High levels of FAD autofluorescence indicate pathology preceding cell death

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ABSTRACT

Flavin adenine dinucleotide (FAD) autofluorescence from cells reports on the enzymatic activity which involves FAD as a cofactor. Most of the cellular FAD fluorescence comes from complex II of the electron transport chain in mitochondria and can be assessed with inhibitor analysis. The intensity of FAD autofluorescence is not homogeneous and vary between cells in tissue and in cell culture types. Using primary co-culture of neurons and astrocytes, and human skin fibroblasts we have found that very high FAD autofluorescence is a result of an overactivation of the mitochondrial complex II from ETC and from the activity of monoamine oxidases. Cells with high FAD autofluorescence were mostly intact and were not co-labelled with indicators for necrosis or apoptosis. However, cells with high FAD fluorescence showed activation of apoptosis and necrosis within 24 hours after initial measurements. Thus, high level of FAD autofluorescence is an indicator of cell pathology and reveals an upcoming apoptosis and necrosis.

Keywords: *FAD, flavoproteins, cell death, neurons, astrocytes, fibroblasts, mitochondria, MAO*

INTRODUCTION

Autofluorescence of biological molecules is used in basic biological and biomedical research and also in medicine as a potential diagnostic tool [1]. In the 1950s, the laboratory of Britton Chance first identified the autofluorescence of metabolic cofactors involved in the metabolism of mitochondrial respiratory enzymes [2,3]. Along with NADH (nicotinamide adenine dinucleotide), FAD (flavin adenine dinucleotide) is a metabolic electron carrier existing in a reduced ($FADH_2$) and in an oxidized (FAD) form that is cycling between these two states by accepting or donating electrons. The oxidized form – FAD, is autofluorescent and emits at a peak of 520–530 nm when excited between 365 and 465 nm.

FAD is a redox cofactor of several important reactions including the formation of the riboflavin moiety (vitamin B2), glutathione synthesis, cholesterol metabolism, etc [4].

In the electron transport chain of mitochondria FAD plays a role of an electron carrier and a donor of electrons mainly to complex II. FAD as a cofactor has to be covalently bound to mitochondrial complex II of the electron-transport chain for the conversion of $FADH_2$ into FAD, that suggests that all FAD fluorescence related to mitochondrial respiration is linked to the activity of complex II [5]. FAD is the predominant cofactor for the succinate-ubiquinone oxidoreductase (SQR; Complex II), an essential component of the aerobic electron transport, and for the menaquinol-fumarate oxidoreductase (QFR)- the anaerobic counterpart, although it is only present in approximately 10 % of all known flavoenzymes.

FAD is also covalently bound to other mitochondrially located enzymes – monoamine oxidase A and B [6]. Monoamine oxidases catalyse monoamines to aldehydes and H_2O_2, using FAD as a cofactor, where $FADH_2$ is formed and at the end of the reaction the reduced flavin is re-oxidized back to FAD [7].

Alterations in energy metabolism or redox status may lead to changes in the FAD level and thus FAD autofluorescence is used as a tool for the assessment of complex II-dependent mitochondrial respiration [8].

One of the forms of programmed type cell death – apoptosis is a complex process which allows for the maintenance of tissue homeostasis. However, induction of apoptosis is not always a process that plays purely physiological role: overactivation of apoptosis in postmitotic cells such as neurons leads to cell death- a histopathological hallmark of neurodegeneration. In some cases apoptosis maybe activated by inflammation or ischaemic processes in the tissues [9].

Although necrosis is not a type of programmed cell death, induction of this process can be triggered by chronic cellular pathology. It can also be activated by metabolic changes that can be detected long before the actual necrosis takes place. Here, using primary co-culture of neurons and astrocytes and human skin fibroblasts we study whether in cells with very high FAD autofluorescence, this process is induced by changes in mitochondrial FAD and whether this higher FAD fluorescence intensity could be used as an indicator of cellular pathology and as a predictor of cell death. We have found that a 3-4 -fold increase in FAD fluorescence is induced by the increased pool of FAD in mitochondrial complex II and monoamine oxidases. Cells with increased FAD autofluorescence were alive and did not show any co-localisation with indicators of necrosis or apoptosis. However, measurement of apoptosis and necrosis in cells with bright FAD fluorescence in the following 24 hours showed that in these cells cell death is already induced, and that cells could at this stage be protected by the application of mitochondrial substrate methyl succinate.

METHODS

Cell cultures

Mixed neuroglial brain cultures were prepared from Sprague-Dawley rat pups 0–3 days postpartum (UCL breeding colony). Animal husbandry and experimental procedures were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986. Subjects were culled following the Schedule 1 procedure and the brain was dissected in ice-cold HEPES buffered salt solution (Ca^{2+}, Mg^{2+}-free; Gibco-Invitrogen, Paisley, UK). The tissue was minced and trypsinized (0.25% for 15 min at 37 °C), triturated and plated on poly-d-lysine-coated glass coverslips and cultured in Neurobasal-A medium (Gibco-Invitrogen) supplemented with B-27 (Gibco-Invitrogen) and 2 mM GlutaMAX using routine protocol [9,10]. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, culture media exchanged once a week and maintained for a minimum of 14 days before experimental use. Neurons were easily distinguishable from glia: they appeared bright using phase contrast, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer. Cells were used at 14–16 DIV.

Additionally, human skin fibroblasts [11] were used as a research object. Skin fibroblasts were cultured in DMEM (Biological Industries, Kibbutz Beit-Haemek, Israel), 10% FBS (Biological Industries, Kibbutz Beit-Haemek, Israel), 1% GlutaMAX (Gibco, New York, USA) Cultures

of cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The confluence of cells during the studies was 40-50% which was taken as a day 0 for some experiments.

Live cell imaging

FAD autofluorescence

FAD autofluorescence was monitored using a using a Zeiss 900 CLSM detection system and a 63x oil immersion objective. Excitation used was the 454 nm Argon laser line and the emission fluorescence was measured after 505-550 nm. Illumination intensity was kept to a minimum (at 0.1-0.2 % of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~2 µm. FAD quantification has been performed on Z-stack images (Figure 1 Ai, Aii). FAD redox index and mitochondrial pools were estimated according previously described [13]**.**

Cell death

***Necrosis*:** For assessment of necrotic cell death, cells were loaded with propidium iodide (PI; 10 µM) and 300 nM Hoechst for 15 min, washed 3x with PBS 1x and analysed using a cooled CCD camera. Hoechst stains the total number of cell (nuclei, measured with excitation/emission ~350/481 nm) while PI stains only cells with a disrupted plasma membrane. Dead cells (PI positive, measured with excitation/emission ~535/617 nm) were counted as a fraction of the total (Hoechst positive). In each experiment, five random fields were examined, and the mean is representative of three independent experiments for each condition.

Apoptosis: NucView™ 488 Caspase-3 Substrate (Biotium) (excitation/emission maxima ~488/515 nm) was applied for detection of caspase-3/7 activity and visualization of morphological changes in the nucleus during apoptosis. Cells were incubated with 5 µM NucView™ 488 Caspase-3 Substrate 30 min at room temperature.

Statistical analysis

Data were analysed with Origin Pro 2019 (MicroCal, Oregon, USA) and are expressed as mean ± SEM. Man-Whitney test was used to estimate the statistical significance between experimental groups. Significance was accepted at a 95% confidence level ($p < 0.05$). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

RESULTS

The level of FAD autofluorescence varies between cells in culture

To study the differences in the level of FAD autofluorescence we used human fibroblasts and primary rat co-culture of cortical neurons and astrocytes. FAD quantification has been performed on Z-stack images (Figure 1Ai, Aii). Cells with FAD autofluorescence 3-10 times higher than the mean basal level of FAD autofluorescence of cells in the culture were attributed to the group corresponding to "cells with high FAD autofluorescence" (Figure 1B-J).

In primary co-culture of neurons and astrocytes the level of FAD autofluorescence was different between neurons and astrocytes but difference was observed also within the same cell type in the same imaging field (Figure 1 B-C). Percentage of neurons and astrocytes with very high (3-fold increase and higher) levels of FAD autofluorescence was dependent on the age and culturing conditions of the primary co-culture and in both preparations increased with age in culture (from 6.4±0.8% at 7 DIV to 27.8±4% at 20 DIV; N=11 experiments; Figure 1 F).

The levels of FAD fluorescence in cultured human fibroblasts was also different between cells despite the cell type homogeneity of the cell line (Figure 1 G, H, J). In a similar way as in primary co-culture the number of fibroblasts with high FAD was dependent on the condition and age of the cell culture.

Thus, we have suggested that the high level of FAD autofluorescence could indicate cell pathology and, possibly cell death.

High levels of FAD are not associated with necrosis or apoptosis at the time of measurement

In order to identify if cells exhibiting high levels of FAD autofluorescence are damaged or dead, we loaded the fibroblasts with Propidium Iodide which is a fluorescent indicator, impermeable for plasma membrane that becomes fluorescent only if the integrity of plasma membrane is compromised; it binds to nuclear DNA and reports for occurrence of necrosis [12]. We have found that the number of dead fibroblasts with high FAD was even lower compared to cells with basal level of autofluorescence (4±0.6% with High FAD, N=4 experiments; 8±0.7% with basal levels of FAD, N=4 experiments; Figure 2 A, B).

In mixed cultures of primary neurons and astrocytes, loading of the cells with PI was similar for cells with high or basal FAD levels (Figure 2 C; N=5 experiments). Importantly, the level

of dead cells with high FAD was low in both neurons and astrocytes (Figure 2C). Thus, high levels of FAD is not a criterion for occurrence of necrosis in these cells.

In order to identify if high level of FAD is associated with the activation of apoptotic pathways we used fluorescent substrate for caspase-3, NucView 488, which becomes fluorescent and binds to nuclear DNA upon activation of the key apoptotic enzyme caspase-3 [12]. Despite the difficulties because of the overlap of FAD and the NucView488 signals, we found that activation of caspase-3 was easily detectable in the nuclear area while the FAD signal arose from different to the nuclear area (Figure 2 D). The number of cells with prevailing caspase-3 activation was equally low in all cell types – fibroblasts (2%), neurons (7%) and astrocytes (4%). Furthermore, the percentage of cells with initiated apoptosis was similar from cells with both, low or high FAD autofluorescence (N=6 experiments; Figure 2E, F).

Hence, high levels of FAD does not indicate activation of apoptosis in these cells. Nevertheless, we cannot exclude that high levels of FAD autofluorescence may indicate cellular pathology which culminates in necrotic or apoptotic cell death.

High levels of FAD lead to cell death in the following 24 hours

To identify if high levels of FAD autofluorescence can lead to cell death in the succeeding 24 hours we used graded coverslips for the identification of the cells with high levels of FAD autofluorescence. On the first day the FAD autofluorescence was measured, the coordinates were set down and 24 hours later the same culture of fibroblasts or neurons and astrocytes was labelled with PI or NucView488 for assessment of cell death percentage (for PI measurements see Figure 3 A -an image before and after 24 hours).

Our measurements revealed high percentage of necrotic fibroblasts (PI positive; Figure 3 A, B) among cells that exhibited high levels of FAD fluorescence 24 hours in advance. It should be noted that not all of them kept the same high levels of FAD and some cells could be detectable only by the red signal from the nuclei. Additionally, about 30% of the cells with high FAD autofluorescence could not be found to be attached to the coverslip on the next day that could also indicate necrotic damage of the cells followed by detachment and wash out (N=5 experiments; Figure 3C). The percentage of necrotic cells in primary co-culture cortical neurons (59±7% of cells, N=7) and astrocytes (56±5%, N=7) 24 hours after initial measurements was also higher (Figure 5 D).

Pathology, associated with high levels of FAD autofluorescence in various cell types can also induce apoptosis within 24 hours. Thus, the number of neurons and astrocytes with activated caspase-3 (NucView488 positive; Figure 3E) dramatically increased after 24 hours (N=6 experiments; Figure 3 F). Importantly, neurons and astrocytes with high FAD levels were often missing in the set coordinates on the next day that indicates as well that the actual number of dead cells could be even higher.

Considering the high percentage of cell death in cells with high FAD we used inductor of apoptosis Staurosporine (0.5 μM) to test if targeted activation of apoptosis induces faster changes in FAD autofluorescence. We have found that application of Staurosporine had almost no effect on the level of FAD in cells with high level of autofluorescence while it increases the fluorescence of FAD in cells with lower FAD (Figure 3 G, H).

Thus, high level of FAD autofluorescence does not correspond to cell death rate at present but reports for a pathology that would lead to cell death in the following 24 hours.

High FAD signal is due to increased levels of FAD turnover in mitochondrial complex II and increased MAO activity

High levels of FAD autofluorescence that lead to cell death may be induced by changes of the activity or expression levels of various enzymes in the cell. In order to identify the cellular source of increased FAD, we measured FAD autofluorescence levels in response to various inhibitors.

Although FAD is involved in multiple processes in the cells, the most abundant autofluorescence signal comes from mitochondrial complex II [3]. FAD autofluorescence coming from the electron transport chain of mitochondria could be subtracted from the total FAD autofluorescence using the mitochondrial uncoupler FCCP (1μM) that gives the maximal rate of respiration and the corresponding maximum in FAD signal (and lowest $FADH_2$). On the other hand, inhibition of mitochondrial respiration by NaCN (1 mM) which blocks the consumption of $FADH_2$ is resulting in the lowest levels of mitochondrial FAD [13] (Figure 4 A-B) [10]. We have compared the levels of mitochondrial FAD in cells with low and high levels of FAD and have found that, expectably, mitochondrial pool of FAD was ~2.5-fold higher in neurons, astrocytes and fibroblasts with higher total FAD signal (Figure 4C). The percentage of mitochondrial FAD signal from the total cellular FAD was significantly higher in cells with high level of total FAD in the cells (Figure 4 B-C). Additionally, the mitochondrial levels of FAD can also report for the relative rate of complex II-associated respiration (redox

index). FAD redox index indicates the balance between the consumption of $FADH_2$ in complex II and FAD production, result of chemical reduction. Higher levels of FAD redox index suggested faster consumption of $FADH_2$ and faster complex II-related mitochondrial respiration. In the case of cells with higher levels of cellular FAD, the elevated levels of FAD in mitochondria was not only associated with the increase in total mitochondrial FAD pool but was also linked to the increased consumption of $FADH_2$ in mitochondria and the increased redox index (Figure 4C, D).

Another area with relatively high levels of FAD signal is also in mitochondria from the enzyme monoamine oxidase (MAO), located on the outer mitochondrial membrane [14]. To estimate the MAO-related pool of FAD we used high concentrations of Adrenaline (1 μM for skin fibroblasts) and 10 μM tyramine (to avoid receptor-dependent processes in neurons and astrocyte) to maximally activate MAO to utilize FAD, followed by inhibition of this enzyme (in both isoforms A and B) by 20 μM Selegiline (Figure 4 E, F). Although MAO A and B activity should be represented as biphasic response in FAD autofluorescence (reduced to $FADH_2$ and oxidised to FAD in the later phase) we clearly observed only a decrease in FAD autofluorescence levels in response to the addition of monoamines followed by inhibition of MAOs by 20 μM Selegiline (Figure E, F). It should be noted that the MAO-related FAD pool (a decrease to the application of monoamines) in cells with increased total FAD autofluorescence was higher compared to cells with lower total FAD signal (Figure 4 G) and as a proportion of MAO-related FAD signal to the total autofluorescence signal in these cells.

Thus, increased levels of FAD autofluorescence are induced by higher levels of FAD turnover in MAOs, and the increased pool and redox index of FAD - from the activity of the mitochondrial complex II.

Application of substrate for complex II partially decreases FAD and protects neurons and astrocytes against cell death

High levels of FAD signal from mitochondria occur not only because of the increased pool of FAD (flavoproteins) but also because of the increased redox state of these cells (see Figure 4C). Considering this, application of mitochondrial substrates may reduce high levels of FAD autofluorescence. Application of membrane permeable analogue of succinate – methyl succinate (5 mM) decreased the levels of FAD in fibroblasts, neurons and astrocytes (Figure 5 A). It should be noted that methyl succinate did not change the total pool of FAD (flavoproteins) but decreased the FAD redox index in these cells (Figure 5A).

Incubation of neurons and astrocytes with 5 mM methyl succinate for 24 hours significantly reduced the number of dead cells in the population of cells with originally high FAD signal (Figure 5A). Interestingly, although monoamines (adrenaline, dopamine or tyramine) reduced FAD levels, incubation of the neurons and astrocytes with dopamine did not change the number of dead cells among the high FAD cell population (Figure 5B). Thus, the increased levels of FAD can be partially restored and moreover, it protects cells against cell death.

DISCUSSION

In this study, we found that high FAD autofluorescence in skin fibroblasts, primary neurons and astrocytes can be used as an indicator of cell pathology which precedes the apoptotic or necrotic cell death. It should be noted that high levels of FAD autofluorescence is not an indicator of immediate cell death; cells with high levels of FAD did not show co-localization with apoptotic or necrotic markers (Figure 2). Higher levels of FAD autofluorescence was previously shown in ethanol- and heat-treated cells suggesting that an increase in autofluorescence is associated with upcoming cell death [15,16]. However, our data are more in agreement with a study which shows that increased autofluorescence in bacteria and human cells is a measure of cell struggle for survival [17]. High levels of FAD autofluorescence was shown in pre-apoptotic cells, insinuating that it is connected with the increase in energy demand of these cells necessary for programmed cell death [18]. Our data confirms that high levels of FAD is a signal that communicates cellular processes that may lead to cell death, and for some cells this state could still be reversible (Figure 5).

Although FAD is involved in multiple reactions in the cells, the major source of FAD autofluorescence is from the mitochondrial complex II that can be confirmed by application of the activator (uncoupler) and inhibitor of mitochondrial respiration in live cells [19]. In cells with higher FAD levels, most of the signal is also associated with mitochondrial flavoproteins (Figure 4). Increased level of autofluorescence is a result of the increased consumption of $FADH_2$ in mitochondrial respiration that is confirmed by the higher FAD redox state (Figure 4). The higher FAD signal reflects generally increased mitochondrial FAD pool which is more likely connected to higher expression levels of flavoproteins (Figure 4D). Interestingly, higher pool of mitochondrial FAD is connected to compensatory mechanisms in mitochondria developed in cells with mitochondrial complex I mutation [20], aged cells with pronounced energy deprivation [13] and in cells with PINK1mutation - familial form of Parkinson's disease

[21]. It should be noted that the difference in the levels of FAD signal and mitochondrial complex II activity may reflect the differences in the metabolic state of neurons and astrocytes from different brain regions [22] or in cells with energy collapse subjected to excitotoxicity [10]. High levels of mitochondrial FAD and high levels of FAD redox index are suggesting limitation of substrates for complex II-related respiration and was confirmed in our experiments (Figure 5). Importantly, Me-succinate not only reduced the FAD redox index but also protected against upcoming cell death.

Another source of the increased pool of FAD in cells with high FAD autofluorescence were monoamine oxidases. It should be noted that monoamine oxidases reduce FAD to "$FADH_2$" and in the second step of the reaction FAD is oxidized back to "FAD" form [7]. However, in our experiments application of catecholamines reduced the fluorescence intensity of the FAD signal suggesting that the oxidation of FAD in MAO is slower than the reduction of FAD to $FADH_2$ that explained the relative difference in MAO FAD pools. Our results suggested that cells with high FAD autofluorescence express more MAO or/and exhibit an increase activity of these enzymes. Application of dopamine did not protect the culture of neurons and astrocytes against cell death, but we should consider the sophisticated responses of cell monoamines, including receptor depending and receptor independent processes [23–25].

Altogether, high levels of FAD autofluorescence in cells could be used as a marker of pathology which will inevitably lead to cell death in the following 24 hours.

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Data Availability

Data underlying the results may be obtained from the authors upon reasonable request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

Conceptualization and design of methodology: PRA, AYV, AYA, AVD. Investigation and data curation: EIB, AD, AYV, PRA, AYA. Formal analysis and visualization: AD, EIB, PRA, AYA. Writing – Original Draft Preparation: EIB, PRA, AYA. Writing – Review & Editing: EIB, PRA, AYV, AVD, AYA. Project administration: AYA, PRA, AVD. Supervision and funding acquisition: AYA, AVD.

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FIGURE LEGENDS

Figure 1. High level of FAD autofluorescence in neurons, astrocytes and skin fibroblasts. Ai-Aii representative images of cortical astrocytes after using Z-stack images, 3D (i) and 2.5D (ii) reconstructions. B-Images of FAD autofluorescence in cortical astrocytes. Red line depicts the trajectory of the profile taken in **C**. D and E are representative images and profile of the FAD autofluorescence intensity in cortical neurons. F- Quantification bar chart showing the percentage of neurons and astrocytes with high autofluorescence signal (3-fold and higher than basic) in different *days in vitro* (DIV). G-Difference in the level of autofluorescence intensity between skin fibroblasts with low (taken as 100%) and high levels of FAD autofluorescence. Representative image (H) of different levels of FAD autofluorescence in human skin fibroblasts from the same focal plane and the profile (J) of their FAD autofluorescence intensity. * $p < 0.05$, ** $p < 0.001$.

Figure 2. High level of FAD autofluorescence is not associated with necrosis or apoptosis in the time of measurement. A -Image of the FAD autofluorescence and PI fluorescence in human skin fibroblasts. Percentage of PI positive fibroblasts (B) and primary rat cortical neurons and astrocytes from the co-culture, 15 DIV (C). D- Representative image of FAD autofluorescence of skin fibroblasts loaded with NucView488. Quantification bar chart depicting the percentage of NucView488-positive fibroblasts (E) and primary rat cortical neurons and astrocytes from the co-culture with high or low FAD fluorescence, at 16 DIV (F). * $p < 0.05$.

Figure 3. High levels of FAD lead to cell death in the subsequent 24 hours. A- Representative image of PI fluorescence in skin fibroblasts with high FAD before (upper panel) and after 24 hours after initial measurements (lower panel). Cells coordinates were identified 24 hours post imaging using graded coverslips. PI- positive fibroblasts with high or low FAD signal (B) or number (%) of cells with high FAD which were detached within 24 hours (C). D- Percentage representation of neurons and astrocytes with high FAD co-localized with PI signal in the following 24 hours. E- Representative image of cortical neurons and astrocytes labelled with NucView488. F- Percentage representation of neurons and astrocytes with high FAD co-labelled with NucView488 signal after 24 hours. G -H Application of activator of apoptosis Staurosporine differentially changes FAD levels in fibroblasts with high or low FAD autofluorescence from the same focal plane. * $p < 0.05$, ** $p < 0.001$.

Figure 4. High level of FAD fluorescence originates from mitochondria. A-Representative image of cortical neurons and astrocytes with high and low FAD autofluorescence. B-Representative trace of the dynamic of complex II-related FAD fluorescence. 1 μM FCCP and 1 mM NaCN were added to cells to maximally activate mitochondrial respiration and completely inhibit it, respectively. C- Quantification bar chart depicting the relative mitochondrial FAD pool of cortical neurons and astrocytes and human skin fibroblasts; cells with low FAD signal were taken as 100%. D- Quantification bar chart of FAD redox index in fibroblasts, neurons and astrocytes. Changes in the level of FAD fluorescence in response to 1μM adrenaline (E, fibroblasts) or 20 μM tyramine (F, neurons) and 20 μM selegeline. G-Quantification of relative MAO-dependent FAD pool in human skin fibroblasts, and cortical neurons and astrocytes; cells with low FAD signal were taken as 100%. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Figure 5. Me-succinate protects cells with high FAD autofluorescence against cell death. A- Changes in FAD signal in response to 5 mM Me-succinate, 1 μM FCCP and 1 mM NaCN in cortical neurons with high and low initial FAD autofluorescence. B -Quantification bar chart depicting the effect of 5 mM Me-succinate or 20 μM dopamine on viability (% of PI-positive cells) of cortical neurons and astrocytes with high FAD signal. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

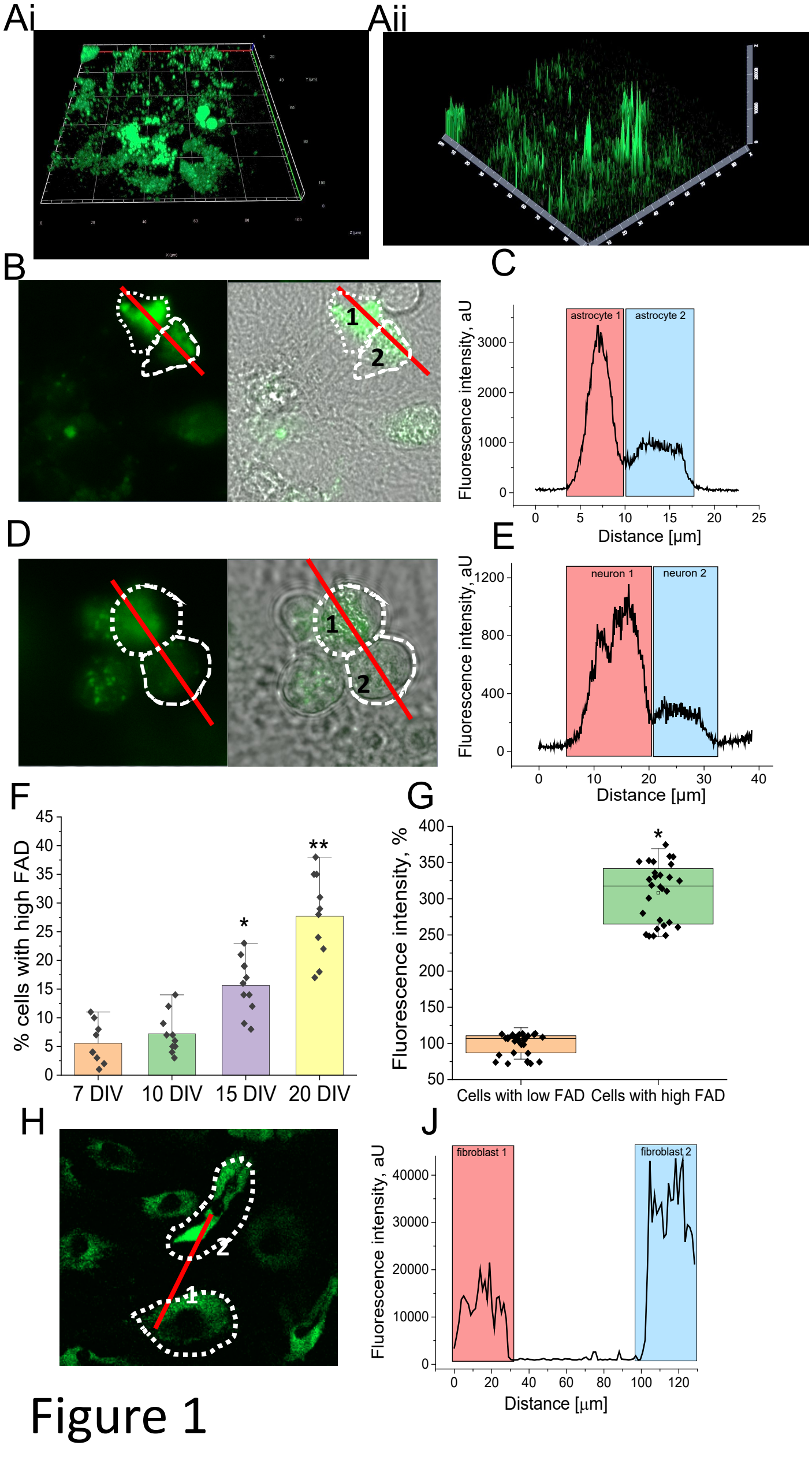
Ai
Aii
B
C
astrocyte 1
astrocyte 2
Fluorescence intensity, aU
Distance [μm]
D
E
neuron 1
neuron 2
Fluorescence intensity, aU
Distance [μm]
F
% cells with high FAD
7 DIV
10 DIV
15 DIV
20 DIV
G
Fluorescence intensity, %
Cells with low FAD
Cells with high FAD
H
J
fibroblast 1
fibroblast 2
Fluorescence intensity, aU
Distance [μm]

Figure 1

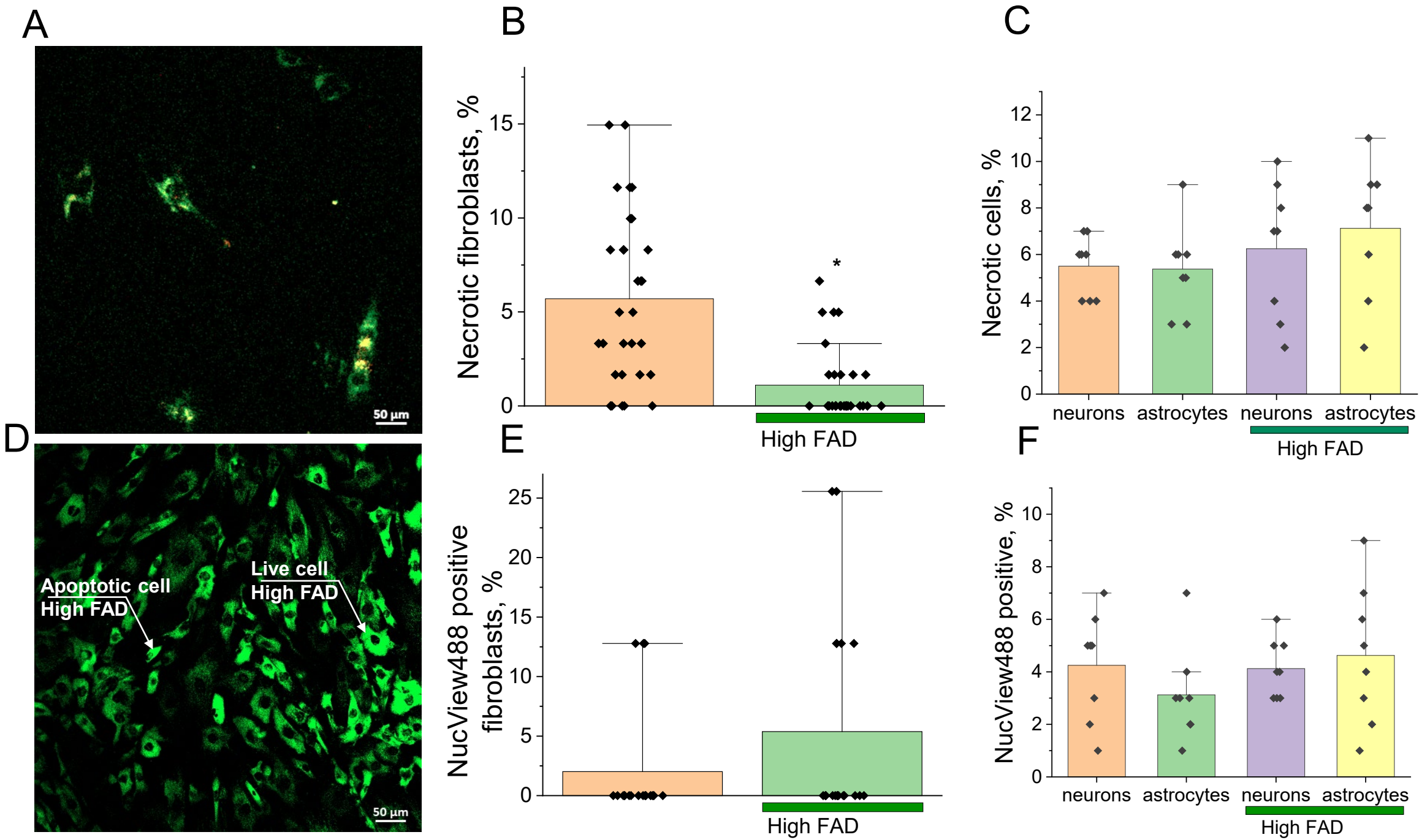
A
50 µm
B
Necrotic fibroblasts, %
0
5
10
15
*
High FAD
C
Necrotic cells, %
0
2
4
6
8
10
12
neurons
astrocytes
neurons
astrocytes
High FAD
D
Apoptotic cell
High FAD
Live cell
High FAD
50 µm
E
NucView488 positive fibroblasts, %
0
5
10
15
20
25
High FAD
F
NucView488 positive, %
0
2
4
6
8
10
neurons
astrocytes
neurons
astrocytes
High FAD

Figure 2

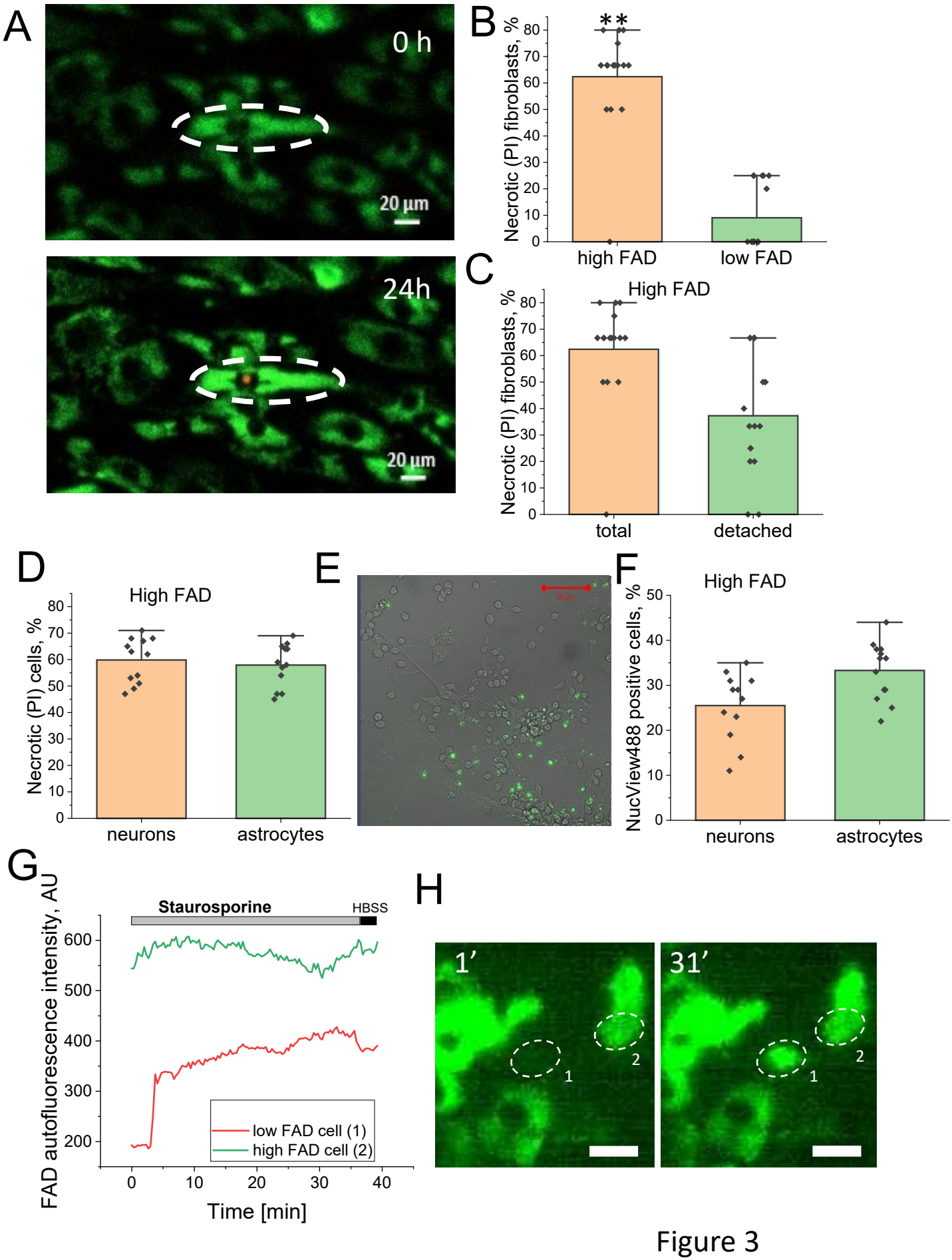
A
0 h
20 µm
24h
20 µm
B
Necrotic (PI) fibroblasts, %
**
high FAD
low FAD
C
High FAD
Necrotic (PI) fibroblasts, %
total
detached
D
High FAD
Necrotic (PI) cells, %
neurons
astrocytes
E
F
High FAD
NucView488 positive cells, %
neurons
astrocytes
G
Staurosporine
HBSS
FAD autofluorescence intensity, AU
Time [min]
low FAD cell (1)
high FAD cell (2)
H
1'
31'
1
2
1
2

Figure 3

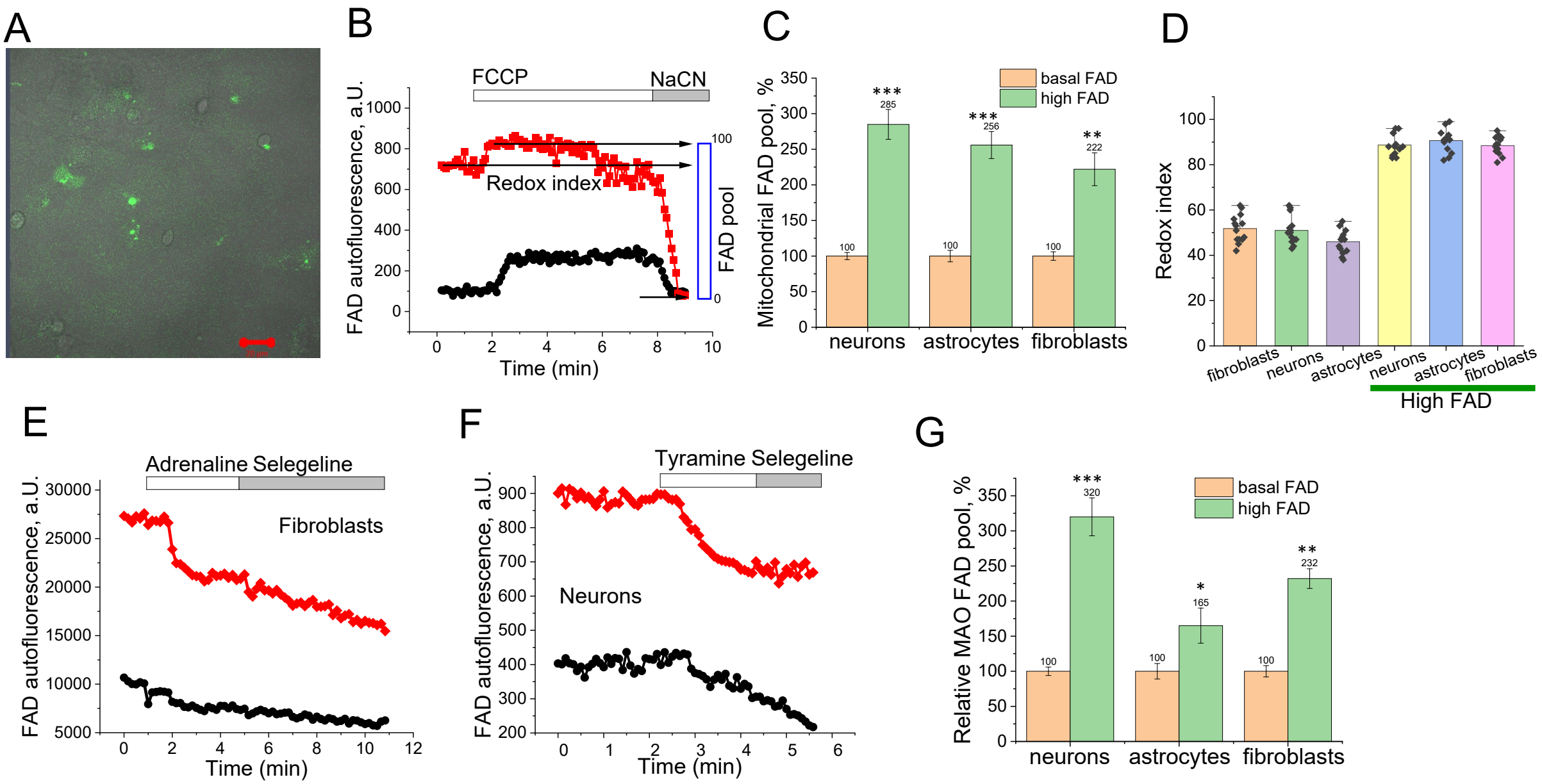
A
B
FCCP
NaCN
Redox index
FAD pool
FAD autofluorescence, a.U.
Time (min)
C
basal FAD
high FAD
Mitochondrial FAD pool, %
neurons
astrocytes
fibroblasts
D
Redox index
High FAD
E
Adrenaline Selegeline
Fibroblasts
FAD autofluorescence, a.U.
Time (min)
F
Tyramine Selegeline
Neurons
G
Relative MAO FAD pool, %

Figure 4

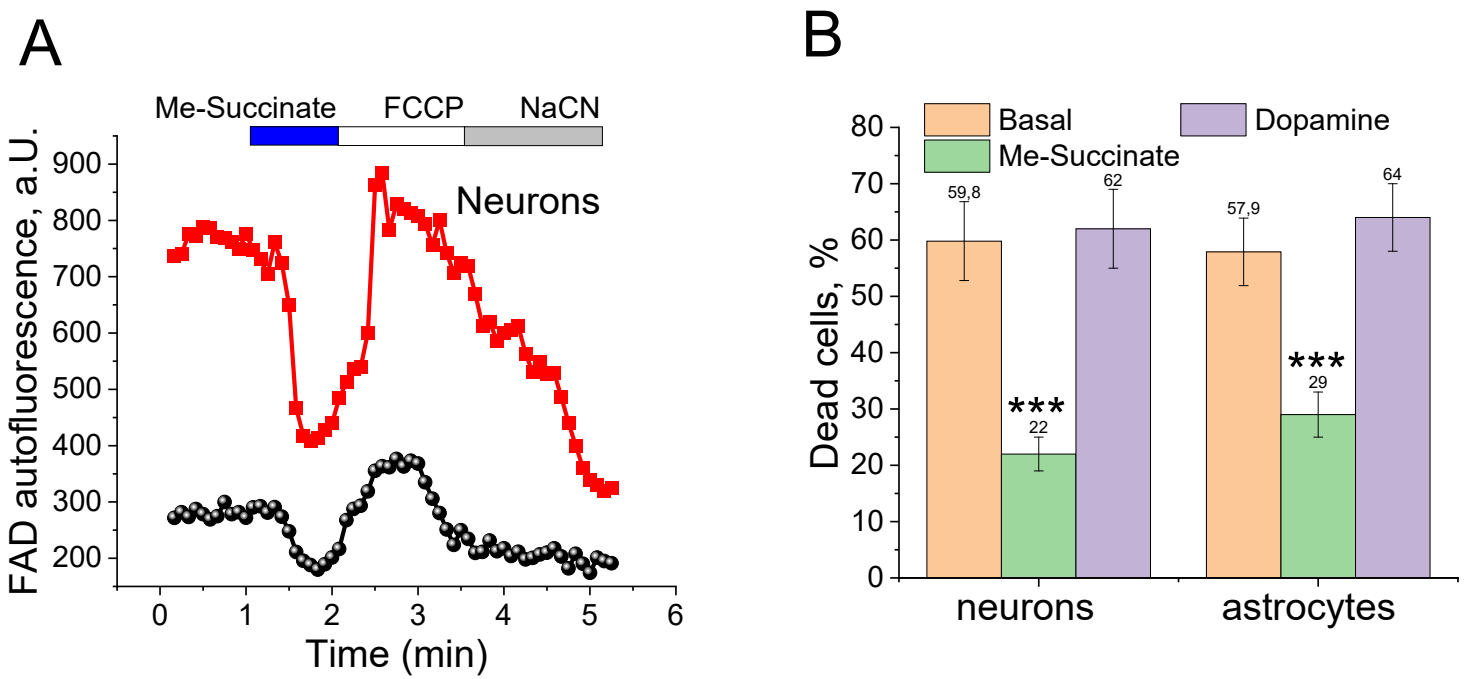
A
Me-Succinate
FCCP
NaCN
Neurons
FAD autofluorescence, a.U.
900
800
700
600
500
400
300
200
0
1
2
3
4
5
6
Time (min)
B
Basal
Dopamine
Me-Succinate
Dead cells, %
80
70
60
50
40
30
20
10
0
59,8
62
57,9
64
***
22
***
29
neurons
astrocytes

Figure 5