

Decoding COVID-19 mRNA Vaccine Immunometabolism in Central Nervous System: human brain normal glial and glioma cells by Raman imaging

H. Abramczyk*¹, B. Brozek-Pluska¹ and Karolina Beton¹

¹ Lodz University of Technology, Institute of Applied Radiation Chemistry, Laboratory of Laser Molecular Spectroscopy, Wróblewskiego 15, 93-590 Łódź, Poland

*Correspondence:

halina.abramczyk@p.lodz.pl;

Tel.: +48 42 631 31 88, H.A.

Abstract: The paper presents the effect of COVID-19 mRNA (Pfizer/BioNT) vaccine on in vitro glial cells of the brain studied by means of Raman spectroscopy and imaging.. The results obtained for human brain normal and tumor glial cells of astrocytes, astrocytoma, glioblastoma incubated with the Covid-19 mRNA vaccine Pfizer/BioNT vaccine show alterations in the reduction-oxidation pathways associated with Cytochrome c.

We found that the Pfizer/BioNT vaccine down regulate the concentration of cytochrome c in mitochondria upon incubation with normal and tumorous glial cells. Concentration of oxidized form of cytochrome c in brain cells has been shown to decrease upon incubation the mRNA vaccine. Lower concentration of oxidized cytochrome c results in lower effectiveness of oxidative phosphorylation (respiration), reduced apoptosis and lessened ATP production. Alteration of Amide I concentration, which may reflect the decrease of mRNA adenine nucleotide translocator. Moreover, mRNA vaccine leads to alterations in biochemical composition of lipids that suggest the increasing role of signaling. mRNA vaccine produce statistically significant changes in cell nucleus due to histone alterations. The results obtained for mitochondria, lipid droplets, cytoplasm may suggest that COVID-19 mRNA (Pfizer/BioNT) vaccine reprograms immune responses. The observed alterations in biochemical profiles upon incubation with COVID-19 mRNA in the specific organelles of the glial cells are similar to those we observe for brain cancer vs grade of aggressiveness.

Keywords: covid-19 mRNA vaccines; SARS-Cov-2; cell lines of astrocytes, human brain cancer; Raman imaging; Raman spectroscopy, cytochrome c, mitochondrial dysfunction

1. Introduction

The COVID-19 pandemics has witnessed an explosion in research in the field of immunometabolism that has revealed that similar mechanisms regulate the host response to infection, autoimmunity, and cancer. The new tools by Raman imaging we present in this paper raise exciting possibilities for new ways to understand pathways of our immune responses, recognize metabolites that regulates these pathways and suggest how we might use them to optimize vaccinations to stimulate the conditions of adaptive immune system.

The pandemic outbreak in 2019 by the SARS-COV-2 virus generating acute respiratory syndrome caused 230 418 451 confirmed cases of COVID-19, including 4 724 876 deaths, reported to WHO. As of 23 September 2021, a total of 5 874 934 542 vaccine doses have been administered [1]. In response to the urgent need for a vaccine, pharmaceutical companies including Pfizer/BioNT in 2020 proposed vaccines based on mRNA technology. The Pfizer/BioNT vaccine (BNT162b2) is more than 90% effective against COVID-19 [2].

In order to enter the host cells, the SARS-COV-2 virus uses the S surface protein, the so-called spike protein (spike S protein). Vaccines based on mRNA technology are designed to produce antibodies to the spike protein. mRNA vaccines are vaccines in which ribonucleic acid (RNA) is used

48 as a template for the production of viral proteins. These proteins are designed to trigger the production
49 of antibodies, which are then transferred to the host's immune system.

50 In the paper, we present effect of mRNA vaccines on the glial brain cells that are involved in
51 tumor microenvironment infiltration by using a novel non-invasive tool such as Raman imaging. Here
52 we demonstrate that Raman imaging reveal new expanses on the role of basic mechanisms of cancer
53 pathology and effect of mRNA vaccines. This approach can monitor interactions in tumor
54 microenvironment and mechanisms related to immune response.

55 Raman spectroscopy and imaging enabling quantitative and non-invasive monitoring of
56 intracellular changes without the need of using external markers. Traditional methods of molecular
57 biology require the destruction of cell membranes and the isolation of intracellular components to
58 study the biochemical changes inside cells. In Raman imaging, we do not need to destroy cells to
59 learn about biochemical composition of intracellular structures (cell organelles). Tracking alterations
60 in biochemical composition in separate organelles is extremely valuable in establishing molecular
61 mechanism of cancer development and mechanisms of infections. Until now, no technology has
62 proven effective for detecting concentration of specific compounds in separate cell organelles.
63 Therefore, existing analytical technologies cannot detect the full extent of biolocalization inside and
64 outside specific organelles.

65 We will concentrate on normal and tumor glial cells upon incubation with mRNA vaccine. The
66 reason is that cancer diseases are the most serious cause of death, exceeding heart disease, strokes,
67 pneumonia and COVID-19. Although at the moment there is no vaccine against most cancers, rapid
68 development of mRNA vaccines may help in development anticancer vaccines.

69 The announcement of effective and safe vaccines for COVID-19 has been greeted with
70 enthusiasm. The vaccines currently used in the global vaccination campaign (3.36 billion doses have
71 been administered across 180 countries, according to data collected
72 [<https://www.bloomberg.com/graphics/covid-vaccine-tracker-global-distribution/>].

73 While COVID-19 vaccines bring potential hope for a return to some kind of normality, many of
74 the fundamental mechanisms by which mRNA vaccines induce strong responses are still incompletely
75 understood and should be continued [2]. The mRNA vaccine encoding the COVID-19 spike (S)
76 protein encapsulated in lipid nanoparticles gain entry into dendritic cells (DCs) at the injection site or
77 within lymph nodes, resulting in production of high levels of S protein.

78 Nevertheless, there is still much to learn. It is not clear which cell specific activation contributes
79 the most to vaccine efficacy and what activation may inhibit the generation of adaptive immunity or
80 lead to poor tolerability of the vaccine [3].

81 There are controversies on harmful effects from spike S protein produced by COVID-19
82 vaccination and long-term effects. Researchers have warned that Pfizer-BioNTech's coronavirus
83 disease 2019 (COVID-19) vaccine induces complex reprogramming of innate immune responses that
84 should be considered in the development and use of mRNA-based vaccines. There are also
85 controversies on biodistribution of mRNA vaccines. It has been reported [4] that intramuscular
86 vaccines (which Pfizer/BioNT vaccine is) in macaques (a type of monkey) remain near the site of
87 injection (the arm muscle) and local lymph nodes, where white blood cells and antibodies are
88 produced to protect from disease. The lymphatic system, lymph nodes also clean up fluids and remove
89 waste materials. Similar results were obtained for a mRNA vaccine against H10N8 and H7N9
90 influenza viruses in mice [4]. However, recent results on interactions between the immune system and
91 the viral proteins that induce immunity against COVID-19 may be more complex than previously
92 thought [5]. Evidence has been found that spike S protein of COVID-19 has remain not only near the
93 site of injection, but also circulate in the blood. COVID-19 proteins were measured in longitudinal
94 plasma samples collected from 13 participants who received two doses of mRNA-1273 vaccine. 11 of
95 13 participants showed detectable levels of COVID-19 protein as early as day one upon first vaccine
96 injection. Clearance of detectable COVID-19 protein correlated with production of IgG and IgA [6].

97 In the view of the recent results it is important to be aware that the spike S protein produced by
98 the new COVID-19 mRNA vaccines may also directly affect the host cells with the long-term
99 consequences. Thus, one should monitor biodistribution and location of spike S protein from mRNA

100 vaccines and the effects of the COVID-19 spike S protein on human host cells in vitro and appropriate
101 experimental animal models.

102 In this paper we will concentrate on central nervous system (CNS) because in addition to
103 pneumonia and acute respiratory distress, COVID-19 is associated with a host of symptoms that are
104 related to the CNS, including loss of taste and smell, headaches, twitching, seizures, confusion, vision
105 impairment, nerve pain, dizziness, impaired consciousness, nausea and vomiting, hemiplegia, ataxia,
106 stroke and cerebral hemorrhage [7].

107 It is unclear whether severe acute respiratory syndrome coronavirus, which causes COVID-19,
108 can enter the brain. It has been postulated that some of the symptoms of COVID-19 may be due to
109 direct actions of the virus on the CNS; for example, respiratory symptoms could be in part due to
110 COVID-19 invading the respiratory centers of the brain [8,9]. Encephalitis has also been reported in
111 COVID-19, and could be a result of virus or viral proteins having entered the brain [7,10].

112 COVID-19 mRNA has been recovered from the cerebrospinal fluid [11], suggesting it can cross
113 the blood–brain barrier (BBB). Other coronaviruses, including the closely related SARS virus that
114 caused the 2003–2004 outbreak, are able to cross the BBB [12], and COVID-19 can infect neurons in
115 a Brain Sphere model [13]. However, COVID-19 could induce changes in the CNS without directly
116 crossing the BBB, as COVID-19 is associated with a cytokine storm, and many cytokines cross the
117 BBB to affect CNS function [9]. It has been found that COVID-19 reaches the brain, infects astrocytes
118 and triggers neuropathological changes that contribute to the structural and functional alterations in the
119 brain of COVID-19 patients [14]. The researchers raised a concern that the lipid nanoparticles (LNPs)
120 that can diffuse quickly, could potentially gain access to the central nervous system (CNS) through the
121 olfactory bulb or blood. However, this needs to be determined with further study. Also, the role of
122 innate memory responses to LNPs needs to be studied [15].

123 Visualization of alterations in single cells upon delivery of mRNA vaccines would help evaluate
124 the efficacy of candidate formulations and aid their rational design for preclinical and translational
125 studies. Here, we show that Raman imaging allows for quantitative, and non-invasive monitoring
126 response to mRNA vaccine in specific organelles without any labeling.

127 In this paper we will study implications for possible consequences of COVID-19 mRNA vaccine
128 (Pfizer/BioNT BNT162b2) on the central nervous system (CNS). We have already studied in detail the
129 biochemical alterations in specific organelles of brain cells vs cancer aggressiveness.[16,17] Thus, we
130 can compare the effect of mRNA on normal and cancer cells with the effect of cancer aggressiveness.
131 As far as we know the mRNA Pfizer vaccine has not been tested for patients suffering of cancer.
132 Therefore this contribution will help monitoring responses in host brain cells similar to a viral
133 infection, because the incubation with COVID-19 mRNA vaccine mimics COVID-19 infection, but
134 instead of the whole virus, only one key protein S for the immune response is synthesized, without
135 causing COVID-19 infection.

136 We will study human brain normal glial cells and glioma cells in vitro: normal human astrocytes
137 (Clonetics NHA), human astrocytoma CCF-STTG1 (ATTC CRL-1718) representing mildly
138 aggressive brain tumor and human glioblastoma cell line U87-MG (ATCC HTB-14) representing
139 highly aggressive brain tumor by Raman imaging. mRNA vaccine mimics COVID-19infection, but
140 instead of the whole virus, only one key protein S for the immune response is synthesized, without
141 causing COVID-19 infection.

142 We will monitor the effect of the mRNA vaccine on biodistribution of different chemical
143 components, particularly cytochrome c, in the specific organelles of a cell: nucleus, mitochondria,
144 lipid droplets, cytoplasm and membrane.

145 In the presented study we will identify dynamics and biochemical composition of the organelles
146 through characteristic Raman spectra upon injection of mRNA vaccine and incubation with the
147 vaccine in vitro cells.

148 We will show also that Raman spectroscopy and Raman imaging are competitive clinical
149 diagnostics tools for cancer diseases linked to mitochondrial dysfunction and are a prerequisite for
150 successful pharmacotherapy of cancer.

151 In this paper we explore alterations in reduction-oxidation pathways related to Cyt c in human
152 brain normal and tumor cells upon incubation in vitro with COVID-19 vaccine (Pfizer/ BioNT
153 BNT162b2).

154 **2. Materials and Methods**

155 *2.1. Reagents*

156 Cytochrome c (C 2506) was purchased from Sigma Aldrich (Poland).

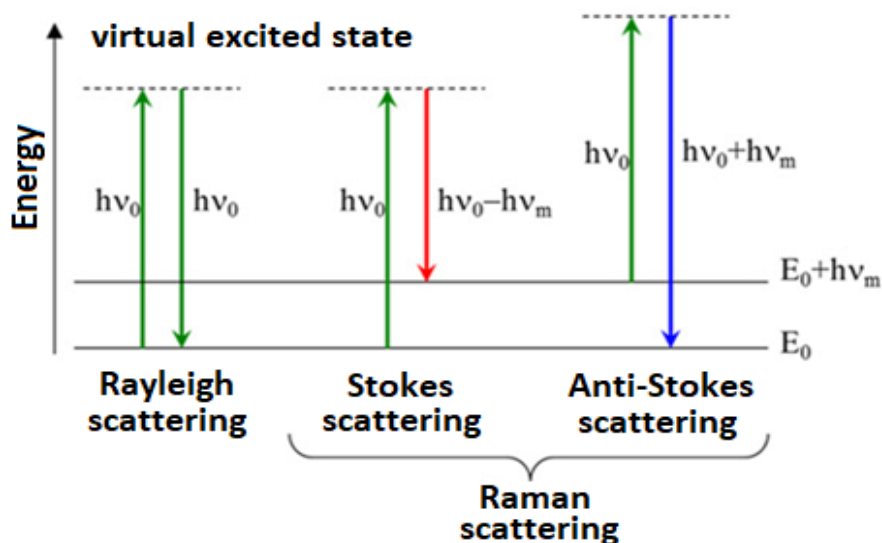
157 *2.2 In vitro cells culturing and incubation with vaccine*

158 The studies were performed on normal human astrocytes (Clonetics NHA), human astrocytoma
159 CCF-STTG1 (ATTC CRL-1718) and human glioblastoma cell line U87-MG (ATCC HTB-14)
160 purchased from Lonza (Lonza Walkersville, Inc., Walkersville, MA, USA) and American Type
161 Culture Collection (ATCC), respectively. The NHA cells were maintained in Astrocyte Medium
162 BulletKit Clonetics (AGM BulletKit, Lonza CC-3186) and Reagent Pack (Lonza CC-5034) without
163 antibiotics in a humidified incubator at 37 °C and 5% CO₂ atmosphere. The U87MG cells were
164 maintained in Eagle's Minimal Essential Medium with L-glutamine (ATCC 30-2003) supplemented
165 with 10% fetal bovine serum (ATCC 30-2020) without antibiotics in a humidified incubator at 37 °C
166 and 5% CO₂ atmosphere. The CRL-1718 cells were maintained in RPMI 1640 Medium (ATCC 30-
167 2001) supplemented with 10% fetal bovine serum (ATCC 30-2020) without antibiotics in a humidified
168 incubator at 37 °C and 5% CO₂ atmosphere. Cells were seeded on CaF₂ window (Crystran Ltd., Poole,
169 UK; CaF₂ Raman grade optically polished window 25 mm diameter × 1 mm thick, no.CAFP25-1R,
170 Poole, UK) in a 35 mm Petri dish at a density of 5 × 10⁴ cells per Petri dish. Upon 24 h of incubation
171 of cells in pure culture medium, cells were supplemented with the vaccine at various time and
172 concentration variants. The COVID-19 mRNA (Pfizer/BioNT vaccine was diluted in 1,8 mL of 0,9%
173 sodium chloride. The total volume of used Petri dishes was 3 mL, so that we added 180 µL of the
174 diluted vaccine to 3 mL of pure culture medium The real dose-of the vaccine- that is administered to
175 patients is equal to 2.25 mL/6= 0.3 mL corresponding to 30 µg per dose [18]. It is difficult to estimate
176 the volume of the body in which the vaccine is diluted in real patients. The doses of 1 µL/mL and
177 60 µL/mL we used correspond to the dose given during the vaccination of real patients assuming the
178 local distribution in the place of injection and around 100 times higher when we use the volume of the
179 fluids in the human body). Before Raman examination, cells were fixed with 4% formalin solution
180 (neutrally buffered) for 10 minutes and kept in phosphate-buffered saline (PBS, no. 10010023, Gibco)
181 during the experiment.
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183 *2.3. Raman imaging and spectroscopy*

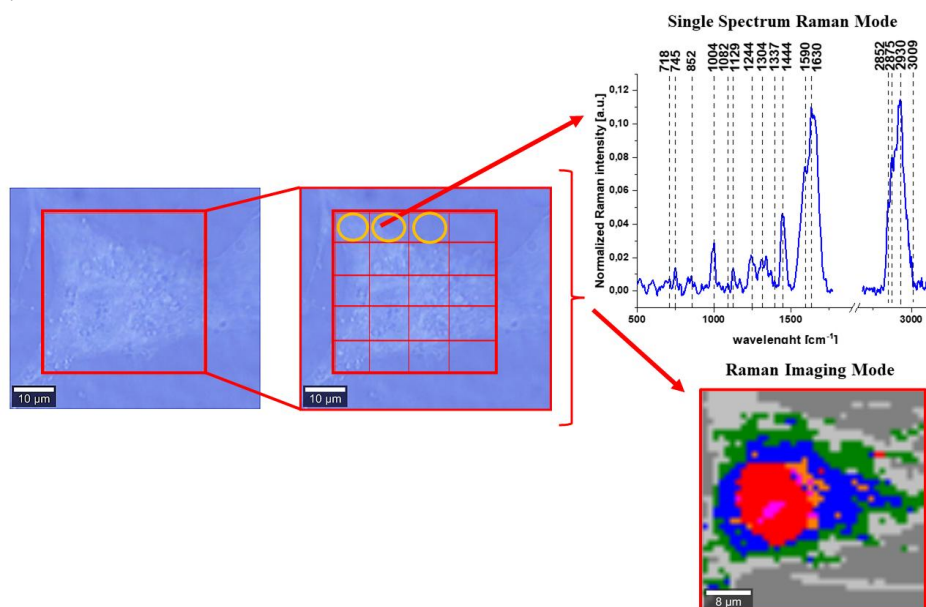
184 Raman spectroscopy is an analytical technique where inelastic scattered light is used to obtain the
185 information about the vibrational energy of analyzed samples. In the vast majority of scattering events,
186 the energy of the molecule is unchanged after its interaction with the photon; and therefore the
187 wavelength, of the scattered photon is equal to that of the incident photon. This is called elastic
188 (energy of scattering particle is preserved) or Rayleigh scattering and is the dominant process during
189 interaction of photon with the molecule. In a much rarer event (approximately 1 in 10 million photons)
190 Raman scattering occurs, which is an inelastic scattering process with a transfer of energy between the
191 molecule and scattered photon. If the molecule gains energy from the photon during the scattering
192 (excited to a higher vibrational level) then the scattered photon loses energy and its wavelength
193 increases which is called Stokes Raman scattering. Inversely, if the molecule loses energy by relaxing
194 to a lower vibrational level the scattered photon gains the corresponding energy and its wavelength
195 decreases; which is called Anti-Stokes Raman scattering. Quantum-mechanically, Stokes and Anti-
196 Stokes are equally probable processes. However, with an ensemble of molecules, the majority of
197 molecules will be in the ground vibrational level (Boltzmann distribution) and Stokes scattering is
198 statistically more probable process. In consequence, the Stokes Raman scattering is always more

intense than the Anti-Stokes component and for this reason, it is nearly always the Stokes Raman scattering that is measured wherewithal Raman spectroscopy. Scheme 1 presents illustration of Rayleigh, Stokes and Anti-Stokes Raman Scattering.



Scheme 1. Schematic presentation of scattering phenomena.

Raman imaging is a technique based on Raman scattering allowing not only a single spectrum acquisition characteristic for a single point of the sample but also the analysis of vibrational spectra of any sample area. The imaging mode allows the analysis of distribution of different chemical molecules inside the sample. Using algorithms such as Cluster Analysis (see section 2.4) based on 2D data obtained by using Raman imaging make possible to create Raman maps to visualize cell's substructures: nucleus, mitochondria, lipid structures, cytoplasm, cell membrane and learn about their biocomposition.



Scheme 2. Schematic comparison of Raman single spectra and Raman imaging modes of data acquisition.

Raman spectra and images were recorded using a confocal Raman microscope (WITec (alpha 300 RSA+), Ulm, Germany) in the Laboratory of Laser Molecular Spectroscopy, Lodz University of Technology, Poland. The Raman microscope consisted of an Olympus microscope (Olympus Düsseldorf, Germany) a UHTS (Ultra-High-Throughput Screening) monochromator (WITec, Ulm, Germany) and a

thermoelectrically cooled CCD camera ANDOR Newton DU970N-UVB-353 (EMCCD (Electron Multiplying Charge Coupled Device, Andor Technology, Belfast, Northern Ireland) chip with 1600×200 pixel format, $16 \mu\text{m}$ dimension each) at -60°C with full vertical binning. A $40\times$ water immersion objective (Zeiss, W Plan-Apochromat $40\times/1.0$ DIC M27 (FWD = 2.5 mm), VIS-IR) was used for cell lines measurements. The excitation laser at 532 nm was focused on the sample to the laser spot of $1 \mu\text{m}$ and was coupled to the microscope via an optical fiber with a diameter of $50 \mu\text{m}$. The average laser excitation power was 10 mW , and the collection time was 0.5 and 1 s for Raman images. Raman images were recorded with a spatial resolution of $1 \times 1 \mu\text{m}$. The Raman microspectrometer was calibrated every day prior to the measurements using a silica plate with a maximum peak at 520.7 cm^{-1} .

2.4. Data processing

Data acquisition and processing were performed using WITec Project Plus software. The background subtraction and the normalization (model: divided by norm (divide the spectrum by the dataset norm)) were performed by using Origin software. The normalization model: divided by norm was performed according to the formula:

$$V' = \frac{V}{\|V\|}$$
$$\|V\| = \sqrt{v_1^2 + v_2^2 + \dots + v_n^2}$$

where:

v_n is the n^{th} V values.

The normalization was performed for low ($500\text{-}1800 \text{ cm}^{-1}$) and high ($2600\text{-}3500 \text{ cm}^{-1}$) frequency spectral regions separately.

For each organelle of a cell we have recorded hundreds of Raman spectra (because we have recorded one single Raman spectrum for each point of the imaging area marked in red in the microscopic image of the Scheme 2 with the resolution of $1 \mu\text{m}$). We used Cluster Analysis method, implemented in WITec project software to calculate the average Raman spectra.

2.5. Cluster analysis

Spectroscopic data were analyzed using Cluster Analysis method. Briefly Cluster Analysis is a form of exploratory data analysis in which observations are divided into different groups that have some common characteristics – vibrational features in our case. Cluster Analysis constructs groups (or classes or clusters) based on the principle that: within a group the observations must be as similar as possible, while observations belonging to different groups must be different.

The partition of n observations (x) into k ($k \leq n$) clusters S should be done to minimize the variance (Var) according to the formula:

$$\arg \min_S \sum_{i=1}^k \sum_{x \in S_i} \|x - \mu_i\|^2 = \arg \min_S \sum_{i=1}^k |S_i| \text{Var} S_i$$

where

μ_i is the mean of points S_i .

Raman maps presented in the manuscript were constructed based on principles of Cluster Analysis described above. Number of clusters was 7 (the minimum number of clusters characterized by different average Raman spectra, which describe the variety of the inhomogeneous biological sample).

3. Results

To learn about alterations in biochemical composition in cell organelles by methods of conventional molecular biology one have to disrupt a cell to release the cellular structure to estimate fractions that are enriched with specific organelles. Using Raman imaging we do not need to break cells to learn about the localization, distribution and bio-chemical composition of specific compounds in different organelles. To properly address alterations in single brain cells upon incubation with COVID-19 Pfizer/BioNT vaccine,

we systematically investigated how Raman spectroscopy and Raman imaging monitor responses to the vaccine in specific organelles.

Figure 1 shows the Raman image of a single cell of glioblastoma (U-87 MG) of highly aggressive brain tumor incubated with mRNA-based Pfizer/BioNT vaccine (dose 60 $\mu\text{L}/\text{mL}$) for 96 hours and corresponding Raman images of specific organelles. The Raman images were created by K-means cluster analysis using 7 clusters. The blue color represents lipids including rough endoplasmic reticulum and lipid droplets (filled with retinoids), the orange color represents lipid droplets (filled with triacylglycerols of monounsaturated type (TAG), magenta color represents mitochondria, red color represents nucleus, green- cytoplasm, and light grey – membrane (the dark grey color corresponds to cell environment).

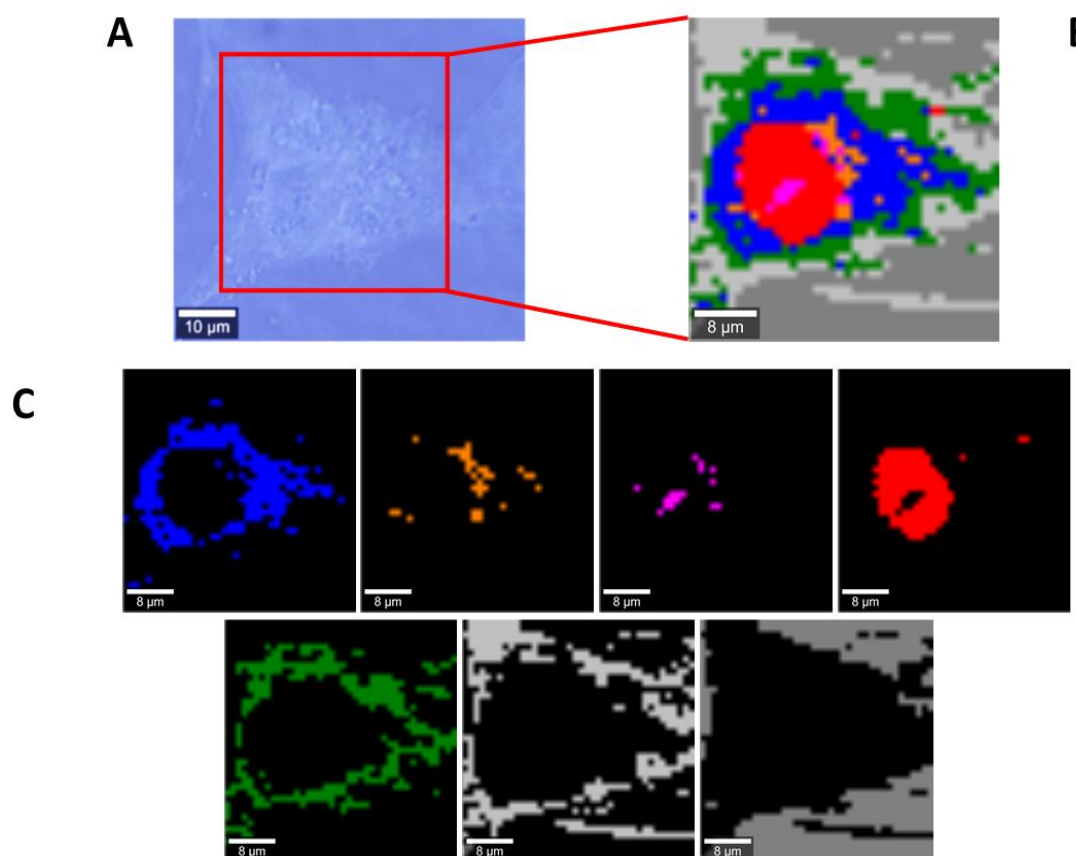
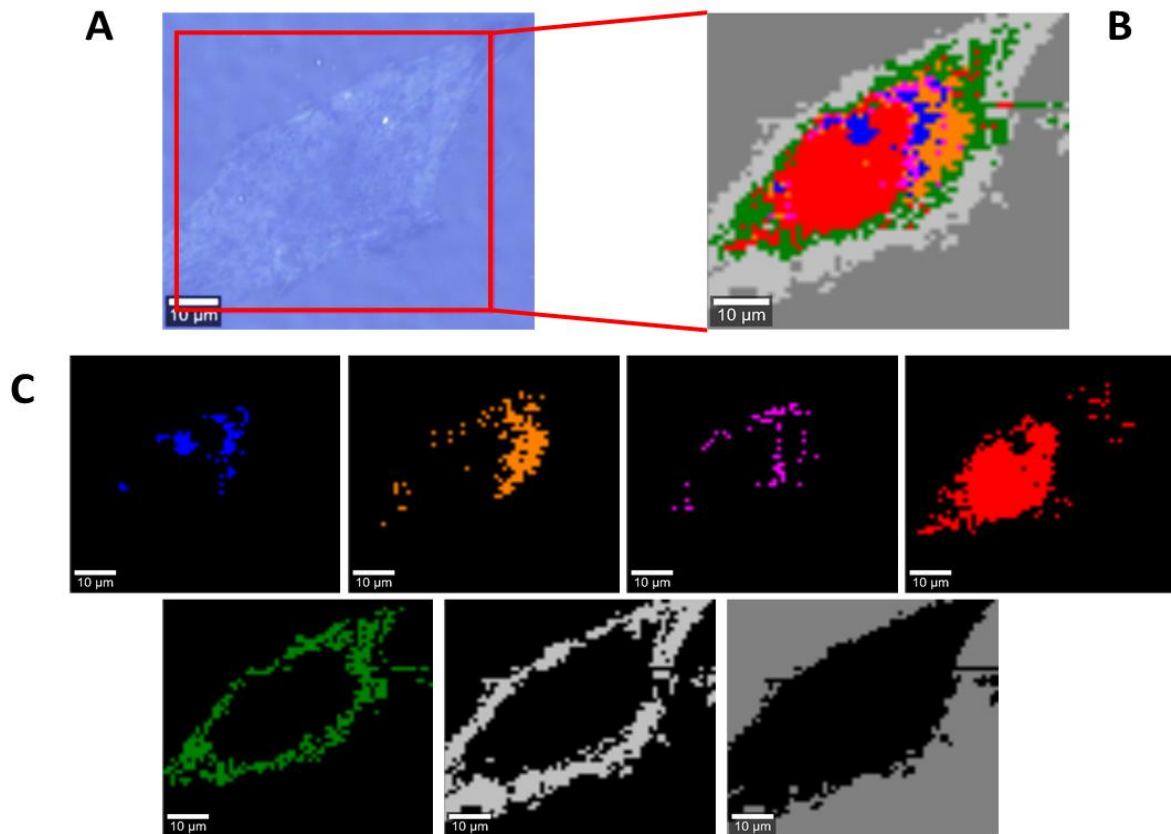


Figure 1. Microscopy image (A), Raman image of glioblastoma (U-87 MG) cell ($30 \times 20 \mu\text{m}$, resolution $1.0 \mu\text{m}$) incubated with Pfizer/BioNT vaccine (dose $60 \mu\text{L}/\text{mL}$) for 96 hours (B) and Raman images of specific organelles: lipids and lipid droplets (blue and orange), mitochondria (magenta), nucleus (red), cytoplasm (green), membrane (light grey), cell environment (dark grey) at 532 nm .

Figure. 2 shows the Raman image of a single cell of astrocytoma (CRL-1718) of mildly aggressive brain tumour and corresponding Raman images of specific organelles incubated with mRNA- vaccine from Pfizer/BioNT (dose $60 \mu\text{L}/\text{mL}$) for 96 hours. The Raman images were created by K-means cluster analysis using 7 clusters with the same coding colours as in Fig.1.



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Figure 2. Microscopy image (A), Raman image of astrocytoma (CRL1718) cell (65x60 µm, resolution 1.0 µm) incubated with Pfizer/BioNT vaccine (dose 60 µL/mL) for 96 hours (B) and Raman images of specific organelles: lipids and lipid droplets (blue and orange), mitochondria (magenta), nucleus (red), cytoplasm (green), membrane (light grey), cell environment (dark grey) at 532 nm.

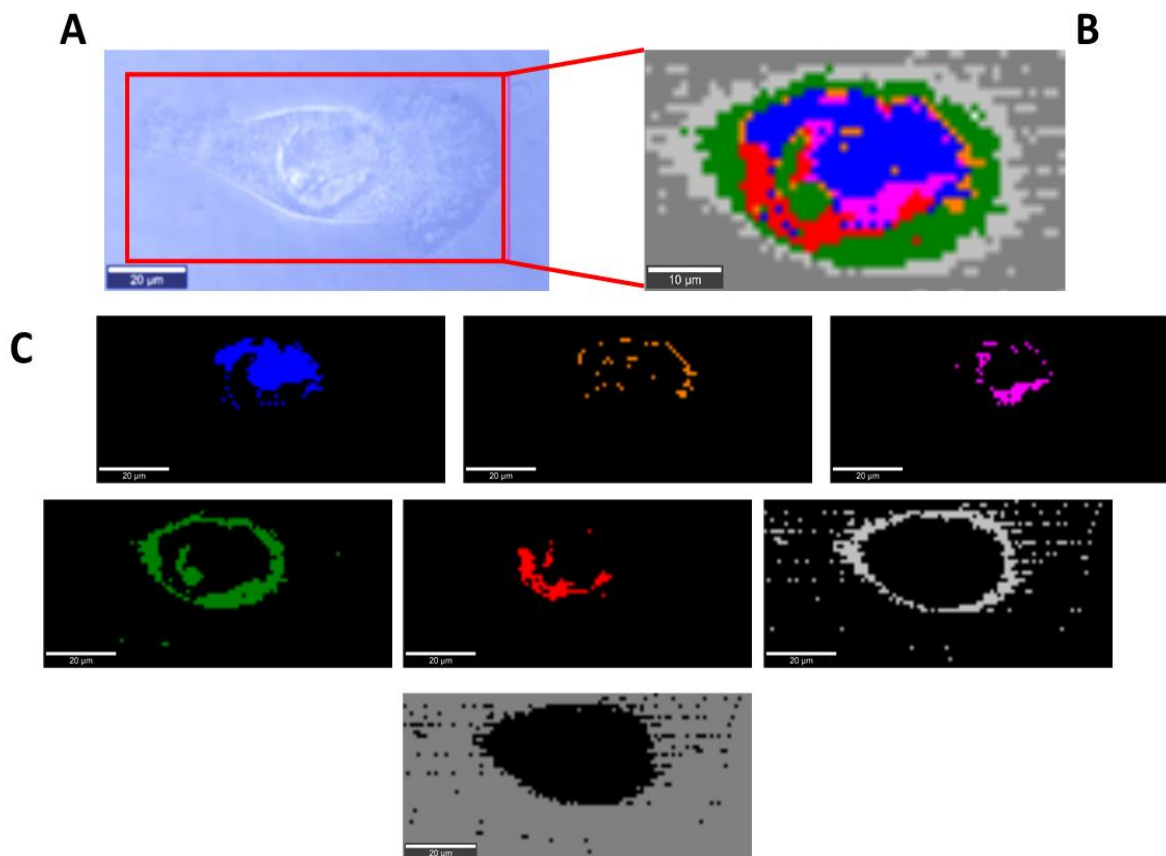
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Figure. 3 shows the Raman image of a single normal cell of astrocyte (NHA) and corresponding Raman images of specific organelles. The Raman images were created by K-means cluster analysis using 7 clusters with same the coding colours as in Fig. 1.



294
295 **Figure 3.** Microscopy image (A), Raman image of astrocyte (NHA) cell (100x45 µm, resolution 1.0 µm)
296 incubated with Pfizer/BioNT vaccine (dose 60 µL/mL) for 96 hours (B) and Raman images of specific
297 organelles: lipids and lipid droplets (blue and orange), mitochondria (magenta), nucleus (red), cytoplasm
298 (green), membrane (light grey), cell environment (dark grey) at 532 nm.

299 To properly address alterations in brain tumor cells upon incubation with
300 COVID-19 vaccine (Pfizer/BioNT) we systematically investigated how the
301 Raman imaging and spectroscopy monitor response of in vitro human brain
302 normal cells and cells of different aggressiveness.
303

304 3.1. Mitochondria-mRNA

305 Figure. 4 shows the effect of the Pfizer/BioNT vaccine on mitochondria in
306 glioblastoma cells (U87 MG), astrocytoma (CRL-1718) and normal cells of
307 astrocyte (NHA) by Raman imaging. Fig 4 shows a comparison of the average
308 Raman spectra (normalized by vector norm) for mitochondria at 532 nm
309 excitation with and without mRNA vaccine.

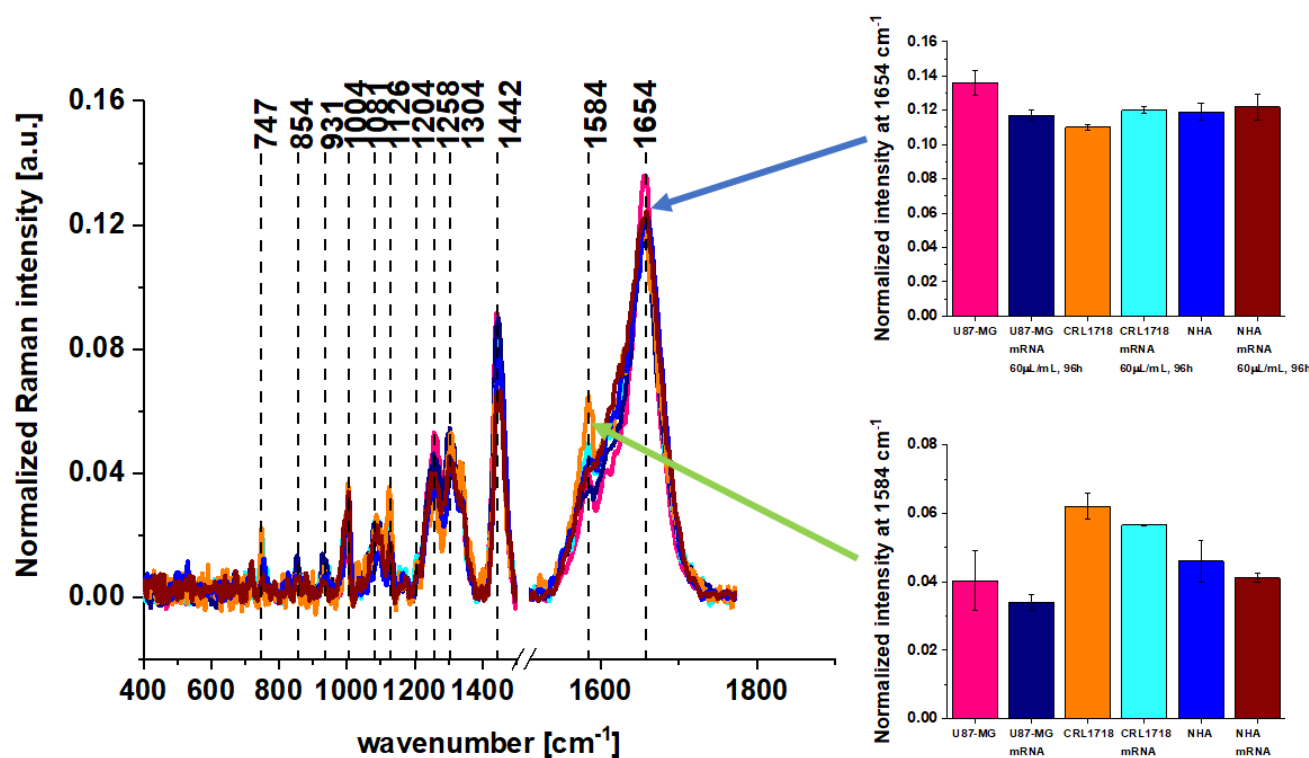


Figure 4. The effect of the Pfizer/BioNT vaccine from on mitochondria in glioblastoma cells (U87 MG) ---, glioblastoma cells (U87 MG) upon incubation with Pfizer/BioNT vaccine for 96h -----, astrocytoma (CRL-1718) -----, astrocytoma cells (CRL1718) upon incubation with Pfizer/BioNT vaccine for 96h -----, normal astrocytes (NHA) -----, and normal astrocytes (NHA) upon incubation with Pfizer/BioNT vaccine for 96h ----- (number of cells for each cell's type: 3, number of Raman spectra for each single cell: minimum 1600, excitation wavelength: 532 nm, laser power: 10 mW, integration time: 1.0sec).

Detailed inspection into Fig. 4 demonstrates that the most significant changes occur at 1584 cm⁻¹. The peak at 1584 cm⁻¹ represents the “redox state Raman marker” of Cyt c concentration. Recently we demonstrated that this Raman vibration can serve as a sensitive indicators cytochrome c concentration and correlates with cancer aggressiveness [16]. We showed that the Raman intensity of the band at 1584 cm⁻¹ corresponding to the concentration of cytochrome c in mitochondria of a cell in vitro decreases with brain tumor aggressiveness.[16,17]

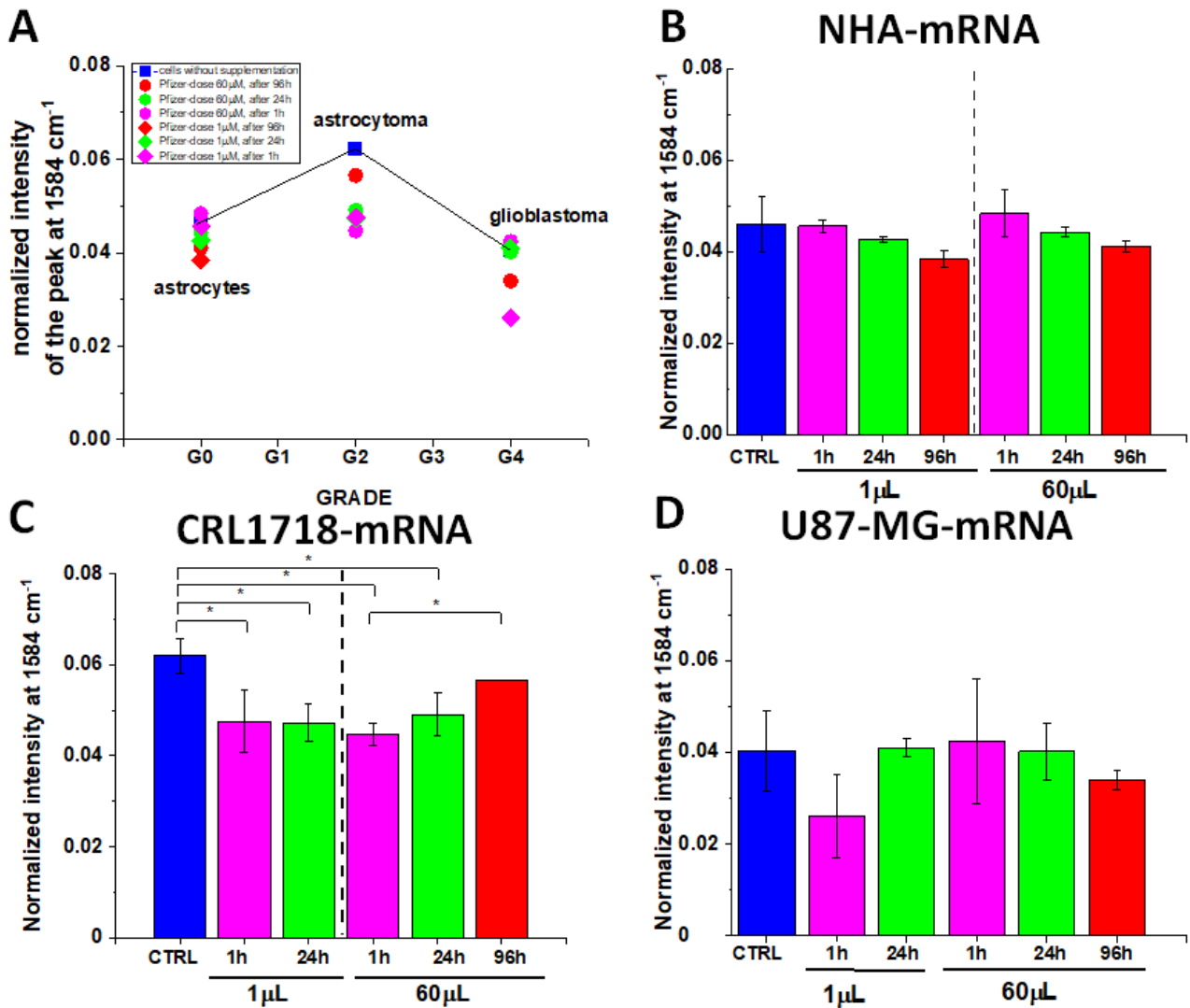
Briefly, cytochromes are classified on the basis of their lowest electronic energy absorption band in their reduced state. Therefore, we can distinguish cytochrome P450 (450 nm), cytochrome c (550 nm), cytochromes b (≈565 nm), cytochromes a (605 nm). The cytochromes are localized in the electron transport chain in the complex known as complex III or Coenzyme Q – Cyt C reductase, sometimes called also the cytochrome bc1 complex (cytochrome b, cytochrome c1). Cytochrome c, which is reduced to cytochrome c Fe²⁺ by the electron from the complex III to complex IV, where it passes an electron to the copper binuclear center, being oxidized back to cytochrome c (cyt c Fe⁺³). Complex IV is the final enzyme of the electron transport system. The complex IV contains two cytochromes a and a3 and two copper centers.

Until now, no technology has proven effective for detecting Cyt c concentration in specific cell organelles. Existing analytical technologies such as enzyme-linked immunosorbent assays (ELISA), Western blot, high performance liquid chromatography (HPLC), spectrophotometry and flow cytometry cannot detect the full extent of Cyt c localization inside and outside specific organelles. Therefore, none of the methods used to control Cyt c concentration can provide direct evidence about

341 the role of cytochrome *c* in apoptosis and oxidative phosphorylation, because they are not able to
342 monitor the amount of cytochrome in specific organelles such as mitochondria, cytoplasm, or
343 extracellular matrix. Raman imaging does not need to disrupt cells to open yjrm to release the cellular
344 structures to learn about their biochemical composition. Recently we showed [16,19] that the
345 concentration of Cyt *c* in cancer tissues increases with cancer aggressiveness. It indicates that the netto
346 concentration of Cyt *c* in mitochondria is higher than release to cytoplasm. This finding reflects the
347 dual face of Cyt in life and death decisions: apoptosis and oxidative phosphorylation. The balance
348 between cancer cells proliferation (oxidative phosphorylation) and death (apoptosis) decide about level
349 of cancer development. The Cyt *c* concentration in mitochondria as a function of cancer
350 aggressiveness reflects its contribution to oxidative phosphorylation and apoptosis.[16,19] In contrast,
351 in single cells in vitro where the interactions with the extracellular matrix are eliminated the trend is
352 opposite than in the cancer tissues. The biochemical results obtained by Raman imaging showed that
353 human single cells in vitro demonstrate a redox imbalance by downregulation of cytochrome *c* in
354 cancers.

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356 One can see from Figure 4 that the Raman signal of oxidized cytochrome *c* is
357 the strongest for astrocytoma control cells and the weakest one for high-grade
358 glioblastoma (U-87 MG). It indicates that the processes of oxidative
359 phosphorylation and apoptosis decrease with cancer aggressiveness.

360 Fig. 4 also shows significant changes at 1654 cm^{-1} corresponding to Amide I
361 vibrations. The intensity of the band at 1654 cm^{-1} decreases for glioblastoma
362 U87 MG upon incubation with mRNA. It has been reported that changes in
363 mitochondrial membrane potential favour functional deterioration of the adenine
364 nucleotide translocator (ANT), which belongs to mitochondrial carrier family
365 [20]. As ANT represents about 10 % of proteins in mitochondria the observed in
366 Fig. 4 decrease upon incubation with mRNA may reflect this deterioration.
367 Figure. 5 shows a comparison of the average Raman spectra (normalized by
368 vector norm) obtained for mitochondria in glioblastoma cells (U87 MG),
369 astrocytoma (CRL-1718) and normal cells of astrocyte (NHA) with and without
370 mRNA vaccine for 532 nm excitation.



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372 **Figure 5.** The normalized Raman intensity of the band 1584 cm⁻¹, (based on the Raman spectra normalized
 373 by vector norm) obtained for mitochondria in normal cells of astrocyte (NHA) (A,B), astrocytoma (CRL-
 374 1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine (control, blue) and with
 375 Pfizer/BioNT vaccine: doses 1 μL/mL and 60 μL/mL, time of incubation 1h-magenta, time of incubation 24h-
 376 green, time of incubation 96h-red. The one-way ANOVA using the Tukey test was used to calculate the value
 377 significance, asterisk * denotes that the differences are statistically significant, p-value ≤ 0.05.

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One can see that the normalized Raman intensity of the band at 1584 cm⁻¹ corresponding to the concentration of cytochrome c in mitochondria of cells in vitro decreases upon incubation with mRNA vaccination when compared with the control samples. This effect depends on brain tumor aggressiveness. The observations illustrated in Figure 5 confirm that incubation with the mRNA vaccine decreases the cytochrome c concentration for astrocytoma CRL1718 with statistical significance. Lower concentration of cytochrome c upon incubation with mRNA observed for astrocytoma leads to reduction in mitochondrial membrane potential, reduction of oxidative phosphorylation (respiration) and apoptosis and lessened ATP production.[16,17]

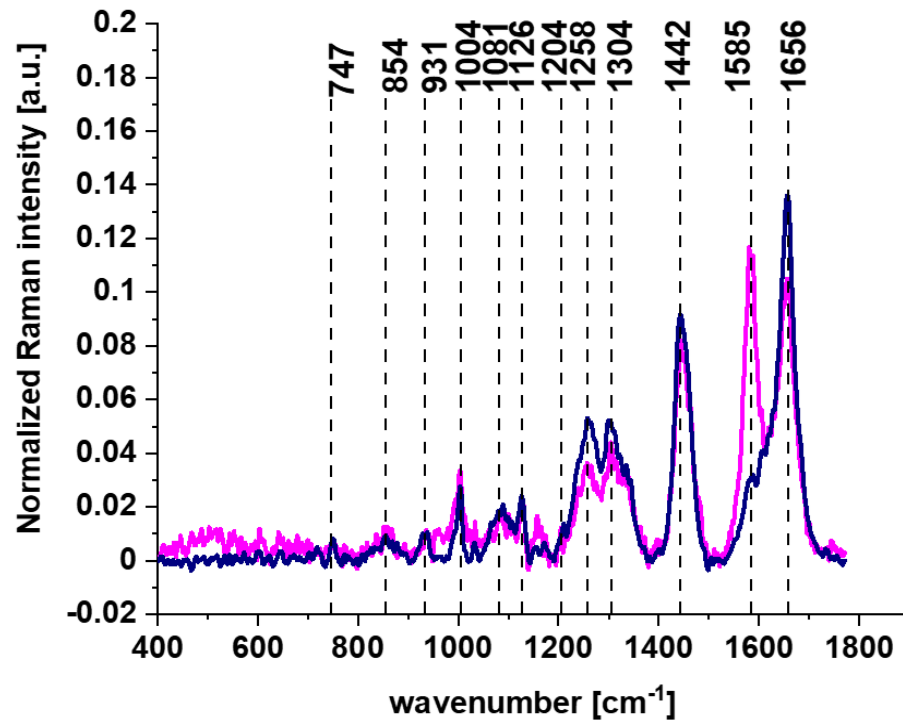
The results presented in Fig. 5 suggest that the Pfizer/BioNT vaccine against COVID-19 reprograms innate immune responses by downregulation of cytochrome c. This conclusion is based on the observation of the cytochrome c may play the role of a universal DAMP molecules (Damage-associated molecular patterns) able of alarming the immune system for danger in any type of cell or

393 tissue and contributing to the host's defense [21]. Damage-associated molecular
394 patterns (DAMPs) are endogenous danger molecules that are released from
395 damaged or dying cells and activate the innate immune system by interacting
396 with pattern recognition receptors (PRRs).

397 Our results presented so far fully support these suggestions [17,21].
398 Cytochrome c is a key protein that is needed to maintain life (respiration via
399 oxidative phosphorylation) and cell death (apoptosis). As a result cytochrome c is
400 a key protein in cancer development. The pandemic has witnessed an explosion
401 in research examining the interplay between the immune response and the
402 intracellular metabolic pathways that mediate it. Research in the field of
403 immunometabolism has revealed that similar mechanisms regulate the host
404 response to infection, autoimmunity, and cancer. Our results suggest that the
405 triangle: cytochrome c- cancer - the immune system are strongly linked.

406 We found that there is very close relations between cytochrome c and
407 immune system through retinoic acid [22]. Retinoic acid (RA) is an essential
408 molecule in the innate immune system that does not stimulate its ATPase and
409 leads to lack of cytokine induction. Fig.6 shows effect of retinoic acid on
410 cytochrome c in U87 MG cells of glioblastoma.

411 Detailed inspection into Figure. 6 demonstrates that the most significant
412 changes occurs at 1584 cm^{-1} corresponding to reduced form of cytochrome c. The
413 Raman intensity of cytochrome c drastically increases upon incubation of
414 U87 MG cells with retinoic acid and represents the reduced form [16,17] in
415 contrast to the oxidized form observed without RA incubation. Incubation in
416 vitro with retinoic acid increases the amount of reduced form of cytochrome c.
417



418

419 **Figure 6.** Average Raman spectra for mitochondria for glioblastoma U87-MG cells without (violet) and
420 upon incubation for 24h with retinoic acid ($c=50\ \mu\text{M}$, pink), (alle spectra represent the arithmetic mean of
421 3 spectra characteristic for mitochondria of analyzed cells, calculated based on the minimum 1600 single
422 Raman spectra, excitation wavelength 532 nm, laser power 10 mW, integration time 1.0 sec).

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Our results from Fig. 6 support the conclusion that retinoic acid is a key player in immunity [23]. Moreover, it has been shown recently that RIG-I (retinoic acid induced gen) triggers a signaling-abortive anti-COVID-19 defense in human lung cells [24].

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3.3. Nucleus -mRNA

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Therefore is particularly important to monitor alterations in nucleus upon incubation with mRNA by Raman imaging. Figure 7 shows results obtained for nucleus without and upon incubation with vaccine by Raman spectroscopy and imaging.

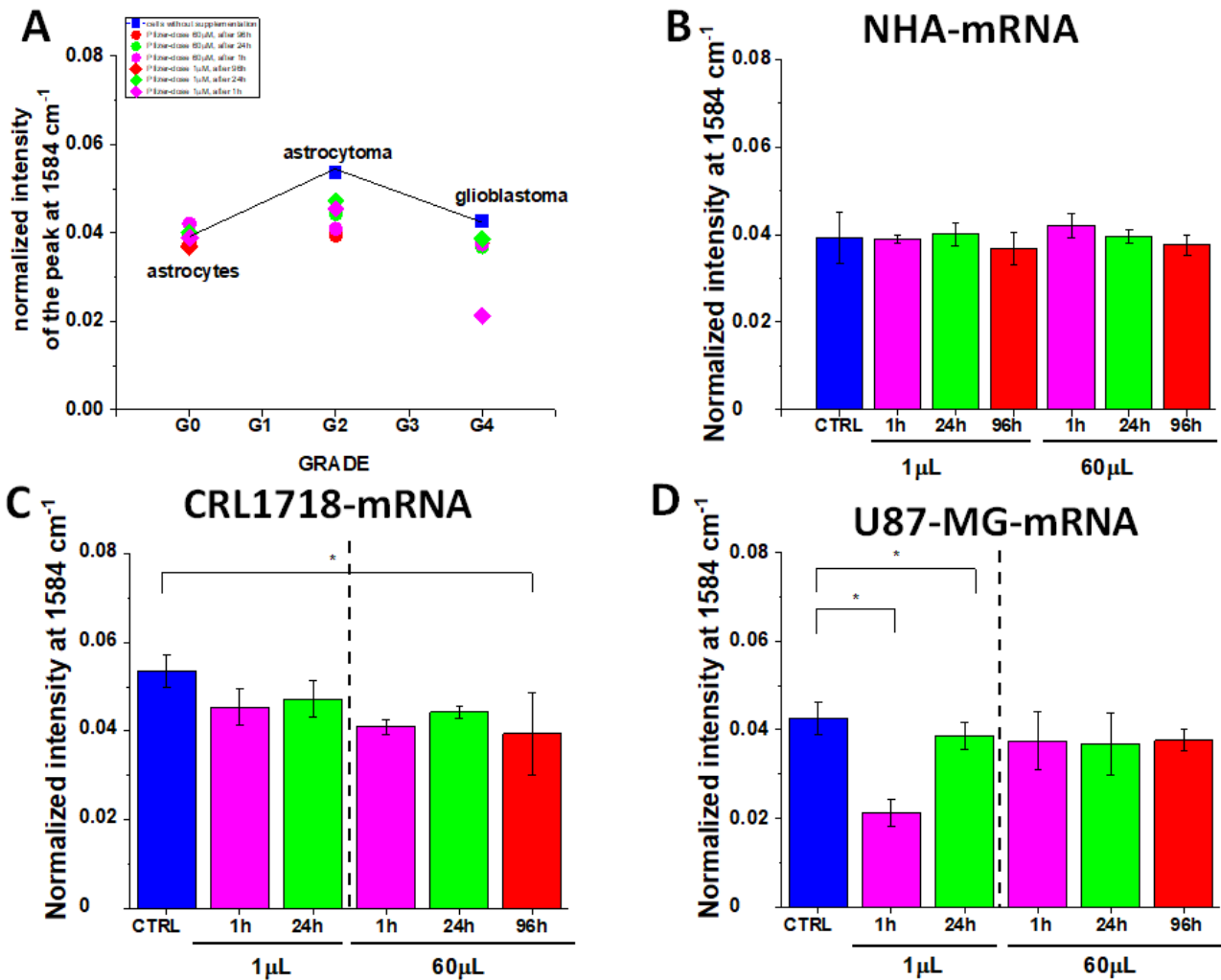


Figure 7. The normalized Raman intensity of the band 1584 cm⁻¹, (based on the Raman spectra normalized by vector norm) obtained for nucleus in normal cells of astrocyte (NHA) (A,B), astrocytoma (CRL-1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine (control, blue) and with Pfizer/BioNT vaccine: doses 1 μL/mL and 60 μL/mL, time of incubation 1h-magenta, time of incubation 24h-green, time of incubation 96h-red. The one-way ANOVA using the Tukey test was used to calculate the value significance, asterisk * denotes that the differences are statistically significant, p-value ≤ 0.05.

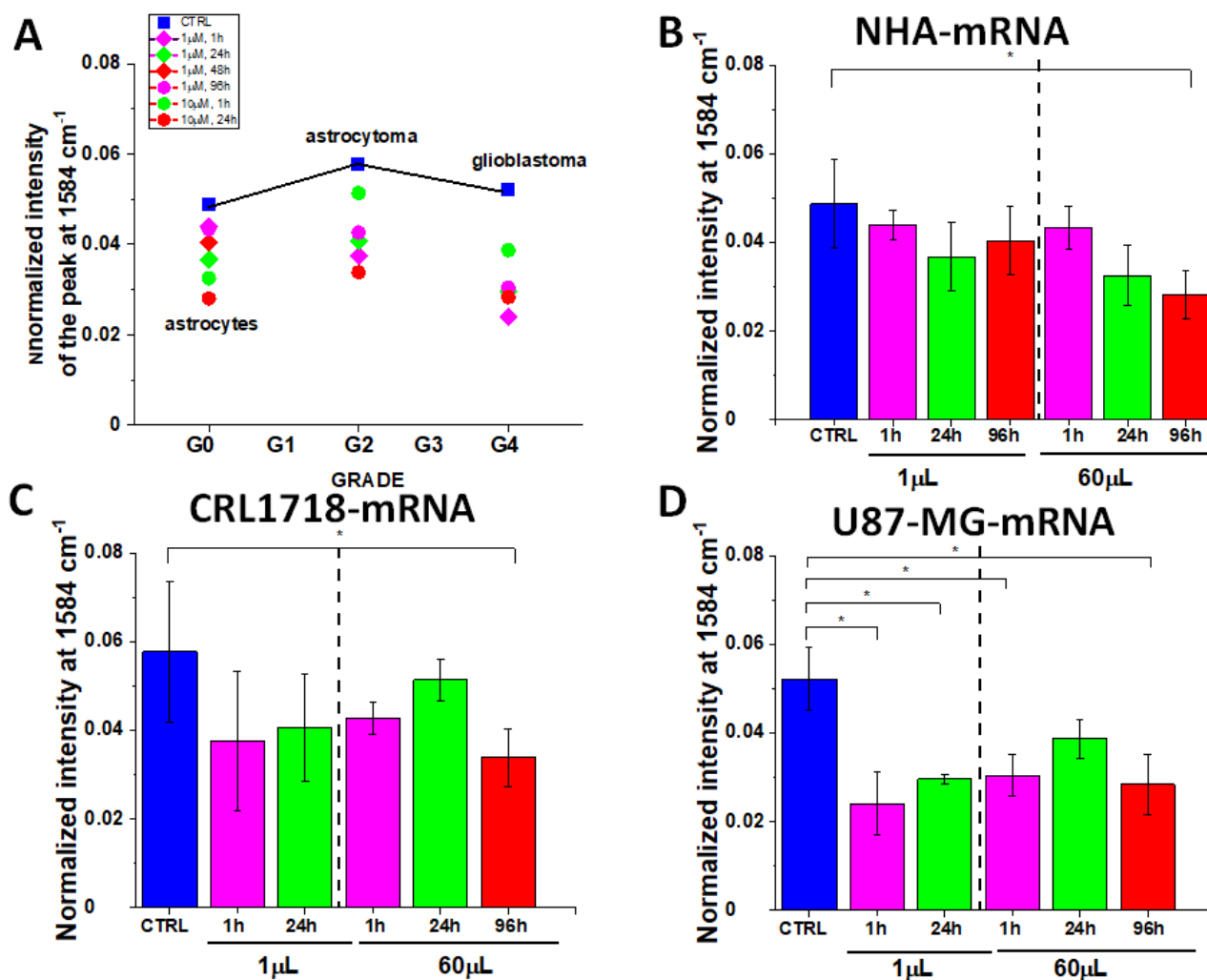
Generally, our results seems to support the conclusions that mRNA vaccine does not enter the DNA of the cell. The Figure. 7 shows that there is no statistical significance for cytochrome c activity for normal astrocytes NHA and U87 MG at 60 μL/mL dose. In contrast, one can observe some statistically significant changes for astrocytoma for the dose 60 μL/mL for the incubation time 96h, and for U87-MG glioblastoma cells for low dose 1 μL/mL for 24h. Because it is believed that mRNA vaccine does not introduce any changes corresponding to DNA, we interpret this result as posttranslational changes in histones, not in DNA. It may indicate that posttranslational changes in histones of the nucleus occur upon incubation with the mRNA vaccine.

3.4. Lipid droplets-mRNA and rough endoplasmic reticulum

The significant changes in cytochrome c concentration occurs not only for mitochondria. Similar alterations in biochemical composition of cytochrome c reflected by the band at 1584 cm⁻¹ are also observed in lipid droplets and in lipid structures of rough endoplasmic reticulum (RER) (Figures 8 and 9) . RER is studded with ribosomes that perform biological protein synthesis (mRNA

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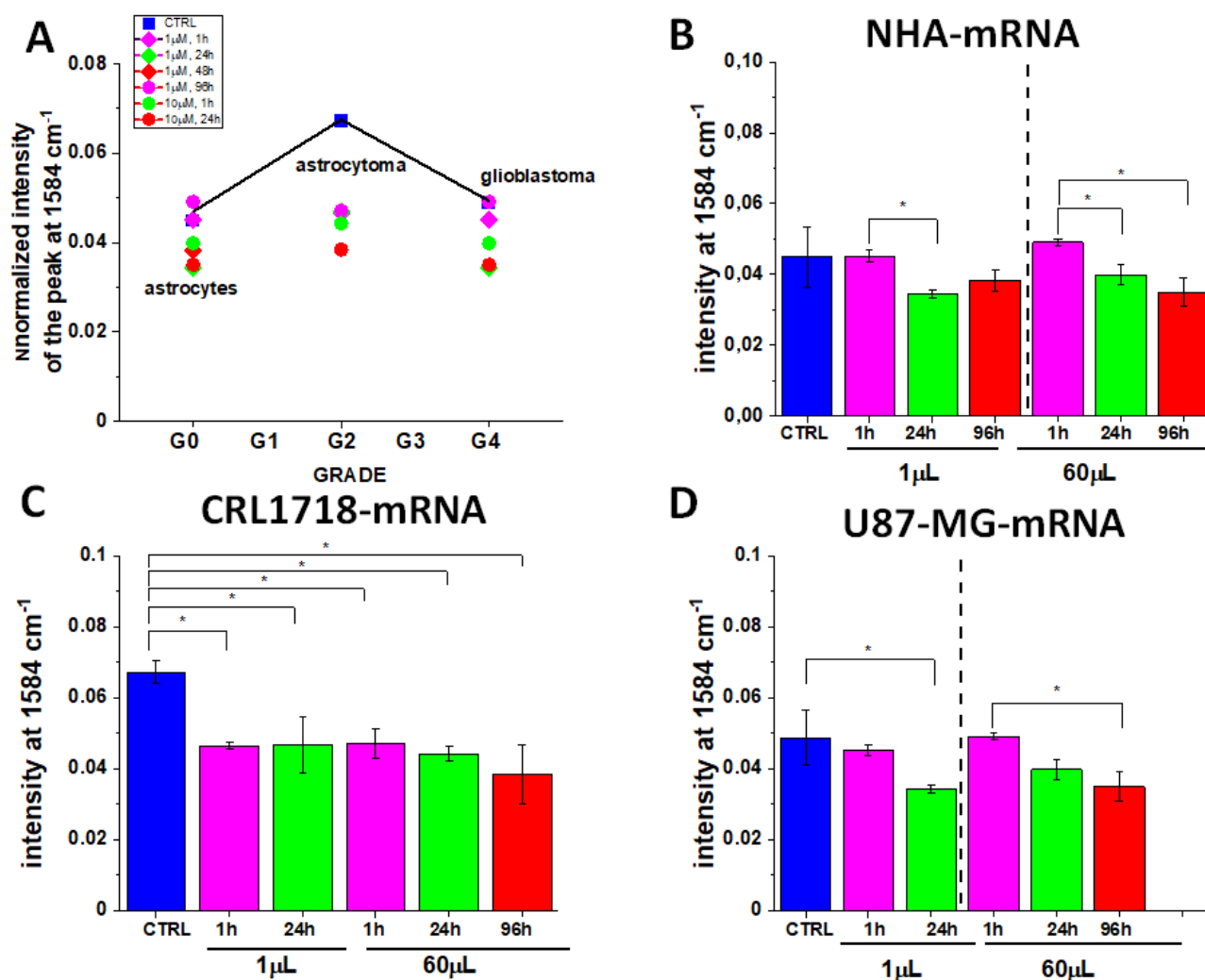
translation). Ribosomes are the sites of protein synthesis for mRNA vaccines. Ribosomes are too small to be seen by resolution of Raman imaging.



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471 **Figure 8.** The normalized Raman intensity of the band 1584 cm⁻¹, (based on the Raman spectra normalized
472 by vector norm) obtained for lipid droplets in normal cells of astrocyte (NHA) (A,B), astrocytoma (CRL-
473 1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine (control, blue) and with
474 Pfizer/BioNT vaccine: doses 1 μL/mL and 60 μL/mL, time of incubation 1h-magenta, time of incubation 24h-
475 green, time of incubation 96h-red. The one-way ANOVA using the Tukey test was used to calculate the value
476 significance, asterisk * denotes that the differences are statistically significant, p-value ≤ 0.05.

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479 **Figure 9.** The normalized Raman intensity of the band 1584 cm⁻¹, (based on the Raman spectra normalized
 480 by vector norm) obtained for rough endoplasmatic reticulum in normal cells of astrocyte (NHA) (A,B),
 481 astrocytoma (CRL-1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine
 482 (control, blue) and with Pfizer/BioNT vaccine: doses 1 μL/mL and 60 μL/mL, time of incubation 1h-
 483 magenta, time of incubation 24h-green, time of incubation 96h-red. The one-way ANOVA using the Tukey
 484 test was used to calculate the value significance, asterisk * denotes that the differences are statistically
 485 significant, p-value ≤ 0.05.

486

487 The Figure. 8 and 9 shows that the cytochrome c signal in lipid droplets and
 488 rough endoplasmatic reticulum at 1584 cm⁻¹ decreases for all types of studied
 489 glial cells: normal cells, astrocytoma, and glioblastoma, all periods of incubation
 490 and doses with statistical significance.

491 This trends in lipid droplets and rough endoplasmatic reticulum correlate
 492 with changes observed in mitochondria (Fig.5) .

493 The alterations in lipid composition can also be monitored by the band at
 494 2845 cm⁻¹ that monitor concentration of triglycerides (TAG) [22,25,26]. Figure. 9
 495 shows a comparison of the average Raman spectra (normalized by vector norm)
 496 obtained from the cluster analysis for mitochondria at 532 nm excitation with and
 without mRNA vaccine in the high frequency spectral region.

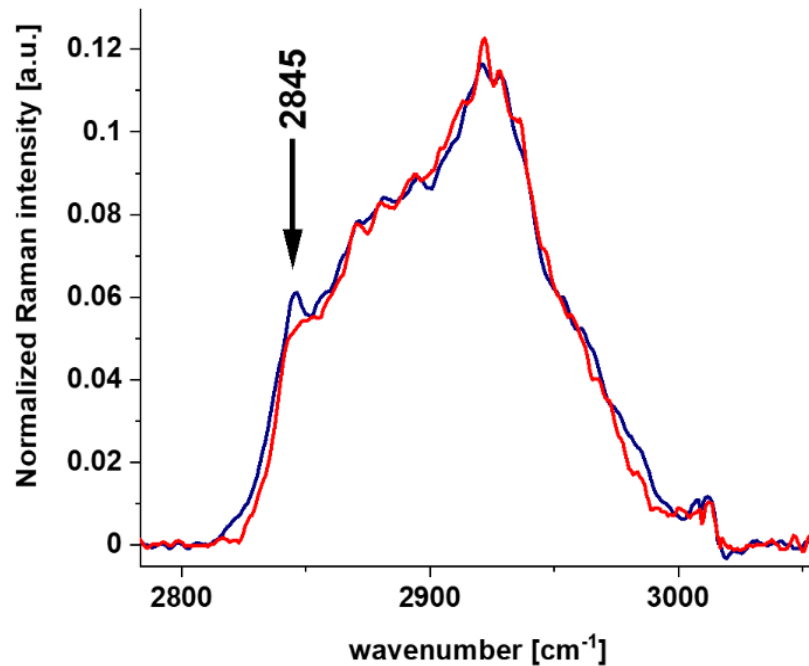


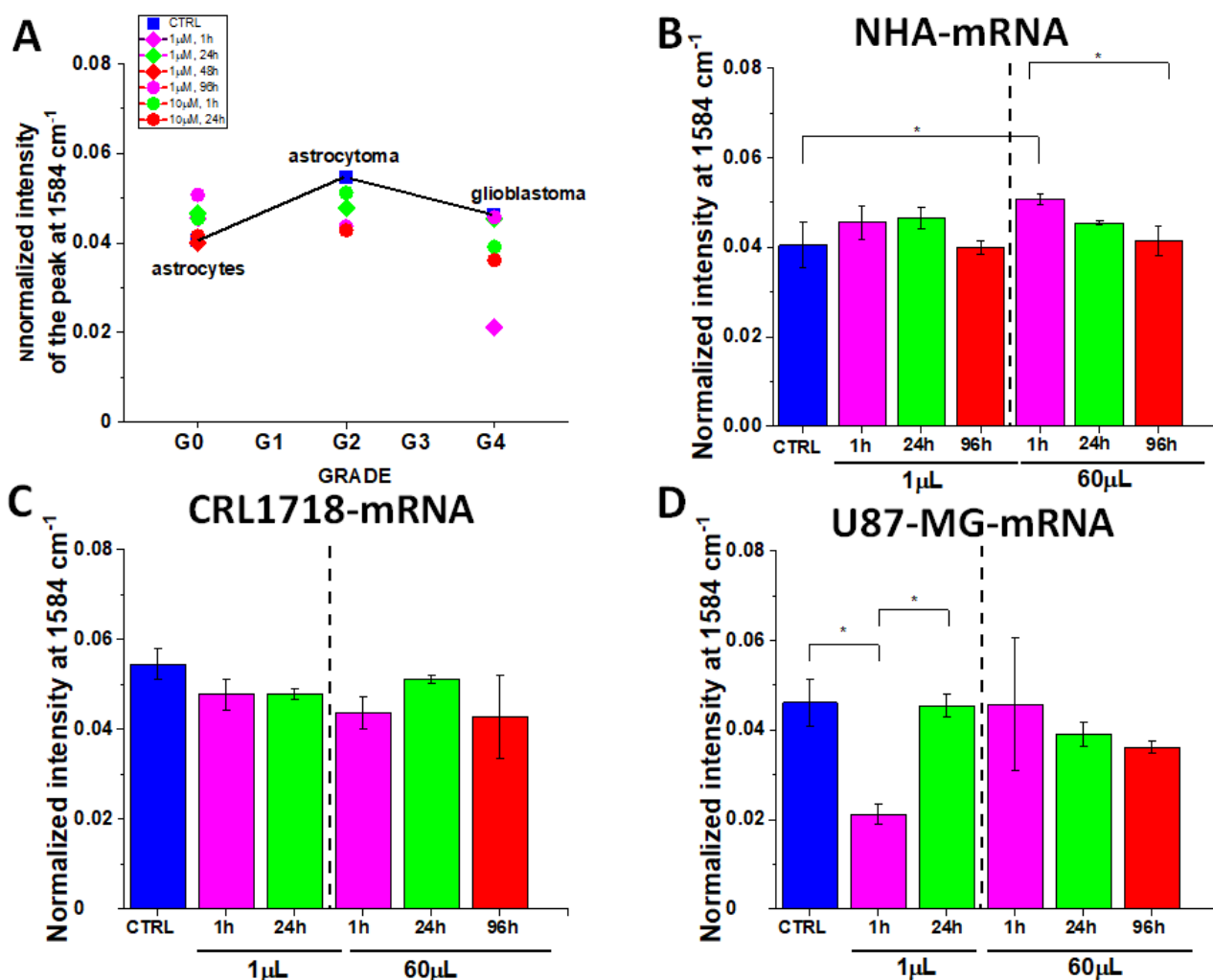
Figure 10. The effect of the Pfizer/BioNT vaccine on lipid droplets in glioblastoma cells (U87 MG) by Raman imaging: for U87-MG control cells without vaccine (dark blue) and upon incubation with vaccine for 96h for the dose 60 $\mu\text{L/mL}$ (red).

Recently we found that the Raman signal intensity of the band at 2845 cm^{-1} in lipid droplets is significantly higher for high-grade cancer of glioblastoma (U87 MG) than for normal astrocytes (NHA), indicating a higher concentration of TAGs in cancer lipid droplets [20]. The higher concentration of TAG was related to the increased amount of cytoplasmic lipid droplets in human glioblastoma cells in comparison to normal astrocytes.

Recently we showed that the lipid droplets in cancer cells are predominantly filled with TAGs and are involved in energy storage. The lipid droplets in normal cells NHA are filled mainly with retinyl esters /retinol and are involved in signaling, especially JAK2/STAT6 pathway signaling [22]. The results presented in Figure 9 suggest that upon incubation with mRNA increases the role of signaling. Our results support those reported recently that COVID-19 spike protein elicits cell signaling in human host cells, which may have serious implications for Possible Consequences of COVID-19 vaccines [27].

3.5. Cytoplasm-mRNA

Vaccine mRNA is translated by ribosomes in the cytoplasm. Therefore is particularly important to monitor alterations in cytoplasm upon incubation with mRNA. Figure. 11 shows effect of incubation with mRNA compared to the control cells without mRNA in cytoplasm.



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523 **Figure 11.** The normalized Raman intensity of the band 1584 cm⁻¹, (based on the Raman spectra
 524 normalized by vector norm) obtained for cytoplasm in normal cells of astrocyte (NHA) (A,B), astrocytoma
 525 (CRL-1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine (control, blue) and
 526 with Pfizer/BioNT vaccine: doses 1 μL/mL and 60 μL/mL, time of incubation 1h-magenta, time of incubation
 527 24h-green, time of incubation 96h-red. The one-way ANOVA using the Tukey test was used to calculate the
 528 value significance, asterisk * denotes that the differences are statistically significant, p-value ≤ 0.05.

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530 One can see that cytochrome c activity at 1584 cm⁻¹ in cytoplasm increases
 531 upon incubation with mRNA for normal astrocytes (NHA) at 60 μL/mL and for
 532 glioblastoma U87 MG at 1 μL/mL. In contrast, for astrocytoma and glioblastoma
 533 U87 MG The Raman signal at 1584 cm⁻¹ decreases upon incubation with
 534 mRNA. Statistical significance is presented in Figures 10 B,C,D.

535 The release of Cyt c from mitochondria to the cytosol activates apoptosis by
 536 the stream of caspase and proteases.[28] The concept that programmed cell death
 537 by apoptosis serves as a natural barrier to cancer development.[29] Therefore, the
 538 results provide new tools to check the role of Cyt c in an anti-apoptotic switch by
 539 phosphorylation of Tyr48.[30]

540 Our results from Fig. 10 show that apoptosis is reduced upon mRNA vaccine
 541 for astrocytoma and glioblastoma which indicate decreased ability to fight
 542 against cancer development by programmed cell death.
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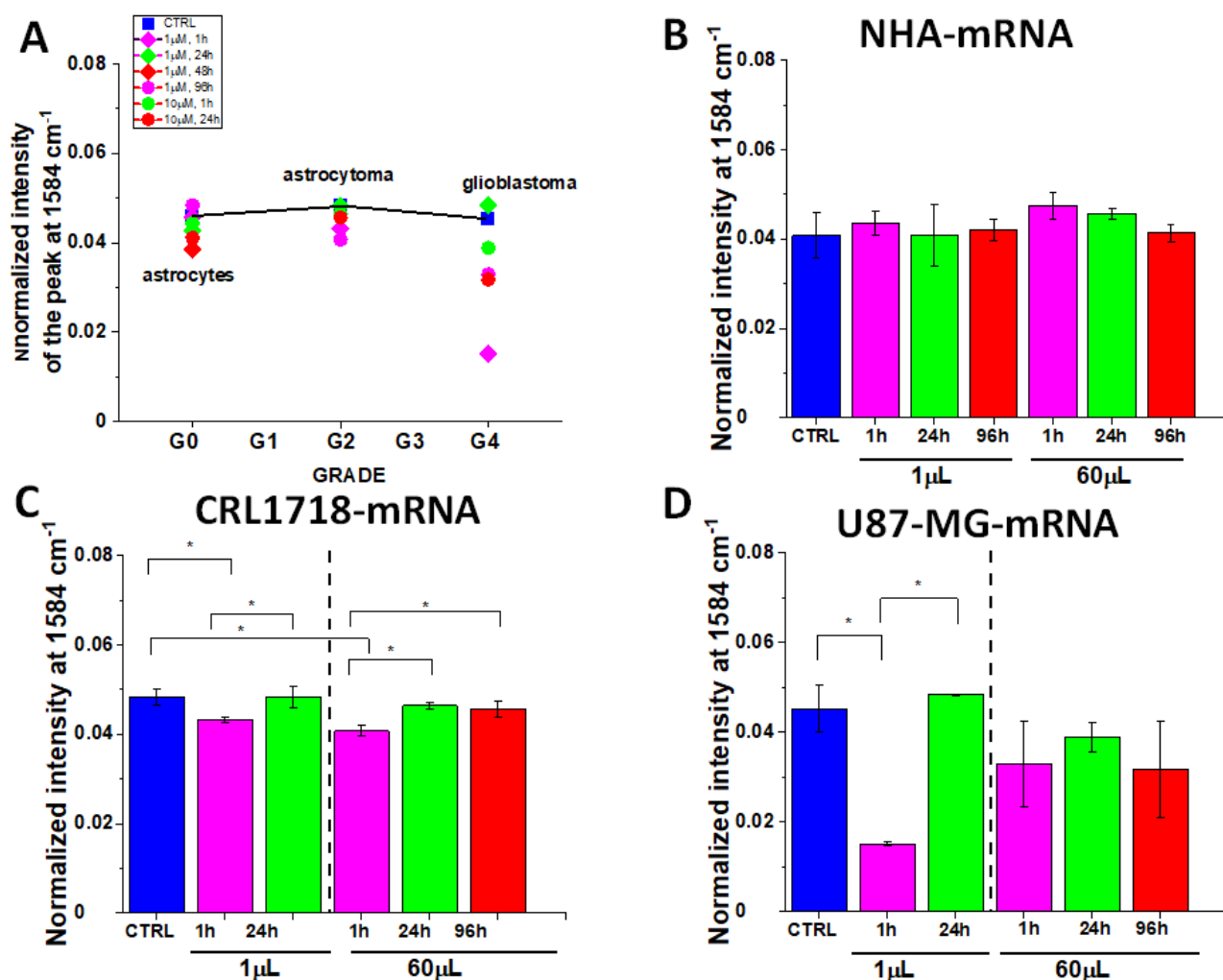
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3.6. Membrane-mRNA

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As it is well known professional antigen-presenting cells (APCs) play a crucial role in initiating immune responses. Under pathological conditions also epithelial cells act as nonprofessional APCs, thereby regulating immune responses at the site of exposure. Therefore it is interesting to monitor alterations at the surface of the cell membranes upon incubation with mRNA.

Figure. 12 shows effect of incubation with mRNA compared to the control cells without mRNA at surface of the membrane.



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Figure 12. The normalized Raman intensity of the band 1584 cm^{-1} , (based on the Raman spectra normalized by vector norm) obtained for cell membrane in normal cells of astrocyte (NHA) (A,B), astrocytoma (CRL-1718) (A,C) and glioblastoma cells (U87 MG)(A,D) without Pfizer/BioNT vaccine (control, blue) and with Pfizer/BioNT vaccine: doses $1\text{ }\mu\text{L/mL}$ and $60\text{ }\mu\text{L/mL}$, time of incubation 1h-magenta, time of incubation 24h-green, time of incubation 96h-red. The one-way ANOVA using the Tukey test was used to calculate the value significance, asterisk * denotes that the differences are statistically significant, $p\text{-value} \leq 0.05$.

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One can see that cytochrome c activity at 1584 cm^{-1} decreases for glioblastoma U87 MG with statistical significance at $p\text{-value} \leq 0.05$.

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Table 1. summarize the results presented above for the Raman intensity band at 1584 cm^{-1} for mitochondria, nucleus lipid droplets, cytoplasm and cell membrane for NHA, CRL1718 and U-87 MG cell lines, presented as the mean \pm SD based on the normalized Raman spectra. (normalization: divided by norm).

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Table 1. The comparison of the Raman intensity band at 1584 cm^{-1} for mitochondria, nucleus, lipid droplets, cytoplasm and cell membrane for NHA, CRL1718 and U-87 MG cell lines, presented as the mean \pm SD based on the normalized Raman spectra (normalization: divided by norm). Raman bands intensity were taken from normalized by vector norm spectra, number of cells=3.

572 **Table 1.**

	Dose	Incubation time	MITOCHONDRIA	NUCLEUS	LIPID DROPLETS	CYTOPLASM	MEMBRANE
NHA			0.04607±0.00613	0.0393±0.00591	0.04865±0.01003	0.04053±0.00515	0.04076±0.00515
	mRNA						
	60 µL/mL	1h	0.04836±0.00512	0.04208±0.00287	0.04337±0.00476	0.05073±0.00107	0.04742±0.00287
		24h	0.04435±0.00104	0.03966±0.00154	0.03254±0.00693	0.04545±4.60241E-4	0.04566±0.0012
		96h	0.04112±0.00127	0.03762±0.00233	0.02808±0.0054	0.04154±0.00332	0.04136±0.002
	1 µL/mL	1h	0.04567±0.00132	0.0389±0.001	0.04387±0.00344	0.0455±0.00387	0.04355±0.00267
		24h	0.04262±5.7E-4	0.04005±0.00267	0.03668±0.00773	0.04657±0.00235	0.0407±0.0069
		96h	0.0384±0.00182	0.03693±0.00371	0.04037±0.00766	0.03996±0.0016	0.04206±0.00246
CRL1718			0.06203±0.00381	0.05355±0.00364	0.05772±0.01587	0.05456±0.00338	0.04844±0.0018
	mRNA						
	60 µL/mL	1h	0.04469±0.00243	0.04091±0.00178	0.04265±0.00358	0.04368±0.00364	0.04071±0.00121
		24h	0.04908±0.00479	0.04429±0.00149	0.05135±0.00475	0.05119±9.37692E-4	0.04637±6.53265E-4
		96h	0.05654±6E-5	0.03941±0.0093	0.03382±0.00646	0.04282±0.00928	0.04566±0.00176
	1 µL/mL	1h	0.04757±0.00683	0.04544±0.00416	0.03745±0.01578	0.04775±0.00352	0.04323±5.60139E-4
		24h	0.04723±0.00405	0.04723±0.00405	0.04069±0.01214	0.04778±0.00121	0.0483±0.0024
U87-MG			0.03106±0.0075	0.03054±0.00809	0.01434±0.00424	0.0326±0.00659	0.04011±0.00312
	mRNA						
	60 µL/mL	1h	0.04242±0.01364	0.03744±0.00657	0.03042±0.00471	0.04573±0.01483	0.03293±0.0096
		24h	0.04014±0.00625	0.03682±0.00696	0.03869±0.00443	0.03911±0.00275	0.03883±0.00339
		96h	0.03397±0.0022	0.03761±0.00247	0.02832±0.00686	0.03616±0.00138	0.03174±0.01082
	1 µL/mL	1h	0.02613±0.00916	0.02123±0.00296	0.02406±0.00701	0.02121±0.00215	0.0151±5.06976E-4
		24h	0.04102±0.00192	0.03867±0.0031	0.02953±9.76761E-4	0.04547±0.00243	0.04836±1.64306E-4

4. Conclusions

We showed that new tools of Raman imaging we present in this paper raise exciting possibilities for new ways to understand links between pathways of cancer, immune responses, and recognize metabolites that regulates these pathways.

We used Raman spectroscopy to monitor changes in the redox state of the mitochondrial cytochromes in human brain cells *in vitro* of normal astrocytes, astrocytoma, glioblastoma upon incubation with mRNA vaccine. We observed the effect of the mRNA vaccine on biodistribution of different chemical components, particularly cytochrome c, in the specific organelles of human brain glial cells: nucleus, mitochondria, lipid droplets, cytoplasm, rough endoplasmatic reticulum and membrane.

We showed that mRNA vaccine (Pfizer) changes mitochondria by downregulation of cytochrome c resulting in lower effectiveness of respiration (oxidative phosphorylation) and lower ATP production. It can lead to lower immune system response.

Decrease of Amide I concentration in mitochondrial membrane potential may suggest functional deterioration of the adenine nucleotide translocator. mRNA vaccine modifies significantly *de novo* lipids synthesis in lipid droplets. The results presented in paper suggest that upon incubation with mRNA the role of signaling function of lipid droplets increases. The observed alterations in biochemical profiles upon incubation with the Pfizer/BioNT in the specific organelles of the glial cells are similar to those we observe for brain cancer vs grade of aggressiveness.

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Author Contributions: Conceptualization-H.A.; funding acquirement- H.A.; investigation-B.B-P, K.B.; visualization- B. B-P.; writing-original draft - H.A., B. B-P.; writing - B.B-P., K.B.; review - H.A; editing-B.B-P., K.B. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: The raw data underlying the results presented in the study are available from Lodz University of Technology Institutional Data Access. Request for access to those data should be addressed to the Head of Laboratory of Laser Molecular Spectroscopy, Institute of Applied Radiation Chemistry, Lodz University of Technology. Data requests might be sent by email to the secretary of the Institute of Applied Radiation Chemistry: mitr@mitr.p.lodz.pl.

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