527 nm (Venus; acceptor). Split the fluorescence emission at 500 nm with an emission image splitter and employ band pass filters at 483/32 and 542/27 to further isolate donor and acceptor fluorescence. Strong expression might result in saturation of the detectors. In this case, you might use a neutral density filter to reduce intensity of excitation.

5.7. Select a region of interest (ROI) apparently devoid of cellular fluorescence for background subtraction. Then, create ROIs delineating cell bodies.

5.8. Set the frequency of image acquisition and the overall recording time. For long (>30 min) experiments, an acquisition frequency of 0.2 – 0.5 Hz is recommended to prevent phototoxicity.

NOTE: Adjust focus of the cell during the recording if needed.

5.9. Start the recording. It is recommended to record at least 5 min under baseline conditions to ensure the stability of the preparation.

5.10. To induce changes in intracellular ATP, transfer the perfusion tube from standard ACSF to a saline containing metabolic inhibitors (e.g. CIS, see **Table 1** and below). Alternatively, use a saline with elevated potassium concentration to mimic release of potassium from active neurons.

NOTE: Application by bath perfusion is a relatively slow process, which globally acts on the entire preparation. Take note of the time at which the new solution actually started to enter the experimental bath. Depending on the distance between the chamber and the saline's reservoir, as well as on the speed of the perfusion, a delay time must be considered.

6. High resolution documentation of cellular ATeam fluorescence

6.1. Directly after the recordings, transfer the recording chamber containing the slice culture to the confocal laser scan microscope.

NOTE: Take special care. Because of potential photodamage, perform this step only after experiments. For documentation purposes, one may exchange the E-ACSF with H-ACSF. Therefore, a perfusion system is not necessarily required.

6.2. Take z-stacks at the highest z resolution possible at the given optical configuration.

6.3. Apply a deconvolution algorithm to increase image resolution.

REPRESENTATIVE RESULTS:

AAV vectors are a reliable tool to selectively express foreign genes in cells within living tissue[16]. Direct application of AAVs containing the sequence cassette of ATeam1.03^{YEMK} and a specific promoter results in a high expression of the sensor in the chosen cell type. At DIV 14 (˜10 days after a transduction), neurons expressing ATeam under the human synapsin promoter are found at high density in the neocortex of cultured tissue slices at depths of up to 50 µm below the slice surface (**Figure 5A**). Comparable results can be achieved in the hippocampus (**Figure 5B**).

For the measurement of ATP levels in astrocytes, ATeam1.03^{YEMK} is expressed under the control of the human glial fibrillary acidic protein (GFAP) promoter. This results in efficient transduction of cells in both neocortex and hippocampus of cultured tissue slices (**Figure 6**). Notably, two different morphological phenotypes can be distinguished, depending on the depth relative to the surface of slice preparations. In the first, superficial layer, cells are characterized by thick primary processes that are predominately arranged in parallel to the surface. These cells exhibit strongly overlapping domains, creating a dense meshwork of apparently reactive astrocytes (**Figure 6**). In deeper layers (30 – 60 µm from the surface), transduced astrocytes exhibit fine cellular processes that form largely spherical domains and their morphology resembles that of astrocytes in situ as reported earlier [19-21] (**Figure 6**). To obtain better transduction of deeper-layer astrocytes as well as better optical access to these deeper layers, the glial scar tissue can be removed as described in Step 4.

Successful expression of ATeam1.03^{YEMK} allows the dynamic measurement of changes in ATP levels in neurons or astrocytes, depending on the promoter used (see above). Experiments were performed in an experimental bath constantly perfused with E-ACSF (bubbled with 95% O_2 and 5% CO_2). In organotypic slices expressing ATeam1.03^{YEMK} in hippocampal neurons, regions of interest (ROIs) were selected before starting the recording, representing the somata of pyramidal cells (**Figure 7A**). Moreover, a region for background subtraction was chosen (**Figure 7A**). Emission of Venus as well as eCFP fluorescence was then collected for each of these ROIs separately and depicted as fluorescence emission level over time (**Figure 7B**). After recording fluorescence under control conditions for several minutes to ensure a stable baseline, cellular metabolism was inhibited by exposing the slice preparation to a glucose-free saline, to which 5 mM sodium azide (NaN_3) was added for one minute (**Figure 7B**). This manipulation induced opposite changes in the emission intensity of the FRET pair (**Figure 7B**, left panels), with a decrease of Venus (527 nm) and an increase of eCFP (475 nm) emission. Calculating the FRET ratio by dividing the fluorescence emission of Venus by that of eCFP (F_{Venus}/F_{eCFP}) resulted in signals that reflect the relative changes in the intracellular ATP levels, the so-called “ATeam FRET ratio” (**Figure 7B**, right panel). In all recorded neurons (n=70 cells in N=5 slices), NaN_3 caused a reversible decrease in the ATeam FRET ratio, indicating a reversible decrease in intracellular ATP levels upon inhibition of cellular metabolism.

To ensure the stability of the preparation and the sensor under long-term experiment conditions,

slices expressing ATeam in neurons or astrocytes were constantly perfused with ACSF for prolonged periods (> 50 min; n=12 cells each, N=3 OTCs from 3 brains). Under these conditions, the ATeam FRET ratio did not change (**Figure 8**). Exposing the preparations to CIS containing metabolic inhibitors ("Chemical ischemia", see **Table 1**), in contrast, again resulted in the expected drop in the ATeam FRET ratio as observed above.

Next, we analyzed the responses of neurons and astrocytes expressing ATeam1.03^{YEMK} to an increase in the extracellular potassium concentration. After establishing a stable baseline, neurons were perfused with a saline in which the potassium concentration was increased from 3 to 8 mM for 3 minutes (**Figure 9A**). This manipulation did, however, not result in a detectable change in the ATeam FRET ratio (n=56 cells in N=5 slices). To ensure that the sensor reacted to a change in ATP levels, slices were then again exposed to a sustained period of chemical ischemia elicited by replacing E-ACSF by CIS. Chemical ischemia resulted in a rapid decrease in the ATeam FRET ratio to a new, stable level, indicating nominal depletion of ATP after 2-3 minutes (**Figure 9A**).

The same experimental protocol was performed with slices, in which ATeam1.03^{YEMK} was expressed in astrocytes. In contrast to what was observed in neurons, astrocytes reacted to the increase in extracellular potassium by a reversible increase in the ATeam FRET ratio, indicating an increase in intracellular ATP levels (n=70 cells in N=5 slices) (**Figure 9B**). Subsequent exposure to chemical ischemia resulted, as expected, in a large drop in the ATeam FRET ratio, indicative of nominal depletion of intracellular ATP (**Figure 9B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Representative transmission images of acute and organotypic brain slice preparations. Wide view comparison of an acutely isolated parasagittal brain slice (**A**) and a parasagittal organotypic brain slice maintained in culture for 12 days (**B**). DG: dentate gyrus; CA1/3: CA1/CA3-region of the hippocampus; E-CTX: Entorhinal cortex; CTX: (neo-) cortex.

Figure 2. Principle of FRET-based ATP imaging in cultured organotypic brain slices using the genetically encoded sensor ATeam1.03^{YEMK}. Schematic representation of the protocol presented in this work. Briefly, parasagittal organotypic slices, cultured for 1-3 days, are transduced with an adeno-associated viral vector containing either, the astrocyte-specific hGFAP- or the neuron-specific hSyn-promoter and the sequence for the expression of ATeam 1.03^{YEMK}. Diluted aliquots of these vectors (1:2-1:4) are directly applied on the top of a slice, which is maintained under culturing conditions for at least 6 more days. Changes in intracellular ATP levels can then be visualized in cells expressing the sensor by exciting it at 434 nm and by acquiring fluorescence emission simultaneously at 527 (acceptor) and 475 (donor) nm.

Figure 3. Schematic illustration of the mechanical removal of the glial scar. The figure shows a hippocampal slice culture which is covered by a glial scar (blueish ellipsoid). By one time shearing the tips of two syringe needles at the smallest pole of the culture and at the edge of the glial scar (blue dashed line), the scar will flip aside.

Figure 4. Configuration of the FRET imaging setup. (**A**) Schematic illustration of the different components and their spatial arrangement required for the FRET imaging setup. The arrangement consists of a monochromator with a xenon lamp as a light source, an upright fixed-stage microscope (1), an image-splitter system (2), a digital CCD or CMOS camera for time-lapse recording (3), and an experimental bath adapted for stable constant perfusion (4). The bath perfusion is realized by a peristaltic pump with adjustable flow rate. (**B**) Image of the experimental workspace. The FRET imaging setup is mounted on a vibration-damped table carrying a x/y-translational stage, into which the experimental bath is embedded. Numbers: see (**A**). (**C**) Schematic view of the light pathway from the monochromator to the digital camera. Indicated is the position of the different filters and the dichroic mirror. Numbers: see (**A**).

Figure 5. Visualization of neurons expressing ATeam1.03^{YEMK} in cultured parasagittal organotypic brain slices. Images on the left correspond to extended focus projections of 43 optical sections (1.05 µm each) of cortical tissue (**A**) and (**B**) of 70 optical sections (0.6 µm each) of hippocampal tissue. Images on the right represent the volume view of the same projection. Cells are color-coded according to their depth relative to the slice surface as indicated by the color scale on the right.

Figure 6. Visualization of astrocytes expressing ATeam1.03^{YEMK} in cultured parasagittal organotypic brain slices. The image on the left corresponds to an extended focus projection of 191 optical sections (0.45 µm each). For illustration purposes, the glial scar was excluded from the projection of astrocytes. Images on the right represent the volume view of the same projection before and after removal of the glial scar. Cells are color-coded according to their depth relative to the slice surface as indicated by the color scales on the right.

Figure 7. Demonstration of time lapse ATeam FRET ratio imaging. (**A**) Top left: Wide-field fluorescence image of the pyramidal layer and stratum radiatum of the CA1 region of a cultured organotypic hippocampal slice expressing ATeam1.03^{YEMK} in neurons. Top right: Enlarged view of boxed section as indicated on the left. White lines delineate regions of interest (ROIs) 1-3 representing cell bodies of CA1 pyramidal neurons chosen for analysis in (**B**). BG represents the ROI chosen for background correction. Bottom: Pseudo-colored images representing fluorescence emission of Venus (green), eCFP (purple) and the ratio of Venus/eCFP. (**B**) Time lapse recording in ROIs 1-3, representing neuronal cell bodies (see **A**). Traces on the left show normalized fluorescence emission of Venus (green) and eCFP (magenta). Traces on the right show the corresponding ATeam FRET ratio. Note that perfusion with 5 mM NaN_3 in the absence of extracellular glucose for 1 minute (grey bar) induces a reversible decrease in the ATeam FRET ratio, indicating a decrease in intracellular ATP concentration.

Figure 8. Baseline experiments employing ATeam. Long-term ATeam FRET ratio imaging in 14 different cells under baseline conditions in neurons (top) and astrocytes (bottom). Data were taken under comparable conditions as other experimental data. At the end of each measurement, chemical ischemia was elicited by perfusion with CIS as indicated by the arrow. NOTE: Baseline ATeam FRET ratios are stable over time under baseline conditions.

Figure 9. Representative experiments illustrating changes in ATP levels in neurons and astrocytes. (**A**,**B**): Images on the left show ATeam fluorescence from neurons and astrocytes located in the hippocampal CA1 region of organotypic slices. Traces on the right represent time lapse recordings of the ATeam FRET ratio obtained from a ROI positioned over a single cell body. In both experiments, slices were first subjected to an increase in the extracellular potassium concentration for 3 minutes (see bar), followed by a final exposure to chemical ischemia. Note that while neurons do not respond to the elevation of extracellular potassium (**A**), astrocytes react with an increase in ATP (**B**).

Table 1. Solution composition

DISCUSSION:

In this methodological study, we demonstrate a procedure for the cell-type specific expression of ATeam 1.03^{YEMK}, a FRET-based, genetically encoded nanosensor[14], for measurement of changes in ATP levels in astrocytes or neurons in organotypic tissue slice cultures of the mouse brain[15]. In exemplary recordings, we show that an increase in the extracellular potassium concentration does not result in a change in ATP concentrations in neurons, while astrocytic ATP levels rise in response to this manipulation. Moreover, our results demonstrate that upon inhibition of cellular metabolism, ATeam 1.03^{YEMK} FRET ratio rapidly drops in both cell types, indicating a rapid decrease in intracellular ATP.

Expression of ATeam 1.03^{YEMK} in organotypic slice cultures requires maintenance of the tissue in culture under controlled conditions for at least 7-10 days. Alternatively, ATeam 1.03^{YEMK} can also be employed for measurement of ATP in acutely isolated brain tissue slices and in optic nerves of mice[13,15]. Measurements in acutely isolated tissue, however, necessitate the generation of transgenic animals or a stereotactic application of viral vectors into the brain, involving animal experimentation and strict animal care protocols. In this regard, ATeam 1.03^{YEMK} expression in organotypic tissue slice cultures represents a useful and valuable alternative[22,23]. For many years now, organotypic tissue slice cultures serve as an established model system to study neural properties, connectivity and development[24-26]. They not only maintain the general tissue architecture and lamination (**Figure 1**), but also host the preferential properties of cell cultures such as superior accessibility and direct control of experimental conditions. Organotypic tissue slice cultures are also routinely employed to express foreign genes by using viral vectors[27]. Several types of viral vectors have been reported to deliver transgenes into brain tissue[16,28]. Adenoviral vectors induce high expression in glial cells, but not hippocampal neurons[16], and might generate glial reactivity[17]. Adeno-associated viral vectors as used here emerge as a good alternative[15], and their effectiveness has also been shown in vivo[29].

While mainly being used for the study of neuronal properties, recent studies have established that organotypic tissue slice cultures can also be employed for the analysis of astrocytes. Cultured slices are usually covered by a layer of reactive astrocytes[19,30] (**Figure 5**), but astrocytes exhibit a more native, non-reactive morphology and cytoarchitecture in deeper layers[19,30] (**Figure 5**). In the present study, we describe a procedure for the mechanical removal of the outer glial scar,

which results in a better experimental and optical accessibility of native astrocytes within the proper organotypic tissue layers. Moreover, its removal improves the expression efficacy in deeper layers of the organotypic slices; if the glial scar is not removed, transduction by AAVs might tend to be restricted to the superficial cell layers.

Several external mechanical factors need to be considered when performing experiments in tissue slices. A variation in bath perfusion velocity can induce movements of the entire preparation and/or induce changes in focus, resulting in artificial transient changes of the sensor signal. Moreover, both astrocytes and neurons have been reported to respond to mechanical deformation such as imposed by high perfusion rates[32,33]. In our hands, using a reliable peristaltic pump, together with maintaining small and stable volumes of saline between the tissue and the objective (meniscus) results in a stable FRET-signal under baseline conditions at the perfusion velocity used here (1.5 - 2.5 mL/min; **Figure 8**).

In the present study, we also demonstrate that FRET-based imaging with ATeam 1.03^{YEMK} can be employed to monitor ATP levels in neurons and astrocytes. An alternative means introduced earlier for measurement of cellular ATP is the so-called luciferin-luciferase assay[34-37]. This approach, however, is based on imaging of bioluminescence and only provides a rather low temporal and spatial resolution partly due to high background noise levels. Another method routinely employed in recent years was the imaging of changes in the intracellular magnesium concentration using the ion-sensitive fluorophore magnesium green[38-40]. This approach relates to the observation that a consumption of ATP results in the release of its co-factor magnesium. Imaging with magnesium green thereby only provides a secondary estimate of changes in ATP levels. Moreover, magnesium green is also sensitive to changes in intracellular calcium, introducing another difficulty when interpreting results obtained with this method.

The recent development of genetically-encoded nanosensors for direct imaging of cellular metabolites, therefore, represented a big step forward[11,12]. Several different sensors were generated that can be employed for measurement of intracellular ATP[36,41-43]. Among those are the ratiometric fluorescent ATP indicator "QUEEN"[41] as well as PercevalHR, which senses the ATP:ADP ratio[42]. While the latter probe is a valuable tool for the study of the energy status of cells, it requires simultaneous measurement of changes in pH[42].

In the present study, we employed ATeam, a nanosensor of which several variants exist, which - among others - differ in their binding affinity for ATP[14]. In vitro, ATeam 1.03^{YEMK} exhibits a K_d of 1.2 mM at 37 °C, which is close to cellular ATP levels determined in different neuronal cell types, ranging from hypothalamus and cerebellum [34] to hippocampus[37,44,45]. In cuvette measurements, lowering the temperature by 10 °C resulted in a significant decrease in the binding affinity of ATeam 1.03^{YEMK} to ATP, suggesting that it might not be ideal for cellular imaging at room temperature[14]. Our earlier study[15], however, demonstrated that the behavior and response of ATeam1.03^{YEMK} expressed in neurons and astrocytes to different manipulations is similar at near physiological and at room temperature, indicating that the sensor allows reliable determination of intracellular ATP levels under both conditions. In addition, our previous experiments addressed the pH-sensitivity of ATeam1.03^{YEMK} expressed inside cells[15], showing that it is

insensitive to changes in intracellular pH by about 0.1-0.2 pH units. If the K_d in the low mM range is a concern, alternative ATeam variants might be used[14], among them red-shifted variants of ATeam ("GO-ATeam")[43].

Our experiments using ATeam 1.03^{YEMK} demonstrate that an increase in the extracellular potassium concentration by a few mM only (from 3 to 8 mM) results in a transient increase in the ATeam 1.03^{YEMK} ratio in astrocytes in organotypic slice culture. This observation confirms earlier studies[15,46] and clearly indicates that astrocytes respond to the release of potassium by active neurons with an increase in their ATP production, mostly likely as a consequence of a stimulation of the Na^+/K^+-ATPase and the Na^+/HCO_3^- cotransporter, respectively[47,48]. In contrast to this, neurons did not show a response, which is in line with previous work as well[15]. Both cell types, however, rapidly and strongly reacted to inhibition of cellular glycolysis and mitochondrial respiration as shown before[15]. Under conditions of chemical ischemia, ATeam FRET ratios fell to a new stable level, indicating a nominal depletion of cellular ATP. The latter result suggests that both neurons as well as astrocytes exhibit a relevant consumption of ATP also under steady-state conditions without additional stimulation by synaptic activation or application of neurotransmitters. Taken together, we conclude that FRET-based imaging with genetically encoded nanosensors, among them ATeam 1.03^{YEMK}, will provide a valuable approach to elucidate the cellular processes that are responsible for changes in intracellular ATP levels and cellular ATP consumption under different conditions.

DISCLOSURES:

The authors declare no competing interests. The authors received financial support enabling open access publication by Nikon Microscope Solutions, Düsseldorf, Germany, which produces instruments used in the video article. The company was neither involved in designing the experiments presented here, nor in their execution, nor in the data handling, nor in the manuscript writing.

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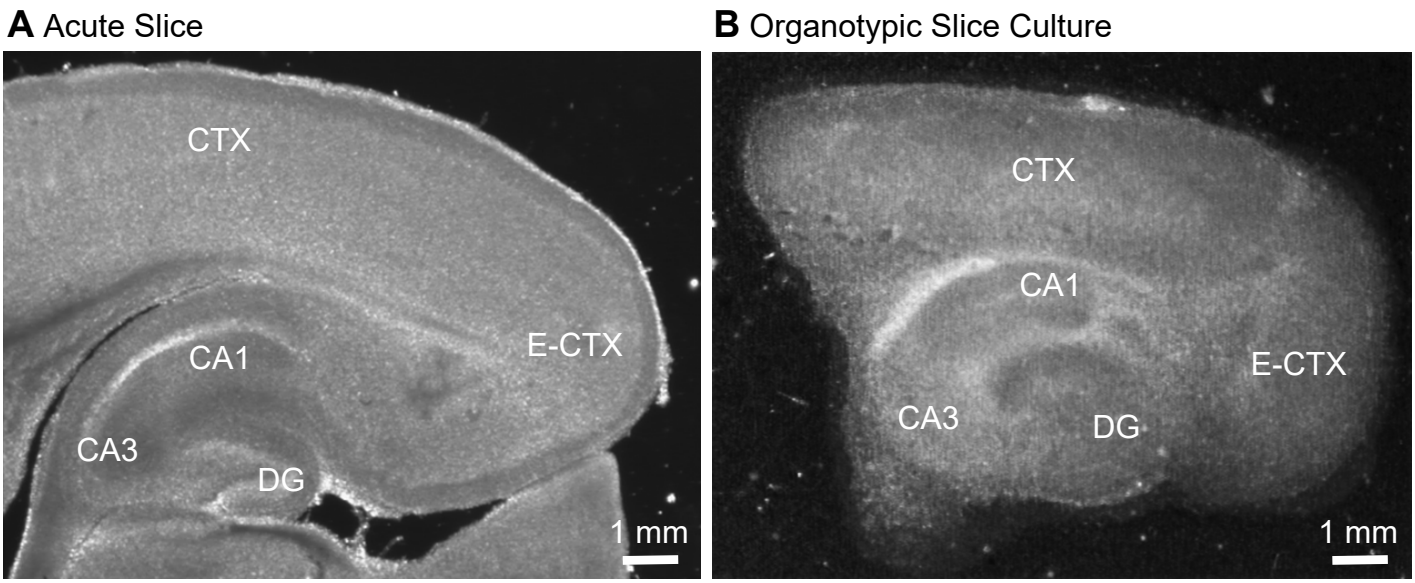

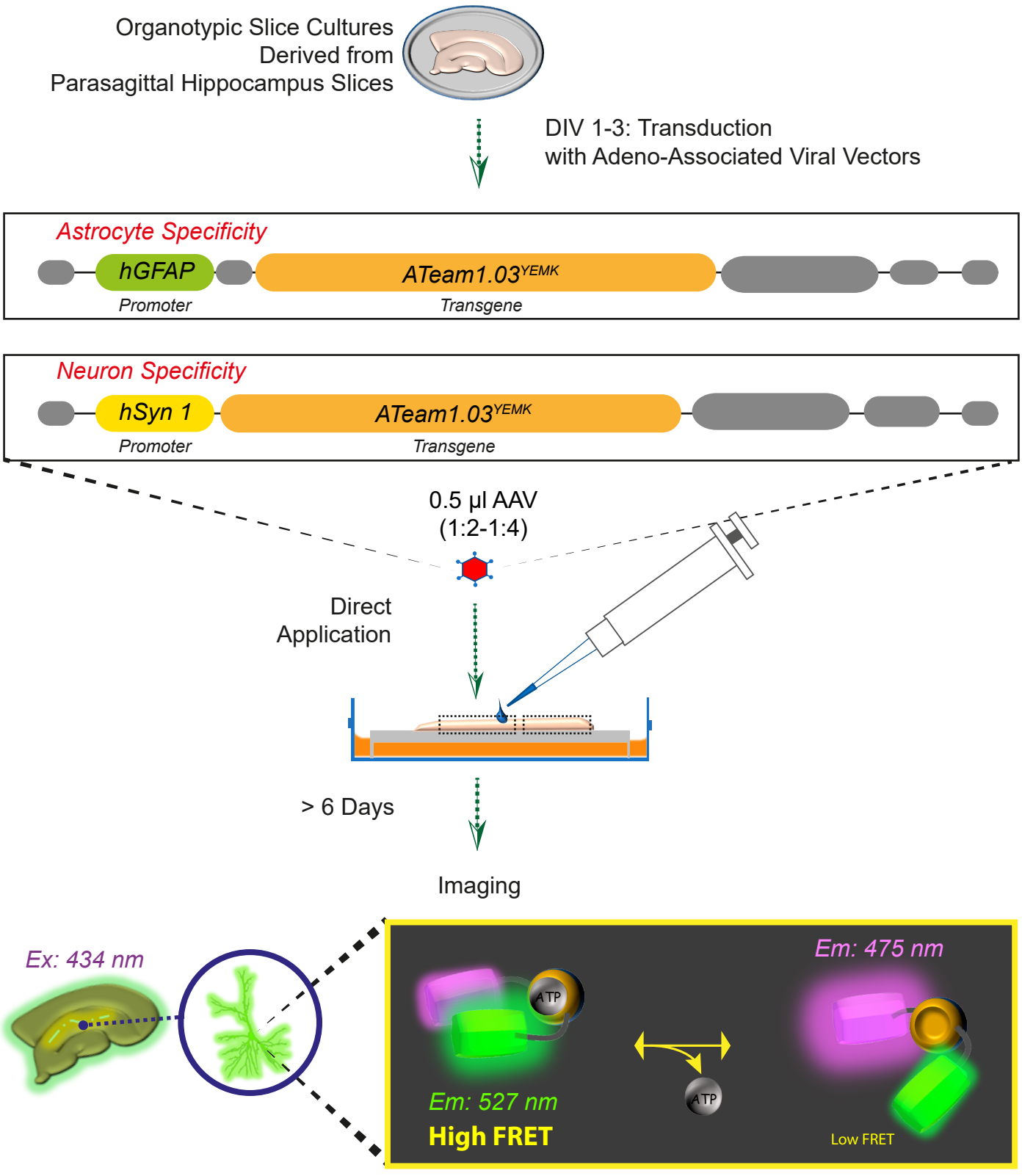

Figure 3

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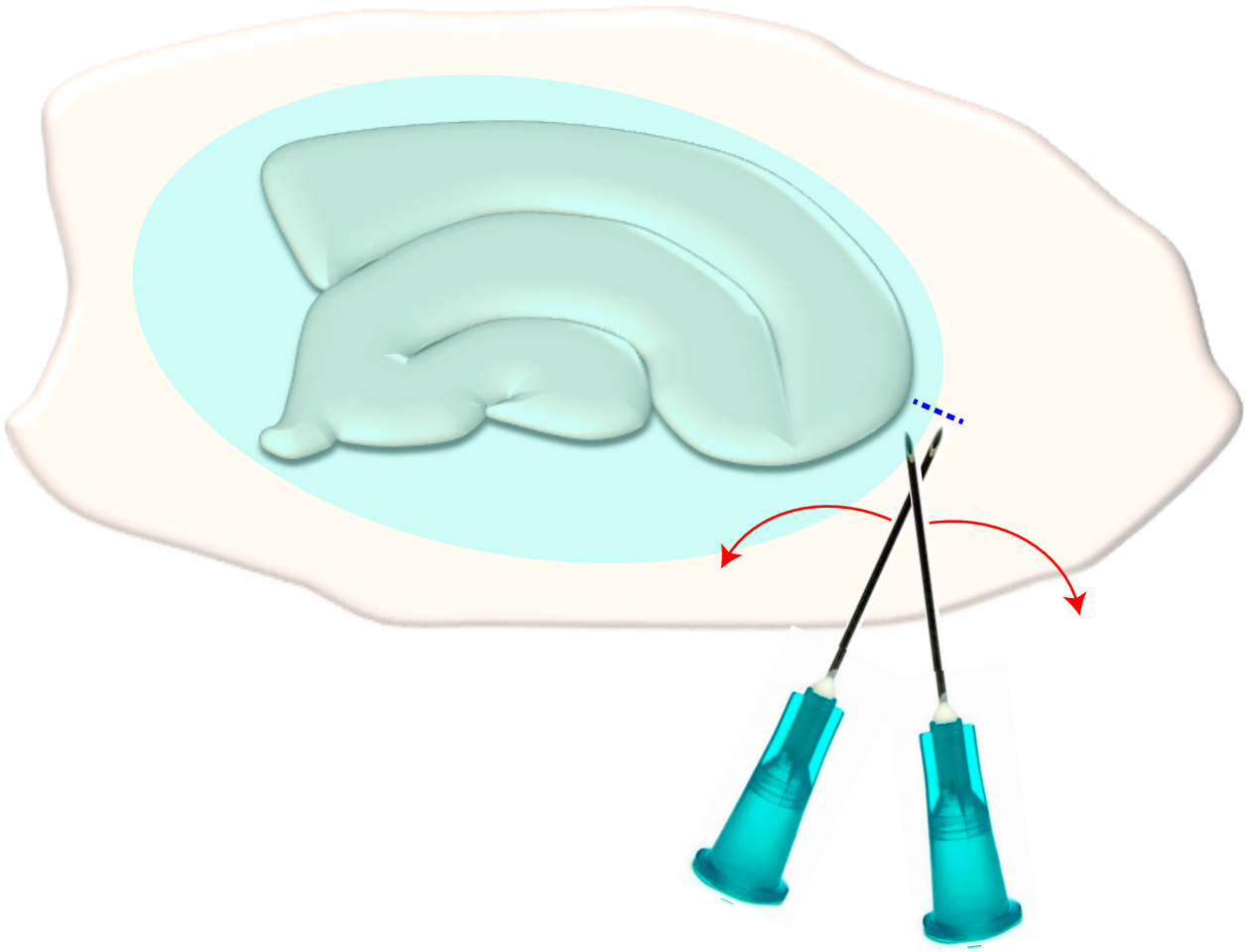

Lerchundi_Fig 4

Figure 4

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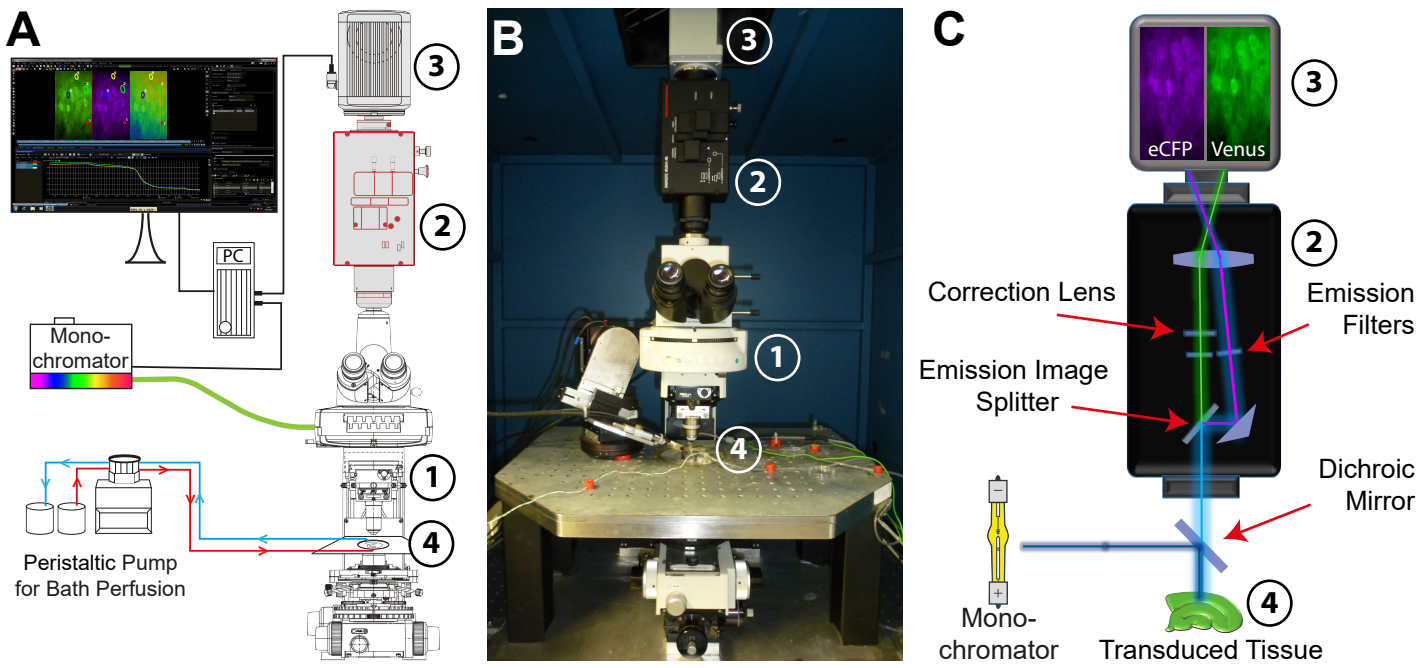

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Figure 5

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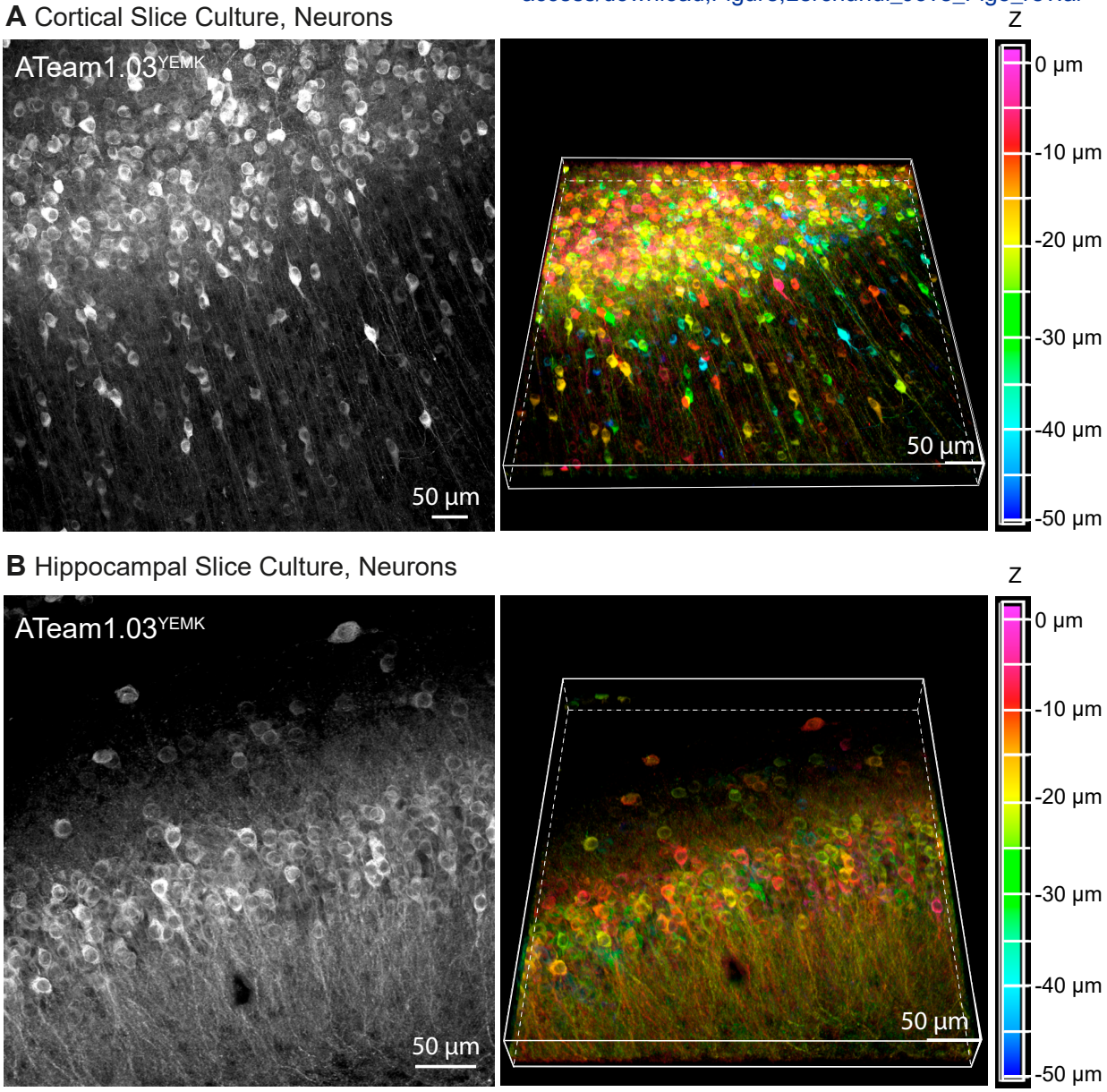

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Figure 6

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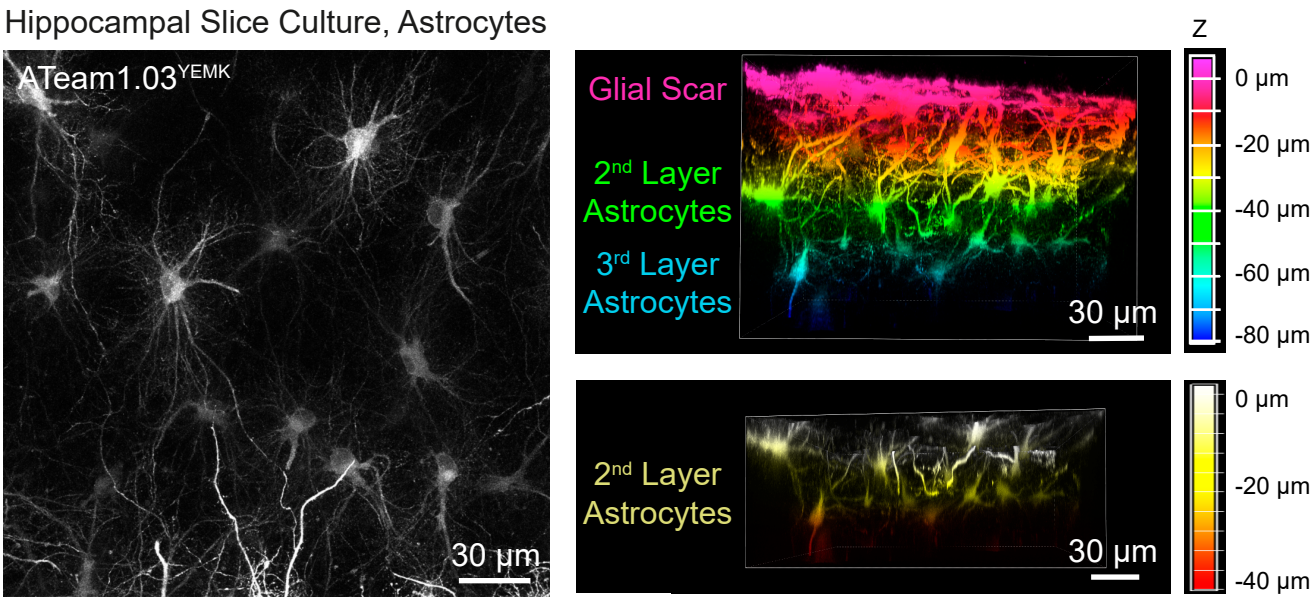

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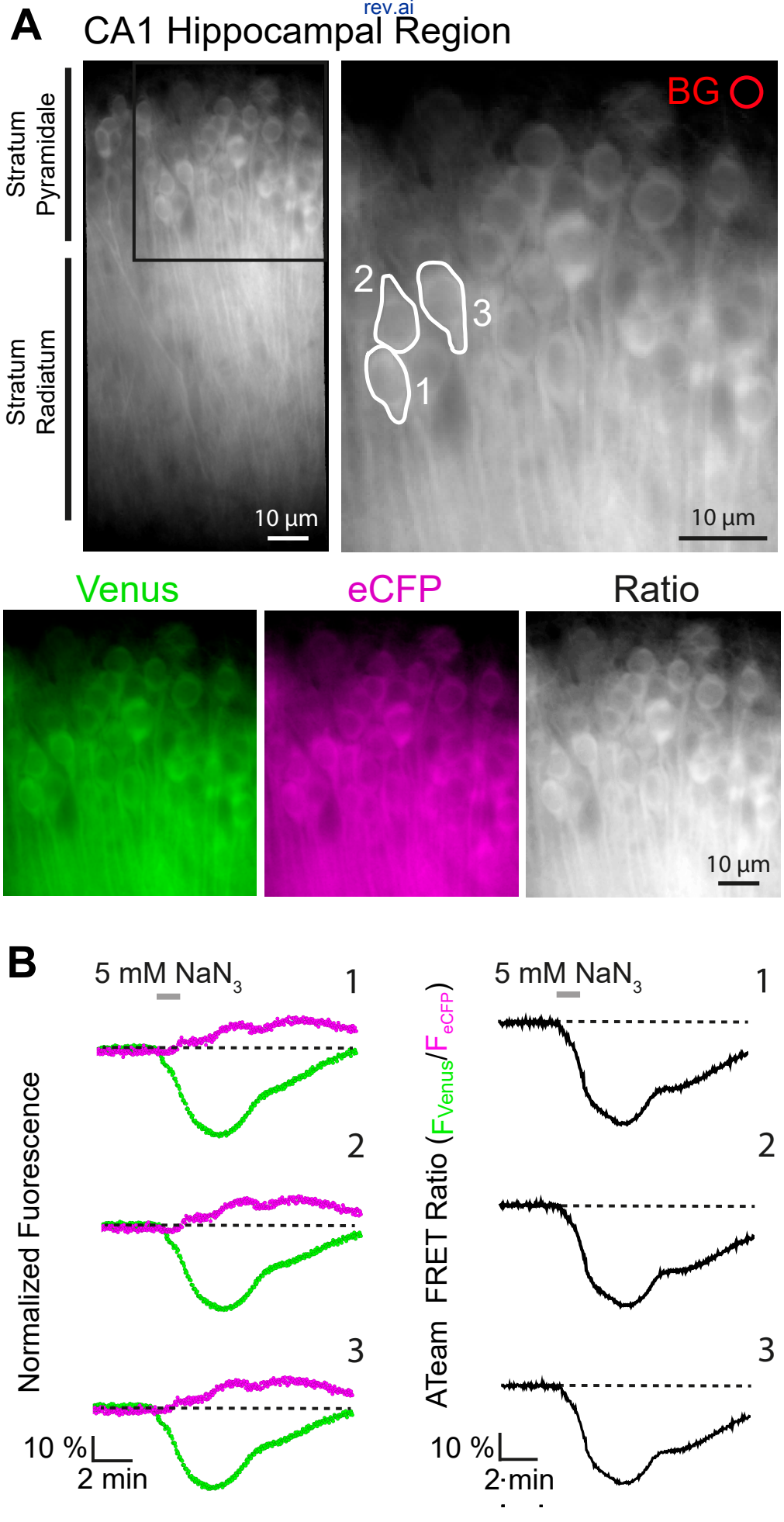

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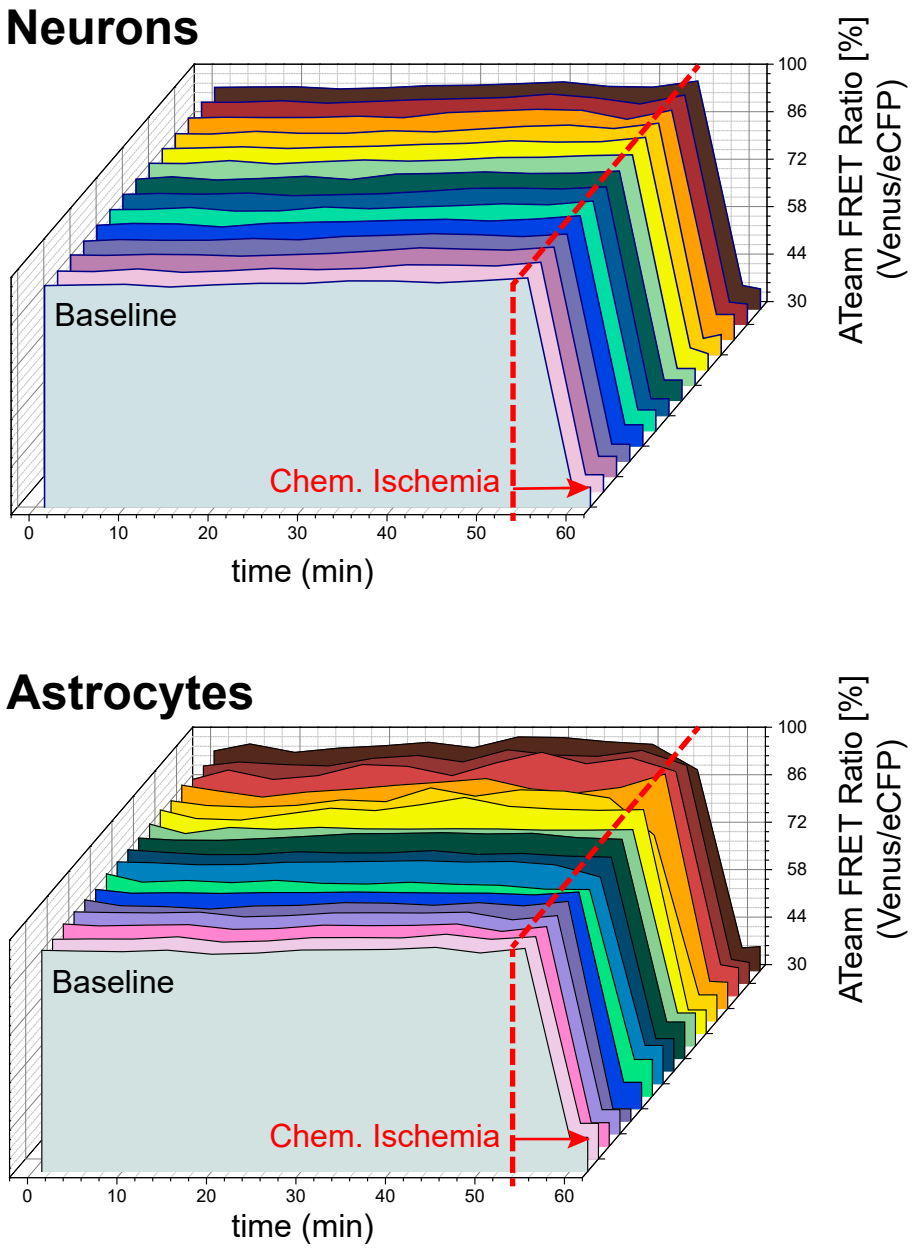

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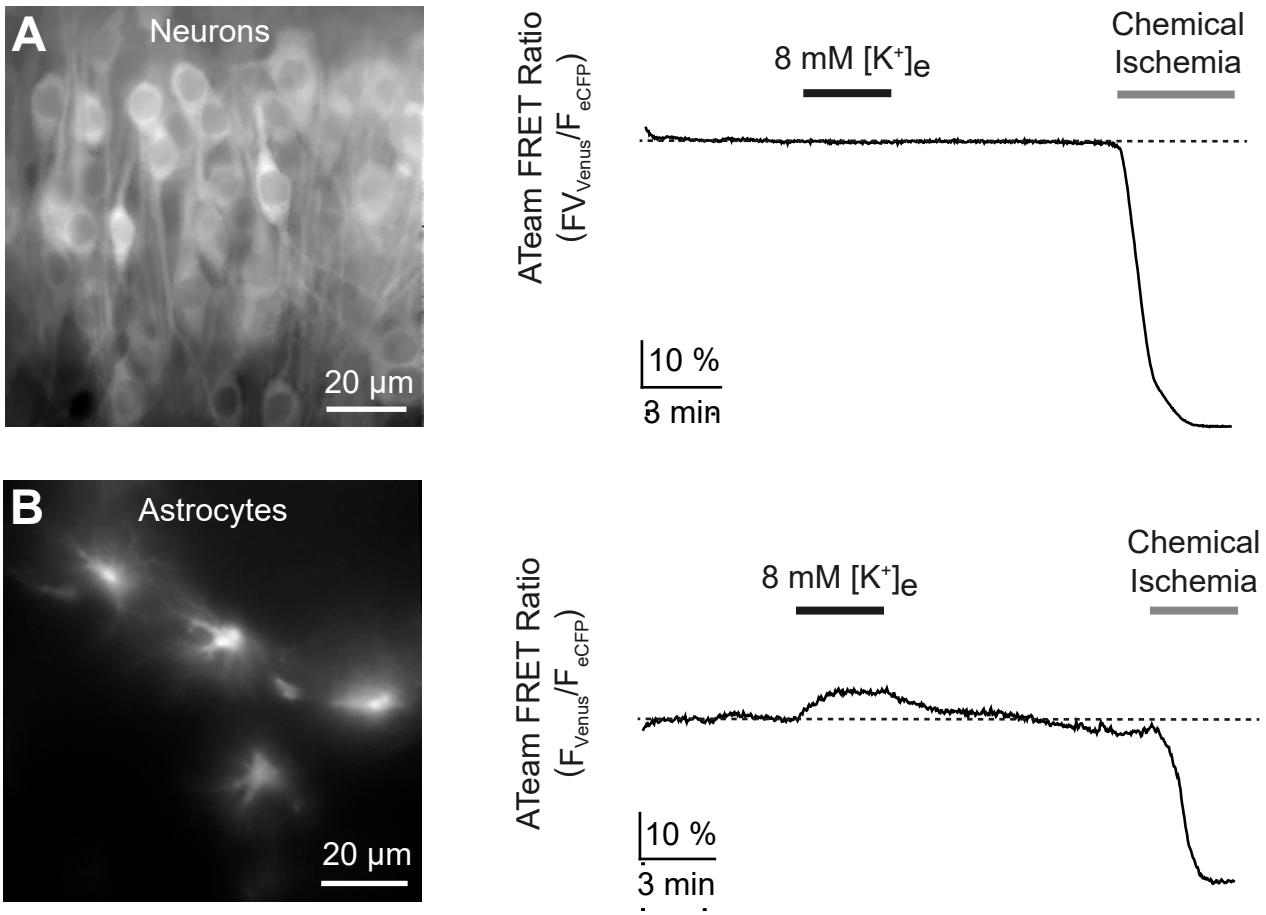

Lerchundi_Fig9

Table 1

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Salines and Media - Formulation

Name	Abbreviation	Composition
Artificial Cerebrospinal Fluid Solution	ACSF	NaCl KCl $CaCl_2$ $MgCl_2$ NaH_2PO_4 $NaHCO_3$ Glucose
Experimental ACSF	E-ACSF	NaCl KCl $CaCl_2$ $MgCl_2$ NaH_2PO_4 $NaHCO_3$ Glucose Lactate
Chemical Ischemia Solution	CIS	NaCl KCl $CaCl_2$ $MgCl_2$ NaH_2PO_4 $NaHCO_3$ 2, 2-Deoxyglucose NaN_3
8 mM potassium ACSF	8 mM K^+ ACSF	NaCl KCl $CaCl_2$ $MgCl_2$ NaH_2PO_4 $NaHCO_3$ Glucose Lactate
Hepes-buffered ACSF	H-ACSF	NaCl KCl $CaCl_2$ $MgSO_4$ NaH_2PO_4 Hepes Glucose
Hanks' Balanced Salt Solution	HBSS	
Dulbecco's Phosphate-Buffered Saline	DPBS	
Organotypic Culture Medium	OTC medium	heat-inactivated horse serum

MEM
L-glutamine
Insulin
NaCl
$MgSO_4$
$CaCl_2$
Ascorbic acid
D-glucose

Concentration [mM]	Comments
125	Bubbled with 5% CO_2/95% O_2, pH 7.4
2.5	Always add glucose right before usage.
2	Do not store for more than one day with glucose
1	~310 mOsm/L
1.25	
26	
20	
136	Bubbled with 5% CO_2/95% O_2, pH 7.4
3	Always add glucose right before usage.
2	Do not store for more than one day with glucose
1	~320 mOsm/L
1.25	
24	
5	
1	
136	Bubbled with 5% CO_2/95% O_2, pH 7.4
3	~318 mOsm/L
2	
1	
1.25	
24	
2	
5	
128	Bubbled with 5% CO_2/95% O_2, pH 7.4
8	~320 mOsm/L
2	
1	
1.25	
24	
5	
1	
125	Adjusted to pH 7.4 with NaOH
3	Always add glucose right before use
2	Do not store for more than one day with glucose
2	~310 mOsm/L (adjusted with sucrose)
125	
25	
10	
	Sigma (catalog number H9394).
	Gibco (catalog number 14287-080)
20%	34°C, 5 % CO_2, pH 7.4, under culturing condition

79% ~320 mOsm/L
1
0.01 mg/ml
14.5
2
1.44
0.00125 %
13

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
2-deoxyglucose	Alfa Aesar	L07338
36-IMA-410-019 Argon laser	Melles Griot	
Ascorbic acid	Carl Roth	3525.1
band pass filters 483/32	AHF Analysentechnik AG	
band pass filters 542/27	AHF Analysentechnik AG	
Beamsplitter T 455 LP	AHF Analysentechnik AG	
Beamsplitter T 505 LPXR	AHF Analysentechnik AG	
Confocal laser scannig microscope C1	Nikon Microscope Solutions	
Data processing Origin Pro 9.0.0 (64-bit)	OriginLab corporation	
D-glucose monohydrate	Caelo	2580-1kg
DPBS	GIBCO/Life	14190250
Eclipse E 600FN upright microscope	Nikon Microscope Solutions	
Eclipse FN1 upright microscope	Nikon Microscope Solutions	
Experimental chamber	custom build	
EZ-C1 Silver Version 3.91	Nikon Microscope Solutions	
Hanks' Balanced Salt solution	Sigma-Aldrich	H9394
HERAcell 150	Thermo Scientific	
HERAsafe KS/KSP	Thermo Scientific	
Horse serum	GIBCO/Life	26050088
Huygens Professional	SVI Imaging	
Image J 1.52i	Wayne Rasban national Institute of Health	
Insulin	Sigma-Aldrich	I6634
IP serie peristaltic pump	Ismatec	
Layout software, Illustrator CS6	Adobe	
L-glutamine	GIBCO/Life	25030024
Microm HM 650 V	Thermo Scientific	

Microscope stage	custom build	
Microsoft Excel 16	Microsoft	
Millicell culture insert	Merck Millipore	PICM0RG50
Minimum Essential Medium Eagle	Sigma-Aldrich	M7278
Monochromator Polychrome V	Thermo Scientific/FEI	
NaN_3 (Sodium Azide)	Sigma-Aldrich	S-8032
Nikon Fluor 40x / 0.80 W DIC M ∞/0 WD 2.0	Nikon Microscope Solutions	
NIS Elements 4.50 advanced Research	Nikon Microscope Solutions	
ORCA-Flash4.0	Hamamatsu Photonics	
Perfusion tubing	Pro Liquid GmbH	
Photoshop CS 6 Version 13.0	Adobe	
Sodium L-lactate	Sigma-Aldrich	71718-10G
ssAAV-2/2-hSyn1-ATeam1.03YEMK-WPRE-hGHp(A)	ETH Zürich	v244
ssAAV-5/2-hGFAP-hHBbI/E-ATeam1.03YEMK-WPRE-bGHp(A)	ETH Zürich	v307
WVIEW GEMINI optic system	Hamamatsu Photonics	

Comments/Description
Non-metabolizable glucose analog
488 nm wavelength argon
Antioxidant, Vitamin C
Splitter compatible emmision filter
Splitter compatible emmision filter
Excitation dichroic mirror
Splitter dichroic
Modular confocal microscope system C1
Scientific graphing and data analysis software
Dulbecco's phosphate-buffered saline
Perfusion chamber for live-cell imaging
Imaging software for confocal microscope
With Phenol Red for pH monitoring
CO_2 incubator HERAcell ® 150 with decontamination routine
Safety Cabinet
Heat inactivated
Deconvolution software
Image processing Software available in the public domain
Insulin from bovine pancreas
High-precision multi-channel pump
Vector graphics editor
Vibration microtome. Thermo scientific discontinued the production of the device in the meantime. Any other slicer or tissue chopper siutabl

Spreadsheet software for basic data processing
Hydrophilized PTFE, pore size 0.4 µm
Synthetic cell culture media
Ultra fast switching monochromator
Mitochondrial inhibitor (complex IV inhibitor). CAUTION: Azide is toxic. Be aware not to accidentally ingest or inhale it, and prevent ist absoption through the skin.

Water Immersion Microscope Objective

Imaging software. Upgraded version for FRET imaging
Digital CMOS camera
Tygon tubing, 1.52 x 322 mm (Wd: 0.85)
Image processing software

Single-stranded AAV vector that induces the expression of ATeam1.03^{YEMK} under the control of the human synapsin 1 promoter fragment hSyn1.
Single-stranded AAV vector that induces the expression of ATeam1.03^{YEMK} under the control of the human glial fibrillary acidic protein promoter fragment ABC1D.
Emission Image Splitter

le for slicing living tissue is fine, too.

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Author(s): Rodrigo Lerchundi, Marcel Fiefer, Karl U Hafitz, Na Huang Christine R Rose

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Detailed response to the editor's and reviewer's comments on our MS: JoVE60294 **"Imaging of intracellular ATP in organotypic tissue slices of the mouse brain using the FRET-based sensor ATeam1.03YEMK"**

All changes are marked with a green background in the body of the text.

Comment	**Action**
Editorial comments	
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.	Done
2. Please provide at least 6 keywords or phrases.	We provided two more key words to meet this limit.
3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points	Done
4. 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.	Done
5. Please ensure the Introduction contains all the following: a) A clear statement of the overall goal of this method b) The rationale behind the development and/or use of this technique c) The advantages over alternative techniques with applicable references to previous studies d) A description of the context of the technique in the wider body of literature e) Information to help readers to determine whether the method is appropriate for their application	Done
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".	Done

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.	Done
8. The Protocol should contain only action items that direct the reader to do something.	Done For some methodological steps, we provide additional information which is required to understand the action item.
9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.	Done
10. Please move the ethics statement before all the numbered step of the protocol section.	Done
11. Please ensure you answer the "how" question, i.e., how is the step performed?	Done
12. 1: Please convert all the solution recipe details to a table. Then upload the table individually as .xlsx file to your editorial manager account. Refer to the table wherever applicable. If you intend to leave it here, then please convert the details to numbered action steps in imperative tense and use complete sentence to describe how to make the solution.	We created a new table 2 as suggested and refer to the table in the body of the text.
13. 2.2.1: Please include age, sex, strain of mice? Do you anesthetize the animal before decapitation, CO2 asphyxiation? Please provide the complete euthanasia procedure.	Done. We clarified the procedure.
14. 2.2.2: How is the brain removal performed? How do you cut the skin, how do you remove the skull, which part of the brain? Do you maintain sterility? Please list all the steps.	We provide some more details. Notably, this part of the methods was already demonstrated in great detail in our two earlier JoVE contributions.
15. 2.2.5: How do you identify the hippocampal formation? How is the isolation performed? How do you visually identify the cerebral cortex? Are all these done on the sections obtained?	We included a reference to Figure 1 for clarification. We do not think that more details are required since this is a standard procedure in neuroscience labs.
16. 4.5: What is the MOI of transduction?	Both viruses are commercially available. The production site of the viruses provides only the virus titer but not the MOI.
17. 6,7: For each step please include how the step is performed.	Done
18. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 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.	We uploaded 3 versions of the MS. 1) the revised MS with no labels at all. 2) the revised MS with changes made according to the comments labelled in green. 3) the highlighted Protocol section (in BLUE) for filming purposes.

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20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol b) Any modifications and troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique	Done
21. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.	Done
22. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.	Done
Reviewer #1:	
*A phototoxicity control (no treatment) for the time and imaging frequency mentioned for live cell fluorescence imaging should be conducted for both neuron and astrocyte experiments and results added to Fig 6 and Fig 7.	We provide the requested data for astrocytes and for neurons. They are included as the new figure 8
*Fig 5. The protocol for mechanical removal of the outer glial scar requires at least a schematic diagram to interpret clearly how this this done.	We included the requested scheme in the new figure 4
	Former figures 4 to 7 are accordingly renamed. New figures are named "rev" at the end of the filename. New figures are referenced accordingly in the body of the text.
*Line 252. The authors describe direct removal of the glial scar 'just before starting the experiment'. Is there any evidence (in house or in the literature) to verify that removal of this layer (i.e. mechanical disruption) does not negatively impact on the physiology of the neuronal and glial cells directly beneath? Please add to discussion.	We included a clarifying sentence in the protocol section 5.3.

*Fig 6. Using a wide-field fluorescence microscope gives the total pooled fluorescence of ATeam-positive cells in the slice through the depth of the tissue at that region. (i.e. from the fluorescence images Fig 6A you can see that the cells behind the cell body of interest will contribute to the signal). Unless captured using a confocal microscope one cannot state that they are recording fluorescent signal from 'single cell'.	We agree and removed the statement “single cell” from the text when describing imaging results.
Would it be possible to perform a z-stack in epifluorescence mode to see how deep the depths of the field reaches with the lens in use? What are the properties of the lens used for the wide-field fluorescence microscope and confocal microscope (Magnification, NA)? This manuscript would benefit from a movie of the cells as a supplementary source.	The lens properties were already provided in the list of materials originally submitted. We plan on including the requested movie as Lab material in the main movie. Therefore, there is no need for an additional movie as supplementary material.
Does the focus remain stable with change of perfusions?	Since we are using precision pumps, there is definitely no change in the focus.
*Fig 6B. Why is there an increase in the absolute eCFP intensity during the recovery phase of the experiment (i.e. when Venus begins to increase towards baseline levels)?	Changes in fluorescence intensity of both channels are related to changes in ATP levels. In addition to that, emission intensity will change due to other factors, e.g. changes in cellular volume. Especially the latter cannot be ruled out following inhibition of metabolism, which is a relatively strong challenge for cells. Calculating the fluorescence ratio of the FRET pair does cancel out such artifacts, which are not related to ATP.
*Can you recommend tubing for the peristaltic pump? Please include details in table of materials. What was the desired pressure/perfusion rate obtained from this system? Please note that this will impact 'sheer stress' across the OTC, which under some circumstances may affect cellular physiology. This point needs to be noted and addressed.	Information on Tubing is included in the materials list. We stated the perfusion rate in the protocol section. We commented on potential ‘sheer stress’ (see 5.2.).
Minor concerns	
*This manuscript describes identification and analysis of ATP in two different cell types (astrocytes & neurons). However this point is not reflective in the title of the paper and should be modified accordingly.	We decided to hold on with the original title, since it already is quite long.
*Line 121. OTC is a standard abbreviation for 'organotypic culture' - change to 'OTC medium' in place of OTC here and line 151.	Done
*Line 150-1. State the 6-well plate containing OTC medium should be kept in incubator until procedure (especially if prepared the day before as noted in point 2.1.).	Done

*Line 153. How many washing plates required? Consistent naming of plates is also required throughout manuscript- Rename 'washing plates' as 'petri dishes' (line 199) or vice versa.	Done
*Is continued carbonation of ACSF required during methods 2.2.2 - 2.2.4?	Clarified
*Line 155. Rephrase to clarify plates stay in incubator for 30min-overnight until required. Current sentence reads as - 'place them in incubator until'…at least 30min prior to preparation'.	Clarified
Line 160. 'Day of preparation' should be annotated as 2.2.	Done
*Line 160-1. Is ACSF continuously bubbled to maintain pH during dissection? Please explicitly state. Also state here to place solution on ice.	Done throughout the text whenever applicable.
*Line 163. 'Dissection and slicing' should be annotated as 2.2.1 & all others changed accordingly.	Done. Please note that the numbering changed anyway during the revision process.
*Line 200. Modify sentence to clarify these are the petri dishes pre-warmed in the incubator for at least 30min/overnight.	Done
*Line 204-206. Consistent naming of dishes required. Replace 'culture plate' with 'Petri dish'.	Done
*Line 253. Replace 'culture medium' with 'OCT medium'.	Done throughout the text if applicable.
*Line 275. Is the E-ACSF continuously bubbled with CO2 to maintain carbonation throughout experiment? Please clarify.	Clarified
*Line 276. Is there a neutral density filter and excitation band pass in place? Which percentage power (watts of the bulb) was used? Was the shutter of the lamp used or was there an extra shutter?	In a few cases we inserted ND filters into the excitation light path. Other optical elements are not needed since the light source is a monochromator which supplies a very narrow band of excitation light. No external shutter was employed.
*Line 278-282. Is there is any difference in ATeam probe dynamics when used at room temperature compared to physiological temperature? Please include.	We already addressed this issue in a former paper and discuss this issue in detail in the discussion
ine 353. "described in chapter 5.1." Replace with: "described in chapter 5."	Done
*Line 357. "constantly perfused with ACSF". Is this continuously carbonated ACSF? Please provide more detail.	Clarified
*Line 365. "Glucose-free saline to which 5 mM NaN3 was added…". Does this refer to the chemical ischemia solution (CIS)? If so, please refer to solutions as outlined in Protocol section 1 - Salines & culture media. If not, please provide details of this glucose-free saline with	Clarified

reference on how you controlled for changes in osmolarity.	
*Line 377. Please refer to solutions outlined in Protocol section 1 - Salines & culture media. Ie. E-ACSF to High Potassium ACSF, as not only is potassium concentration increased, sodium chloride concentration is decreased to accommodate for changes in osmolarity.	Clarified
*Line 382. Reference 'CIS' here to explicitly show which solution was used from the list in 'Protocol section 1 - Salines & culture media'.	Clarified
*Line 384. Is this corrected for time for solution to pass through tubing (as mentioned in chapter 6.10)?	Yes
*Line 413-423. Is the bath connected to the peristaltic pump continuously carbonated to maintain constant pH? If so please state and represent on illustration of Fig 3.	Clarified (lines 267/268) and added to Fig. 3A
*Line 457. Is this the pooled time lapse recording from a population or just one cell? Please state.	Clarified (Just from one cell, no averaged data)
*Line 461. Change from 'increase in ATP' to 'increase in ATeam FRET ratio'. Please also change y-axis on Fig 7 to 'ATeam FRET ratio' as 'ATeam signal' is misleading.	Done throughout the text if applicable.
*Line 473. 'ATeam 1.03YEMK signals rapidly drop'. Please reword to ATeam FRET ratio as the CFP signal actually increases.	Done throughout the text if applicable.
*Fig 5. The protocol for mechanical removal of the outer glial scar requires at least a schematic diagram to interpret clearly how this this done.	Done, see above
*Fig 6. Using a wide-field fluorescence microscope gives the total pooled fluorescence of ATeam-positive cells in the slice through the depth of the tissue at that region. (i.e. from the fluorescence images Fig 6A you can see that the cells behind the cell body of interest will contribute to the signal). Unless captured using a confocal microscope one cannot state that they are recording fluorescent signal from 'single cell'.	See above
*Would it be possible to perform a z-stack in epifluorescence mode to see how deep the depths of the field reaches with the lens in use? What are the properties of the lens used for the wide-field fluorescence microscope and confocal microscope (Magnification, NA)?	We did not include this experiment and quantification since it would exceed the topic of the MS. The properties of the lenses used were already stated in table of materials of the first submission.
*This manuscript would benefit from a movie of the cells as a supplementary source. Does the focus remain stable with change of perfusions?	See above.
*Please also change y-axis on Fig 6.B to 'ATeam FRET ratio' as 'ATeam signal' is misleading.	Done

*Please provide details of 'filter membrane' in table of materials section for that mentioned on line 175.	Done
*The authors should clarify that for high resolution single-cell imaging experiments, confocal microscopy should be used instead of standard fluorescence microscopy. However the depth into the cultured slice would need to be consistent to ensure reproducible penetration of the drugs/solution conditions and corresponding responses.	This is not entirely true. We have shown in other publications that one is able to gain subcellular resolution with wide-field imaging, too. For thin tissue and for cells in culture, wide-field imaging even allows for a higher spatial resolution than confocal imaging (depending on the wavelengths used).
Tissue sectioning may also be carried out using a McIllwain tissue chopper as described in other methods papers relating to OTCs.	We added a clarifying sentence in Table 1.
Reviewer #2	
1. In the introduction, please discuss the sensor's ATP Kd and hill slope. Then relate this to expected intracellular ATP concentrations in the discussion.	Information on the sensors expected binding affinity in relation to intracellular ATP levels is presented in detail in the discussion.
2. I do not like the word carbogenated. Please replace this with 95% O2 and 5% CO2	Done
3. Please state the osmolarity of the solutions when first mentioned in the protocol section	Done; the osmolarity of all solutions is stated in the newly generated list (c.f. concerns of reviewer 1)
4. Please refer to the 8mM K+ solution as "8 mM K" rather than high K.	Done
5. In the abstract AAV not AVV	Done
6. Introduction, please also cite work by Gary Yellen that does not support the idea that astrocytes provide energy to neurons. This is tangential to this paper, but should be cited.	Done
7. In figure 2, please change the color of CFP to cyan rather than purple to match its wavelength	We prefer to stick to the colors used, because they represent our overall color scheme. Most importantly, people with a red-green visual impairment may otherwise not be able to identify colors.