Explant-Like Passaging of Cells Growing on Portable Substrata Permits the Avoidance of Enzyme Application and Facilitates the Passage Procedure

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The experiments presented in this paper show how growing cells on portable substrats can be useful to facilitate and accelerate the passaging (subculture) of anchorage-dependent cells. Experiments have shown that portable substrats are cheap, commercially available, and transparent. They are easily cut into various shapes and sizes, and are easy to sterilize. Portable substrats are also friendly to cells and permit faster than usual cell passaging procedures. Anchorage-dependent cells growing on the bottom of a culture vessel made of glass or polystyrene can be quickly passaged with previously-cut small fragments of glass fiber or nylon meshes or small fragments of polyester foil as well as nylon fishing lines and biodegradable surgeon threads that have been inserted into the vessel. The surfaces of such fragments of portable supports are quickly overgrown with cells and can be easily transferred to a new culture vessel. As with tissue explants, cells migrate and grow over the bottom of the new culture vessels. Using cell viability tests, analyses of proliferation and fluorescence microscopy, we confirmed the utility of the investigated substrats for cell culture. In addition, the passaging cells, together with a portable support (like explants), eliminate the need for an application of proteolytic enzymes which modify numerous cell properties and activities and would keep the cell from detaching from the substratum which would lead to the cell rounding and changes in the cell's cytoskeleton architecture.

Key words: Passaging, enzyme free, portable substrata, anchorage dependent cells, trypsin, EDTA, explant, scaffold.

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The development of methods for culturing animal and human cells in vitro permitted the expansion of research into a variety of fields of molecular and cellular biology, biotechnology, and biomedicine. Maintaining a constant supply of cells in culture requires cell passaging. This is the process of transferring a small number of cells into new vessels and diluting them in a larger amount of new media. This is relatively easy for cancer, embryo, and immune system cells which are able to proliferate suspended in fluid or semi-solid media (MOSCONA *et al.* 1965; EASTY 1967b; WYLIE 1967; MEER *et al.* 1978). It is much more difficult to transfer anchorage-dependent cells to new vessels as they need to be attached to solid substrata in order to grow. This requirement is a common feature of almost all tissue cells described usually as normal, i.e. unable to form tumors when implanted into multicellular organisms (WRIGHT *et al.* 1977). Many types of cancer cells show the ability to grow in both conditions i.e. in suspension and when attached to solid substrata.

Subculturing anchorage-dependent cells usually requires cell detachment, cell suspension, followed by cell centrifugation, concentration and lastly, dilution to the desired cell densities. Meth-

ods commonly used for the harvesting and detachment of cells growing on the surface of solid substrata are based on the use of the following proteolytic enzymes: trypsin, papain, dispase or collagenase. Trypsin, the most commonly used enzyme, modifies the epitopes of cell surface markers causing changes in a cell membrane's physical properties and shows mitogenic activity (Burger 1970, 1971; Sefton & Rubin 1970, 1971; Russell & Pastan 1973; Molinari & Platt 1975; Anghileri & Dermietzel 1976; Furcht & Wendelschafer-Crabb 1978; Gray *et al.,* 2001; Huang *et al.* 2010). Due to the negative side-effects caused by cell trypsinization and the scraping of cells with a silicone rubber, attempts have been taken to develop alternate methods of viable cell detachment (Rabinovitch & Destefano 1975; Korohoda *et al.* 2015).

The purpose of the experiments described in this paper was to develop methods that could be used to accelerate and facilitate the process of cell passaging while minimizing cell injury. The experiments were based on the assumption that, similarly to tissue explant fragments (Abercrombie & Heaysman 1954; Easty 1967a; Bereiter-Hahn *et al.* 1981), anchorage-dependent cells could be easily transferred together with pieces of the supports they are grown on. The goal was to examine various, easily available substrata onto which animal cells would attach and spread, and to determine which of these materials would be the most convenient for cell culturing and passaging.

Materials and Methods

Cell lines

Experiments were performed on rat 3T3 fibroblasts, AT-2 rat prostate adenocarcinoma cells of the Dunning R3327 series cell line , normal mouse astrocytes C8-D1A, and human glioblastoma multiforme T98G cells. Before experimentation, cells were cultured in 25 cm^2 Falcon dishes at 37°C and 5% CO_2 while maintaining high humidity as described previously (Musialik *et al.* 2013; Korohoda *et al.* 2016; Pudełek *et al.* 2019; Ryszawy *et al.* 2019; Ryszawy *et al.* 2020). AT-2 and C8-D1A cells were grown in RPMI-1640 medium (Sigma). 3T3 and T98G cells were grown in DMEM with a high glucose concentration of 4500 mg/l (Sigma). All media were supplemented with heat-inactivated, 10% fetal bovine serum (FBS, Gibco) and 1% Antibiotic-Antimycotic solution (Sigma).

Cell passaging

For explant-like cell passaging, cells were grown in Petri dishes (Falcon, polystyrene 3 and 6 cm in diameter) containing fragments of the different types of portable supports being tested (1-1.5 mm long fibers, 0.25 x 1 cm polyester foils). For the investigated portable supports, materials were cut to the proper size needed to cover the bottom of a dish and were inserted into Petri dishes 6 cm in diameter. The supports were sterilized with UV light (15 min, each side). After adding the supports to the cell cultures, the Petri dishes were placed in a cell incubator for the next 24 hours. Afterwards, the supports were placed in new Petri dishes using tweezers. The procedure was repeated several times using the same supports.

Cell growth rate

To determine the proliferation rate curves, cells were seeded at a density of 5 x 10^4 per well of a 12-well polystyrene cell culture plate (Falcon) (three plates for each substrat). The initial number of cells was adjusted to the available surface area. Cells were counted using a Beckman Coulter Z2 Particle Counter after 24, 48, 72, and 96 hours (Ryszawy *et al.* 2020b). Before counting, cells were detached with a trypsin/EDTA solution (0.25% trypsin, 1mM EDTA in PBS without Ca^{2+} and Mg^{2+}, Sigma, ST. Louis, MO).

Cell viability

Cell viability was tested using fluorescein diacetate (FDA) and ethydium bromide (EtBr) tests and an epi-fluorescence microscope (Jenavert, Carl Zeiss, Jena), as described in literature (Szydłowska *et al.* 1978; Grys *et al.* 2017). Before being tested, cells were detached with trypsin, stained and transferred onto glass slips. The cells were photographed under an epi-fluorescence microscope (Jenavert, Carl Zeiss, Jena) or a phase contrast Olympus CK40 microscope with a Canon digital camera EOS 4000D. All experiments were conducted in triplicate.

Substrats

For the following four types of materials, quantitative experiments were carried out:

1 – Polystyrene Petri dishes, 3 and 6 cm in diameter (Falcon; Fig. 1A).

2 – Polyester foil used for xerography (Folie fûr Kopiegerate Igepa, Reinbeck, Germany, thickness 180 µm; Fig. 1B, M).

3 – Glass fiber materials used for modeling (JINO, Bohemia, nets of different mesh 49 g/m^2 to 110 g/m^2; Fig. 1 C, D, K, L).

4 – Nylon nets used for stiffening curtain carpets (Magam, Ariadna, Poland; Fig. 1E, F, I).

For three substrata only photographic documentation was made:

– Glass fiber wallpaper Primacol Flis (Unicell Poland) (fiberglass net, 40±5 g/m^2; Fig. 1 G, H);

– Nylon strings and nylon fishing lines of various diameters (100-500 µm, Fig. 1 J, N);

– Surgical biodegradable threads; Novosyn DSMP 19 Braun Poly(glycolide-co-L-lactid), Safil DSt, and Safil Violet (Braun) (Fig. 1 O, P).

Additionally, a preliminary examination of the utility of four other materials was carried out:

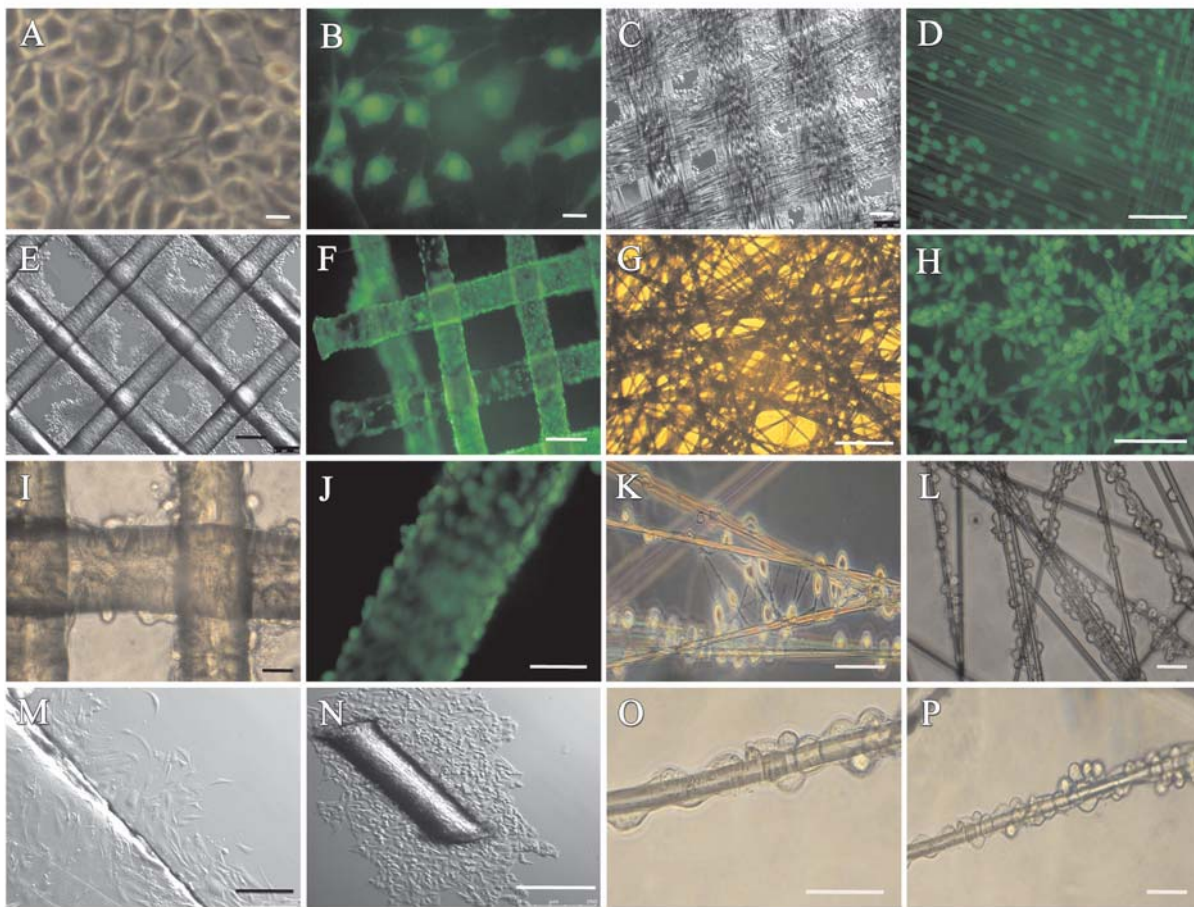– 100% polyester microfibers (50-200 µm in diameter), single threads or bundles;

Fig. 1. A-P. 3T3 rat fibroblasts and AT-2 rat prostate adenocarcinoma cells attached, spreading and growing on a variety of portable supports and on the bottom of a control polystyrene Petri dish. A – 3T3 cells growing in a Falcon Petri dish (polystyrene), magnification 200x, Bar = 20 µm; B – 3T3 cells stained with FDA growing on polyester foil (xenography folie, Reinbeck) observed under an epi-fluorescence microscope, magnification 200x, Bar = 20 µm; C – AT-2 cells growing on modeling glass mesh (JINO), epi-light microscope, magnification 32x. Bar = 100 µm; D – AT-2 cells stained with FDA growing on modeling glass mesh (JINO), epi-fluorescence microscope, magnification 32x, Bar = 100 µm; E – AT-2 cells growing on a nylon net (Magam), inverted light microscope, magnification 40x. Bar = 500 µm; F – FDA stained AT-2 cells attached and growing on a nylon net (Magam), epi-fluorescence microscope, magnification 125x, Bar = 20 µm; G – AT-2 cells growing on irregular glass fiber mesh (Primacol Flis), epi-light microscope, magnification 32x, Bar = 100 µm; H – FDA stained AT-2 cells growing on irregular glass fiber mesh (Primacol Flis), epi-fluorescence microscope, magnification 32x, Bar = 100 µm; I – AT-2 cells attached and growing on a nylon net (Magam), epi-light microscope, magnification 125x, Bar = 200 µm; J – FDA stained AT-2 cells attached and growing on nylon fishing line, epi-fluorescence microscope, magnification 200x, Bar = 250 µm; K – FDA stained AT-2 cells attached and growing on glass modeling microfibers, epi-fluorescence microscope, magnification 125x, Bar = 100 µm; L – AT-2 cells growing on modeling glass fibers, epi-light microscope, magnification 125x, Bar = 100 µm; M – AT-2 cells moving from polyester foil (xenography folie, Reinbeck) onto a clean polystyrene surface, inverted microscope, magnification 100x, Bar = 250 µm; N – AT-2 cells moving from the surface of a nylon fishing line onto the clean polystyrene surface of a Petri dish, inverted microscope, magnification 80x, Bar = 250 µm; O, P – 3T3 cells growing on a surgical thread (Novosyn), inverted microscope magnification 200x, Bar = 100 µm. All pictures are representative of at least 3 independent experiments. For visualization the cells were grown on the investigated substrats for 72 hours.

– Steelon fluorocarbon-coated monofilament fishing lines (Konger, Germany) and Siglon, Balzer (0.230 μm in diameter) and thicker (0.2-1 mm in diameter) nylon strings from a few different producers;

– Gelatin capsules (Parke Davis & Co., Detroit, Michigan, USA) used as drug containers; empty capsules of different shapes and thicknesses;

– Fiberglass nets of Duct Tape Gewebe-Klebeband (Germany); clean net fragments of irregular fiber thickness.

Statistical analysis

All data were presented as mean ±SD from at least three independent experiments (N ≥3). The statistical significance of the differences (p<0.05) was tested using the non-parametric Mann-Whitney U-test. In order to measure the proliferation rate on the 4th day of the experiment, each substratum cell culture was compared to the control cultures in polystyrene Petri dishes. The viability test comparison was carried out between the 1st and 4th day of each substratum culture separately. Data processing was performed using GraphPad software.

Results

In all of our experiments, cells grew on the portable substrata with kinetics comparable to the way they grew on the bottom of commercially available, disposable polystyrene Petri dishes.

Figure 1 presents examples of 3T3 rat fibroblasts and AT-2 rat prostate adenocarcinoma cells attached and spread on tested portable substrata. When fragments of the supporting adhesive substrata, together with the cells growing on them, were transferred to a new vessel or onto an adhesive portable substratum, the cells easily passed from the old substrata to the new, clean material, very much like tissue explants, adhering and flattening onto the surface (Fig. 1 M, N).

Figure 2 illustrates the growth rates of four different cell lines on four different adhesive supports: a polystyrene Petri dish (control), polyester foil, glass fiber mesh, and nylon mesh. For the normal mouse astrocytes C8-D1A, there were no statistically significant differences between cell growth on the control and on the three tested substrata after 96 h of cell incubation (Fig. 2 B). Polyester foil and nylon mesh substrata yielded larger cell numbers compared to the control for AT-2 and T98G cells only (Fig. 2 C, D). The number of cells growing on glass fiber mesh were significantly higher for three of the tested cell lines (3T3, AT-2 and T98G cells), among them AT-2 cell numbers were almost two-fold higher than those growing on the polystyrene dishes (Fig. 2 A, C, D). The
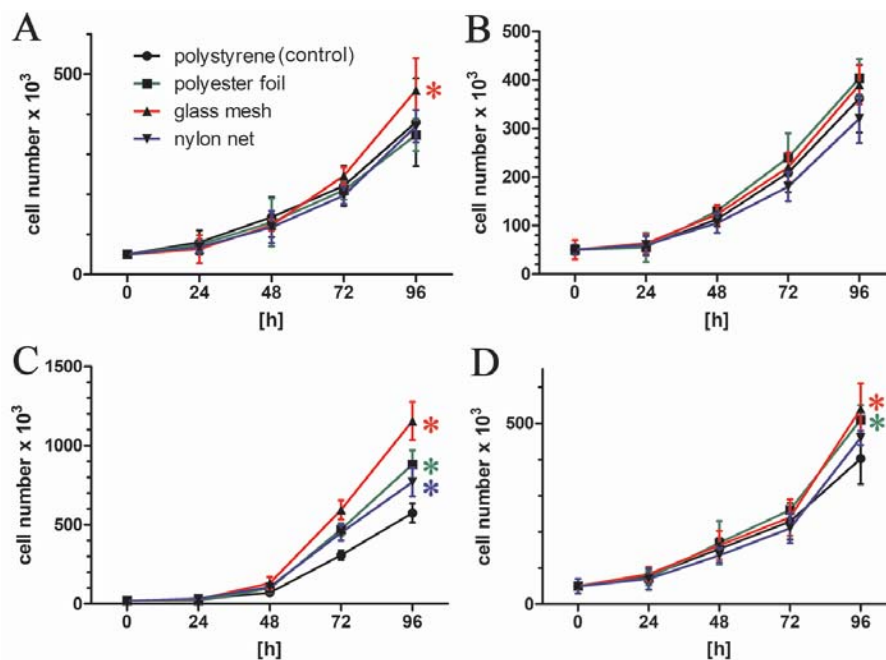

Fig. 2. A-D. The growth curves of cells cultured on polystyrene (the bottom of Falcon Petri dishes), polyester foil, glass fiber mesh, and nylon net. A – normal rat 3T3 fibroblasts; B – normal mouse astrocytes C8-D1A; C – rat prostate adenocarcinoma AT-2 cells; D – human glioblastoma multiforme T98G cells. For statistical analyses, the non-parametric Mann-Whitney U-test was used (each condition vs. control), an asterisk denotes any statistically significant differences at p<0.05.
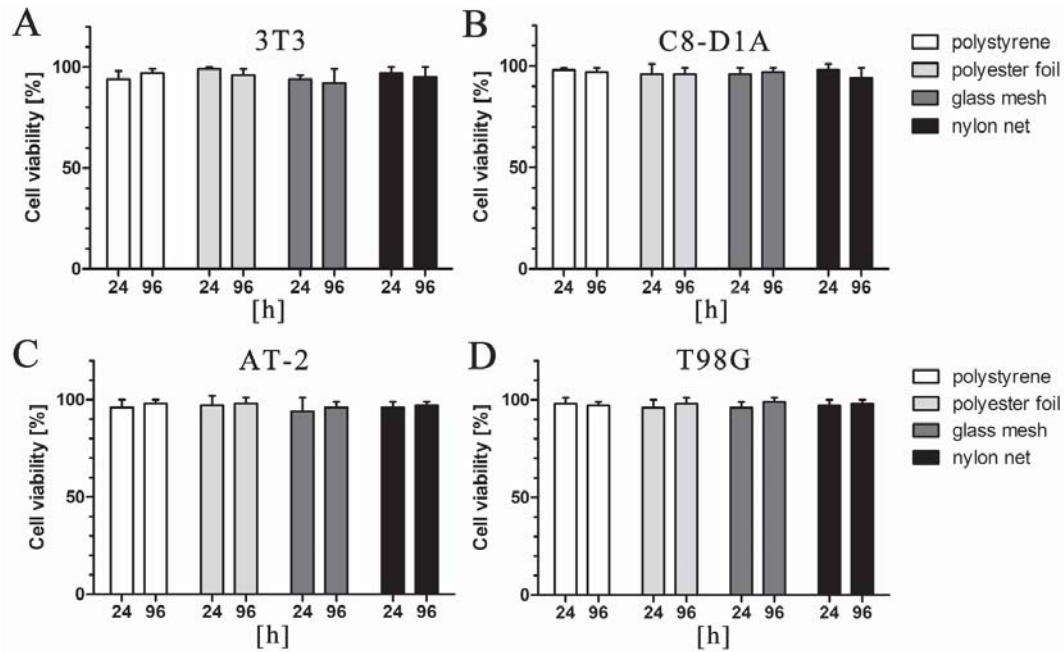
Fig. 3 A-D. Viability of cells cultured on polystyrene, polyester foil, glass fiber mesh, and nylon net. The cells were harvested with trypsin-EDTA solution, stained using FDA/EtBr test and counted under a fluorescence microscope. Each time at least 200 cells were counted in three independent experiments. The results are given as mean ±SD. Note the viability never decreases under 92%. Abscissa – time in hours, Ordinate – cell number in thousands/viability in %. For statistical analyses the non-parametric Mann-Whitney U-test was used, no statistically significant differences were observed ($p>0.05$).

relatively high dynamic cell growth on the glass fiber mesh may be caused by the 3D structure of this material.

Regarding the viability of the cells, there were no differences between all cell lines and all substrata when comparing the first and fourth day of incubation (Fig. 3).

The growth kinetics of the examined cells on the other substrata was not tested quantitatively, however, our observations revealed that growth was similar to that of cells on the substrata described above. These preliminary observations regard nylon nets, nylon fishing lines, steelon fluorocarbon-coated lines, bovine gelatin capsules, and three types of surgical threads (Novosyn DSMP 19 polylactic acid, Safil DSt, and Safil Violet).

Discussion

In the first years of animal cell culture, glass culture vessels were used. Such vessels had various shapes and sizes, required a special washing procedure, and the passaging of cells was associated with the need to detach them from the surface of the glass (MOSCONA et al. 1965; EASTY 1967a; WYLIE 1967). For cell cloning, the photography of cells, or the separation of migrating cells, coverslips were put into vessels or used as bottoms in

Rose's chambers (EASTY 1967a; HAM 1972; PRESCOTT 1972; BEREITER-HAHN et al. 1981; JANIK et al. 1992; MIŁOSZEWSKA et al. 1998). In these cases, coverslips could sometimes be used as portable substrats. Coverslips, however, are uncomfortable to use. They are fragile and it is not easy to cut them into a variety of shapes. In addition, when used as inserts, they adhere to the bottom of vessels and their detachment is very difficult without the use of special suction cups.

Routine cell passaging procedures based on the use of proteolytic enzymes require the centrifugation of cell suspensions. In addition, the detachment of cells from substrats is associated with cell rounding and the disassembly of stress fibers followed by great changes in cell cytoskeleton architecture due to the release of cell isotonic contractions (KOROHODA & WOHLFARTH-BOTTERMANN 1976; ANGHILERI & DERMIETZEL 1976; FURCHT & WENDELSCHAFER-CRABB 1978; KOROHODA & KAJSTURA 1982; KOROHODA et al. 2015). It has been shown that, proteolytic enzymes should not be used in analytical or immunological studies requiring the intact composition of the cell surface. These enzymes have been commonly used since the 1950s for cell sub-culturing and detachment from solid substrata, but they change cell activities and properties, which are later examined using modern methods of molecular and cellular biology (BURGER 1970, 1971; SEFTON & RUBIN 1970,

1971; Russell & Pastan 1973; Molinari & Platt 1975; Anghileri & Dermietzel 1976; Furcht & Wendelschafer-Crabb 1978; Gray *et al.* 2001; Huang *et al.* 2010). Taking into account the abovementioned drawbacks of procedures based on the use of proteolytic enzymes, the portable supports on which anchorage-dependent cells grow have a significant advantage over routine procedures for cell passaging and cell culture. Since we weren't able to find any information on commercially-available and easily-accessible substrata suitable for cell culture and subculture and how it compared to using polystyrene Petri dishes, which would eliminate the need for proteolytic enzymes, we decided to start experiments in this field.

Previously, we observed that anchorage-dependent fibroblasts adhered to thin (8-10 µm in diameter) glass fibers of glass wool, and migrated over them, growing into 2D and 3D cultures (Korohoda 1971; Czyż *et al.* 2004). Glass fibers are easy to cut and sterilized, and they do not fluoresce, but glass wool is irregular in thickness, and can be from 4 to 20 µm in diameter. In our experiments, therefore, we used different glass materials. The glass fiber used in modeling has the form of a mesh with uniform mesh density and fiber or bundle thickness, and is available in a range of parameters. It is easy accessible, non-fluorescent, and easy to cut with scissors into sheets and fragments of the desired size and shape. This glass fiber proved to be very convenient as a 2D and 3D support for cell culture, cell passage, and for usage in various experiments. In all our experiments cells grew on the glass fiber with comparable or better kinetics than on the bottom of commercially available, disposable polystyrene Petri dishes and flasks.

Pieces of the glass fiber wallpaper used to protect walls from cracking can be used as fiberglass supports as well. We used the fiberglass wallpaper, Primacol Flis (produced by Unicell Poland) and Duct-Tape produced in Germany, with the same results. Since glass fiber mesh is rather irregular, the cells rapidly attach to its surface and can be easily transferred to new vessels.

Another kind of portable substratum convenient for the passage of anchorage-dependent cells are transparent polyester foils. Anchorage-dependent cells attach to polyester materials, flatten, migrate, and grow on the surface of fibers and other materials used as inserts in cell culture vessels (Raetz *et al.* 1982; Black & Hall 1985; Collins 1986; Roessger *et al.,* 2009). Polyester is easy to sterilize, shows low fluorescence in blue light, and is transparent in white light. The polyester discs proved to be a convenient substrat to transfer replicas of cell clones (Black & Hall 1985; Collins 1986). Polyester foils are easy to sterilize with UV light or in an autoclave. In our experiments, we examined flat, thin and transparent polyester foils used for xerography and the preparation of transparencies. We observed that all of the tested cells adhered to them, flattened on their surface and grew with similar kinetics as on polystyrene, glass fiber and nylon meshes (nets). These foils can also be cut into fragments and transferred to new culture vessels while cells are growing on them similarly to cell explants. The cells very easily migrated from the foil fragments onto new, clean substrats, clean foils, glass and nylon meshes, or onto the bottoms of polystyrene dishes.

It has been shown many times that cells attach to nylon threads. Numerous experiments have been carried out particularly to determine the difference in the adhesiveness of various white blood cells (Schiffer *et al.* 1977; Barbe *et al.* 2001). There are various nylon threads and meshes of different mesh sizes (densities) and different diameters available commercially. In our experiments, we found that the cells being examined stuck to all nylon nets, flattened on them and grew. They also grew on nylon threads and fishing lines 100 to 500 µm in diameter. Nylon threads and lines, as well as steelon fluorocarbon-coated monofilament fishing line (Konger, Germany) appeared to be very adhesive for all of the tested cell types. The growth kinetics of the examined cells on nylon meshes was similar to that on the glass fiber nets and polystyrene dish surfaces. This allows for the use of fragments of nylon nets with cells growing on them to passage the cells identically as on glass fiber mesh fragments, and similarly to tissue explants for primary cell culture.

When anchorage-dependent cells are transferred to new vessels for 3D culture, the cultivation of organoids, or implementation to living organisms, the use of biodegradable surgical threads or collagen capsules appears to be much cheaper and more convenient than the use of expensive and specially synthesized substrats for such purposes (Kleiman *et al.* 1981; Drumheller & Hubbell 1994; Płończak 2008, 2012; Chlupáč *et al.* 2014).

Biodegradable surgical threads can be used as portions of portable substrata. Of the dozen types of surgical threads we examined, three proved to be useful for cell passage and possible further cultivation of cells in 2D and 3D cultures. They are: Novosyn DSMP 19 Poly(glycolide-co-L-lactid), and Safil DSt or Safil Violet. These are surgical threads made up of a twist of thin filaments, and are slowly hydrolysable. All of the cells growing on glass and polystyrene that we examined also flattened on the surface of these three types of surgical threads and grew on them. What's more, these threads are relatively durable and yet dissolve after only 3-4 days.

Bovine collagen capsules produced as containers for orally administered drugs proved useful as well, for the same purposes as biodegradable surgical threads. Capsules of various sizes produced from beef collagen by different companies vary in durability in secondary cultures. It is possible to select soluble capsules which are dissolved after a few hours or a few days. Depending on the needs, more durable capsules can be selected for cloning or 2D and 3D cultures, or quickly biodegradable capsules for cell passage.

All of the substrata types listed above on which cells grew, can be used for the rapid passaging and transferring of cells to new vessels. Unlike flat, bottom-sticking coverslips that were previously used by some researchers, threads and nets are easy to grasp with tweezers normally used for coverslips (or stamps) and transfer to a new vessel without the requirement for special Handi-Vac Coverslip Pick-Up Tools. When passaging with the use of small pieces of portable substrata, the pieces can be reused many times: we used the abovementioned substrats for continuous cell culture for over two years with no signs of a decrease in cell viability, proliferation rates, or phenotypic changes. Moreover, due to avoiding the trypsinization process, the viability of cells produced using this process is better (94% $\pm$ 2 for trypsin, 97% $\pm$ 2 for explants). Depending on the number and size of fragments of the portable supports, the required dilution rate and number of cells transferred to secondary cultures can be estimated. Fragments of a portable substrata can be used not only for cell passage, but as the pre-analytical preparation of cell samples used later for various experiments and analytical tests. To summarize, the usage of portable substrata has many advantages over routine procedures based on the use of proteolytic enzymes and requiring the centrifugation of cell suspensions.

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Author Contributions

Research concept and design: Z.M., W.K.; Collection and/or assembly of data: D.R., J.P., A.T., J.T., M.J.; Data analysis and interpretation: W.K.; Writing the article: W.K.; Critical revision of the article: D.R.; Final approval of article: D.R., W.K.

Conflict of Interest

The authors declare no conflict of interest.

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