



Article

Vibrational Markers of a Model Circulating Metastatic Cells LLC-R9

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Abstract: Metastasis in oncological diseases remains one of the main reasons for negative prognosis regarding treatment. Any new data on the biophysical and biochemical characteristics of circulating metastatic cells will help to develop a concept for antimetastatic therapy. In this study, we found a number of differences in the spectroscopic and morphological features of circulating metastatic cells. FT-IR and Raman spectra cultivated by adhesive and de-adhesive methods (with the latter used as a model for metastatic cells) have shown spectroscopic features, namely in FT-IR spectra in the region of CH stretching vibrations, which are associated with structural rearrangements in the cell membrane, as well as changes in the intensity and position of the PO_2^- group vibration bands correlated with proliferative activity. The spectral features in the regions of OH stretching and Amide I vibrations as well as other spectral markers of the metastatic cells grown under different cultivation conditions were derived. Raman spectra showed a redistribution of the amino acid Tyr/Trp (tryptophan to tyrosine) ratio and in Tyr doublet intensity in the region of $500\text{--}900\text{ cm}^{-1}$, as well as varying glycogen levels in different cells. The spectroscopic markers are in accordance with biochemical data. CARS and confocal optical microscopy were applied to determine the state of the cells and the F-actin expression level, which turned out to be higher for adhesive cells in comparison with de-adhesive cells. The shape and the morphological properties of the cells differ drastically. The correlation of vibrational markers with biochemical data and the cytofluorometric method was discussed.

Keywords: metastatic cells; Lewis lung carcinoma tumor cells (LLC-R9); adhesive and de-adhesive cultivation; spectroscopic and microscopic markers; FT-IR; Raman; CARS spectroscopy; confocal microscopy; F-actin expression



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1. Introduction

Metastases are known as the final stage of tumor progression and significantly complicate the course of cancer development. Most deaths (>90%) of cancer patients are not caused by the primary tumor but by metastatic damage to vital organs. Metastasis is a process during which cancer cells spread from the primary tumor to distant organs and

tissues in the patient's body. At the first stage of this process (metastatic cascade), the tumor cell must separate from the primary tumor and enter the bloodstream, becoming a circulating metastatic cell [1,2].

Studying the biochemical and morphological characteristics of such cells is an extremely difficult task because when they enter the blood, most of them die via apoptosis. However, a small part still survives and reaches another healthy organ, where secondary tumors (metastases) develop.

Carcinoma is the most common type of cancer, accounting for 80% to 90% of all neoplasm diagnoses. Carcinoma forms in the epithelial tissue that lines various organs, internal body cavities, and the skin. One of the properties that distinguishes normal epithelial cells from tumor cells with metastatic potential is their adhesiveness. Cell adhesion is the process where the cells interact and attach to the neighboring cells through the specialized cell's surface molecules. When adhesion is disrupted, it leads to the death of normal epithelial cells by apoptosis. Metastatic tumors contain metastatically active cells that can survive by detaching from the main body of the tumor.

The latest research has made it possible to identify the main biological phenomena that characterize the process of metastasis, including the epithelial–mesenchymal transition of cells, during which the epithelial intercellular contacts are lost (adhesion disorders, reorganization of the cytoskeleton, and changes in the shape of cells), while the epithelial markers (cytokeratins, occludins, and E-cadherins) are replaced by mesenchymal ones (N-cadherin and vimentins). Also important is the formation of clusters of circulating metastatic cells with other cells, including normal cells, as well as the important influence of the microenvironment [3,4]. The task of developing an effective model of circulating metastatic cells is important both for studying the mechanisms of metastasis and for developing new antimetastatic drugs. In our previous works [5,6], it was shown that the transition from adhesive to non-adhesive cell growth is accompanied by differences in glucose consumption, lactate production, intracellular level of ROS (reactive oxygen species), and proliferative heterogeneity of the LLC cells.

From the end of the last century, biomedical vibrational spectroscopy began to develop actively and became a separate field of biophysical research [7]. The methods of infrared and Raman spectroscopy are widely applied for testing of the molecular structure and conformation states of biological polymers [8] as well as the organization of complex molecules; drug influence on the organelles, cells, tissues, and viruses; diagnostics of diseases [9,10], etc. New methods, such as surface-enhanced infrared absorption (SEIRA) [11,12] and surface-enhanced Raman spectroscopy (SERS) [13] drastically enhanced the sensitivity of the methods of vibrational spectroscopy. The application of non-linear optical methods such as second harmonic generation (SHG) [14,15] and coherent anti-Stokes Raman scattering (CARS) spectroscopy and imaging [16,17] introduced new possibilities by recording not only the spectrum but also the object image in a mapping mode, identifying organelles [18], proteins, lipids, DNA, etc. [19,20], with greater sensitivity than in linear optics.

Spectroscopic data are analyzed and processed with different mathematical approaches, including cluster analysis, principal component analysis, neural networks, etc., that are fundamental for various databases and artificial intelligence technologies now and in the near future [7].

Despite the fact that much attention has already been paid to studying tumor cells and tissues by spectroscopic methods and significant results have been obtained [21,22], there are practically no data on metastatic cells. Here, we present vibrational and microscopic data on model metastatic cells arising from oncological disease. The evaluation of the biophysical and biochemical characteristics of circulating metastatic cells can give us a new concept for antimetastatic therapy as well as clear up the mechanism of disease.

2. Materials and Methods

Cells from the National Bank of Cell Lines and Tumor Strains of the IEPOR (R.E. Kavetskiy Institute of Experimental Pathology, Oncology, and Radiology) of the National

Academy of Sciences of Ukraine were used in this study, namely the Lewis lung carcinoma cells LLC/R9. This tumor cell strain was obtained as a result of a number of chemotherapy courses and is resistant to cisplatin, has a high angiogenic potential, and is less metastatically active than the original LLC strain [6,23,24]. The cell cultures were grown *in vitro* at 37 °C in a humidified atmosphere with 5% CO₂ in an RPMI 1640 incubation medium containing 10% fetal calf serum, 40 µg/mL gentamicin, and 2 mM L-glutamine. The LLC/R9 cells at 0.3×10^6 were seeded in 60 mm Petri dishes. These dishes were either pretreated with poly-HEMA solution to model de-adhesive growth or left untreated to model adhesive cell growth (Figure 1). The cells were then incubated for 3 days without changing the incubation medium. The original cells were taken as an initial reference point.

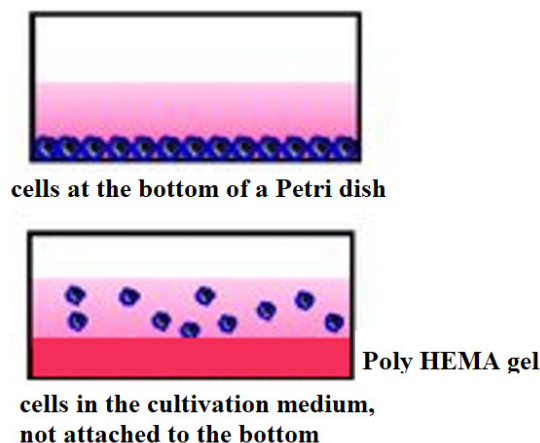


Figure 1. Picture of incubation of tumor cells during their adhesive and de-adhesive growth. For the latter, Petri dishes were treated with poly-HEMA solution.

Staining of the LLC/R9 cells during adhesive growth was performed when the cells were grown on coverslips. The staining of the LLC/R9 cells under de-adhesive growth was carried out in suspension (1.5×10^6 cells) to preserve the native shape of the cells. Coverslips with the cells were fixed in 4% formalin in FSB at 37 °C for 15 min, after which the cells were washed 3 times with 3 mL of FSB and permeabilized in 0.5% Triton X-100 in FSB at room temperature for 1 h for phalloidin staining (Fluor488), after which FSB was washed 2 times. Blocking of nonspecific binding was performed by incubating coverslips or the cells in 10% ETS in FSB for 1 h at room temperature. After blocking, the cells were stained with phalloidin in 10% ETS/FSB at a dilution of 1:1000 at room temperature for 1 h. After staining, the coverslips and the cells were washed with FSB 3 times, and then the drugs were embedded in a ProLong Glass Antifade Mountant with NucBlue (cat. number P36983, Invitrogen, Waltham, MA, USA) for their polymerization overnight, after which the microscopic studies of the cells were performed.

The Raman spectra of the cells were recorded at room temperature in the backscattering geometry using a Horiba Jobin-Yvon T64,000 triple Raman spectrometer ($200\text{--}1700\text{ cm}^{-1}$) with excitation by solid-state laser Spectra Physics EXLSR-532-150-CDRH (532 nm).

The FTIR spectra of the cells were recorded at room temperature using INVENIO-R (Bruker, Ettlingen, Germany) instrument in the $3800\text{--}600\text{ cm}^{-1}$ spectral region in the attenuated total reflection mode. For these measurements, we used Bio-ATR attachment, which was created by Bruker Optics GmbH, especially for the investigation of biomolecules, cells, and tissue. The wavenumber accuracy was about 0.01 cm^{-1} , and the absorbance accuracy was about 0.1%. For registration of ATR spectra, the samples were deposited on a working surface of the Bio-ATR attachment and then dried in the nitrogen flow at room temperature. All FT-IR spectra were baselined and normalized to a maximum of Amide A absorption band at 3290 cm^{-1} . The positions of the absorption bands were determined using OPUS 8.2 software (Bruker). For spectra deconvolution, the PeakFit4.0 program was used, and the band contours were approximated by a Gaussian curve.

The images of the cells stained with fluorescent dyes were obtained on a laser scanning confocal microscope Carl Zeiss (Oberkochen, Germany) LSM 510 META. The research was carried out in three modes: in the light microscope mode (in the light of a halogen lamp, which illuminated the sample), in the fluorescent microscope mode (in the light of an ultraviolet mercury lamp HBO-103 W/2, which excited the fluorescence of dyes), as well as in the confocal microscope mode (excitation of fluorescence from the dyes using lasers). A Plan-Apochromat 40 $\times$ /1.30 Oil DIC objective was used to obtain overview images of the cells, and a Plan-Apochromat 100 $\times$ /1.4 Oil DIC objective was used for obtaining more detailed ones. Confocal images were obtained in the MultiTrack mode, when the fluorescence of the dyes was excited and recorded. A diode laser (λ = 405 nm, 25 mW) was used to excite the Hoechst 33342 dye emission, and a BP 420–480 nm emission light filter was used to record the fluorescence. An argon laser (λ = 488 nm, 30 mW) was used to excite the iFluor 488 dye, and a BP 505–570 nm emission light filter was used to record the fluorescence.

We used a unique home-made CARS system assembled at the Lithuanian Centre for Physical Research and Technology, Vilnius, Lithuania (FTMC). The system consists of a simple and compact picosecond pulsed Nd:YVO₄ laser (EKSPLA Ltd., Vilnius, Lithuania) with a pulse repetition rate of 1 MHz and an optical parametric oscillator with a tunable frequency from 700–4500 cm^{−1}.

3. Results

Fourier-transform infrared spectroscopy (FT-IR) is a powerful tool for studying biological objects, such as biomolecules, cells, and tissues. Unlike staining procedures and other biochemical analysis methods, this technique is fast, non-invasive, and does not require additional treatment with drugs or dyes. Spectral differences between cancerous and normal cells indicate the differences in metabolic processes and allow them to be distinguished. FT-IR spectroscopy provides us with a tool that allows one to establish a molecular-level correlation between spectral markers and morphological features in the structure of the objects under study. However, the widespread use of infrared spectroscopy in biology, biochemistry, and clinical practice faces certain difficulties in identifying and deciphering complicated spectral information. The infrared absorption spectrum of a cell is a superposition of the absorption of its molecular components, such as proteins, lipids, and nucleic acids [25]. In our previous works, we performed spectral analysis of cellular components [10,11] of normal and pathological cells [26,27].

The following model was used to analyze the FT-IR absorption spectra (see Figure 2). The high-frequency region of 3800–2400 cm^{−1} refers to the absorption of hydrogen-bonded OH and NH molecular groups. The region of CH stretching (the bands at 2950, 2920, 2880, and 2850 cm^{−1}) is related to the absorption of lipids, while CH bending vibrations (the bands 1460–1320 cm^{−1}) are mainly related to the absorption of proteins [28]. Proteins are the major contributor to 950–1700 cm^{−1} absorption, whereas lipids are the major contributor to 2800–2950 cm^{−1}. DNA, RNA, and glycogen only make substantial contributions in the absorption between 950 and 1300 cm^{−1}, where protein is a major contributor, too. There are no DNA or RNA absorbance bands that do not overlap with those of other components.

The ratio of the peak height at 1400 cm^{−1} to the peak height at 2852 cm^{−1} is used as a measure of protein/lipid content. The absorption of the protein fraction is analyzed by the Amide I (at 1645 cm^{−1}) and Amide II (at 1545 cm^{−1}) bands. The protein component of the cell is characterized by the absorption of the amide group, i.e., the peptide bond-CO-NH-vibration bands. Amide I and Amide II bands for α -helix, β -sheet, and the disordered polypeptide chain structure have clearly distinguished frequencies, which allows to use them for conformational analysis of the secondary structure of the protein fraction.

For polypeptide chain-ordered conformations, the absorption band at 1658 cm^{−1} consists of several components due to specific interactions between neighboring peptide groups. For antiparallel β -sheets, the most intensive component is observed at 1632 cm^{−1} and the weakest one at 1685 cm^{−1}. The A-helix structure is characterized by a strong

band at 1650 cm^{-1} and a weaker band at 1646 cm^{-1} [29]. The contribution from nucleic acid fraction to the FT-IR spectrum of a cell overlaps with the absorption of protein in the $1800\text{--}1500\text{ cm}^{-1}$ region and lipid fractions in the PO_2^- region, but in the latter, the contribution from the phosphate backbone of nucleic acids [30] dominates.

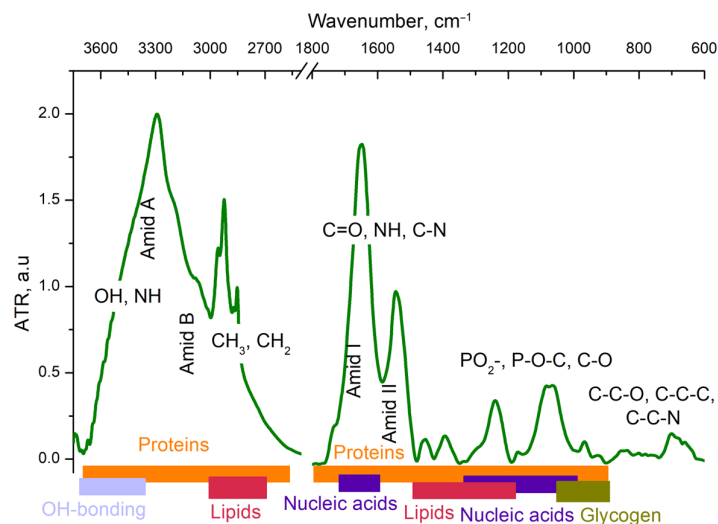


Figure 2. Regions of FT-IR absorption spectrum of LLC cells that can be attributed to the absorption of lipid and protein fractions, and nucleic acids.

3.1. IR Spectral Analysis of LLC-R9 Tumor Cells

Significant differences in the FT-IR absorption spectra of LLC-R9 cells grown under adhesive or de-adhesive conditions were registered in the regions of hydrogen bonds ($3600\text{--}3000\text{ cm}^{-1}$), lipid fraction ($3000\text{--}2850\text{ cm}^{-1}$), proteins ($1700\text{--}950\text{ cm}^{-1}$), and nucleic acid ($1350\text{--}950\text{ cm}^{-1}$) vibrations.

In the region of stretching vibrations of hydrogen-bonded OH groups, an increase in the contribution of weak hydrogen bonds is observed for de-adhesive LLC-R9 cells, which is manifested as a broadening of the Amide A band, while the position of the band remains unchanged (3290 cm^{-1} , NH stretching vibrations) for all three samples (Figure 3).

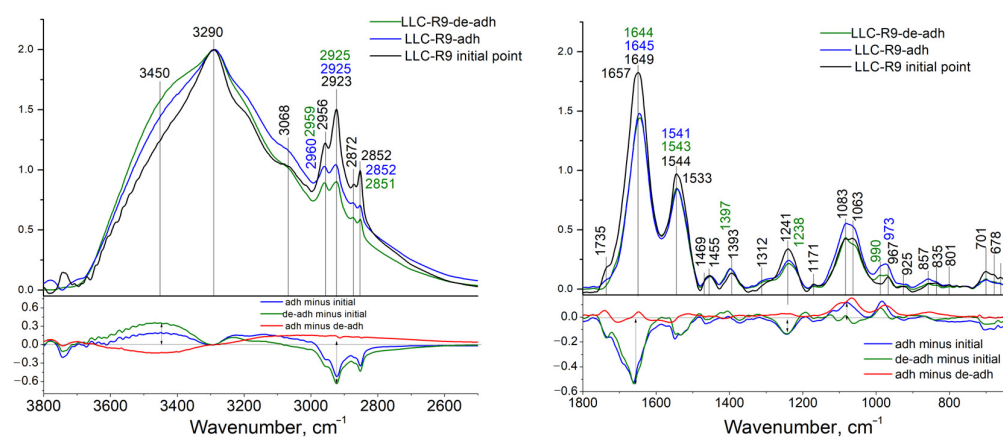


Figure 3. Normalized FT-IR absorption spectra of LLC-R9 cells grown under adhesive (blue curve) and de-adhesive (green curve) conditions. The black curve refers to initial cells. The bottom part of the figure shows the difference spectra. Photometric accuracy—above 0.001.

The area under the low-frequency shoulder of this band (from $3400\text{ to }3700\text{ cm}^{-1}$) for the cells grown using de-adhesive method is 399, 312 for the adhesive method, and for the zero point, this indicator is 308.

According to the hydrogen bond theory, a weakening of hydrogen bonding can be assumed for de-adhesive growth (we register an increased intensity of 3450 cm^{-1} shoulder of the OH stretching band centered at 3290 cm^{-1}). The appearance of a shoulder at 3450 cm^{-1} corresponds to a decrease in the enthalpy of H-bonding according to [31,32], Equation (1):

$$-\Delta H (\text{kcal/mol}) = 0.3 (\Delta\nu_{\text{OH}} - 40)^{1/2} \quad (1)$$

According to [33], the frequency of unbound free OH is 3700 cm^{-1} . An estimation of H-bond enthalpy using Equation (1) shows that the enthalpy of new H-bonding is, on average, 1.4 kcal/mol less for de-adhesive cells than for adhesive ones.

In the region of CH stretching vibrations, we registered more intense CH_2 vibration bands (centered at $2923, 2852\text{ cm}^{-1}$) for LLC-R9 initial cells, while the contribution of CH_3 vibrations remained unchanged. In this case, the method of cultivation—adhesive or de-adhesive—does not significantly affect the region of CH stretching vibrations; however, as compared to the initial point cells, we see a decrease in the contribution of CH_2 and an increase in that of CH_3 group absorption, which suggests changes in the structure of the cell membrane (Figure 3a).

In the Amide I absorption region (Figure 4), we noted the differences in the width and position of this band for LLC-R9 cells, both for their adhesive and de-adhesive growth. In particular, for de-adhesive cells, the Amide I band has a symmetrical shape with a maximum near 1644 cm^{-1} , which can be attributed to the predominant contribution from α -helical protein components. Such features may be directly related to the proliferative heterogeneity of the two cell strains. In particular, this observation is consistent with previous findings that, on the third day of cultivation, the LLC-R9 strain had a predominant contribution from the G2/M phase, characterized by active protein synthesis [6]. For a detailed analysis of this spectral region, the Amide I band was decomposed into components. The following model assignment of the components was used: α -helix 1645 cm^{-1} , β -sheet folded structures 1690 cm^{-1} and 1630 cm^{-1} , turns 1675 cm^{-1} , side groups 1615 cm^{-1} , and disordered structure 1659 cm^{-1} (see Figure 4 and Table 1 [29]). The component analysis confirms a larger contribution from α -helical protein for LLC-R9 cells grown using both adhesive and de-adhesive methods, but the contribution from disordered structures is greater for the adhesive growth method.

While fitting this region, for de-adhesive cells, we noted an increase by 0.5% in contributions of turns, and a decrease by 1.5% of the disordered phase, while a larger amount of disordered forms was registered for adhesive cells. Therefore, in de-adhesive cells, the protein is better ordered, compacted in the form of microfilaments, and so the content of the disordered phase is smaller. These data are in good agreement with confocal microscopy results, which confirm the compaction of F-actin and the formation of microfilaments in de-adhesive cells.

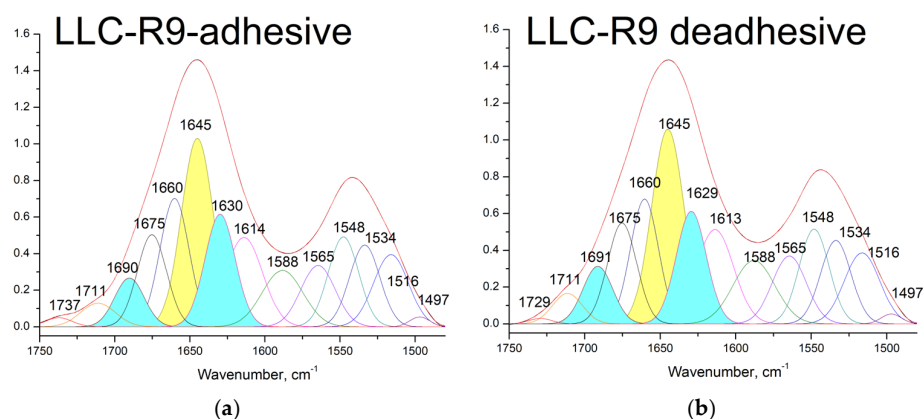


Figure 4. Decomposition of the Amide I band of LLC-R9 cells grown under adhesive (a) and de-adhesive (b) conditions. $r^2 = 0.999$. Yellow area— α -helix, blue area— β -sheet structures, red line—calculated curve, black line—experimental curve.

Table 1. The LLC-R9 cells Amide I IR absorption band components.

Secondary Structure	LLC-R9 Adhesive		LLC-R9 De-Adhesive		Differences (De-Adh minus Adh)
	Position, cm^{-1}	Contribution in% of Total	Position, cm^{-1}	Contribution in% of Total	
α -helix	1645	26.2	1645	26.4	0.2
β -sheet	1630, 1690	21.3	1629, 1691	21.5	0.2
Turns	1675	11.8	1676	12.3	0.5
Side groups	1614	14.6	1613	14.8	0.2
Disordered form	1660	16.3	1660	14.8	−1.5

The analysis of the PO_2^- asymmetric vibration band in the region of $1340\text{--}1180\text{ cm}^{-1}$ (Figures 3 and 5) shows a lower-frequency shift to 1237 cm^{-1} and to 1238 cm^{-1} for adhesively and de-adhesively grown LLC-R9 cells, respectively. However, in the case of adhesive growth, there is a major contribution from the main absorption band corresponding to the α -form of DNA, with weaker lateral components corresponding to other forms of DNA. And, in the case of the de-adhesive growth, the contribution of the component at 1218 cm^{-1} increases significantly.

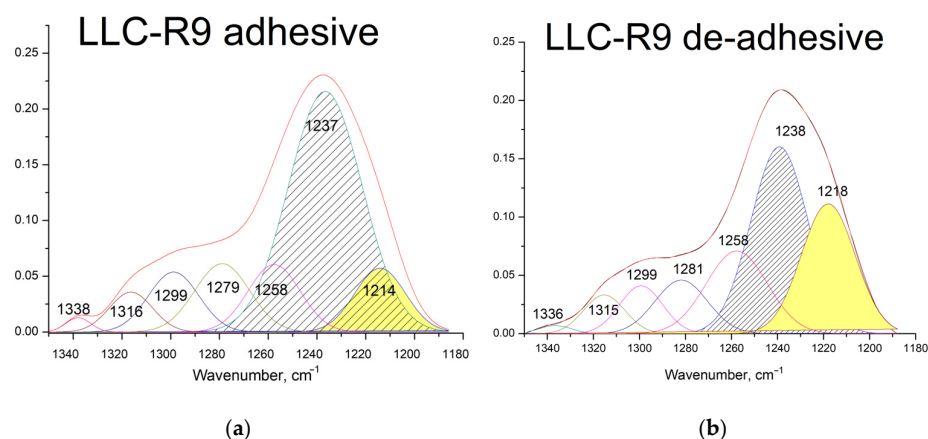


Figure 5. Decomposition of PO_2^- asymmetric stretching vibration band of LLC-R9 cells: (a) adhesive and (b) de-adhesive growth. $r^2 = 0.999$. Yellow and shaded area— PO_2^- asymmetric vibration for different forms of DNA, red line—calculated curve, black line—experimental curve.

Thus, de-adhesively grown cells show an increased content of proteins and DNA conformations, as well as membrane reorganization in comparison with adhesive cells. Spectral indicators of this transformation are the redistribution of H-bonds, changes in CH stretching vibrations, Amide I, and phosphate absorption bands, as well as transformations in the low-frequency region responsible for vibrations of big parts of the molecules (Table 2).

Table 2. The LLC-R9 cells IR absorption band assignment. The frequencies with the major differences are marked by colors.

LLC-R9			
De-Adhesive	Adhesive	Initial Point	Assignment
3450	3450	3450	ν OH
3288	3286	3290	Amide A, ν NH,
3066	3068	3068	Amide B, Fermi resonance Amide II
2959	2959	2956	ν as CH_3
2923	2925	2923	ν as CH_2 asym
2873	2872	2872	ν s CH_3 sym
2851	2852	2852	ν s CH_2 sym

Table 2. Cont.

LLC-R9			
De-Adhesive	Adhesive	Initial Point	Assignment
	1735	1735	ν C=O
-	-	1657	Amide I, ν C=O, ν C-N, δ N-H
1644	1645	1649	Amide I, ν C=O, ν C-N, δ N-H, δ OH
1543	1542	1544	Amide II, ν C-N, δ N-H
-	-	1533	Amide II, ν C-N, δ N-H
1451	1454	1455	δ CH ₂
1398	1396	1393	δ CH ₃
1238	1237	1240	ν PO ₂ -asym
1218	1214		ν PO ₂ -asym
1170	1171	1171	ν C-C, δ C-OH, ν C-O, C-OH
1084	1081	1083	ν PO ₂ -sym
1058	1062	1063	ν C-O deoxyribose
990	987		C-C, C-O DNA and deoxyribose
970	973	967	C-O DNA and deoxyribose
917	917	925	C-C, C-O
858	858	854	C _{3'} endo/anti (A-helix of DNA)
834	835	835	C _{2'} endo/anti (B-helix of DNA)
778	780	782	Out-of-plane bend
	699	701	Out-of-plane bend
		678	C-C-H out-of-plane bend
	655	660	C-C-O out-of-plane bend

The frequencies with the major differences are marked by colors.

3.2. Analysis of Raman Spectra

In the CH stretching vibration region, we do not observe any significant differences between the two cell cultivation methods. However, in the low-frequency region of 400–900 cm⁻¹, there are different contributions attributed to individual amino acids: tyrosine (Tyr), tryptophane (Trp), etc. For example, the bands at 856–830 cm⁻¹ are attributed to tyrosine out-of-plane and in-plane ring breathing (854 cm⁻¹ and 825 cm⁻¹, respectively), and the band in the region of 780–750 cm⁻¹ to tryptophane. The ratio between the bands 854 cm⁻¹ to 825 cm⁻¹ is a spectral marker for the H-bonding environment changes influencing the structural transformation in proteins (Figure 6, Table 3). The Tyr/Trp ratio increase from 1.33 to 1.67 suggests a transition of Tyr from an electron acceptor in H-bonding to an electron donor [34]. Tyr can indicate changes in intermolecular interactions due to environmental changes and the reorganization of proteins under the de-adhesive method of cell growth.

Another band at 484 cm⁻¹ refers to glycogen uptake, which is more intense for de-adhesive growth. Such changes may indicate the differences in metabolic processes. This is consistent with previous conclusions drawn from the IR spectroscopic studies.

Raman spectra features in the region of 400–900 cm⁻¹ are assigned to different amino acids, DNA, and glycogen. We observed different Tyr doublet intensity ratios, 854/825, Tyr/Trp and Trp/DNA ratios, as well as different glycogen levels for adhesive and de-adhesive cells. We can also see the difference in the mid-range region; however, there is a strong overlap of spectral bands.

Table 3. The Raman intensities ratio Tyr/Trp, Tyr/Tyr, Trp/DNA and glycogen band intensity (arb.u.) for the cells of different cultivation.

	825 Tyr/750 Trp	854Tyr/825 Tyr	750 Trp/785 DNA	Glycogen, a.u.
De-adhesive	0.43	1.67	1.0	4.77
Adhesive	0.50	1.33	0.85	1.85
Initial—point	0.60	1.33	0.83	-

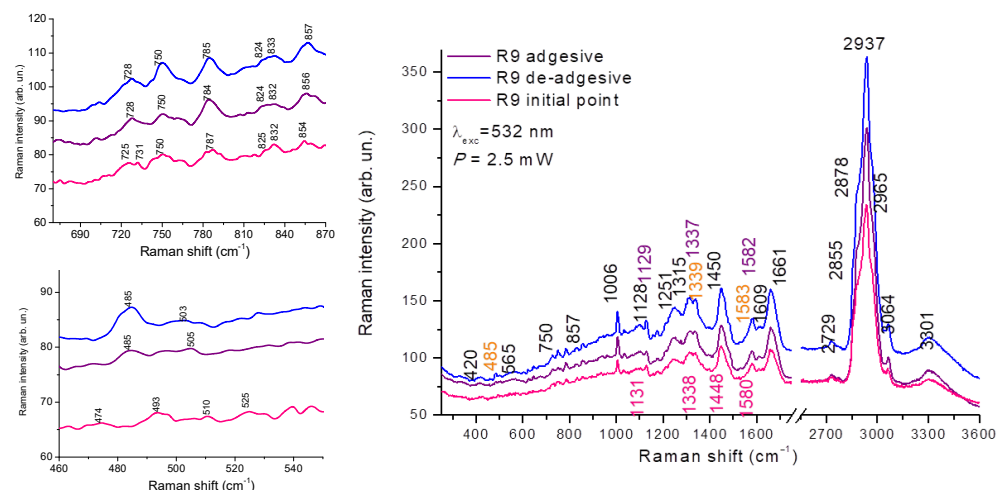


Figure 6. Raman spectra of LLC-R9 cells grown under adhesive and de-adhesive method in the region of $3600\text{--}250\text{ cm}^{-1}$.

3.3. Experimental Data from CARS Microscopy

For the registration of CARS images, adhesive LLC/R9 cells were grown on a glass slide surface. After culturing the cells for 1 day, the slides were washed with phosphate buffer and then dried under a fume hood, then used for CARS spectroscopy and imaging [35].

Due to the high non-resonant background at the Lithuanian CARS facility, it was not possible to detect structural components in the cells. That is why we used only one type of model cell, namely the adhesive ones.

CARS images were registered at 1600 cm^{-1} in forward and epi modes. The CARS image of the cell was clear, with high contrast. The membrane is seen particularly clearly, but the internal morphology of the cell is difficult to discern. Some heterogeneity in cell volume is noticeable, but it is difficult to clearly distinguish the nucleus or other organelles. At the first registration, a single bright spot is observed on the image of the cell. During the second pass, it becomes larger and noticeably brighter. In epi mode, the cell looks more contrasted. The heterogeneity in the structure of the membrane is visible (Figure 7).

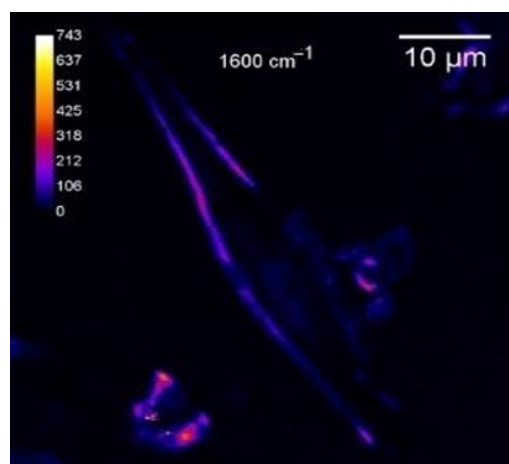


Figure 7. Epi-mode CARS (E-CARS, $940\mu\text{W}_1\text{H}_15400\mu\text{W}$) image of LLC/R9 cells on the cover glass surface. Resonance at 1600 cm^{-1} .

According to Hamaguchi [36], the Raman line near 1600 cm^{-1} is a characteristic of the vitality of cells, so-called “Raman spectroscopic signature of life”, and, really, a strong CARS line was observed at 1595 cm^{-1} only when the cells were under good nutrient conditions. A sudden disappearance of the band near 1600 cm^{-1} followed by the change in the shape and intensity of phospholipid bands was observed, indicating a strong relationship between

the cell activity and the intensity of this band. We, therefore, after Hamaguchi, can call this band “CARS spectroscopic signature of life”. A difference in positions of the lines in Raman and CARS spectra is usually about $3\text{--}15\text{ cm}^{-1}$ [35]. We registered only a weak Raman band (Figure 6, the Raman line at 1609 cm^{-1}) contrary to CARS, which is seemingly due to near-resonant conditions in CARS. After repeated scans, we see the bright dots in the cell (Figure 8), which previously were not observed (Figure 7). We suppose that the bright spots are attributed to Cytochrome C.

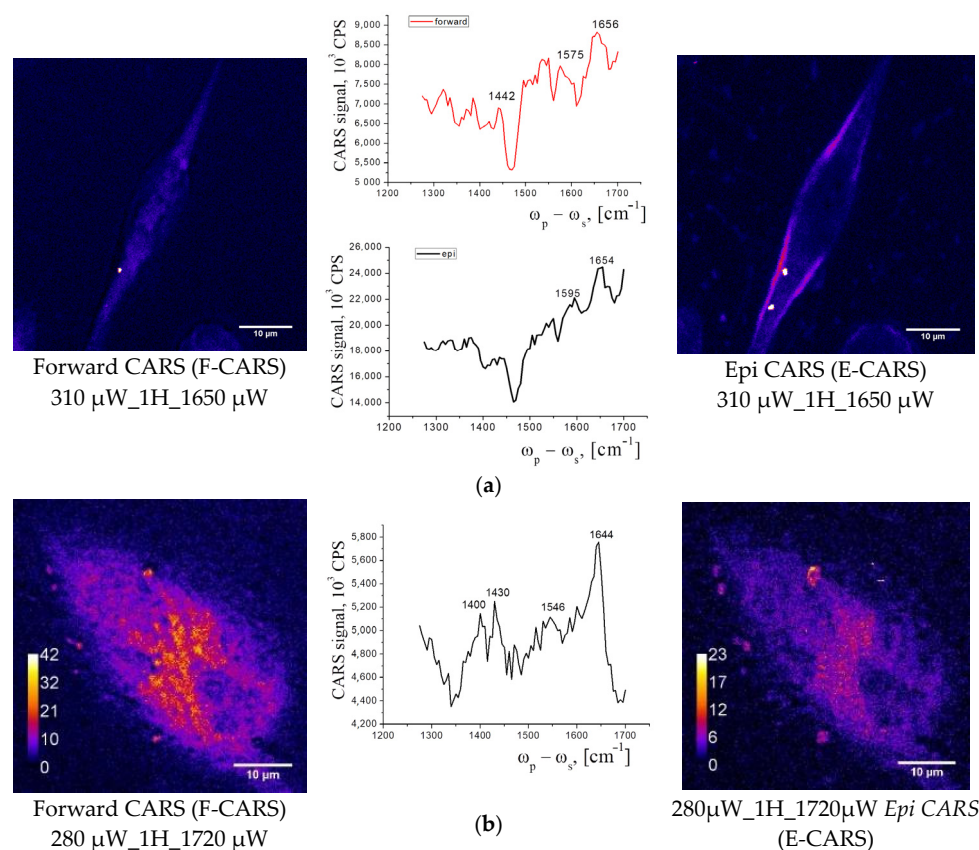


Figure 8. CARS spectra and images of LLC/R9 adhesive cells on cover glass surface. Resonance at 1600 cm^{-1} . The scale bar is $10\text{ }\mu\text{m}$. (a) CARS image of normal LLC/R9 cell. (b) CARS image of necrotic LLC/R9 cell.

Indeed, we found round-shaped cells in the adhesive cell population and assigned them to the early stage of apoptosis or necrosis. However, it is possible to assume a change in the functional activity of these cells while they are alive.

From the overall appearance of CARS spectra (Figure 8), we can say that the images obtained are off-resonant but close to it. However, there are certain features in the spectra that are characteristic of the cells. Namely, the bands in the $1500\text{--}1600\text{ cm}^{-1}$ region can be attributed to the C=O and C=C vibrations of proteins and lipids. In the case of necrosis sample, the cells look damaged, the membrane is broken, and the position of the Amide I band is shifted to the low-frequency region from $1654\text{--}1656\text{ cm}^{-1}$ (α -conformation) in the upper images to 1644 cm^{-1} , indicating an increase in β -protein conformations (Figure 8, image and spectrum). The samples appear to be completely flat and have no volume. The image is not of a high contrast and the signal intensity is low. Deformation vibrations of CH_2 at 1400 and 1430 cm^{-1} were registered and belong to proteins. A characteristic minimum at 1470 cm^{-1} seems to be associated with the electronic subsystem excitation.

Thus, CARS images and spectra with higher sensitivity and contrast are supplemental to Raman spectra; however, it should be noted that this home-made instrument is not

sensitive enough to provide information about the structural components of the cell, and that is why we applied confocal microscopy and staining below.

3.4. Confocal Microscopy Data

It is known that the process of the transition of cancer cells to a metastatic state is accompanied by the formation of invasive structures in the cell, which, in turn, leads to rearrangements of the cytoskeleton. Therefore, the study of F-actin expression is an important stage for understanding epithelial-to-mesenchymal or/and mesenchymal-to-epithelial transitions and, therefore, for the development of new approaches for antimetastatic therapy [37,38]. Visualization using the method of confocal microscopy allows one to study the distribution and shape of actin fibers.

Immunostaining data recorded with a confocal microscope can be used to quantify the relative levels of a test molecule by measuring the average fluorescence intensity in the region of interest (ROI), the number of cells, and the percentage of the cells in the sample that are “positive” for staining with a fluorescent probe. Thus, in these cases, the MFI (mean fluorescence intensity) value in the ROI, calculated as the sum of pixels in the ROI divided by the total number of pixels, is likely to accurately detect whether the cell/tissue sample has markers that differ from the control.

Other similar methods, such as Western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and flow cytometry (FC), have long been used to assess and quantify the relative expression of protein in cultured cells and tissues. However, WB and ELISA have a limited ability to reliably quantify relative protein levels in tissues with complex cellular composition, while tissue dissociation followed by FC is not possible when tissue is limited and/or cells are difficult to isolate. While the detection of protein in tissue using immunofluorescent (IF) probes has traditionally been considered a qualitative technique, advances in probe stability and confocal imaging make IF data easy to quantify, although reproducible quantification of relative protein expression requires careful attention to appropriate controls, experimental design, and data collection.

In the mouse aortic endothelial cells (MAECs), which can be used as reference non-tumor cells, the F-actin fibers are uniform in size, evenly distributed, located in narrow angle ranges, crossing the nucleus lines, and their clusters are closer to the nucleus. Alexa Fluor488 dye was used in the experiment (Figure 9).

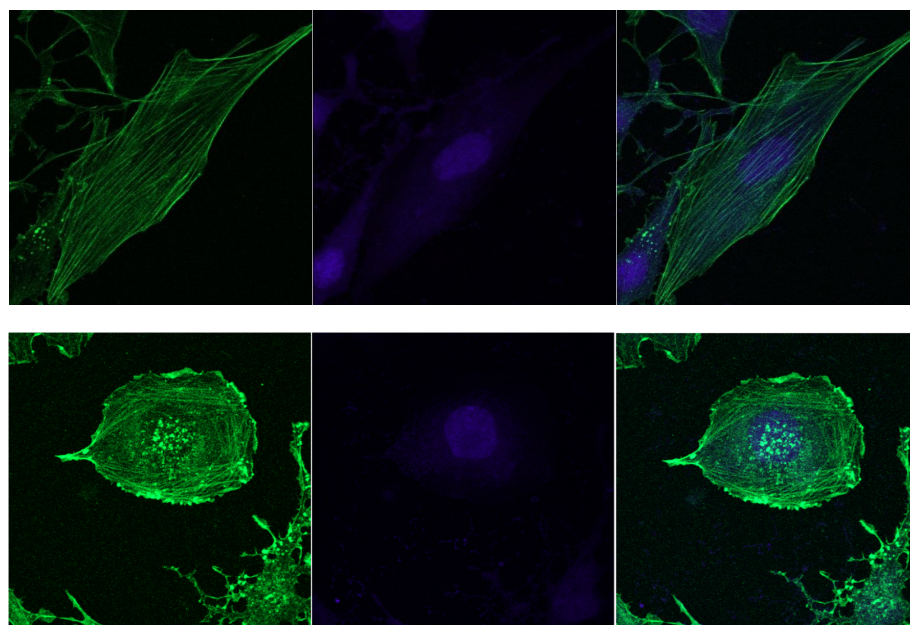


Figure 9. Confocal images of the mouse aortic endothelial cells. F-actin, Alexa Fluor 488 staining, cell nuclei stained by Hoechst 33342.

In contrast to MAEC, adherently grown LLC-R9 cells show a less developed network of actin fibers, with smaller diameters and shorter lengths. The nucleus also occupies a significant part of the cell, which suggests that the cells are in the G2 phase (Figure 10).

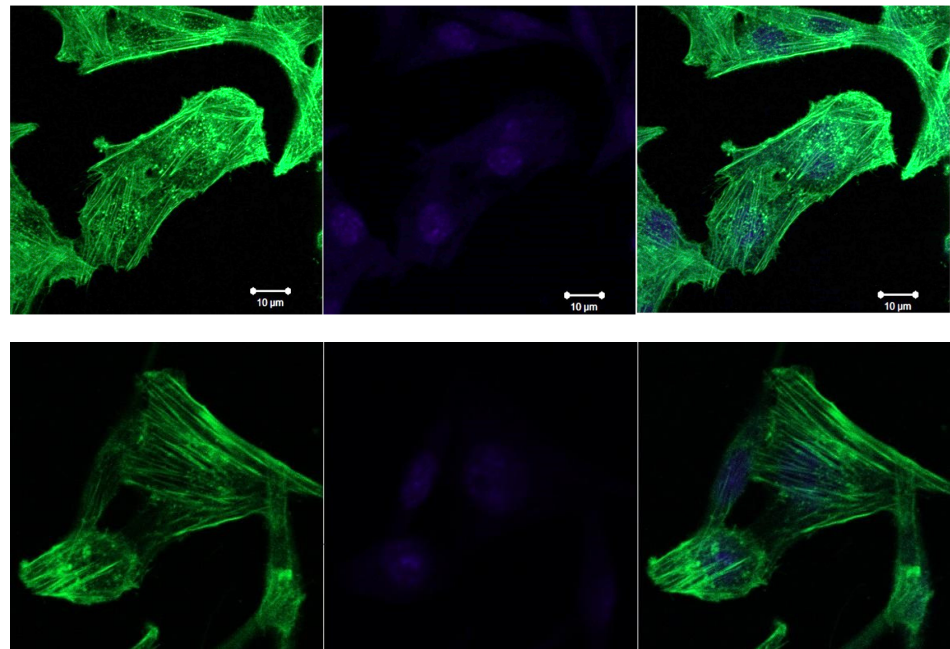


Figure 10. Confocal images of F-actin staining of LLC-R9 cells during the adhesive growth, cell nuclei stained by Hoechst 33342.

In the case of de-adherent LLC cells, we can see the location of F-actin fibers in close proximity to the nucleus; the fibers are tightly packed, tightly covering the nucleus. In de-adherent LLC-R9 cells, F-actin fibers also tightly encircle the nucleus, but it can be argued that, as in the case of the adherent growth, they have smaller diameters and shorter lengths (Figure 11).

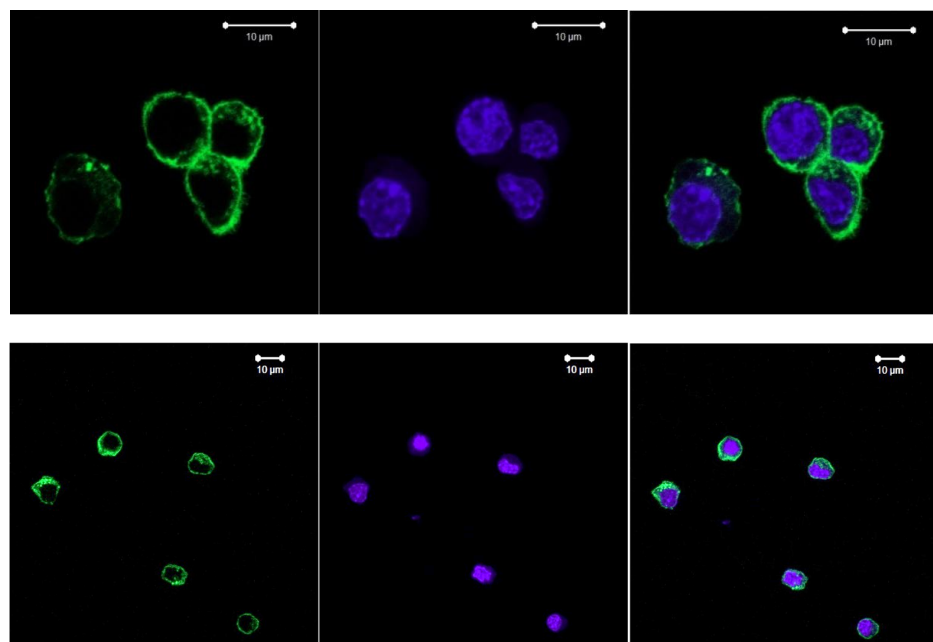


Figure 11. Confocal images of F-actin staining in LLC-R9 cells under de-adhesive growth, cell nuclei stained by Hoechst 33342.2.

In contrast to LLC cells, adhesive LLC-R9 cells show a less developed network of actin fibers with smaller diameters and shorter lengths. The nucleus also occupies a significant part of the cell, which suggests that the cells are in the G2 phase.

The fluorescent images obtained from confocal microscopy were processed using Fiji (ImageJ, v.1.53o) software [39] to measure mean fluorescence intensity (MFI) in a region of interest (ROI). The method was previously described to analyze the samples with varying counts and shapes of the cells [40]. The green-color channel was extracted from each image to focus on the Alexa Fluor488 staining. The ROIs were manually outlined by visible cell verges, and the MFI was measured.

According to Table 4, the MFI in MAEC cell image quality is generally lower compared to LLC-R9. Adhesively grown LLC-R9 tend to contain higher staining signals than de-adhesive ones.

Table 4. The MFI of the cell fluorescent images obtained from confocal microscopy.

Cell Type	MFI
MAEC-2	45.6
MAEC-5	42.1
LLC-R9-adhesive-2	72.1
LLC-R9-adhesive-4	67.3
LLC-R9-de-adhesive-1	46.1
LLC-R9-de-adhesive-2	38.0

Table 4 shows that the level of F-actin in reference cells is in the region of 45.6 and 42.1%, for adhesive cells 67.3–72.1%, and for de-adhesive cells 38.0–46.1%. This means that the level of F-actin is higher in the adhesively grown cells. Now, we are in the process of developing a new method for the accurate estimation of the actin and vimentin level with confocal microscopy. Due to different light focusing on the plane of the cell of different thicknesses, the data could be different even for the same cell. This is why a Z-scan should be applied in this case.

According to flow cytometry, the level of F-actin in de-adhesive LLC/R9 cells is statistically significantly reduced by more than 38% compared to the adhesive cells. The distribution of F-actin in de-adhesive and adhesive LLC/R9 cells is also significantly different: F-actin is, respectively, fixed in the form of microfilaments or threads. The de-adhesive growth of metastatic cells is accompanied by a decrease in the level of F-actin. Thus, the data obtained from the analysis of confocal images are in accordance with the data obtained using flow cytometry.

Therefore, the morphological properties of the cells grown using the adhesive and the de-adhesive ways are different. The adhesively grown cells have elongated forms while the de-adhesively grown ones are rounded; however, among the adhesively grown cells, you can find other shapes, contrary to de-adhesive cells that all are of a rounded shape. The amount of F-actin increases by approximately 40% in the adhesive cells compared to the de-adhesive ones, which was proven by biochemical methods and using a confocal microscope. As a rule, a more branched network of fibrils is observed in the adhesively grown cells. The biochemical method also indicates an increase in vimentin in these cells.

4. Conclusions

In this study, two models of circulating metastatic cell cultivation, de-adhesive and adhesive, were tested on Lewis lung carcinoma tumor cells (LLC-R9). Various physical and biological methods applied to these cells showed differences in the spectroscopic and morphological properties between the cells. Now, we are at the experimental stage of testing antitumor drugs—paclitaxel, antimetastatic oxamate and entosis inhibitor (agent Y-27632) on the cells. Due to the fact that epithelial tumors make up a significant percentage of

oncological diseases, the significance of practical application in the diagnosis and choice of treatment strategy is unquestionable. However, our study was conducted on only one cell line, which is why it is desirable to perform experiments on other cell lines with different metastatic potential. Focusing on similar properties of circulating metastatic cells, their spectroscopic features may be close. The spectroscopic and morphological markers of circulating metastatic cells LLC-R9 are summarized below:

1. FT-IR spectra of adhesively and de-adhesively grown cells show protein conformation transformation, membrane changes, and H-bond reorganization.
2. From Raman spectra in the region of 400–900 cm^{-1} , Trp, Tyr, amino acids, and glycogen levels were derived and characterized special features, which indicate changes in the environment for adhesive and de-adhesive cells, H-bond redistribution, reorganization of proteins, etc.
3. CARS data correlated with the Raman one and with confocal microscopy indicate that only a small number of round cells can be detected in the population of adhesive cells, which suggests apoptosis or necrosis or changes in cell functional activity under the influence of external stimuli or the environment.
4. The de-adhesive LLC cells are rounded, significantly reducing the cell surface. During adhesive growth, thin F-actin threads are clearly visible as a part of the contractile tissue and the migration apparatus. During de-adhesive growth, F-actin is fixed in the form of microfilaments in the cytoskeleton of the cells. The F-actin level in de-adhesive LLC/R9 cells is statistically significantly reduced by more than 38% compared to adhesive ones. The nature of F-actin distribution in de-adhesive or adhesive LLC/R9 cells is also significantly different: F-actin is fixed in the form of microfilaments or threads, respectively.

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Conflicts of Interest: Taras Vasyliovych Voitsitskyi is currently employed by “Receptor.AI Inc.”, 20-22 Wenlock Road, London N1 7GU, UK, and was employed by “Receptor.AI Inc.” while contributing to this manuscript. His contributions to this work and manuscript were made independently without any requirement, guidance or input by his employer. He received no financial compensation from any source for the contributions he made to the scientific work and manuscript. The other authors declare no conflicts of interest.

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