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Development of 3D Glioma Models Based on Cell Spheroids Embedded in Pectin-Collagen Matrix

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Abstract. Three-dimensional cell culture is a modern tool for cancer research. This cell-based model combines the simplicity and accessibility of two-dimensional systems with mimicking *in vivo* conditions via supporting cell-cell and cell-matrix interactions. This allows it to be used both for studying the mechanisms of cancerogenesis and for preclinical testing anticancer therapeutics. In this research, we propose a simple and detailed technique for the preparation of spheroids composed of human glioblastoma cells embedded in a biopolymer matrix based on modified pectins supplemented with collagen I. The protocol also includes long-term cell monitoring with comprehensive analysis of cell viability, migration and behavior in culture using conventional optical imaging, laser scanning microscopy and flow cytometry

Keywords: glioma, cell-based model, spheroids, biomaterials.

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Разработка 3D-моделей глиомы на основе клеточных сфероидов, встроенных в пектин-коллагеновую матрицу

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Аннотация. Трехмерная клеточная культура – современный инструмент для исследования злокачественных новообразований. Такие клеточные модели сочетают в себе простоту и доступность двумерных систем с имитацией условий *in vivo* благодаря воссозданию взаимодействий клетка-клетка и клетка-матрикс. Это позволяет использовать их как для изучения механизмов канцерогенеза, так и для доклинического тестирования противоопухолевых терапевтических средств. В этом исследовании мы предлагаем простую методику приготовления сфероидов из клеток глиобластомы человека, встроенных в биополимерный матрикс на основе модифицированных пектинов, с добавлением коллагена I. Протокол также включает длительный мониторинг клеток с комплексным анализом жизнеспособности, миграции и поведения клеток в культуре с использованием традиционной оптической визуализации, лазерной сканирующей микроскопии и проточной цитометрии.

Ключевые слова: глиома, клеточные модели, сфероиды, биоматериалы.

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Introduction

Gliomas are the most common type of primary brain tumor, which originate from glial cells [1]. Gliomas have several features: they diffusely infiltrate surrounding healthy tissues and have significant intratumor and intertumor heterogeneity [2], which limits the effectiveness of surgical approaches and complicates the development of a universal drug effective against all molecular subtypes of gliomas. The consequence of this is a low percentage of five-year patients' survival [3]. The key to solving these problems is personalized selection of therapy.

There are many approaches for mass screening of candidate substances for antitumor activity, and today screening using 3D cell culture systems is coming to the forefront. This approach allows to mimic the conditions which exist in tumor *in vitro* and represents a cost-effective and ethical model for assessing the anticancer activity of substances. The simplest and most convenient 3D model of tumor growth is spheroid cultures. Spheroids are round-shaped aggregates of cells. Cultivation of cells in spheroids allows to make the conditions existing in the tumor, to provide intercellular interactions and to support the population of tumor stem cells [4]. This form of cultivation also reduces the diffusion of nutrients and oxygen and forms three zones of the tumor: an outer proliferating zone, an inner quiescent zone and a necrotic core [5].

In addition, the extracellular matrix plays an important role in tumor morphology. The interaction between cells and matrix molecules modulates their behavior, affecting proliferation and invasion [6]. The embedding of spheroids in biomaterials with properties similar to the native tumor matrix makes these tissue-engineered structures more attractive than scaffold-free spheroid cultures and the drug-screening results more representative [7].

This paper offers an original protocol for the production of a three-dimensional model based on spheroids generated from a human glioblastoma cell line and a biocompatible hydrogel material made of low-esterified pectin and native ECM proteins. Pectin is a structural analogue of some glycans in the brain ECM, including polyuronic acid chains and can partially mimic them [8]. This makes pectin matrix materials a good environment for embedding tumor neurospheres. Tissue-engineered constructs based on materials developed can become a platform for studying tumor biology, the metastatic ability of cells, and drug screening. We also offer an optimized protocol for vital analysis of cell behavior in the described models using conventional optical imaging, laser scanning microscopy and flow cytometry.

Materials and methods

1. Cell culture

Human glioblastoma model cell line U 87MG (HTB-14TM, ATCC) was cultured in a mixture of Dulbecco's modified Eagle's medium and F12 with L-Glutamine (DMEM/Ham's F-12, with L-Glutamine, Capricorn, Germany) supplemented with 10 % fetal bovine serum (FBS, Capricorn, Germany), 1mM sodium pyruvate (Gibco, USA), and antibiotic mixture comprises 50 U/ml penicillin and 50 ug/ml streptomycin (Pen Strep; Gibco, USA) at high humidity, 37 °C, and 5 % CO_2; the medium was replaced every 3–4 days. Passages were performed every 6–7 days.

2. Glioma cell spheroid preparation

To prepare spheroids from cells, the hanging drop method and culturing on low-adhesive plates were tested. Hanging drops were formed on the lid of a Petri dish. The drop volume was 50 μl. To form spheroids on low-adhesive coatings, four types of plates were used: 96-well cell culture plate with anti-adhesive surface (Sarstedt, Germany), as well as 96-well plates with different base shapes (flat-, round- and conical-base plate were used), coated with an agarose solution. To prepare flat-bottom plates, sterile 1 % agarose solution was added to the wells in a volume of 50 μl. To create an agarose coating on round- and conical-bottom plates, 50 μl of agarose solution was added and the excess was quickly removed. The coatings were polymerized at room temperature for 15–25 minutes.

Three cell concentrations were used: 2000, 4000, 8000 per drop or well. The formation of spheroids was monitored daily using a Primo Plan-Achromat 10x phase-contrast objective with a numerical aperture of 0.25 and a ZEISS Primovert stereomicroscope with an Axiocam 105 camera (ZEISS, Germany). Subsequently, spheroids formed on conical-bottom plates from 4000 cells were used for embedding in gel.

3. Embedding of spheroids in biopolymer hydrogels

To create tissue-engineered structures, spheroids were cultured in hydrogels imitating the extracellular matrix. Modified pectin with an esterification degree of 27 % was used as a major component of the materials. Final concentration of pectin in the gel was 1 % (10 mg/ml). The preparation of collagen type I isolated from rat tail tendons was used as a supplement to prepare composite hydrogels. Final concentration of collagen in the gel was 1000 μg/ml. The gelation of of 1 % pectin solution was done by adding gelation initiator in order to reach following final concentrations of several components: 4 mM $CaCl_2$, 8 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.4, and 150 mM NaCl.

The hydrogels were prepared in drops with a total volume of 300 μl. The components were mixed on the cooled culture plastic surface. 30 μl collagen I stock solution (10 mg/ml) cooled to 4 °C or its solvent (30 mM acetic acid), 5 μl 180 mM NaOH solution and 8 μl gelation initiator were added sequentially. Then a mixture of neutralized pectin and 10–15 cell spheroids in the volume of the medium, prepared in another tube, was added. For gelation, samples were incubated at 37 °C for 30 minutes or left at room temperature for 5 minutes, after the dish with a drop of gel was quickly and carefully turned upside down and incubated at 37 °C until final gelation for 25 minutes. After gelation, the required volume of medium was added. Spheroids in gel drops were cultured for 5 days at high humidity, a temperature of 37 °C and a CO_2 level of 5 %, with the medium being replaced every 2–3 days.

4. Spheroid imaging and cell migration analysis

Visualization of samples was performed using a phase-contrast objective Primo Plan-Achromat 10x with a numerical aperture of 0.25, a stereomicroscope ZEISS Primovert with a camera Axiocam 105 (ZEISS, Germany). On the fifth day, the zone of cell invasion from the spheroid into the gel was recorded. To assess the level of invasion, we used an index obtained by dividing the diameter of the circumference of the migration zone (which was constructed based on the cells most distant from the spheroid center) by the diameter of the spheroid.

Morphometric parameters of spheroids were measured using the original ZEISS ZEN 3.8 software. The average diameter, cross-sectional area, and shape-factor were entered into a separate file intended for analysis. FIJI (ImageJ) software was also used for the analysis. This option was used when analyzing the average area and number of cell aggregates during optimization of spheroid formation protocols, as well as when calculating viability. Basic graphic editing functions and the particle analysis tool and its basic recommendations for contrast enhancement (threshold adjusting) were used. The data from the summary table were also entered into the database for statistical analysis.

Statistics was processed in GraphPad Prism by Kruskal-Wallis test, and statistical significance by Holm-Sidak method with α = 0.05; GP: 0,1234 (ns), 0,0332(*), 0,0021(**), 0,0002(***), <0,0001(****); Error bars presented as Confidence Intervals (± 95 % CI).

5. Viability analysis by laser scanning microscopy

To visualize the spheroids and analyze their viability, we used laser microscopy to obtain three-dimensional reconstructions of the spheroids. Vital staining after 5 days of culturing the spheroids in gel was performed using 10 μg/ml fluorescein diacetate (FDA, Invitrogen, USA), 20 μg/ml propidium iodide (PI, Invitrogen, USA), and 5 μg/ml Hoechst (Hoechst 33342, Invitrogen, USA). The latter was used for correct counting of living cells by nuclei. Staining with a mixture of dyes and medium was carried out for 40 minutes at 37 °C. A series of optical sections of the spheroids in gels were obtained using an Olympus FV1200 laser scanning microscope (Olympus, Japan) equipped with a UPLSAPO 10x objective with a numerical aperture of 0.40. Cell viability was assessed using Imaris 7.6.5 software.

6. Spheroid disaggregation, viability analysis by flow cytometry

To disaggregate the pectin component of the hydrogels, a 50 mM disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA), pH 7.7 was used as a chelating agent for divalent calcium ions. After adding Na_2EDTA, the samples were incubated for 5 minutes at room temperature.

Subsequently, several enzymatic processing protocols in combination with mechanical disaggregation were tested for spheroid disaggregation. Solutions of trypsin (Trypsin 1:250, Gibco, USA), collagenase type I (Collagenase, Gibco, USA) and collagenase/dispase enzyme mixtures (Collagenase/Dispase®, Roche, Switzerland) were used with different concentrations of the components 0.25 %, 0.5 % and 0.75 % for trypsin; 60 U/ml, 80 U/ml and 100 U/ml for collagenase, 50 U/ml, 60 U/ml and 80 U/ml for dispase. These three enzymatic preparations were tested separately as well as in pairs of trypsin preparation with other ones. Incubation time varied from 20 to 80 min. The prepared cell suspension was stained according to the method described in subchapter 5 and analyzed using a MoFlo Astrios EQ 5L sorter (Beckman Coulter, USA).

Result

This paper describes a method for creating a three-dimensional model of glioma based on spheroids and biocompatible matrix materials. To optimize this method, various approaches to the spheroid formation and the development of tissue-engineered constructs were used.

1. Spheroids formation and embedding in hydrogels

The hanging drop method and cultivation on low-adhesive coated plates were used to form spheroids. The disadvantage of the hanging drop method was the formation of a large number of different size aggregates reaching more than 90 in some drops (Fig. 1B-E). This forced us to focus on using low-adhesion coated plates to generate separated spheroids. Agarose-coated plates with flat or round bottom generate stable cell aggregates on day 5. The minimum number of cells required to form spheroids in this way is 8000. Spheroid sizes ranged from 300 to 650 micrometers. Commercial low-adhesion plates and agarose-coated conical-bottom plates can produce spheroids of 2000, 4000, 8000 cells from those sampled in 24 to 48 hours (Fig. 1F-H). The diameter of the spheroids varied from 100

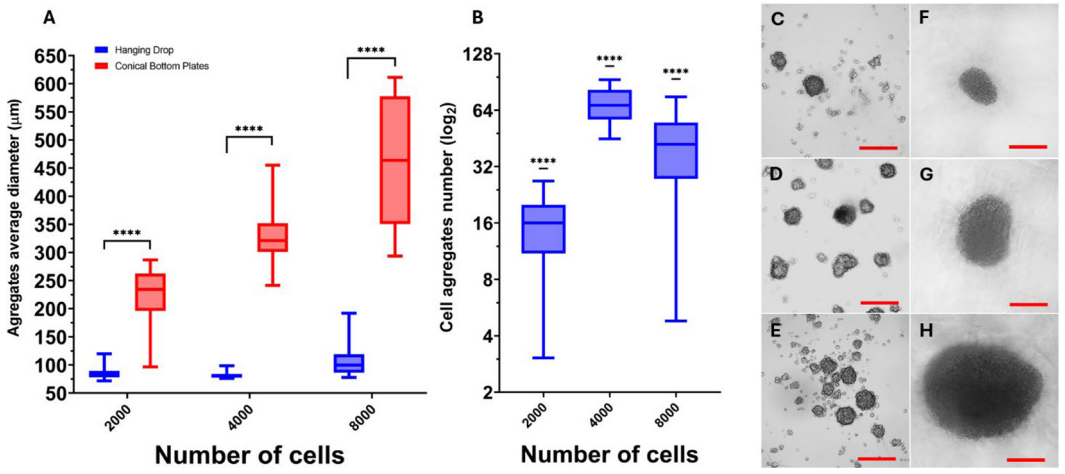

Fig. 1. (A) Represents the average diameter of cellular aggregates produced by different amounts of cells in the hanging drop. For comparison we append data for spheroids from the agarose layer. (B) Shows the aggregate number per droplet or well of the cell culture plate. Spheroids obtained by the hanging drop method from 2000 (C), 4000 (D) and 8000 (E) cells. Spheroids obtained by culturing on low-adhesive conical bottom plates from 2000 (F), 4000 (G) and 8000 (H) cells. Scale bar 200 μm

to 300 μm with a mean of 220 μm for 2000-cell spheroids, from 235 to 470 μm with a mean of 300 μm for 4000-cell spheroids, and from 285 to 635 μm with a mean of 460 μm for 8000-cell spheroids (Fig. 1A). We recommend making spheroids from 4000 cells. Such spheroids have the most convenient sizes for manipulation, since they are visible to the naked eye.

To create three-dimensional culturing systems, spheroids were cultured in pectin and pectin-collagen hydrogels. The described order of gel components mixing is optimal. A different sequence of mixing the components leads to gel heterogeneity due to the collagen precipitation.

Since the polymerization time of the gel prepared according to our protocol is 30 minutes, some of the spheroids have time to sink to the culture dish bottom. This leads to the cells spreading over the plastic surface and interferes with the analysis of migration in the matrix (Fig. 2F-G). We modified the polymerization protocol to avoid this.

Pectin hydrogels do not support cell migration (Fig. 2B-C). To assess the invasive potential, we recommend using matrix materials with the addition of collagen (Fig. 2D-E). The invasive index for pectin-collagen gels ranged from 1.3 to 3.4 with a mean of 2.

2. Live-dead assay

For spheroid visualization and live-dead assay, we chose the method of vital staining with fluorescent tracers followed by laser scanning microscopy. However, we found that this approach does not provide a whole-mount visualization of spheroids of any size (Fig. 3G). This may be due to insufficient fluorophore emission inside the spheroid and the dense packing of cells inside it, which prevents dye diffusion.

Additionally, we tested the disaggregation protocols followed by counting live and dead cells using flow cytometry. The best protocol includes sequential processing with 50 mM Na_2EDTA for 5 minutes and a 0.25 % trypsin with 100 U/ml collagenase I.

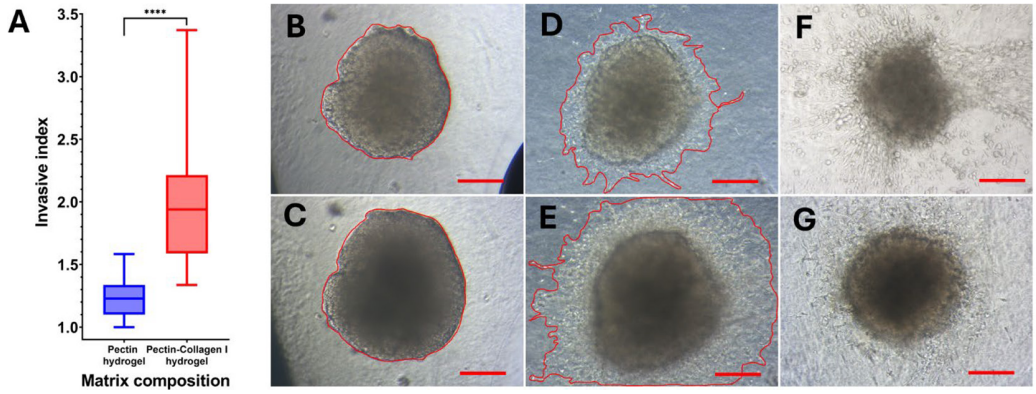

Fig. 2. Spheroid imaging and analysis of cell invasion. (A) Comparison of cell migration through hydrogels of different compositions. (B) Spheroids embedded in a pectin (B) and pectin-collagen (D) matrix on the first day of cultivation. Spheroid embedded in a pectin (C) and pectin-collagen (E) matrix on the fifth day of cultivation. Cell migration along the bottom of the culture plastic (F) and in the thickness of hydrogels (G). Scale bar 200 μm

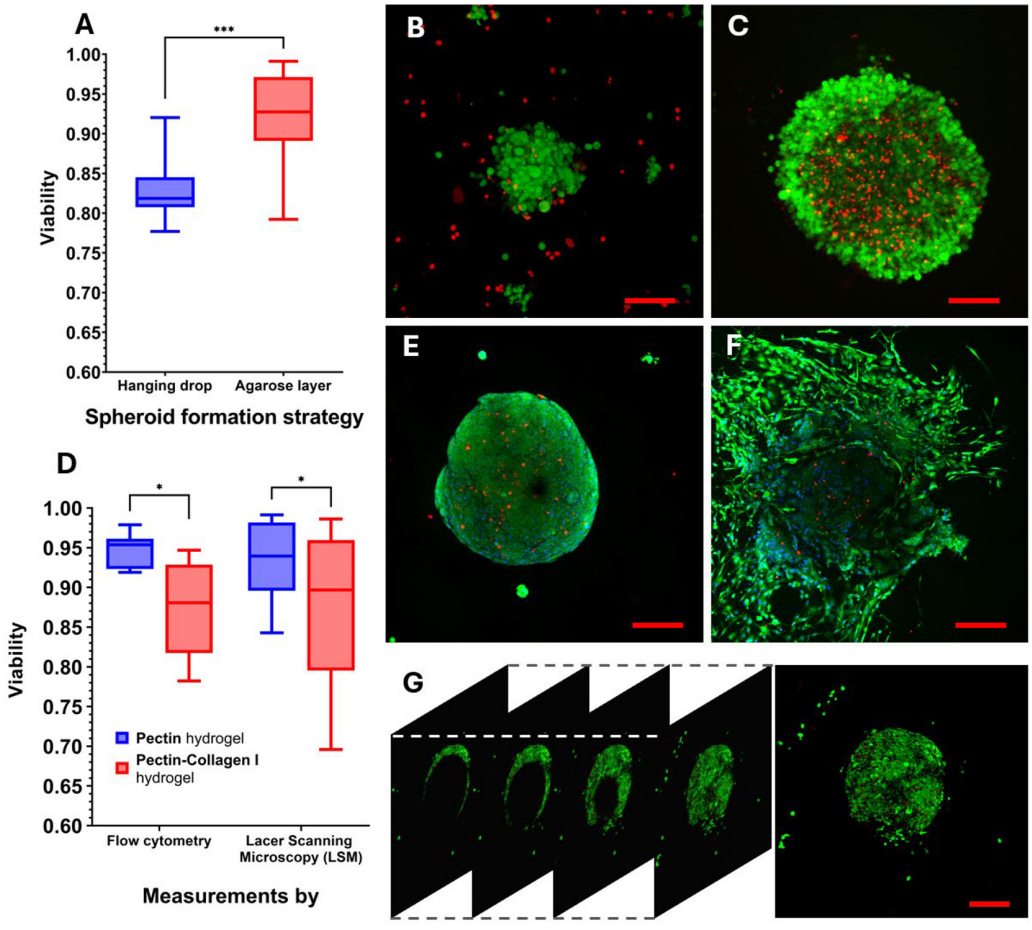

Fig. 3. Analysis of cell viability in spheroids. Comparisons of cell viability of spheroids obtained by different protocols (A) and viability assessment methods (D). Images of spheroids obtained by hanging drop (B) and culturing on low-adhesion plates (C). Images of spheroids after 5 days of culturing in pectin (E) and pectin-collagen hydrogels (F). (G) Disadvantages of quantitative analysis of spheroid cells by laser scanning microscopy. Scale bar 200 μm

The viability of cells in spheroids generated using low-adhesion coated plates (79–99 % with a mean of 86 %) is higher than those formed in a hanging drop (78–92 % with a mean of 82 %). This may be due to the lack of medium in the drop (Fig. 3A).

The cell viability in spheroids was in range of 92–98 % with an average value of 95 % when cultured in pectin hydrogels, 80–95 % with an average value of 88 % when cultured in pectin collagen hydrogels (Fig. 3D).

Discussion

The main factor that determines the phenotype of glioma cells is the microenvironment. The combined effect of the extracellular matrix, tumor and tumor-associated cells activates patterns of gene expression responsible for invasive growth, resistance to hypoxia and nutrient deficiency, maintenance of actively proliferating cells pool of and tumor stem cells, as well as drug resistance. This makes it impossible to use classical two-dimensional cell cultures as a valid platform for studying the mechanisms of gliomagenesis and searching for effective anti-tumor agents [9].

Tumor spheroids are a form of three-dimensional cell cultures and can be generated by a variety of methods. The most common methods used to create spheroids are the hanging drop method, culturing on low-adhesion coatings, microfluidic devices, magnetic levitation, and cell culture rotation [6]. In our study, we focused on two of the simplest, most accessible, and most popular methods for creating spheroids. The application of the hanging drop method to many type of cancer cell cultures was described [10]. There is evidence of successful use of the hanging drop method to create spheroids from glioma cells [11]. However, we encountered difficulties in testing this method, associated with the formation of several different sizes spheroids in one drop, which forced us to focus on the method of culturing on low-adhesive coatings. There are many low-adhesion plates on the market that have been successfully used by researchers to create spheroid cultures [13]. Their disadvantage is their high cost, which makes it difficult to conduct large-scale studies that require a large statistical sample. Hand-made plates with agarose coating [13] or agarose microwells can be a good alternative [14]. According to our observations, conical plates coated with agarose gel allow rapid generation of standard-sized spheroids.

To create 3D models, various matrix materials, which resemble the extracellular matrix in terms of biochemical and mechanical properties are used [15]. Since synthetic polymers are biologically inert, protein and carbohydrate biopolymers are most often used to create tissue-engineered constructs [16]. The advantage of these biomaterials is their adjustable composition and tunable stiffness, as well as batch-to-batch reproducibility, unlike Matrigel. In our work, low-esterified pectin was used as the major component of the matrix material. The advantages of pectin are biodegradability, biocompatibility and low toxicity [17]. Possibility of using pectin in brain tissue engineering demonstrated by researchers [8]. It is important to note that pectin is similar in chemical structure to brain ECM carbohydrates, which are the main component of the brain matrix and plays an important role in stimulating the progression of gliomas. However, as a component of a biocompatible matrix material, pectin has an undeniable advantage since it is subject to simple and physiological ionic gelling without additional modifications, unlike hyaluronic acid [18]. Gelatin is most often used as an additive that stimulates cell migration into the pectin gel [19]. In our study, collagen I was used as an agent for stimulating migration.

To visualize spheroids and analyze cell behavior in 3D cultures, researchers most often use a similar set of methods: light microscopy to assess the state of spheroids during cultivation and calculate

the area of invasion, fluorescent dyes for Live/Dead analysis in combination with layer-by-layer laser scanning microscopy to assess viability [20]. BrdU or Ki67 staining is also used to assess cell proliferative activity [21], and propose protocols for the preparation of sections of spheroids cultured outside of biomaterials for histological or immunohistochemical staining [22]. We encountered difficulties in whole-mount spheroid visualization using fluorescent labeling and confocal microscopy and could recommend using flow cytometry for quantitative analysis of cells in spheroids.

The final version of the protocol for the creation of a three-dimensional cell model of gliomas based on spheroids and pectin-collagen biomaterials and subsequent analysis of cell behavior in culture is presented in supplementary materials.

Conclusion

The ability to use modern cell-based biomedical technologies has become highly accessible to many research laboratories and health facilities around the world over the past decade. However, many research protocols require further fine-tuning before they reach practical use. We hope that our research will help to strengthen the foundation of future approaches to treating patients' tumors based on a personalized paradigm using modern cell biology techniques. The simplicity and rapidity of our protocol allows manipulation not only with cell cultures, but also with patients' samples and primary cell cultures. We recommend following the main steps, so the remaining steps can be modified in accordance with your research goals.

Supplementary Information

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