



JAGIELLONIAN UNIVERSITY
IN KRAKÓW

PHD THESIS

ARCHITECTURE OF THE VIMENTIN CYTOSKELETON IN
MOUSE EMBRYONIC FIBROBLASTS CULTURED ON
VARIOUS BIOPOLYMERS

KAROLINA MARIA FELIKSIAK, M. Sc

SUPERVISED BY:

DR HAB. ZENON RAJFUR, PROF. UJ

DR TOMASZ WITKO, UEK

FACULTY OF PHYSICS, ASTRONOMY AND APPLIED
COMPUTER SCIENCE

DEPARTMENT OF MOLECULAR AND INTERFACIAL
BIOPHYSICS

KRAKÓW, 2024

The most important things in life aren't things

Anthony J. D'Angelo

Wydział Fizyki, Astronomii i Informatyki Stosowanej

Uniwersytet Jagielloński

OŚWIADCZENIE

Ja niżej podpisana Karolina Feliksiak (nr indeksu: 1044563), doktorantka Wydziału Fizyki, Astronomii i Informatyki Stosowanej Uniwersytetu Jagiellońskiego oświadczam, że przedłożona przeze mnie rozprawa doktorska pt. „ARCHITECTURE OF THE VIMENTIN CYTOSKELETON IN MOUSE EMBRYONIC FIBROBLASTS CULTURED ON VARIOUS BIOPOLYMERS” jest oryginalna i przedstawia wyniki badań wykonanych przeze mnie osobiście, pod kierunkiem **dr hab. Zenona Rajfura, Prof. UJ** oraz **dr Tomasza Witko, UEK**.

Pracę napisałam samodzielnie.

Oświadczam, że moja rozprawa doktorska została opracowana zgodnie z Ustawą o prawie autorskim i prawach pokrewnych z dnia 4 lutego 1994 r. (Dziennik Ustaw 1994 nr 24 poz. 83 wraz z późniejszymi zmianami).

Jestem świadoma, że niezgodność niniejszego oświadczenia z prawdą ujawniona w dowolnym czasie, niezależnie od skutków prawnych wynikających z ww. ustawy, może spowodować unieważnienie stopnia nabytego na podstawie tej rozprawy.

Kraków, dnia

.....

.....

podpis doktoranta

Tę pracę doktorską dedykuję przede wszystkim Mężowi i Rodzicom, którzy wielokrotnie wspierali mnie w osiągnięciu celu, pomimo utrudnień i komplikacji, i bez których poświęcenia i czasu, nie mogłabym dokończyć eksperymentów.

Pragnę serdecznie podziękować P. Dr Hab. Zenonowi Rajfurovi za prowadzenie mnie w naukowej drodze, podjęcie opieki nad moimi badaniami i wielką cierpliwość.

Składam wyrazy wdzięczności P. Dr Tomaszowi Witko, za współpracę badawczą i nieocenione wsparcie w finalizacji publikacji naukowych.

Najserdeczniejsze podziękowania dla Koleżanek i Kolegów z Zakładu Biofizyki Molekularnej i Międzyfazowej – dla Olgi Adamczyk, Zbigniewa Bastera, Darii Solarz-Keller, Tomasza Kołodzieja, za wielokrotną pomoc w pracy i wspólną walkę ze złośliwością rzeczy martwych (oraz żywych) na laboratoryjnym polu.

Dziękuję!

Table of Contents

ACRONYMS AND ABBREVIATIONS LIST	8
LIST OF PUBLICATIONS	11
ABSTRACT	12
STRESZCZENIE	13
1. Introduction	14
2. Cytoskeleton	15
2.1 Actin filaments	16
2.2 Microtubules	17
2.3 Intermediate filaments	18
2.3.1 Vimentin	19
3. Nucleus	23
3.1 Nuclear deformations and distortions	25
4. Mechanotransduction	25
4.1 Vimentin in mechanotransduction	26
4.2 Nucleus in mechanotransduction	27
5. Biopolymers	28
5.1 PLA	31
5.1.1 PLA – synthesis	31
5.2 Polyhydroxyalcanoates	34
5.2.1 P(3HO)	36
6. Confocal Microscopy	37
Scope of the study	40
7. Materials & Methods	40
7.1 Preparation of P(3HO) and PLA substrates for cell culturing	40
7.2 Cell culturing	41
7.3 Cells preparation and staining	41
7.4 Microscopic observations of vimentin cytoskeleton of MEF 3T3 cells on polymeric films and glass	42
7.5 Statistical analysis	43
8. Results	43
8.1 PLA and PHO substrates characterization	43
8.2 Analysis of vimentin cytoskeleton architecture on PLA and P(3HO) substrates	45
8.3 Analysis of the structure of vimentin cytoskeleton	50
8.4 Vimentin association to nuclear grooves	54

8.5	Occurrence of nuclear blebs in association to the substrate used for cell culturing	58
8.6	Vimentin – Nuclear Grooves coalignment review	59
9.	Discussion	61
9.1	Influence of the substrate used for cell culturing on formation of classic vimentin vs “nutshell” structure	61
9.2	Influence of the substrate for cell culturing on the occurrence of nuclear grooves, invaginations and blebs	62
10.	Summary of the results and comments	64
10.1	Vimentin association with nuclear grooves in normal MEF 3T3 cells	65
10.2	Vimentin cytoskeleton architecture analysis on polylactide and polyhydroxyoctanoate substrates for cell culturing	66
11.	Final remarks	67
12.	List of Figures	69
13.	References	70

ACRONYMS AND ABBREVIATIONS LIST

AF – Actin Filaments

BPA - Bisphenol A

BSA - Bovine Serum Albumin

CR – Regenerated Cellulose Films

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle's Medium

ECM – Extracellular Matrix

EMT - Epithelial-Mesenchymal Transition

ER - Endoplasmic Reticulum

FBS - Fetal Bovine Serum

HIV - Human Immunodeficiency Virus

IF – Intermediate Filaments

INM – Inner Nuclear Membrane

lcl – long chain length

LINC – Linker of Nucleoskeleton and Cytoskeleton Complex

LSM – Laser Scanning Microscope

KASH - Klarsicht, ANC-1, Syne Homology proteins

SUN - Sad1p/Unc-84 domain

mcl – medium chain length

MEF – Mouse Embryonic Fibroblasts

MF – Microfilaments

MSW – Municipal Solid Waste

MT – Microtubules

MTOC - Microtubule Organizing Center

NE – Nuclear Envelope

NPC – Nuclear Pore Complex

ONM – Outer Nuclear Membrane

PGA - Polyglutamic Acid

PHA – Polyhydroxyalkanoate

PHB - Polyhydroxybutyrate
PLA – Polylactide
PBAT- Polybutylene Adipate Terephthalate
PBS - Phosphate Buffered Saline
PBT - Polybutylene Terephthalate
PCL - Polycaprolactone
PE - Polyethylene
PP – Polypropylene
PVC - Polyvinyl Chloride
P(3HO) – Polyhydroxyoctanoate
ROI – Region of Interest
ROP – Ring Opening Polymerization
scl – short chain length
SCPC – Starch Containing Polycaprolactone
TGF - Transforming Growth Factor
ULFs- Unit-Length Filaments
VIF – Vimentin Intermediate Filaments

LIST OF PUBLICATIONS

This dissertation has been written based on the scientific results previously reported in the following articles:

- [1] K. Feliksiak, D. Solarz, M. Guzik, A. Zima, Z. Rajfur, T. Witko, Vimentin cytoskeleton architecture analysis on polylactide and polyhydroxyoctanoate substrates for cell culturing, *Int. J. Mol. Sci.* 22 (2021). <https://doi.org/10.3390/ijms22136821>.
- [2] K. Feliksiak, T. Witko, D. Solarz, M. Guzik, Z. Rajfur, Vimentin association with nuclear grooves in normal MEF 3T3 cells, *Int. J. Mol. Sci.* 21 (2020) 1–13. <https://doi.org/10.3390/ijms21207478>.

The authorship in the remaining articles:

- [1] T. Witko, D. Solarz, K. Feliksiak, K. Harażna, Z. Rajfur, M. Guzik, Insights into In Vitro Wound Closure on Two Biopolyesters—Polylactide and Polyhydroxyoctanoate, *Materials (Basel)*. 13 (2020) 2793. <https://doi.org/10.3390/ma13122793>.
- [2] T. Witko, D. Solarz, K. Feliksiak, Z. Rajfur, M. Guzik, Cellular architecture and migration behavior of fibroblast cells on polyhydroxyoctanoate (PHO): A natural polymer of bacterial origin, *Biopolymers*. 110 (2019) e23324. <https://doi.org/10.1002/bip.23324>.
- [3] K. Zamora*, D. Dziob, J. Nowak, W. Piekarczyk, Z. Rajfur, J. Laska, Elastyczne podłoża poliakryloamidowe do badań komórkowych Elastic polyacrylamide substrates for cell studies, *Engineering of Biomaterials*, 124 (2014) 11–18.

*K.Zamora is maiden name of K.Feliksiak

ABSTRACT

Cell cytoskeleton is a dynamic structure of three main networks of fibres, among which the most well studied are microfilaments (actin filaments, AFs), and microtubules (MTs). The third type of network, cooperating with the AFs and MTs, is a group of intermediate filaments (IFs), including different proteins depending on the cell type. An intermediate filament present in mouse embryonic fibroblast cells (MEF) is vimentin. MEF cells are valuable material in multiple studies focusing on cellular differentiation, gene targeting, toxicology and drug screening, wound healing process, or cell cycle studies.

Vimentin intermediate filaments (VIFs) are mostly known for maintaining cell integrity and protecting it from external mechanical stress. Nevertheless, VIFs are highly dynamic structures, that are reported to change their architecture upon certain conditions (for instance in different timepoints of adhesion). The involvement of vimentin in transmission of different signals from the exterior of the cell to the areas of nuclear envelope is now evident, yet little is known about the exact mechanisms of these signalling pathways, and the consequences appearing in a single cell, tissue or finally whole organism.

Knowing that the involvement of vimentin in transferring signals from the exterior to the interior of the cell can vary on the base of the microenvironment, the first steps of investigation considered inclusion of the mechanical stimuli, i.e. application of different substrates for cell culturing. The substrates varied in terms of the material of origin, microstructure and physicochemical features, which allowed observing both the behaviour of the cell as a whole, and its internal elements of cytoskeleton.

Also, the choice of the substrates was thoroughly reviewed, and turned towards sustainable materials, that are safe for patient's use, but also leave no harm to the environment. Therefore, MEF cells were cultured on matrices made from materials with high potential of use in tissue engineering applications, namely: polyhydroxyoctanoate (P(3HO)), polylactide (PLA). Glass substrate was used as a reference. Both of these polymers have excellent biocompatibility, and their components might be obtained from the sources of natural origin. Methods of their production are quite different. PLA, that may be obtained from organic resources, like whey, skim milk, corn starch, or sugar cane cellulose, is eventually synthesized in a chemical process of polycondensation or ring opening polymerization (ROP). P(3HO), on the other hand, is produced and stored by bacteria, as their energy reservoir, in a form of intracellular granules, insoluble in water. The polymer is only gathered, purified, and ready for further processing, which opens new possibilities of manufacturing fully natural polymers with no environmental harm. Both chosen polymers are being actively used in medicine (PLA), and PHAs are currently investigated for their possibilities of use as for instance matrices for wound healing. They are proven to have features, that influence cells' morphology, and interdependencies on the elements of cytoskeleton. Therefore, the topic of this thesis is the analysis of the influence of PLA and P(3HO) on vimentin cytoskeleton in relation to cellular behaviour and mechanotransduction of signals from the exterior of the cell to the nuclear envelope area.

STRESZCZENIE

Cytoszkielecik komórki to dynamiczna struktura składająca się z trzech głównych sieci włókien, spośród których najlepiej zbadane są mikrofilamenty (filamenty aktynowe, AFs – actin filaments) i mikrotubule (MTs - microtubules). Trzecim rodzajem sieci współpracującej z AFs i MTs są filamenty pośrednie (IFs – intermediate filaments), obejmujące różne białka w zależności od typu komórki. Filamentem pośrednim obecnym w komórkach fibroblastów embrionalnych myszy (MEF – mouse embryonic fibroblasts) jest wimentyna. Komórki MEF są cennym materiałem w wielu badaniach skupiających się na: różnicowaniu komórek, celowaniu genowym, toksykologii i badaniach leków, procesie gojenia ran, czy też na badaniach nad cyklem komórkowym.

Filamenty pośrednie wimentyny (VIFs – vimentin intermediate filaments) są znane przede wszystkim z utrzymywania integralności komórki i jej ochrony przed zewnętrznym stresem mechanicznym. Niemniej jednak VIFs są strukturami o wysokiej dynamice, które zgodnie z doniesieniami zmieniają swoją architekturę w określonych warunkach (na przykład w różnych etapach adhezji). Zaangażowanie wimentyny w przekazywanie różnych sygnałów z zewnątrz komórki do obszarów otoczki jądrowej jest obecnie oczywiste, ale niewiele wiadomo na temat dokładnych mechanizmów tych szlaków sygnalizacyjnych i konsekwencji występujących w pojedynczej komórce, tkance lub ostatecznie całym organizmie.

Zważywszy, że rola wimentyny w przekazywaniu sygnałów z zewnątrz do wnętrza komórki może zależeć od mikrośrodowiska, w pierwszych etapach badań uwzględniono bodźce mechaniczne, tj. zastosowanie różnych podłoży do hodowli komórkowej. Substraty różniły się pod względem materiału pochodzenia, mikrostruktury i cech fizykochemicznych, co pozwoliło na obserwację zarówno zachowania komórki jako całości, jak i jej wewnętrznych elementów cytoszkieletu.

Wybór podłoży został również dokładnie przeanalizowany i skierowany ku materiałom zrównoważonym, bezpiecznym dla pacjenta, a jednocześnie nie szkodzącym środowisku. Komórki MEF były zatem hodowane na matrycach wykonanych z materiałów o wysokim potencjale zastosowania w inżynierii tkankowej, a mianowicie: polihydroksyoktanie (P(3HO)), polilaktydzie (PLA). Podłoże szklane zostało użyte dla referencji. Obydwa te polimery mają doskonałą biokompatybilność, a ich komponenty mogą być pozyskiwane ze źródeł pochodzenia naturalnego. Metody ich produkcji są dość zróżnicowane. PLA, który można uzyskać z organicznych źródeł, takich jak serwatka, odtłuszczone mleko, skrobia kukurydziana czy celuloza trzciny cukrowej, jest ostatecznie syntetyzowany w procesie polikondensacji lub otwarcia pierścienia polimeryzacji (ROP). Z kolei P(3HO) jest produkowany przez bakterie jako ich materiał zapasowy w postaci granulek wewnątrzkomórkowych, nierozpuszczalnych w wodzie. Polimer jest następnie zbierany, oczyszczany i gotowy do dalszej obróbki, co otwiera nowe możliwości produkcji w pełni naturalnych polimerów bez szkody dla środowiska. Oba wybrane polimery są aktywnie stosowane w medycynie (PLA) i obecnie badane pod kątem możliwości zastosowania, np. jako matryce do gojenia ran. Udowodniono, że posiadają one cechy wpływające na morfologię komórek i współzależności między elementami cytoszkieletu. Dlatego też tematem niniejszej pracy jest analiza wpływu PLA i P(3HO) na cytoszkielecik wimentyny w powiązaniu do zachowania komórki i mechanotransdukcji sygnałów z zewnątrz komórki do obszaru otoczki jądrowej.

1. Introduction

Sensing and transmitting of biosignals is a process that determines the outcome of an event occurring in the environment [1]. The complicated nature of detecting and responding to the signals sent between multiple organisms, can be observed in both, macro and micro scales. Synchronicity of the movement of fish in a shoal, or birds flying in the key not only displays a kind of a unity but may also be noticed in a microscale. In the microscopic universe similar phenomena may be observed in the world of tissues and fluids – the living organism. The cells communicate through chemical, and biochemical signals, and receptors on cell membranes manage coordination of that intercellular dialogue, allowing multiple complex mechanisms to occur, like for instance: migration of the cells creating a tissue towards the closure of the wound, or the movement of single cells towards the more favourable conditions on a given substrate in a culture [2]. These observations lead to the deeper insights into the exact determinants of some cellular “decisions”. The considerations begin with the basic senses that the single cell could be equipped in, and that brings the idea of a “sense of touch” however, the exact term that we are looking for is “mechanotransduction”.

Mechanotransduction is the concept, that covers the communication of the cell and an external stimulus (i.e. tension forces, pressure change, matrix stiffness, shear stress, stretching and compression, etc.) that is converted into a biochemical signal, and gives a desirable effect [3]. The more the inner cellular structure is researched, the better the pathway of this complicated process and the role of each separate cellular organelle are known. Undoubtedly, the crucial element, being the base of the whole process, is cytoskeleton, which spans throughout the cell and links the external environment with the cellular core, the nucleus. Actin cytoskeleton – being broadly described in literature - is known to be one of the most important elements in the whole process. There are numerous studies that also confirm the involvement of microtubules in transferring signals to the interior of the cell [4–6]. The least known for their function though, are intermediate filaments, that connect not only microtubules and actin filaments, but also are the key determinants of cell stability, integrity, and can influence cellular functions, if not working properly.

This work focuses on the intermediate filaments of mouse embryonic fibroblast cells – vimentin IFs, and their formation throughout the cell – from the edge – to the nucleus, observed on different culture substrates.

2. Cytoskeleton

The cytoskeleton is a complex, multifunctional mechanical structure of the cell, involved in a variety of crucial cellular processes, such as: cellular division, motility, transport, endocytosis, mechanotransduction, transmission of force, adhesion, reaction to external stimuli and arrangement of cell shape [7]. This dynamic biopolymer network comprises of three cytoskeletal subtypes, namely: actin (microfilaments), microtubules and intermediate filaments. The individual subtypes are highly dynamic and multifunctional structures, with capabilities of reorganization upon receiving external or internal signals. It is known as one of the most important cell structures in terms of resisting deformations, maintaining cell's form and influencing its mechanical properties [8].

Nowadays it is hard to believe, that just over 30 years ago, this structure has only been recognized in eukaryotes, excluding bacteria and archaea [9]. However, interest in cytoskeleton has grown in recent years, among others, due to its significant contribution to three key aspects of cancer development: cell cycle, morphogenesis and migration [10]. Current research focus on the analysis of the structures, that are directly responsible for signalling, movement, wound healing, metastasis (considering cancer), and mechanotransduction. This brings the attention to the cytoskeleton and internal cooperation of its elements [11].

Cytoskeleton comprises of three main types of structures: actin filaments, microtubules, and intermediate filaments, which work together, influence and control mechanics and integrity of the cell. These three groups of polymers have common features, i.e. network-like construction, resistance to deformations, and constant reorganization. Actin filaments (microfilaments - MFs, also known as "F-actin") and microtubules (MTs) directly influence cell shape and guide the organization of the cellular structures. MTs, that are supported by molecular motors (kinesin and dynein), moving along them in the opposite direction, are also crucial in cellular transport, and separation of chromosomes during mitosis. Both, actin forming MFs and tubulin forming MTs are highly conserved globular proteins. The structure of those two networks is led and regulated by different factors:

- a) nucleation-promoting factors - initiating formation of the filaments
 - b) capping proteins - terminating growth of the filaments
 - c) polymerases - controlling the pace of the growth of filaments
 - d) depolymerizing and severing factors - disassembling the network
 - e) crosslinkers and stabilizing proteins - determining higher order structures
- [10,11].

All the above-mentioned factors are vulnerable to mechanical signalling both from internal and external environment of the cell, which may lead to changes in their activity, and in consequence to reorganization of filaments in the networks.

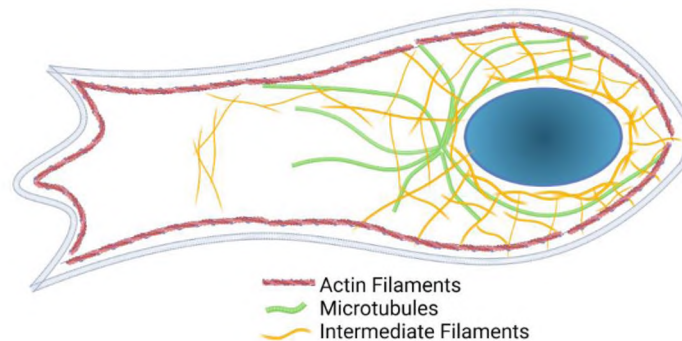


Figure 1 Example of distribution of different elements of the cytoskeleton in the cell. Figure created in Scientific Image and Illustration Software | BioRender.

Despite the common features, cytoskeleton structures may be easily distinguished, as they differ in size, architecture, mechanical stiffness, polarity, assembly dynamics and molecular motors, that are linked to them [10].

2.1 Actin filaments

Actin - a 43kDa highly conserved protein, found in all eukaryotic cells, is not only present in the cytoplasm, but also in the nucleus, taking part in organization of its structure, transcription and transferring signals [12]. The main feature of actin, that allows it to contribute to a wide range of above-mentioned processes, is the ability to polymerize and interact with various proteins. Globular, monomeric actin (G) and actin filaments (F), coexist together in a dynamic balance [13].

Actin filaments are the structures of a diameter size of 9-10 nanometers, comprised of two subunits formed in helical chains. Actin is mostly located in the cortical part of the cell, in the form of short fibrils, creating microfilament network, however MFs might also be organized in parallel, ordered bundles, known as stress fibres [10,14,15].

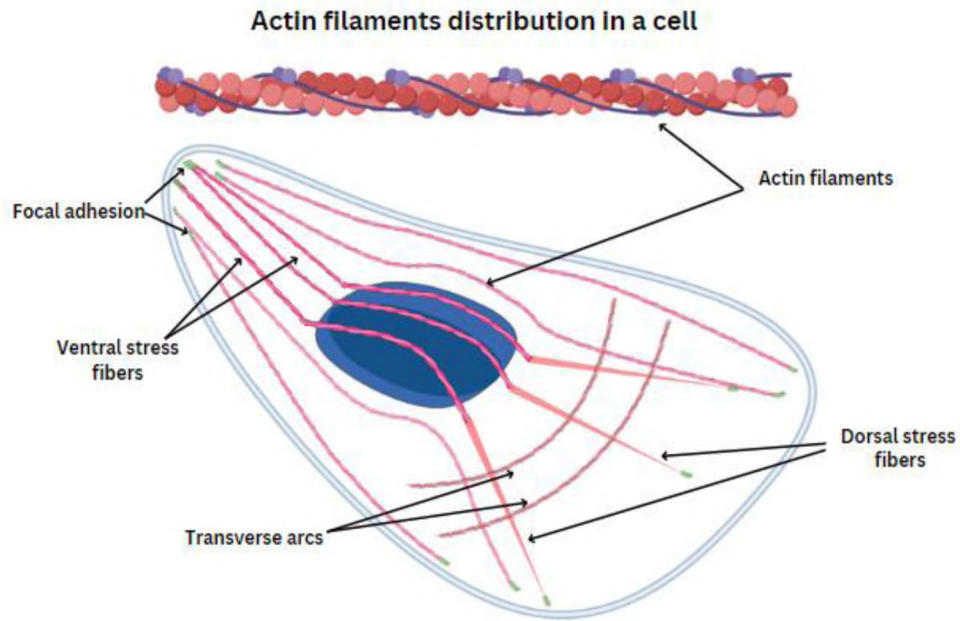


Figure 2 Microfilaments distributed in a cell. Figure created in Scientific Image and Illustration Software | BioRender

MFs can reorganize dynamically, and thus change the shape of the cell membrane, as well as cause creation of filopodial protrusions. That feature is the result of the presence of crosslinkers that bind to actin filaments, which also causes creation of stiff, highly organized structures, such as bundled networks, branched networks and isotropic networks. MFs are dynamic structures, that constantly assemble and disassemble depending on the signals received. There are 6 isoforms of actin present in vertebrates, two of which - β and γ , can be found in all types of [10,16]. β and γ isoforms vary in terms of the dynamics of polymerization process and are located in different areas of the cell. Whereas β actin is present in stress fibres, circular bundles, and cell-cell contacts, γ isoform is expressed in dependence of cell activity. In motile cells γ -actin is found as a mesh in cortical and lamellipodial structures, but also can be seen in a leading edge of migrating cells. In non-migrating cells, γ -actin is also present in stress fibres [16,17].

2.2 Microtubules

Another basic element of the cytoskeleton are microtubules (MTs), the stiffest structures of cellular interior, built of 13 parallel protofilaments of tubulin - a globular protein, comprising of dimers of two subunits: α (55-57 kDa) and β (53 kDa). Tubulin, beside taking

part in spatial organization of cell's cytoplasm, plays a crucial role in mitosis - being a significant protein of mitotic spindle - thus it is directly involved in division and proliferation of the cells [8].

MTs have the most complex dynamics of assembly and disassembly process. Their importance in cell's main activities, such as transport of vesicles, formation of cilium and cell polarity is acknowledged, however complete understanding of some of those processes is still progressing [4].

The organization of MTs in the cell starts close to the nucleus - in the MTOC (microtubule organizing center), and the fibres, depending on the cell type, might be single or multiple parallel bundles alongside with the long axis of cytoplasmic spikes. MTs also can be found freely distributed in the cytoplasm [16].

2.3 Intermediate filaments

Cytoplasmic intermediate filaments (IFs) – another compound of the cytoskeleton - are involved in multiple roles significant for maintaining appropriate organization of cell structure. There are over 70 highly conserved proteins included in the superfamily of IFs (near 65 genes) and despite that they are the least stiff elements of the cytoskeleton, and were initially recognized as stable proteins, it is currently known that IFs are dynamic structures. IFs name refers to their size, that is intermediary between microfilaments and microtubules (diameter near 10 nm), and their role in cellular processes is not only limited to reinforcement of cell structure, but also, they are involved in important cellular processes as cell growth, ageing, stress and homeostasis [18].

IFs are a group of proteins, that vary depending on the tissue origin, expression pattern structure, and sequence identity, thus they can be distinguished into five different gene families (with sixth IF family, that includes lamins residing in the nucleus - type V):

- I. Acidic keratins (ectodermal and endodermal cytokeratins, 28 human genes)
- II. Basic keratins (present in muscle and non-muscle cells, 26 genes in humans)
- III. Homodimerizing intermediate filaments (some with capability of heterodimerizing, in non-muscle endodermal cells - Vimentin, Desmin, GFAP)
- IV. IFs expressed in neurons and muscle (Neurofilaments, Nestin, Synemin)
- V. Nuclear IFs (Lamins)
- VI. Lens-specific beaded IFs (Pakinin, Filensin) [16,19].

Intermediate filaments are organized as pairs of homo- or hetero-dimers which create antiparallel tetramers, that are associated in a final form of rope-like filaments. All IFs have a tripartite molecular structure:

- conserved central α -helical conserved domain (near 310 aminoacids)
- variable non α -helical head (amino terminal) and tail (carboxy terminal) domains[19].

IFs appear in the cytoplasm as a network, connecting cellular and nuclear membrane with microfilaments and microtubules by proteins called plectins, which they also use to connect to each other. IFs are anchored to desmosomes at the adhesion points between cells, and to hemidesmosomes at the adhesions of cells with substrates. While the main function of IFs is to maintain cell morphology and mechanics, they are also known to resist to tensile forces much more effectively, than compressive forces. IFs are assembled by many cell types in response to mechanical stress, however, due to the fact of being unpolarized, they are not involved in supporting directional movement of molecular motors [8,19,20].

Despite differences in structure, features and designation, polymers that create cytoskeleton are providently connected and dependent on each other [21]. Organization of the cytoskeleton elements directly influences the quality of sensing of the mechanical microenvironment and the transmission of compressive and tensile stress. Rheological experiments revealed the ability of IFs to become flexible at low strain, and stiff at high strains which is another proof, that IFs provide mechanical integrity within cell structure and influence cell signalling pathways and mechanotransduction. Considering the above-mentioned discoveries, IFs have recently become a popular subject of further investigation [19,22].

2.3.1 Vimentin

Vimentin, a 466 amino acid intracellular protein, encoded by VIM gene, is one of the intermediate filaments, that contributes to various cellular processes, such as cell adhesion and migration. The name's "vimentin" origin comes from Latin word "vimentum", which means "wickerwork". Vimentin IFs are basically responsible for plasticity of mesenchymal cells although recently their role has been appreciated for contribution to various crucial cellular processes, such as EMT - epithelial-mesenchymal transition, a process, that directly connects to wound healing or cancer metastasis [23,24].

Studies on vimentin IFs are relatively novel, due to the fact, that the early experiments on vimentin-depleted mice suggested, that there were no significant changes in phenotype [25]. It resulted in a long-term understanding, that despite vimentin's high conservation in vertebrates, it is not physiologically important. Recent studies show that motility and directional

migration in vimentin-depleted fibroblasts is affected and results in damaged wound healing and cell migration [23]. Vimentin's importance in many processes is being continuously discovered - i.e. mechanotransduction of shear stress (resistance of arteries), homing, transmigration and extravasation of lymphocytes or maintaining integrity of vascular endothelium [25,26]. Of all vimentin features, the most interesting in terms of this thesis are those, connected to intracellular arrangements:

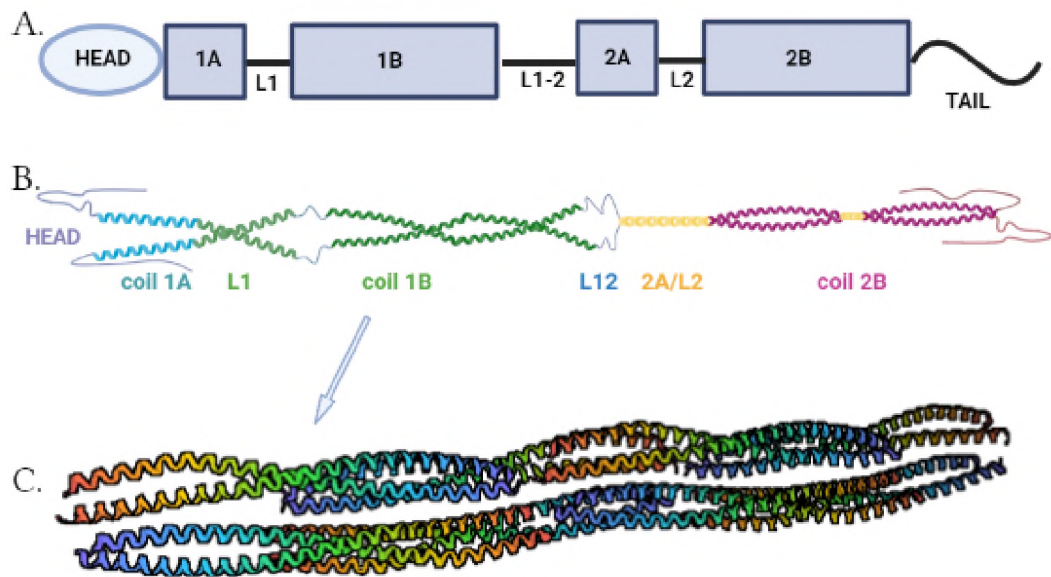
- anchoring, distribution and organization of cellular organelles
- linking cell nucleus to the cell surface
- influencing cell shape by distribution of network
- localizing intracellular organelles by tethering
- preserving mechanical properties of cells
- transduction of mechanical signals and transmitting them in the cytoplasm
- participation in epithelial-mesenchymal transition
- determining traction stress orientation.

On the other hand, lack of vimentin causes defects in polarity of cells, which prohibits single cell migration. Novel research show, that vimentin IFs are participating in vast majority of cellular processes, that are critical in cell's life cycle, whereas motile and invasive cells express higher levels of vimentin, thus the interest in discovering the exact mechanisms of work of these structures increases [22,23,25,27].

2.3.2 Structure and architecture of vimentin network

Vimentin is a 57kDa polypeptide, and as for all intermediate filaments, its structure comprises of α -helical central "rod" domain, and non α -helical domains: amino head (77-residue) and carboxyl tail (61-residue). Two polypeptide chains are associated parallelly forming coiled-coil dimers, the presence of which is in consequence allowing to form homopolymers and heteropolymers. Coiled-coil domain helps achieving high stability of polymers, which is controlled by phosphorylation status of integral proteins. Dimers are associated in antiparallel tetramers, which finally assemble into "unit length filaments" (ULFs). ULFs contain 8 tetramers and serve as ends in elongation phase of yielding non-polar filaments. Mature vimentin IFs' diameter is 10nm, and that final structure provides them much better elasticity and flexibility comparing to actin filaments and microtubules [22,25].

VIMENTIN DOMAINS SCHEMA



CRYSTAL STRUCTURE OF VIMENTIN COIL1B IN FILAMENTOUS FORM

Figure 3 Structure of vimentin. A. Basic construction of vimentin – block chain. B. Coiled-coil dimer of vimentin formed by a pair of parallel chains Coiled-coil segments 1A, 1B, 2A, and 2B are connected by a short linkers (L1, L12, L2). C. Crystal structure of vimentin coil 1B packed in a high-order filamentous form (Source: – <https://www.rcsb.org/structure/5whf>).

The structure of vimentin was initially thought to be a stable, stiff network, yet nowadays it is known, that VIFs are very dynamic structures, which undergo significant organizational changes during different crucial cellular processes - i.e. transition from polymerized network into non-filamentous particles during progression of cells from late prophase into metaphase [28]. Considering that, there are three main forms of vimentin intermediate filaments, that are identified in the cell cytoplasm:

- non-filamentous particles (single or small aggregates of unit length filaments)
- short IFs (IFs (end-to-end linkages of ULFs)
- long/mature VIFs (for which precursors are short filaments and particles).

The last ones create vimentin network present throughout the cell cytoplasm [29].

Vimentin is usually expressed in fibroblasts, endothelial cells, eye lens epithelium and dendritic reticulum cells of lymphoid follicles. Its distribution in the cell cytoplasm starts from large number of IFs surrounding the nucleus; from that point the IFs spread into the trailing edge of the cell. In the cell's leading edge there are only short vimentin filaments in the lamellar region, and particles in the lamellipodium. Vimentin intermediate filaments are known to polymerize longitudinally and laterally without polarity, which suggests that architecture of

VIFs could be a key determinant of the network's function. Two types of the vimentin network can be distinguished, when observing cell migration: fibrous structures - long filaments, and mesh-like structures. The fibres are aligning to the actin's anisotropic orientation of traction stresses [22,27].

2.3.3. Role of vimentin in biological processes

Vimentin's role in crucial biological processes is just being appreciated, as the research show, that it contributes to EMT (epithelial-mesenchymal transition), regulation of transduction pathways, migration of cells, positioning and anchoring of organelles, wound healing, invasion of cancer cells, lipogenesis, and cell-cell or cell-substrate adhesion [30]. Beside the filamentous vimentin residing in the cytoplasm, there is also a fraction of an extracellular vimentin that is secreted to the ECM, which is linked to the processes such as: support of the neural repair, mediation of the viral or bacterial invasion of the host cells or marking tumour cells [5]. More and more recent findings suggest, that vimentin's role in the appropriate functioning of cells and tissues translates directly to the development of the specific diseases, namely: Crohn's disease, cancers, cataract, or HIV [31].

One of the most important processes, that are regulated by vimentin, is cell migration. It has been proven, that vimentin is required for the proper motility by contribution in acto-myosin contractility, interactions with the ECM, and engagement in moving forwards the cell body [31,32]. Also, research show that vimentin deficiency causes problems with adhesion, migration and invasion of lymphocytes, and similarly – in motility and directional migration of astrocytes and fibroblasts. A consequence of impaired migration might be quite easily noticed in defective wound healing process [31,33].

Wound healing assay is a convenient and reproducible model for conducting experiments on cells sheet migration [34]. It is also one of the first experiments in which the presence of extracellular vimentin has been observed. The simplified description of the mechanism is, that upon injury, the cytoskeletal vimentin is excreted into the extracellular space, where it is attaching to the surface of the mesenchymal leader cells at the edges of the wound in the native matrix environment. It has been noticed, that in pro-fibrotic environments vimentin might determine the designation of cells to gain myofibroblast phenotype. This phenotype, characterized by contractile properties and matrix synthesis, has a significant role in tissue repair processes. The extracellular translocation, that vimentin undergoes suggests, that it might have significant influence on closing the wound [35].

Recent research suggest that vimentin plays a crucial role in mediating cell - repair during wound-healing process. Its function as a signalling integrator in regeneration and healing of tissues has been proven, as it is known to control proliferation of fibroblasts and differentiation of keratinocytes in the process of wound healing via TGF- β Slug signalling. Vimentin depleted fibroblasts were observed to have a serious deficiency in growth, which is directly involved in activation of the abovementioned two major factors of epithelial-mesenchymal transition (EMT): TGF- β and Slug protein [36].

Besides that, in mock-cataract surgery model, vimentin has shown its crucial role in activating mesenchymal leader cell population for migration towards closing the wound and promoting population in the injured edge of the epithelium. Furthermore, cells activated at the very moment of wounding gain an invasive phenotype, which also is vimentin dependent and determined [37–39]. Finally, vimentin has also been proven to be highly expressed in cells of fibrotic phenotype, leading to formation of a scar tissue [35].

3. Nucleus

The core of the cell, the repository of genetic information and the Holy Grail for researchers, exploring the essence of life – all this is embraced in an oval-shaped 20 μm ball – the nucleus. The nucleus is the stiffest and the biggest organelle in eucaryotic cells. It carries a tightly packed genetic information, in a form of densely clumped chromatin. The fibres of chromatin are composed of repeating nucleosomes, that are wrapped around histone octamers, and a linker DNA region [3,40]. The DNA in the nucleus is protected by the nuclear envelope (NE), a barrier for the genome, that also provides communication between the cytoplasm and the nucleus. Nuclear envelope is basically a specialized continuation of endoplasmic reticulum (ER), and it comprises of three layers:

- nuclear lamina
- double membrane, separated by perinuclear space, including:
 - inner nuclear membrane (INM)
 - outer nuclear membrane (ONM)
- nuclear pore complexes (NPCs) [41].

Cell Nucleus

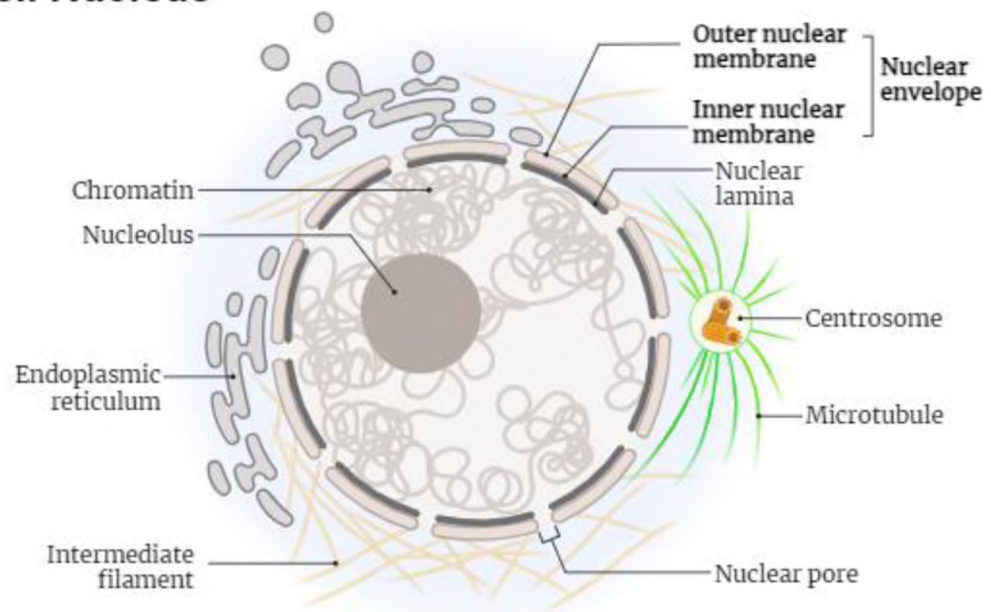


Figure 4 Cell nucleus schema with the crucial elements indicated. Figure created in Scientific Image and Illustration Software | BioRender.

The nuclear lamina is the element that stands the nearest to the DNA – linking the nuclear envelope and the chromatin. It is composed of lamins (A- and B- type) – proteins, that belong to the group of intermediate filaments, and lamin-associated proteins. Nuclear envelope is a dense meshwork engaged in chromatin organization, transcription and replication. It also secures the nucleus structural stability [42].

The outer nuclear membrane is contiguous with the endoplasmic reticulum, and also with the inner nuclear membrane [43]. This permeation of the layers of ONM and INM is associated with the nucleo-cytoskeletal coupling in terms of cellular migration. The presence of the strong anchorages between the cytoskeleton and the nucleus enables movement of cells and is based on LINC complex (linker of the nucleoskeleton and cytoskeleton), that connects the ONM and INM. LINC complex is a group of two classes of transmembrane proteins: KASH (Klarsicht, ANC-1, Syne homology) domain, that has proteins at the ONM, and SUN (Sad1, and UNC-84) domain, with its proteins on INM [40,44].

The last structure – nuclear pore complexes, are practically the fusions of the outer nuclear membrane with endoplasmic reticulum. NPCs serve as channels for selective transport of micro and macromolecules, as well as they enable the diffusion of small molecules [41].

3.1 Nuclear deformations and distortions

Nuclear envelope, as an extension of endoplasmic reticulum, is connected to the cytoskeleton in a variety of ways. First of all, the nuclear lamina itself is a protein of the group of intermediate filaments. Significantly important is also the LINC complex. The domains of LINC – KASH and SUN – interplay in the perinuclear space. Also, KASH proteins (nesprins) interact with the cytoskeleton (microtubules, microfilaments and intermediate filaments), and SUN domain proteins interact with nuclear lamina [44]. All the connections of the nucleus with the proteins that protrude to the cytoplasm, have impact on nuclear stability, deformations and finally – genes expression [3].

The nucleus is the biggest cell organelle. Its stiffness is 5-10 times higher, than the cytoskeleton's, which is provided by the structural protein 'cage' that surrounds it. During migration, cells are exposed to the external factors, like sheer stress (when moving through narrow spaces), or stretching, and the nucleus is forced to resist changes in shape [45]

Common phenomenon in migrating cells is the deformation of the nucleus, namely: narrowing during movement through tight areas, and occurrence of nuclear blebbing. The research confirms the importance of the nuclear stability, and its influence on the development of the cell. What is also interesting, is the fact, that the intermediate filaments are significantly engaged in the process of protection of the nuclei. Vimentin intermediate filaments are involved in regulation of nuclear shape, and cellular motility. VIFs protect nuclei from large deformations and rupture, thus they play an important role in the development of different diseases [46].

The exact mechanism standing behind the cooperation of intermediate filaments, and cell nucleus is yet to be elucidated, however the understanding of the importance of these structures is crucial step for directing research into NE – IFs dependencies.

4. Mechanotransduction

Mechanotransduction is the ability of the cells to detect and respond to mechanical forces by converting them into biochemical and biological signals. As a result, external or internal stimuli trigger cellular responses and generate changes in cells' inner structure[44,47]. The forces that can be converted into biochemical signals can start in the culture matrix, like for example elastic modulus and stiffness of the substrate. Also, other factors may affect cells,

for instance compressive, shear and tensile stress, that directly forces cell reshaping by cytoskeleton inner arrangements. All the stimuli received by the cell from the external environment, activate the transduction of signals from the edges of each cell to the core – the nucleus [47]. Recent studies suggest that the nucleus itself is also a mechanoresponsive organelle and is reacting to the cytoskeletal forces by conformational changes, partial unfolding, phosphorylation of nuclear envelope proteins or modulation of chromatin organization. The result of nuclear alterations might cause changes in transcription [48].

Considering that mechanotransduction is a critical communication way in cellular environment, it plays a crucial role in multiple processes, such as: cell and tissue differentiation, motility and migration, maintenance and disease, adhesion and proliferation [47,48]. The changes in physical environment are detected and adapted to by the cells rapidly, as the speed of propagation of mechanical forces throughout the cell can reach 30 μ m/s, which is much faster than, for instance, molecular motor transport (1 μ m/s)[48]. Hence, any disruptions in transmission of signals also affect the cell promptly, and might lead to diseases like muscular dystrophy, cardiomyopathy, or cancer [44,49].

4.1 Vimentin in mechanotransduction

Despite various research on the cytoskeleton, there are still uncertainties regarding the exact role of many cellular structures in the communication process. Novel research, for example, revealed the influence of extracellular vesicles on proliferation of neural stem cells, that has so far been unknown [50]. Certainly, intermediate filaments present an intriguing subject warranting further exploration, as our understanding of the specific roles vimentin plays in sensing and transmitting various mechanical forces remains limited [51]. However so far, vimentin has been noticed to take part in cell migration, contraction, positioning of organelles, and proliferation [52].

Although not quite accurately, intermediate filaments role used to be connected only to cellular stiffness, and they were thought to provide stability, protection and mechanical integrity to the interior of the cell. Whereas vimentin filaments are able to bear large deformations of around 240% of stretching under tension, they also remain very sensitive to the forces at low levels [53,54]. The tension applied on cellular vimentin is causing changes in its structure prompting at first linearly elastic response – stretching of hydrogen bonds. For the strains that reached a critical value, hydrogen bonds break, and α -helical, coiled-coil unfolds, turning into β -sheets, which allows vimentin molecules to multiply their initial length without

breaking. Finally, if the further stretching of covalent bonds, and uncoiling is no longer possible, the molecule becomes strain hardened [55]. This brilliant ability might lead to an explicit effect on cell behaviour – vimentin depleted cells are reported to have reduced stiffness, whereas in cells with an overexpression of vimentin – the stiffness increases [22]. Moreover, reducing the amount of filamentous vimentin in the cell, causes increased occurrence of actin stress fibres, which leads to higher contractility [56].

Despite the fact, that nowadays vimentin is known to be a highly dynamic network, its role is also recognized in the arrangement of cellular organelles such as mitochondria or endoplasmic reticulum [19]. That function is based on a cross-talk between intermediate filaments, microtubules and microfilaments. Microtubule-dependent motor proteins take part in transporting vimentin's ULFs to the periphery of the cell, where they can be linked into longer filaments. Actin can link to vimentin in two ways: directly, and indirectly with the help of plectin. This allows interaction between vimentin and actin stress fibres and transverse arcs, which gives possibility of retrograde transport of vimentin from the front of the cell [21,57]. The cooperation of vimentin and plectin is also influencing nuclear positioning and shaping. Intermediate filaments are linked to the nuclear envelope by plectin, and depletion of plectin has been proven to lead to nuclear deformations and misshapen nuclei [58].

Despite the abundance of research following the topic of mechanotransduction, there are still multiple aspects that need further insight. For instance – the involvement of vimentin in duroensing (ability of cells to sense stiffness of the extracellular matrix) is suggested to be negligible. The recent studies show that vimentin might be involved in modulation of traction forces in cell, but not in sensing the substrate stiffness [51]. At the same time, other research suggests, that the stiffness of substrate might induce changes in the assembly state of vimentin filaments. Besides, the changes in stiffness of substrate lead to the changes of the amount of soluble vimentin in the cell [59]. Nevertheless, the occurring contradictions leave space for the deeper investigation of the role of IFs in mechanotransduction.

4.2. Nucleus in mechanotransduction

One of the most important and well-studied connection of the nucleus and cytoskeleton is the LINC complex. Also, the nuclear envelope layers – INM and ONM cooperate together in nucleus-cytoskeleton interactions, and in selective transport of proteins. Mechanical forces can be transferred through LINC complex from the actomyosin network to the nuclear envelope, however the force can also be transferred to the nucleus with the nesprin-1 – emerin cooperation, which is a pathway, that skips the LINC complex [44].

Also, recent studies suggest that nuclear lamina, a fibrillar network in the interior of the nucleus, can be involved in mechanotransduction. The ruptures of the lamina were proven to have impact on cellular DNA damage, shortening of telomere, and cell cycle suspension [60].

Both nuclear lamina and nuclear envelope are involved in mechanotransduction by transferring signals between the nucleus and the cytoplasm, and also by providing mechanical protection to the nucleus. The signals from the nucleus are received by the cytoskeleton, and so far, the studies show, that actin filaments are the main cytoskeletal structure, that is involved in nuclear mechanotransduction [44]. Perinuclear actin comprises of:

- actin cap (dorsal stress fibres, that are connected to nuclear envelope and focal adhesions on both ends; direct cooperation with the LINC complex; responsible for regulating the shape of the nucleus)
- dorsal actin cables (not attached to focal adhesions but linked to actin retrograde flow; responsible for movement and positioning the nucleus) [61,62].

Moreover, nuclear mechanotransduction can also occur in nuclear pore complex (NPC), a structure formed by proteins, to create channel allowing transport between the nucleus and the cytoplasm. NPC can bind to chromatin, nuclear envelope, and LINC complex, and is another element that's role is not quite well understood in terms of transferring signals to the core of the cell. Nevertheless, currently LINC complex is the key element of interest, due to its engagement in the cross talk of the nucleus, and the outer organelles of the cell [44,48].

5. Biopolymers

“Biopolymers” is a term describing materials of natural origin, being a product of living organisms. Increasing attractiveness of these polymers is due to their excellent features, i.e.: biocompatibility, biodegradability, bioavailability, or such demanded in nowadays science – sustainable manufacturing. For cellular studies, one of the most important aspects of using biopolymers is their capability to mimic different conditions of a living organism in vitro in terms of the physicochemical characteristic. For instance, biopolymers can be synthesized to imitate structural complexity of the tissues, their hydrophilicity, electrical conductivity or porous structure, which in turn provides the cultured cells the microenvironment, that resembles their real surroundings as much as possible [63,64] Biopolymers created for tissue engineering are developed to gain certain features, and to create a test environment, that could either resemble normal tissue, or quite the opposite – disease (or stress) conditions[65,66].

For the purposes mentioned above, both: materials that are very well known, and novel biopolymers are actively used. The well-known polymers are also thoroughly researched and provide a solid base for experiments. On the other hand, novel polymers give the opportunity to fill the gap in missing features or drawbacks of the existing materials. In this work I focused on the exact set of the materials serving as substrate for cell culturing – PLA (polylactic acid), and P(3HO) – polyhydroxyoctanoate. PLA, a very broadly researched polymer is currently widely used in medical applications and tissue engineering, while P(3HO) is just being introduced into the field of biological applications [67,68]. Both materials have a huge potential of use in wound healing treatment, and since vimentin is one of the critical proteins in that process, the choice of the materials for the experiments becomes obvious. The possibility of creating thin films for cell culture studies, eco-friendly availability of the materials, biodegradability, biocompatibility, and non-toxicity for the cells were crucial features of P(3HO) and PLA to be considered as promising materials for the experiments described in this particular work. The features mentioned above made PLA such a popular material, that currently it is one of the top bioplastics being used in terms of the number of tonnes produced. In 2023 PLA production share of bioplastics worldwide reached 31,0%, which is the highest value among other materials. For the sake of comparison - the use of PHA in the market of bioplastics reached around 4.8% in 2023, and both materials – PLA and PHA, are anticipated to increase their share significantly by 2028 [69]. This suggests that the old, well-known bioplastics, like PLA itself, will remain the top seller in the near future. Interestingly, the biodegradable materials will gradually displace the use of the non-biodegradable ones. It is forecasted, that the use of biodegradable polymers will increase from around 52.0% in 2023, to about 62.0% in 2028, according to the global analysis of European Bioplastics, nova Institute in years 2023-2028 [69]. Considering this, seeking for new, environmentally friendly solutions is a proper direction.

GLOBAL PRODUCTION CAPACITIES OF BIOPLASTICS IN 2023

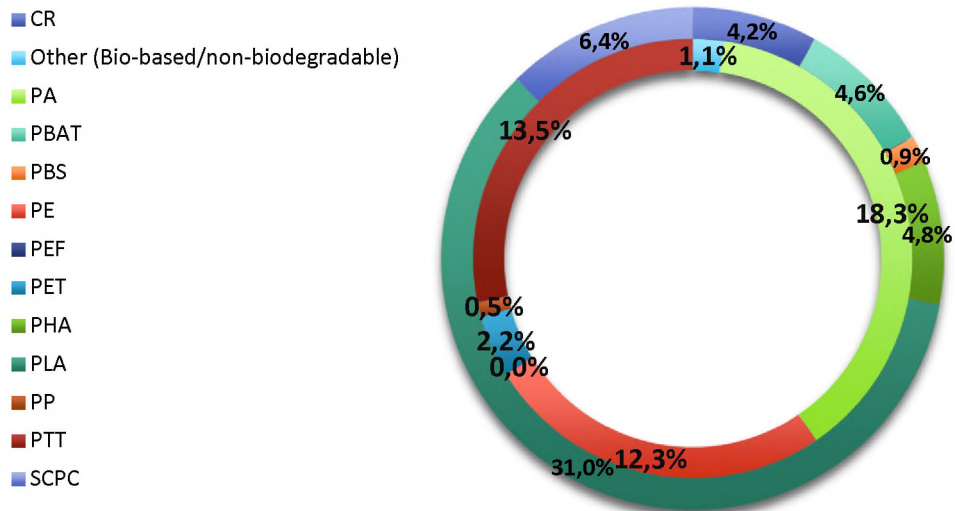


Figure 5 Material share of global biopolymer production capacity (Source: European Bioplastics, nova Institute (2023) [69] Inner doughnut – Biobased/ non-biodegradable materials. Outer doughnut – Biodegradable materials.

Also, interesting to watch will be the development of a brand-new material, PEF (polyethylene furanoate), that is marked on Figure 5 as having 0%. PEF, being 100% biobased, is also comparable to PET, and is planned to be released on the market in 2023 – replacing traditional plastics for food packaging bit by bit [69,70].

The reasons behind the increased interest in seeking and use of bioplastics seem to be quite obvious: environmental benefits of biodegradable plastics, huge problems with traditional plastic wastes management, but also the influence of traditional plastics on our health. Well known bisphenol – A (BPA), that has been broadly used in everyday life, has also extremely harmful influence on the humans health (disruption of endocrine system, etc.), and its adverse effects have been known for years [71]. Unfortunately, next components of plastics are identified to cause as much harm as BPA, and are equally popular in everyday life, for example phthalates. The recent studies show that exposure to these “everywhere chemicals”, might have increased risk of cancer in children by 20% [72]. Since these results have been published recently (2022), the effect they will have on the resignation from the use of traditional plastics, might only be forecasted, yet it seems inevitable that the focus on harmless, bio-based and biodegradable plastics will only increase.

The use of traditional plastics has also been influenced by COVID-19 pandemic, as it postponed the implementation of the UE directive on the reducing the use of traditional plastics (SUP – single use plastics). The directive was supposed to enter into force in July 2021,

whereas, due to the global problems caused by pandemic, and increased demand of use of disposable medical supplies, the directive still is to be implemented. Nevertheless, the worldwide fight against the contamination of the environment with plastic is continued, and the good news is, that the production of bioplastics is predicted to increase from 2.42 million tonnes in 2021, to 7.59 million tonnes in 2026. The future of cleaner Earth is in our hands [73–75].

5.1 PLA

PLA - polylactide (polylactic acid) is a thermoplastic polyester, that can be resourced from renewable raw materials, like corn starch, cassava, etc. Currently PLA is the leading material of use in medical applications, and also has a high potential to replace numerous industrial petrochemical-based polymers [76]. PLA phenomenon is based on its great features – high strength, thermoplasticity, and ease of modification of its physicochemical characteristics. PLA, under certain modifications, can serve as a thin, transparent film with great organoleptic features, or can become thick, firm plastic CD box (as a polystyrene replacement) [77]. Besides that, PLA is not only biostable, but also bioabsorbable, and the rate of its biodegradation may be regulated [78]. Being compostable material, PLA may be a solution to restrain municipal solid waste (MSW), by introducing end-of-life scenarios.

The main producer of the PLA worldwide is Nature Works LLC, which synthesizes it by a polycondensation or a ring-opening polymerization [79]. Since obtaining of the PLA has been relatively expensive, its first applications were strictly in a medical field, however, due to the great interest in the material potential of use, and the development of the methods of obtaining (thermoforming, fiber spinning, molding, sheet and film extrusion), its popularity increased, and cost of manufacturing dropped [77].

5.1.1 PLA – synthesis

The basic monomer of PLA is lactic acid (LA), that can occur in two isoforms – L-LA and D-LA. Lactic acid can be obtained either by chemical synthesis or by bacterial fermentation, which is the preferred method for many manufacturers. Chemical synthesis is less favourable process of obtaining LA, due to its expensiveness, limited production capacity, and lack of possibility of production only the desired L-LA isoform of the monomer [76,80].

The more attractive method of production of LA monomer is bacterial fermentation – that can be distinguished into homo- and heterofermentation, based on the bacteria used for the process. Both methods are used to efficiently produce around 1.8 moles of LA out of 1 mole of hexose,

however homofermentative method is also producing significantly less by-products, than heterofermentative method [81].

In homofermentation bacteria species of *Lactobacillus* genus are used in the conditions of pH 5.4 – 6.4, 38 to 42°C, and placed in cell recycle reactor under low levels of oxygen. The medium for feeding bacteria contains simple sugars, vitamin B, amino acids, and nucleotides. The obtained LA monomer is purified by distillation, however the process of fermentation is being currently adjusted by the producers, to obtain a monomer of a higher purity.

Finally, Lactic acid can be used for synthesis of PLA of high molecular weight, that can occur in three different manners:

- A. Direct condensation polymerization (polymerization (least expensive)
- B. Azeotropic dehydrative condensation of LA
- C. Polymerization through the formation of lactide (ring – opening polymerization – ROP).

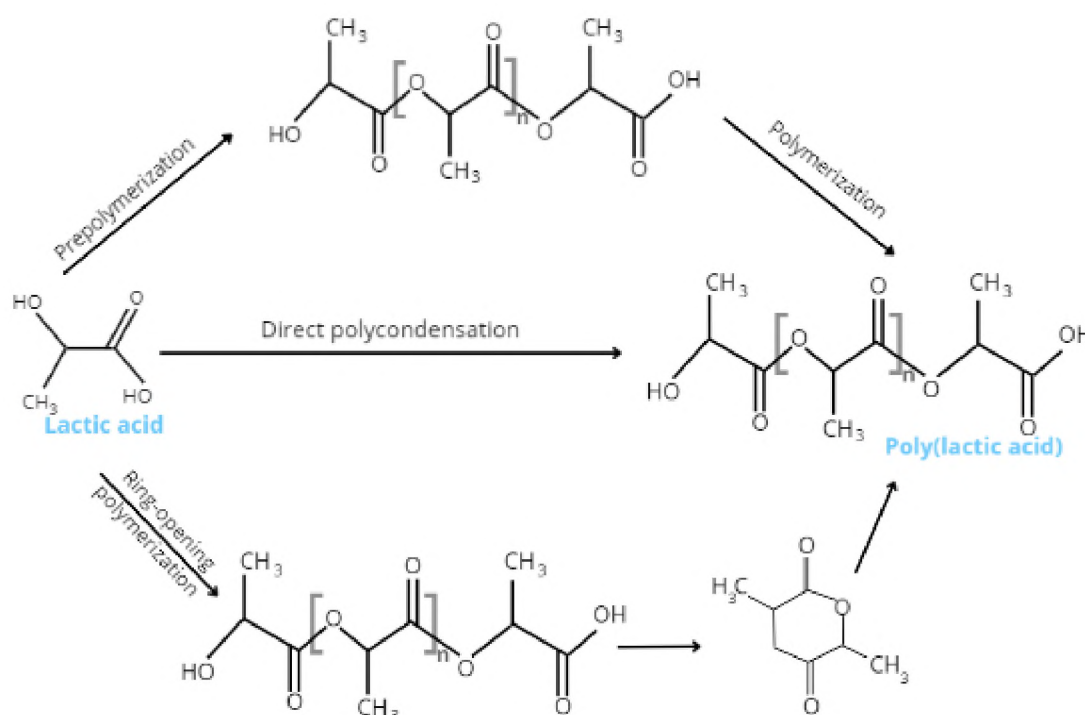


Figure 6 Three methods of obtaining poly(lactic acid).

The cheapest method is direct condensation polymerization method. However, it does not provide a solvent-free PLA of high molecular weight, so there are chain coupling agents and adjuvants needed, which eventually influence the increase in cost of the process. The

second method - direct polycondensation in azeotropic solution allows obtaining high molecular weight PLA without adjuvants or chain extenders, only by adjusting pressure of distillation of LA for a certain time. The third method – polymerization through the formation of lactide – uses LA isomers from fermentation, which are prepolymerized into intermediate low molecular mass PLA, and subsequently converted catalytically into the mixture of lactide stereoisomers. Lactide mixture is then distilled in vacuum, and ring opening polymerization occurs, resulting in production of high molecular mass PLA [80].

The PLA is extremely versatile material, due to its properties, that can be controlled by the polymer architecture, and molecular mass (by adding hydroxylic compounds) [82]. The significant factor in determination of PLA properties is the speed of crystallization, that is dependent on the stereochemical architecture. This gives opportunity to control material's mechanical properties, processing temperatures, and finally - crystallinity, that determines also the degradation rates of PLA [77,80].

The table below shows the physicochemical properties of PLA and other popular plastics, used nowadays:

Table 1 Physical properties of various popular biopolymers [68,76,83,84]

Properties	Unit	PLA	PGA	PCL	PP	PE	PVC	PHB	P(3HO)
ρ – polymer density	g/cm ³	1.21-1.25	1.50-1.71	1.11-1.15	0.9	0.95	1.33	1.18-1.262	-
σ – tensile strength	MPa	21-60	60-99.7	20.7-42	27	20-30	48	40	4.3
E – Young's modulus	GPa	0.35-3.5	6.0-7.0	0.21-0.44	1.3	0.7	3.4	3.5-4	12.6-25.4
ϵ – ultimate strain	%	2.5-6	1.5-20	300-1000	200-700	20-100	200	5.0-8.0	162
T _m – melting temperature	°C	150-162	220-233	58-65	160	110	100-260	168-182	52.8

PLA – poly-L-lactide, PGA – poly(glycolid acid), PCL – poly(γ -caprolactone), PP – polypropylene, PE – polyethylene, PCV – polyvinyl chloride, PHB – polyhydroxybutyrate, P(3HO) - polyhydroxyoctanoate

PLA, in comparison to other popular plastics used in everyday life, has greater range of tensile strength, than PCL, PP or PE. Such a set of properties encourages researchers to pursue the modifications of this polymer to fit the specific parameter needs. Thanks to its characteristics, the material is widely used in multiple industries.

5.2 Polyhydroxyalcanoates

Polyhydroxyalcanoates (PHAs) are a group of polyesters produced by different bacteria species, as a carbon and energy storage materials. PHAs are biodegradable, biocompatible and environmentally friendly thermoplastics, that have a high potential to successfully replace multiple petrochemical plastics used nowadays [85].

The discovery of PHAs reaches 1920s, when poly(3-hydroxybutyrate) was reported to accumulate in bacteria, yet due to the high cost of production, and better availability and price of petrochemical – based plastics, caused lack of interest in PHAs for a while [86]. Nowadays, when there is a growing need of displacement of plastics, that are being harmful for the environment, the interest in polymers of biodegradable features, and eco-friendly background, led to reconsidering bacterial polymers – such as PHAs. Although the cost of manufacturing PHAs has been a problematic matter, there are some resolutions in adjusting the technology of fermentation process, that currently allow optimize the cost of production to become affordable and competitive to the petrochemical plastics [87,88].

PHAs may be categorized, by consideration of different features. One of the important characteristics, that helps to classify that group of polymers, is the length of the monomer chain. Considering that, there are three groups of PHAs: short chain length (scl – PHA; C3-C5), medium chain length (mcl – PHA; C6-C14), and long chain length (lcl – PHA; >C14). Another important feature of PHAs is the source of origin, as they can be biosynthetic or chemical. Also, the type of monomer present in PHA chain determines the category of the polymer. Figure 7 shows the basic categories of biopolymers, based on the source of origin, including PHA monomer length.

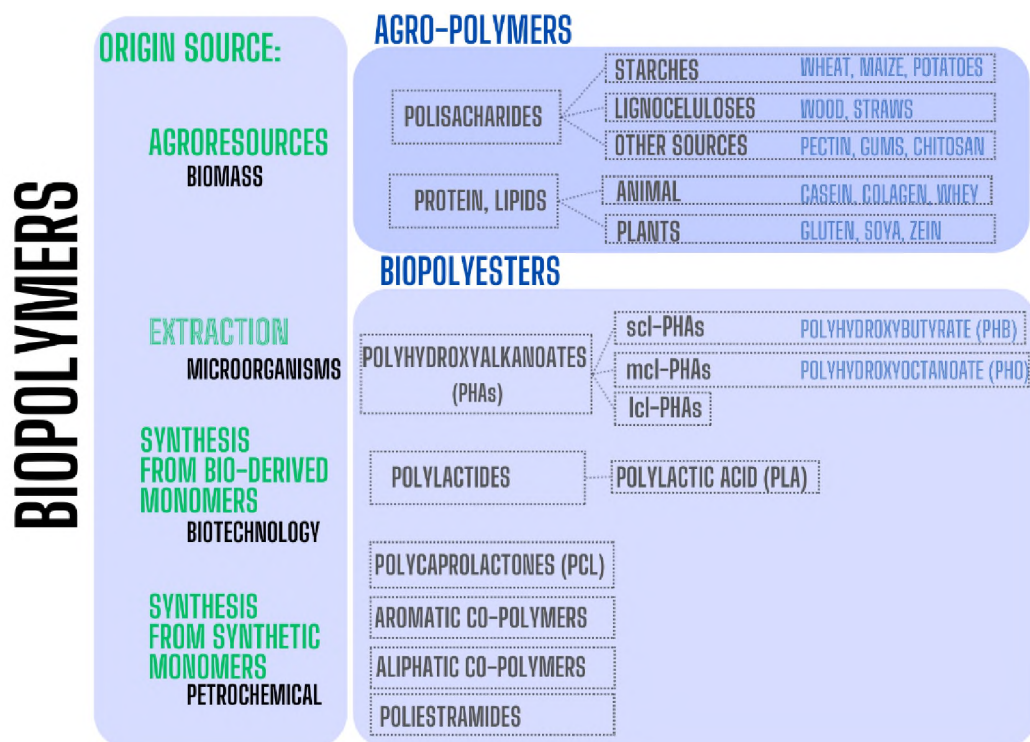


Figure 7 Classification of biopolymer types in different subgroups [89,90].

Among polyhydroxyalkanoates, the most common, naturally occurring homopolymer is short chain length poly(3-hydroxybutyrate) [91]. 3-hydroxybutyric acid, and 4-hydroxybutyric acid are both found to be normal elements of human blood, thus PHAs are generally of a good biocompatibility in vivo. Besides that, PHAs characterize with a wide range of physicochemical properties; they can be identified as both: thermoplastics of high crystallinity, and elastic materials, which allows attempts of applications in multiple indications. The most promising field for PHAs is medicine – as they have been successfully tested as heart valves, or microparticles for drug carrying [85,92]. What is also interesting is the possible effect of hydroxybutyrate on senescence – research suggest that β -HB circulating in blood, might have an anti-aging effect and positive influence on ageing-related neurodegeneration [93].

Besides short-chain-length PHAs, the promising subgroup of polyhydroxyalkanoates, are medium-chain-length (length (mcl-PHA), that are optically active, biodegradable polyesters, and have a potential to be used in different medical applications, including tissue regeneration, or wound healing [94]. In the last two decades, more than 100 mcl-PHAs were identified, investigated and characterized. These monomers usually consist of 6-14 atoms of carbon, linked by 3-hydroxy ester bonding [68].

The production of PHAs can be divided into two main parts. The “upstream” process begins with the appropriate storage of bacteria, that are later used in the fermentation process (i.e. 4°C refrigeration of the bacteria on agar substrates). To ensure the continuous availability of the bacteria, banking of the culture in -80 °C is recommended. Before starting the fermentation, the bacteria refrigerated on agar, are moved to flasks where proliferated in the appropriate medium in 30°C. The second stage of PHAs production is called “downstream”, and it is initiated with the fermentation process. So far, the strain of *Pseudomonas putida* KT 2440 is quite well studied and known to produce a decent amount of mcl-PHA within limited nutrients environment. After setting up appropriate conditions of fermentation for *P. putida* culture (i.e. carbon limited exponential feeding of nonanoic acid), the mcl-PHAs is being produced, and accumulated [95]. Generally, the process of biosynthesis and accumulation of PHA in bacteria is based on three enzymatic reactions, driven by three different enzymes. The first of them – 3-ketothiolase (PhaA) – is a catalyst in reaction of synthesis of two acetyl-CoA molecules into acetoacetyl-CoA. The next enzyme – acetoacetyl-CoA dehydrogenase (PhaB), that is NADPH-dependent, leads to obtaining (R)-(3)-hydroxybutyl-CoA, after reduction acetoacetyl-CoA. The third enzyme, PHA polymerase (PhaC) – allows obtaining the final product – scl-PHA, by initiation of polymerization process. When the demanded quantity of PHA is amassed, it is then harvested, and the process of recovery begins. The gathered PHA is then processed into a suitable form for further work. This process is being constantly investigated, and slightly modified, to achieve the best results, and become more, and more efficient, yet by now, it is known that production of PHA polymers on an industrial scale is possible, and thus PHA become competitive materials for the petrochemical based plastics [87,96].

5.2.1. P(3HO)

P(3HO) – polyhydroxyoctanoate, is one of the mcl-PHAs, that is produced by the *Pseudomonas Putida* KT2440 bacteria strain. The material is with a high potential of use in biomedical applications, due to its biocompatible and bioresorbable characteristics. The detailed research confirms the beneficial features of P(3HO) in terms of adsorption of proteins into the surface of the polymer, and – thus – for cellular adhesion.

Due to its advantageous features, P(3HO) is being examined for prospective heart valve replacement, in investigations on wound-healing, and for joint endoprostheses. The advantageous elasticity (Young's modulus 11.4-34 MPa), endurance (friction coefficient 0.198-0.202), and low fragility (hardness 3.2-4.5 MPa) of P(3HO) make it a serious competitor against the other, well-known polymers. Multiple studies confirm that P(3HO) could be used in a variety of tissue engineering applications, such as bone scaffolds, soft tissue repair and

regeneration, or vascular and nerve tissue engineering [97]. Also, P(3HO) has been confirmed to have beneficial influence on the process of wound closure, when used as a substrate for cell culturing. Considering the fact, that its manufacturing is in an eco-friendly manner, the material has a great potential of use and in replacing other polymers in multiple biomedical applications, yet further research is to be performed [67].

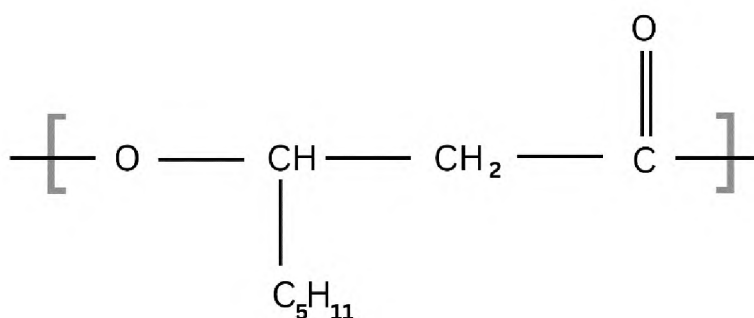


Figure 8 Chemical formula of P(3HO) monomer. – polyhydroxyoctanoate [98].

6. Confocal Microscopy

The microscopic images that were obtained for the purpose of this work, were taken with a confocal microscope. Confocal microscopy is a type of fluorescence microscopy, in which a laser beam is illuminating the sample, and the pinhole aperture is used to reject the out-of-focus light from detection, resulting in obtaining images of higher resolution contrast. The basic rule of confocal microscopy is to focus the optics of the illumination and detection on the same diffraction-limited point, that is scanning the sample, by moving over it. This allows to build a full image of the sample on the detector [99].

The conventional light microscopy only allows illumination and visualization of the entire field of the sample at the same time. This results in creating a “background noise”, that influences the quality of an image. The confocal microscopy, on the other hand, scans the whole sample point by point, and although the entire field of view is also illuminated during the scan, only the light coming from the focal plane is detected, which results in much less bias, and higher quality of the images captured. Also, the samples for the confocal microscopy may be thicker, than in the conventional microscopy, as the devices allow performing optical sectioning for 3D reconstruction images [100].

The basic components of confocal microscope include:

- Source of light (typically a laser, that emits a narrow, and focused light beam of a specific wavelength, to illuminate the sample)
- Scanning system used to raster the laser beam across the scanned sample in an ordered manner (most often it is conducted with the use of 2 mirrors, that precisely control the deflection of the laser sample in x and y directions)
- Pinhole aperture – that filters the light, that passed through the sample, and blocks the light that is out-of-focus
- Photodetector – that converts the detected signal into an electrical one, that can be further processed into visualization
- Image acquisition and processing systems - the signals detected by photodetector are collected by the computer, that creates an image basing on the intensity of the light detected. Additional techniques are often added here, such as fluorescence imaging or time-lapse imaging.
- Optical module with a set of lenses and mirrors that serve for:
 - Appropriate focus and directing the laser beam on the sample
 - Collection of the emitted light
 - Creating a high-quality image
 - Minimizing aberrations
- Sample stage – for holding the position of a sample chosen for imaging, and precise moving of the specimen in the x, y, and z axis, for 3D imaging,
- Computer software, controlling all the above-mentioned elements, i.e.: scanning, movement of the sample, acquisition of the image data, processing of the obtained scans [101,102].

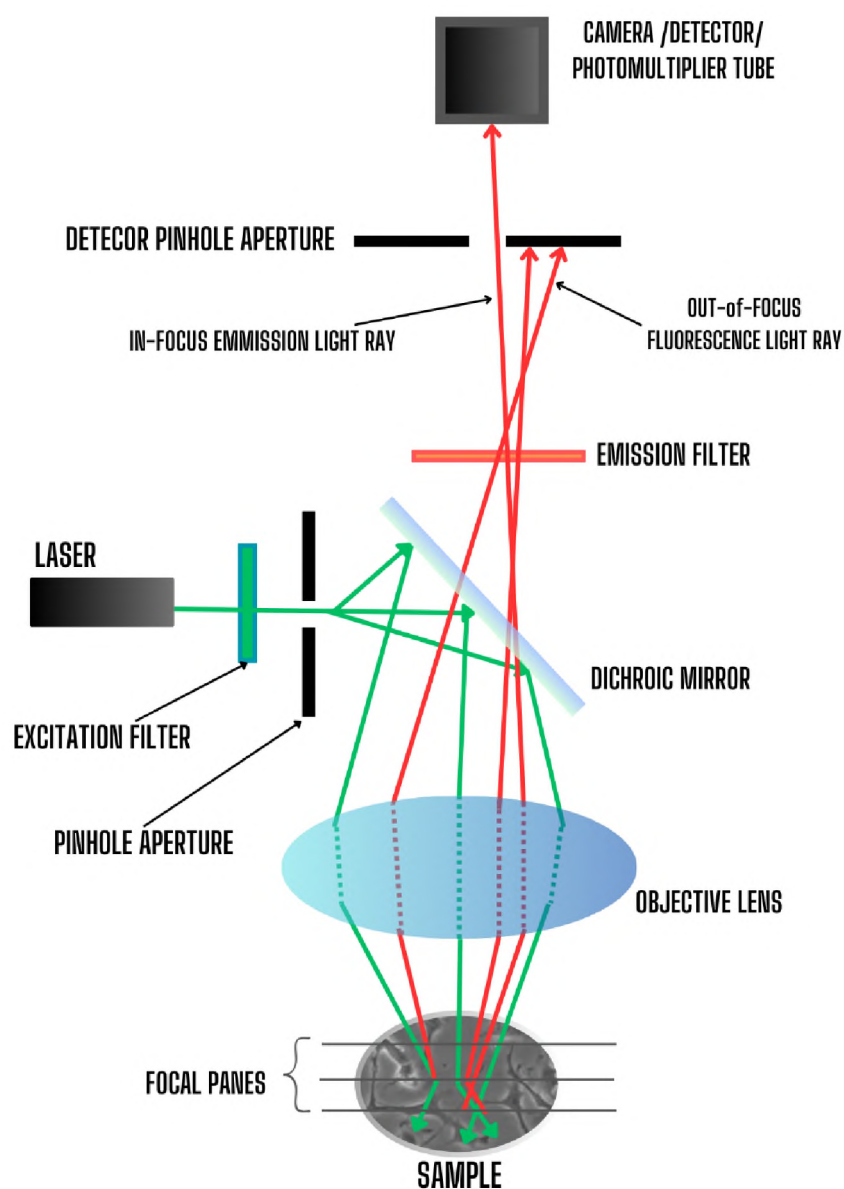


Figure 9 Schematic picture of a standard confocal microscope.

The pictures obtained for the purpose of this thesis were captured by Zeiss Axio Observer Z.1 microscope, with LSM 710 confocal module. The device allowed to perform observations of interior of the cells that were cultured on a thin layer of polymeric film ($\sim 70\mu\text{m}$ of thickness).

Scope of the study

The goal of the study was to analyse the structure and arrangement of vimentin – an intermediate filament present in mouse embryonic fibroblast cells:

- i) Characterization of the vimentin cytoskeleton morphology and analysing the influence of the physicochemical characteristic of the substrates for cell culturing, on the final vimentin assembly y in mouse fibroblast cells
- ii) Analysis of the arrangement of vimentin in the perinuclear area, on the base of observation of interdependencies of VIFs and cell nucleus in mouse fibroblast cells.

7. Materials & Methods

7.1 Preparation of P(3HO) and PLA substrates for cell culturing

The substrates for cell culturing that were used in the experiments supporting this thesis, were both prepared as thin polymeric films, that allowed easy microscopic observation and image capturing.

Bacterial strain of *Pseudomonas putida* [96,103] KT2440 was used to produce PHO in a 5L fermenter. Bacteria was revitalized from a -80°C stock by suspending it in a fresh solid minimal salt medium (MSM) supplemented with octanoic acid sodium salt (20 mM). Bacterial growth continued overnight at 30°C. A single colony was transferred into liquid MSM supplemented with sodium octanoate (20 mM). Culture was left to grow for 12-15 hours and then used as a seed for a fermentation (1:25). Decaying exponential feeding strategy of octanoic acid developed by Maclean *et. al* [104] was adapted to the used fermentation system (Sartorius Biostat B Twin 2x5l in order to produce P(3HO) polymer. Following, the fermentation biomass was separated from the medium by centrifugation, gathered and dried. The first step after gathering the biomass was purification of the extracted polymer in ethyl acetate, with the use of activated charcoal and 0,2µm filter. The obtained P(3HO) was rinsed in cold methanol, and dried, in preparation for further use.

PLA was purchased in a form of 1,75 mm diameter filament for 3D printing – commercial grade (MG Chemicals, Burlington, ON, Canada, PLA17TL1 – of a density 1.24g/cm³, Young's modulus 3.5 GPa, tensile strength 1MPa).

The polymeric films were obtained from both, PLA and P(3HO) in the following steps:

- 1) Dissolving of 0.5 g of polymer in 10ml of ethyl acetate
- 2) Casting 70µl of each solution on glass bottom dish, to obtain flat, thin surface
- 3) Drying the casted films in the oven for 48 hours (50 °C)
- 4) Rinsing the prepared films with the sterile PBS, before use for culturing.

The above-mentioned protocol allowed obtaining films, that were approximately 100µm thick, which allowed appropriate microscopic observations of cultured cells.

7.2 Cell culturing

MEF 3T3 cells were cultured on dishes, with previously prepared polymeric films (PLA, P(3HO)) casted on the dish surface and on plain glass for reference. The samples were held under sterile conditions in an incubator, with the pre-set, stable conditions: 37°C, and the atmosphere containing 5% of CO₂. As a culture medium DMEM Low Glucose solution (Dulbecco's Modified Eagle Medium, Biowest, Nuaille, France), with 10% (v/v) of FBS (Fetal Bovine Serum, Gibco Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics: 1% (v/v) of penicillin and 1% (v/v) of streptomycin (Sigma-Aldrich, Poznań, Poland) was used. The cells were passaged regularly, when achieved around 80% of confluency, and for the experiments, only the cells between 3rd and 9th passage were used.

7.3 Cells preparation and staining

For the standardization of the conducted experiments, cells were cultured on each substrate (P(3HO), PLA and glass) for 12 hours. After that time, cells were fixed in 4% formaldehyde solution in PBS for 10 minutes. The next step was permeabilization in Triton-X solution in PBS (0,1% (v/v)) for 5 minutes. After permeabilization, cells were rinsed in 3% (w/v) BSA solution to block unspecific bonds (Sigma-Aldrich, Poznań, Poland). After these initial steps, the staining procedure was done. Firstly, an overnight staining of vimentin in 5 °C

was performed, with the use of Abcam Recombinant Anti-Vimentin Antibody cytoskeleton marker ab92547. After that, a 3-hour staining of the nucleus with DAPI dye (Thermo Fisher Scientific, Waltham, MA, USA), and a secondary staining of vimentin with Alexa Fluor 633 goat anti-rabbit IgG (H+L) (2 mg/mL) (Thermo Fisher Scientific) occurred at room temperature. The next step was staining of actin and/or microtubules. Actin filaments were stained with the use of Alexa Fluor 488® phalloidin (1 hour in room temperature). In case of microtubules the first step of staining was performed with the use of Monoclonal Anti- α Tubulin mouse antibody (Sigma-Aldrich, Poznań, Poland), and after 1 hour in room temperature, the secondary staining with Alexa Fluor 488 fluorophore Goat anti Mouse (Thermo Fisher Scientific).

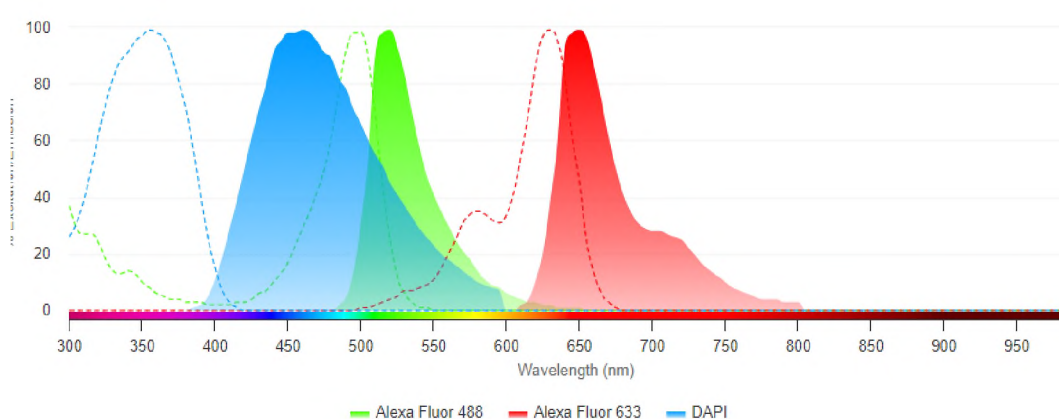


Figure 10 Excitation and emission spectrum of the fluorophores used in the experiments: Blue – DAPI, Green – Alexa Fluor 488, Red – Alexa Fluor 633. The dashed lines present excitation spectrum, while the solid line with the background – presents the spectrum of emission of the given fluorophore.

The protocol of staining each sample was always performed due to the following order:

- Anti-Vimentin antibody (overnight)
- Secondary staining of vimentin (Alexa Fluor 633) + DAPI (3 hours)
- Staining of actin (1 hour) or staining of microtubules (2 hours).

7.4 Microscopic observations of vimentin cytoskeleton of MEF 3T3 cells on polymeric films and glass

The main device, that allowed gathering most of the data obtained in the experiments, was Zeiss Axio Observer Z.1 microscope, with LSM 710 confocal module. The captured images were analysed in Zeiss ZEN Black version 8.1.0.484, PALM Robo version V 4.6.0.4 (Carl Zeiss Microscopy, GmbH Carl-Zeiss-Promenade 10 07745 Jena, Germany), and Fiji app

(ImageJ 1.52p, Wayne Rasband National Institutes of Health, Bethesda, MA, USA) software (<https://imagej.net/>). The images were captured on single cells with an immersion objective (40x magnification, numerical aperture 1.4).

7.5 Statistical analysis

To verify the results of the captured data, the statistical analysis with the χ^2 test was performed. The χ^2 test allowed verification of:

- Dependencies between the classic/nutshell vimentin networks and the substrate used (statistically significant at probabilities of χ^2 0.01)
- Dependencies between the number of grooved nuclei and the substrate (statistically non-significant at probabilities of χ^2 0.05)
- Dependencies between the substrate and the number of nuclei with blebs (statistically significant at probabilities of χ^2 0.01)

The experiments were performed 5 times for each substrate. Per sample, 15-25 cells were analysed. The results of the statistical analysis were calculated and visualized in Origin Pro 2019 Software (OriginLab Corporation, Northampton, MA, USA).

8. Results

8.1 PLA and PHO substrates characterization

To characterize surface structures of PLA and P(3HO) films, the polarized-light observations were performed. This allowed to identify irregularities on the polymeric substrates and comparing two polymers appropriately. The observations were conducted in 5x, 10x, and 40x magnifications.

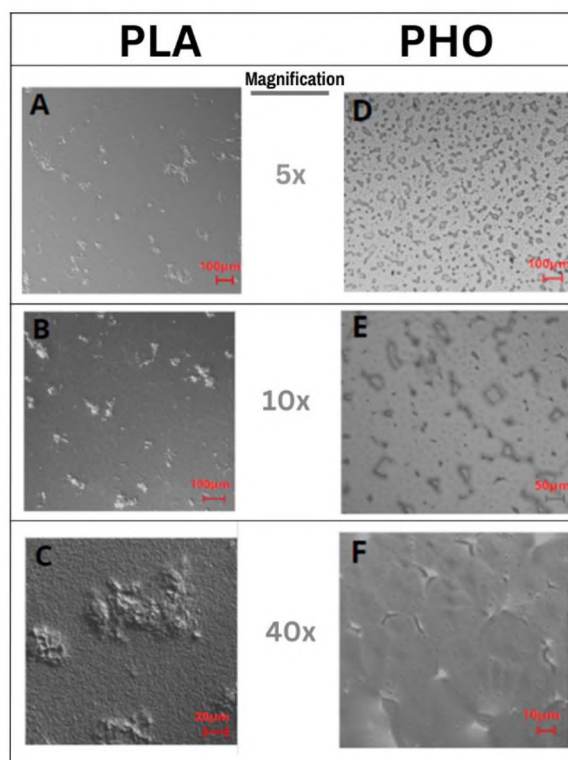


Figure 11 Polarized light microscopic images of PLA (A–C) and P(3HO) (D–F) films in 5× magnification (A, D), 10× magnification (B, E), and 40x magnification (C, F).

In Figure 11 (A, B) there can be seen polarized – light pictures of PLA in 5x and 10x magnification, where some thickenings and rough bulges can be spotted irregularly. In a 40x magnification (Figure 11 C), these bulges can be observed as bumps of different sizes (i.e., 10–400 µm width), that occur on the whole film area. At the same time, the surface of the whole PLA film is rough, and coarse.

Slightly different is the topography of P(3HO) film. The images in Figure 11 (D-F) present a homogenous topography, with a high amount of stable nucleation seeds. There is a low number of irregularities in the z-axis, and also a few defects of the surface, i.e. microcracks. The P(3HO) topography shows irregularly distributed micro- and nano- scale ridges and bumps, which might indicate the random direction of crystallization, and polymeric chains distribution throughout the film.

Comparison of PLA and P(3HO) films, gives a notion, that PLA substrates are with the higher roughness features, especially in the z-scale, although both types of films are of an irregular roughness distribution.

8.2 Analysis of vimentin cytoskeleton architecture on PLA and P(3HO) substrates

Vimentin structure is described in literature as an irregular network, spreading throughout the cell, reaching from the nucleus to the cell edges. It is known to assemble in three forms of filaments:

- Particles of unit – length filaments (ULFs)
- Short ULFs (squiggles)
- Long fibers.

The vimentin network mostly consists of long fibers, that surround the nucleus, and expand to the periphery of the cell. From there, the fibers gently pass into the squiggles, and these short ULFs eventually transform into particles of ULFs. To characterize this basic, and well-studied architecture of vimentin in MEF cells, the analysis of microscopic images was performed.

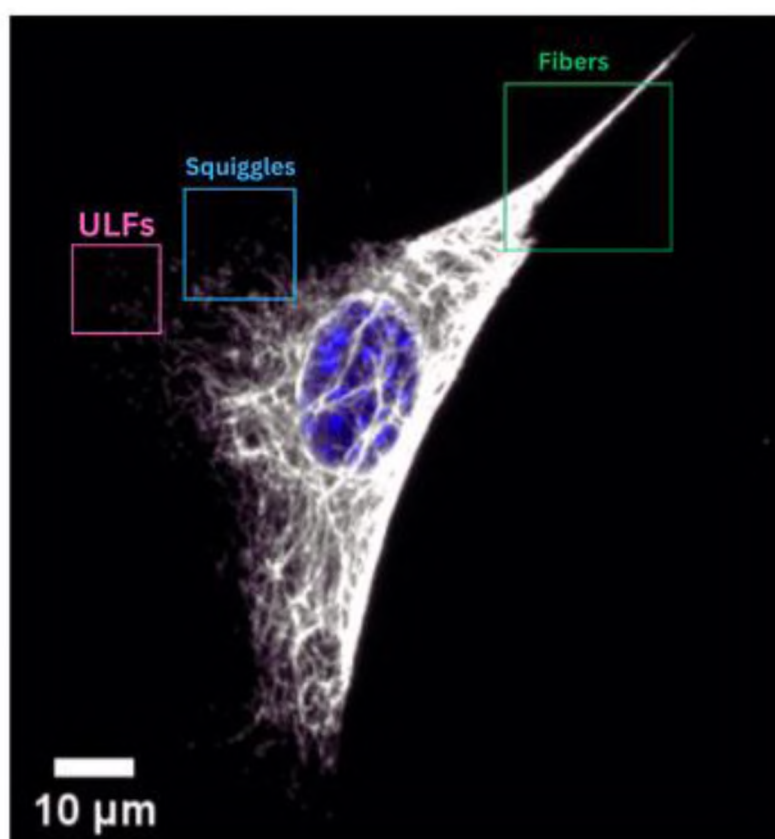


Figure 12 Basic architecture of vimentin fibers types in a single MEF cell, cultured on glass. (Green Square) – Fibers - long vimentin filaments occurring above the nucleus, and in cell's tail and edge. Squiggles (Blue Square) – shorter, than fibers, but longer than particle-length ULFs (Pink Square). Image captured with the use of confocal microscopy.

The network described in Figure 12 is a “classic” form of vimentin, that can be found in literature. In this work, however, the observations were performed, that led to an identification of the other form of vimentin “assembly”. Due to a resemblance to a walnut, for the purpose of this work, the newly discovered architecture structure was called a “nutshell”.

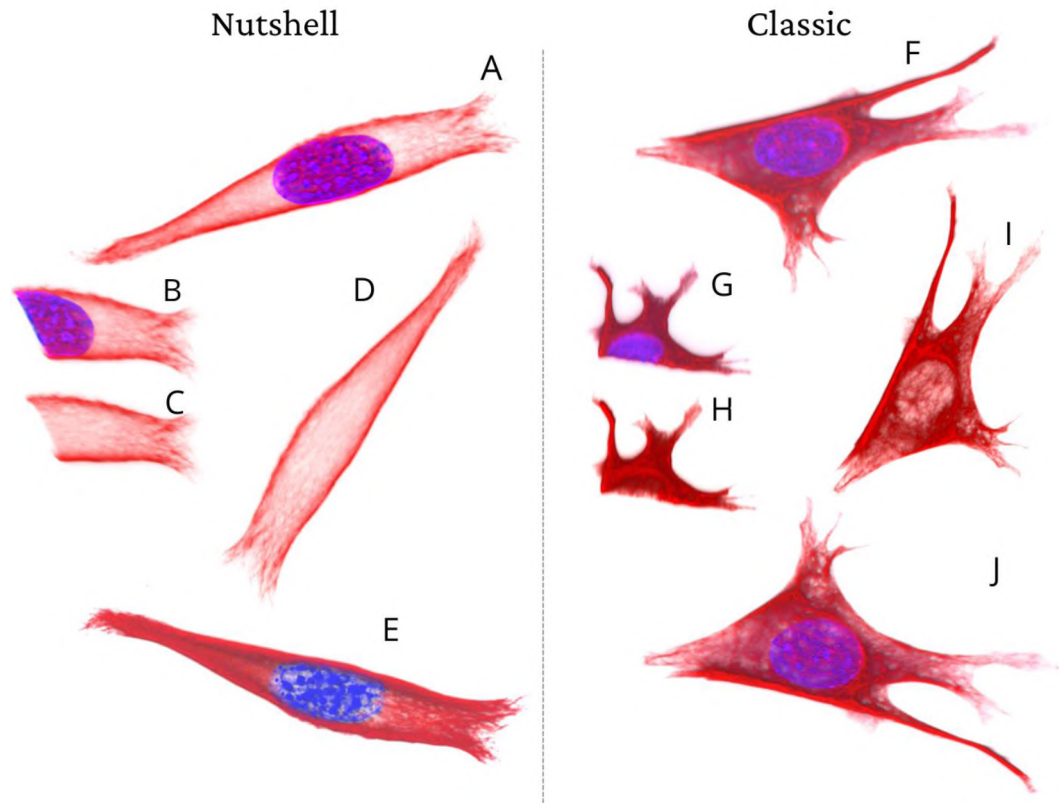


Figure 13 The comparison between the “nutshell” (A-E) and “classic” (F-J) structure of vimentin. Vimentin-Red, Nucleus – Blue. B, G, - slice of vimentin image, in the nuclear area. C, H- slice of vimentin image, without DAPI channel.

In Figure 13 there are presented examples of a “nutshell” and “classic” structure of vimentin cytoskeleton. The “nutshell” network at a glance is much less dense, especially in the nuclear area, when compared to the “classic” structure. In the “nutshell” vimentin, the thickest network occurs on the cell’s edges, not in the perinuclear area. For the “classic” network, the thickest fibers occur on the cell’s contractile edge as well, but also are present in the perinuclear area. The fibers of vimentin in the “classic” network are adhering to the nucleus so densely, that they easily form a halo, surrounding the nucleus on its edges. In the “classic” network, there also can be noticed a slight, but clear thickening of fibers right above the nucleus.

During the experiments, the data was gathered for 288 MEF cells altogether, cultured on:

- P(3HO) – 95 cells
- PLA – 80 cells
- Glass – 113 cells.

For 4% of the reviewed cells, the images captured did not allow to perform the proper analysis, due to the poor quality of these pictures.

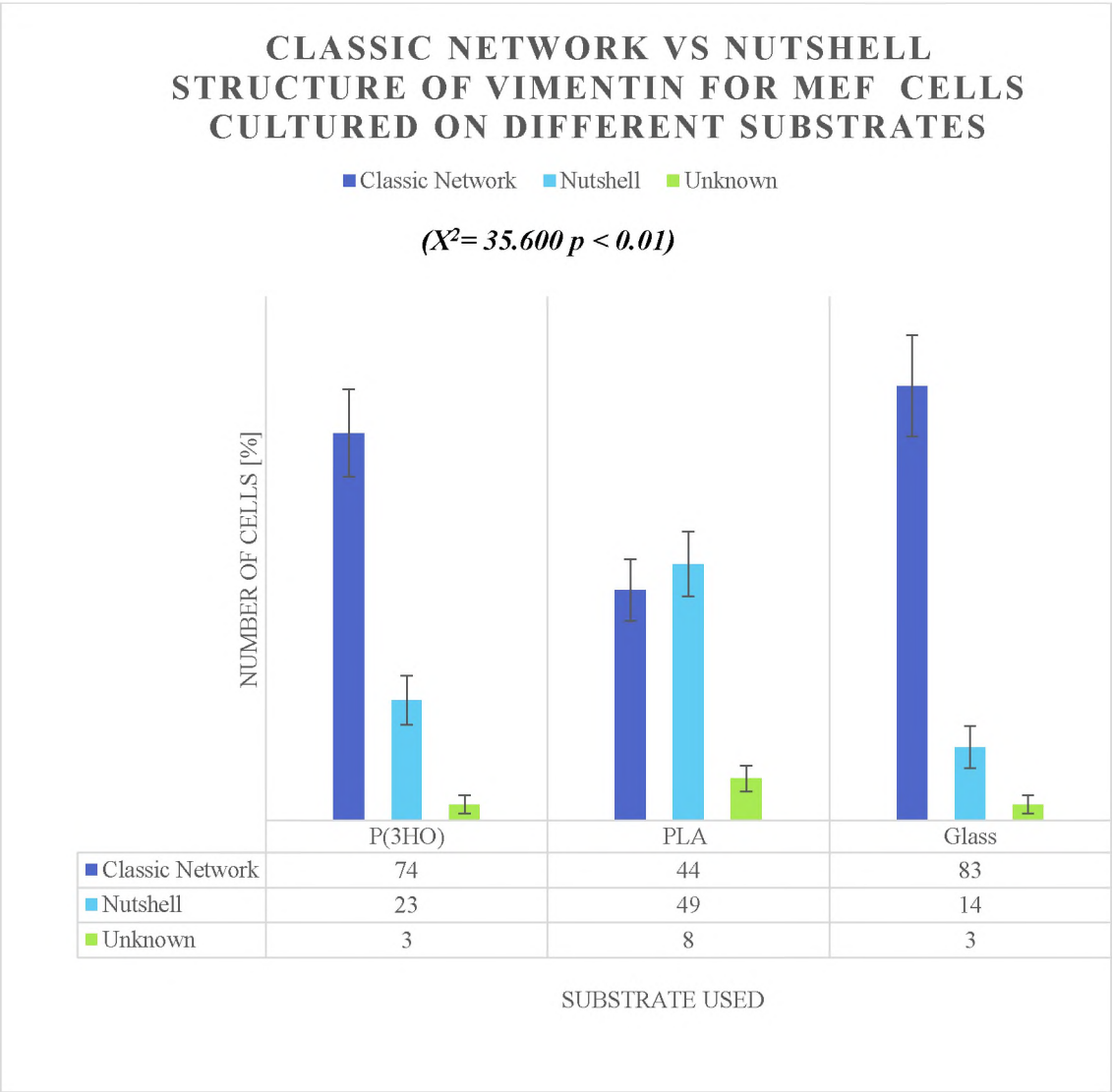


Figure 14 The proportion of cells with "classic" or "nutshell" vimentin cytoskeleton, cultured on glass, P(3HO), and PLA.

The majority of the classic network was seen on glass, where 83% of the cells displayed that phenotype. The proportion of cells with the classic network was equally high for P(3HO) (74%), whereas it was much lower for PLA (44%). Also, there were 49% cells on the PLA with a nutshell structure, indicating that the substrate had a significant impact on the vimentin cytoskeleton's shape. The X^2 test was used to confirm the statistical significance of the findings, demonstrating that the type of vimentin cytoskeleton depends on the substrate used ($X^2=35.600$ $p < 0.01$). Since multiple works prove the relation of formation of actin cytoskeleton depending on Young's moduli of the substrate, we also considered that such influence is maintained for

vimentin. The results suggest, however, that for the vimentin cytoskeleton subtype, factors other than the substrate's Young's modulus are determining the formation of VIFs.

To analyse these two types of vimentin networks deeper, the comparison of their cross sections was performed.

Classic Network | PHO | Semi-crystalline | XC,DSC = 37% | Contactangle: 100 °

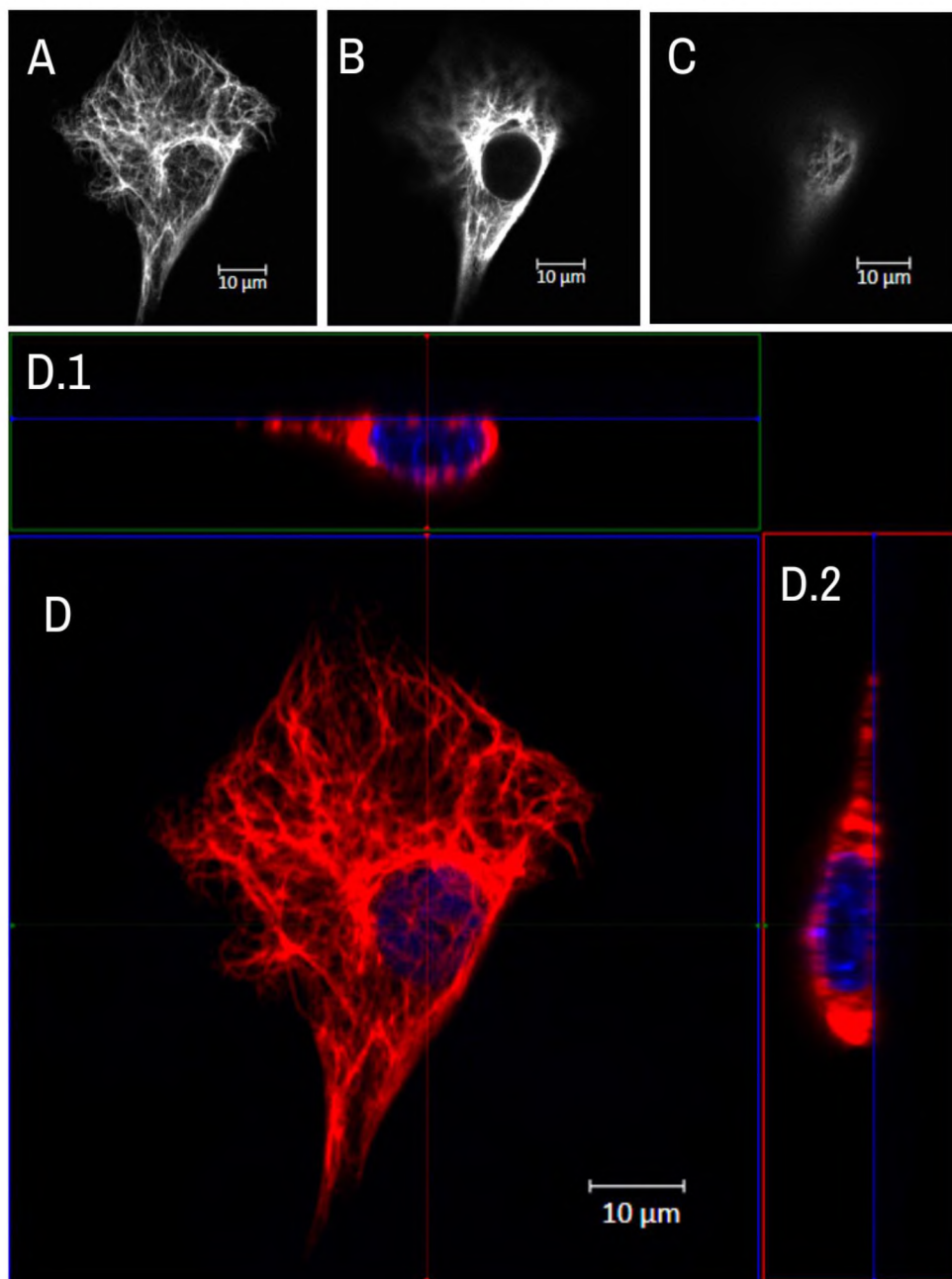


Figure 15 Example of cross section for “classic” vimentin structure. A - single z-stack image of the bottom of the cell. B- z-stack image of the central part of the cell. C – z-stack image of the top of the cell. D, D.1, D.2- orthogonal view.

In Figure 15 and Figure 16 there are shown examples of vimentin networks for MEF cells grown on P(3HO) and PLA. As it was also visible in Figure 12, the “classic” network has vimentin fibers spanned throughout the cell, that closely attach to the nuclear area, and spread to the cell edges in an irregular, intricate arrangement. The “nutshell” network arrangement in relation to the nucleus, is quite the opposite.

Nutshell Structure | PLA | Less-Crystalline | XC,DSC = 4% | Contact angle: 68 °

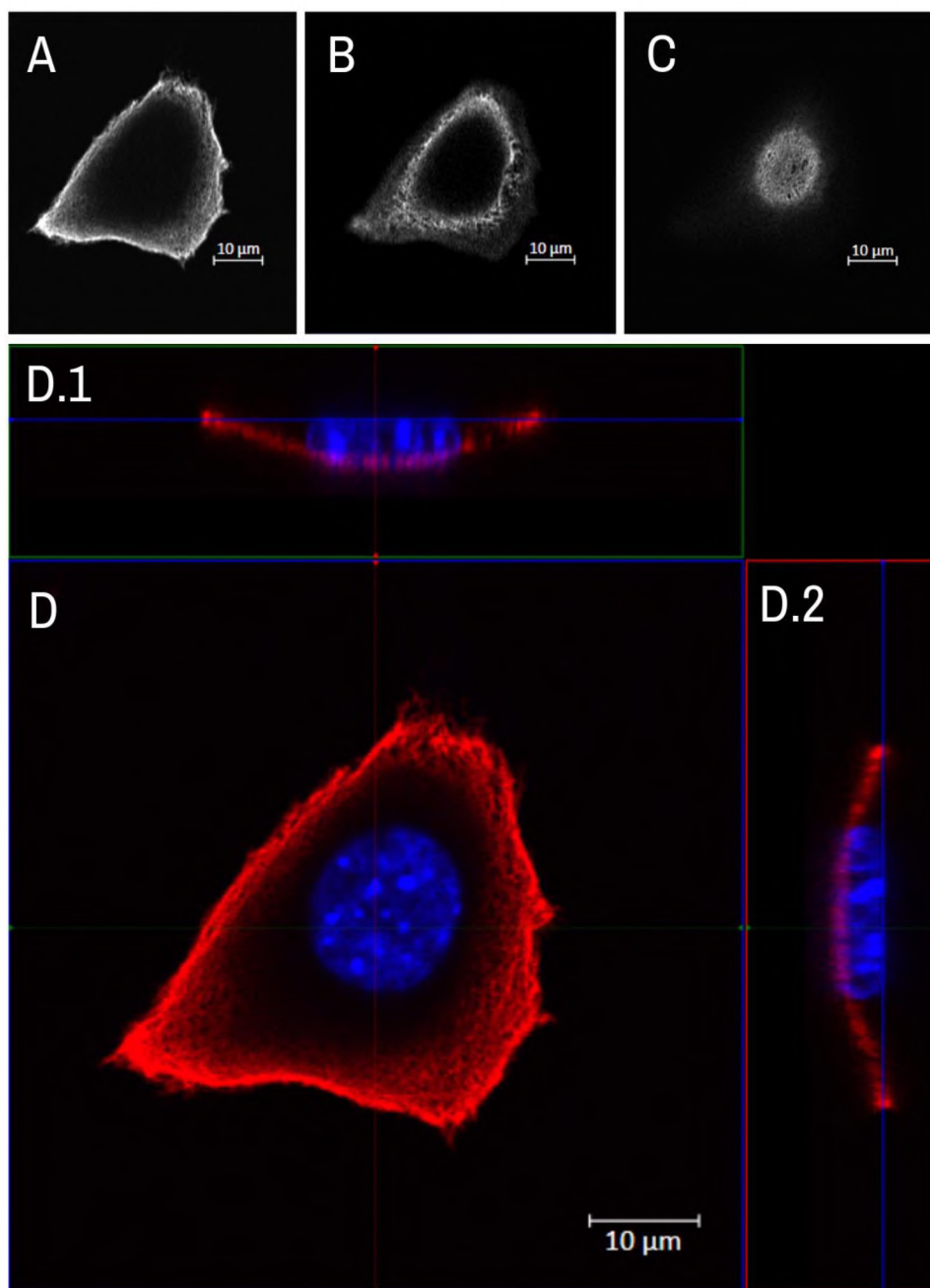


Figure 16 Example of cross section for “nutshell” vimentin structure. A - single z-stack image of the bottom of the cell. B- z-stack image of the central part of the cell. C – z-stack image of the top of the cell. D, D.1, D.2- orthogonal view.

As it may be seen in the Figure 16, the nucleus is only attached to vimentin in its upper area, whereas the side edges of the nucleus, and its bottom, are distant from vimentin. The “nutshell” network only covers the nucleus on the top, which suggests the resemblance to a shield, or a shell (or a nutshell). In the “nutshell” architecture, vimentin occurs as a single layer, not present either under the nucleus, or in the inside of the cell body. The differences in the appearance of these two subtypes are clear and open a field for discussion on the root cause.

8.3 Analysis of the structure of vimentin cytoskeleton

Although the analysis of cell images of vimentin network subtypes allowed making some basic assumptions, there was still precise evaluation needed to be conducted. For this purpose, ImageJ (Fiji) ver 1.5 software was used, and the analysis of the area occupied by vimentin in the cell was performed. The aim was to estimate the amount of vimentin in the following cases:

- the whole area occupied by vimentin
- the amount of vimentin in the selected 10 x 10 μm square regions:
 - above the nucleus
 - in the middle of the cell height
 - below the nucleus.

This approach allowed to determine if the amount of vimentin in “classic” and “nutshell” network differ, in terms of the area occupied by vimentin. The selected regions – under and above the nucleus, and in the middle of the cell height – were chosen to measure and confirm the observations, that were previously made – mainly, that “nutshell” vimentin cytoskeleton is not present under the nucleus, and significantly differs from the “classic” structure.

To start the analysis, each single cell image was at first adjusted, and region of interest (ROI) was selected on the basis of thresholding. Then, a 16-bit maximum intensity picture was selected, ROI was marked, and the amount of vimentin in the chosen area was measured (percentage value).

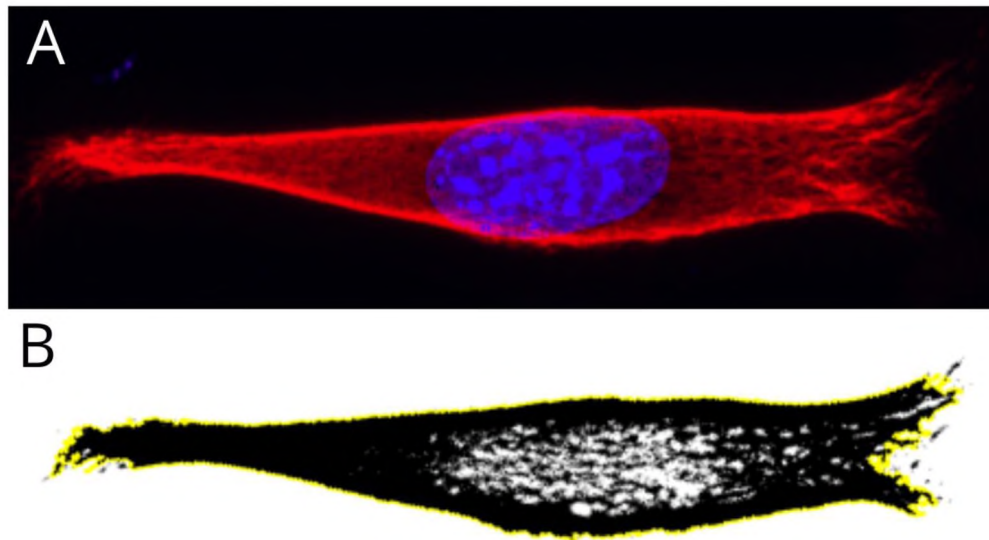


Figure 17 Adjustment of the Region of Interest for the “whole area occupied by vimentin” analysis. MEF cell cultured on PLA, with a ‘nutshell’ vimentin structure. A) Initial adjustment of Image parameters for cropped cell image. B) Selected ROI (Region of Interest) – vimentin cytoskeleton 16-bit (B&W) image – outlined with the yellow line, based on colour thresholding.

The same technique was used for the $10 \times 10 \mu\text{m}$ square regions. Since the aim was to evaluate the presence of vimentin above and below the nucleus, the squares selected for the analysis were placed in the area closest to the center of the nucleus.

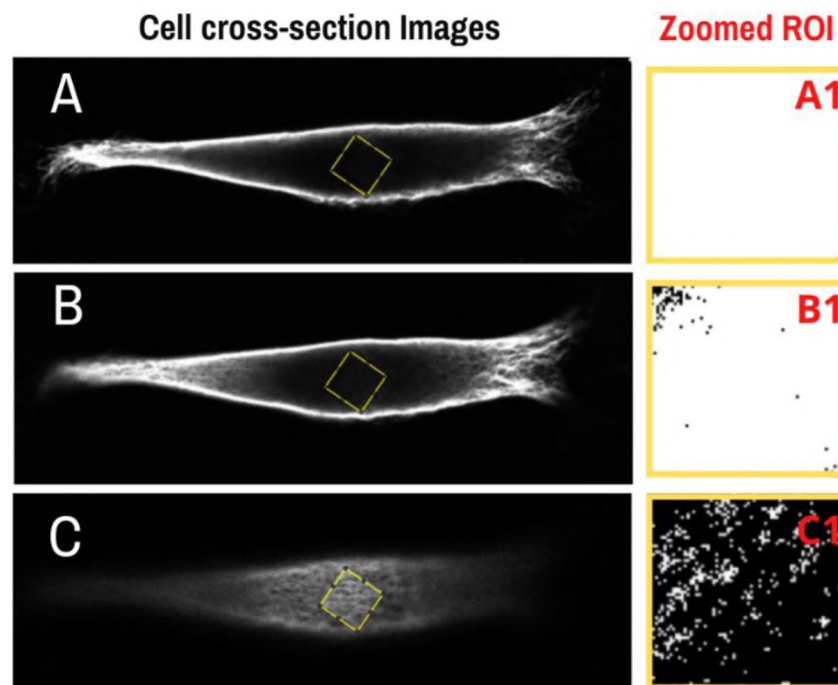


Figure 18 Steps performed during the analysis of vimentin cytoskeleton in the selected $10 \times 10 \mu\text{m}$ square ROIs. Indicating regions under the cell nucleus (A), in the middle of the cell height (B), and below the nucleus (C). A1, B1, C1 – magnified regions of interest, after image thresholding, showing the final amount of vimentin in the selected regions.

The analysis of the original microscopic pictures of vimentin cytoskeleton subtypes led to preliminary assumptions, that the “nutshell” structure differs significantly from the “classic” network, especially in terms of the amount of vimentin under the nucleus. The quantitative analysis, presented in Figure 17 and Figure 18 provided measurable evidence, that the assumptions were correct. The calculations showed that in the “nutshell” structure vimentin was almost completely missing below the nucleus, and in the middle of the cell height. The “classic” network, however, has different amounts of vimentin both – below and above the nucleus, and in the middle of the cell height, where multiple vimentin fibres cross the nucleus, squeezing into the nuclear grooves or invaginations. The analysis was performed for PLA and P(3HO) cultures, and in both cases, the classic network is occupied by vimentin in $91 \pm 5\%$ of the area, whereas in the nutshell variant – $84 \pm 10\%$. These values are not statistically important, and also not significantly different. For the $10 \times 10 \mu\text{m}$ squares however, the values confirm differences, dependent of the substrate used.

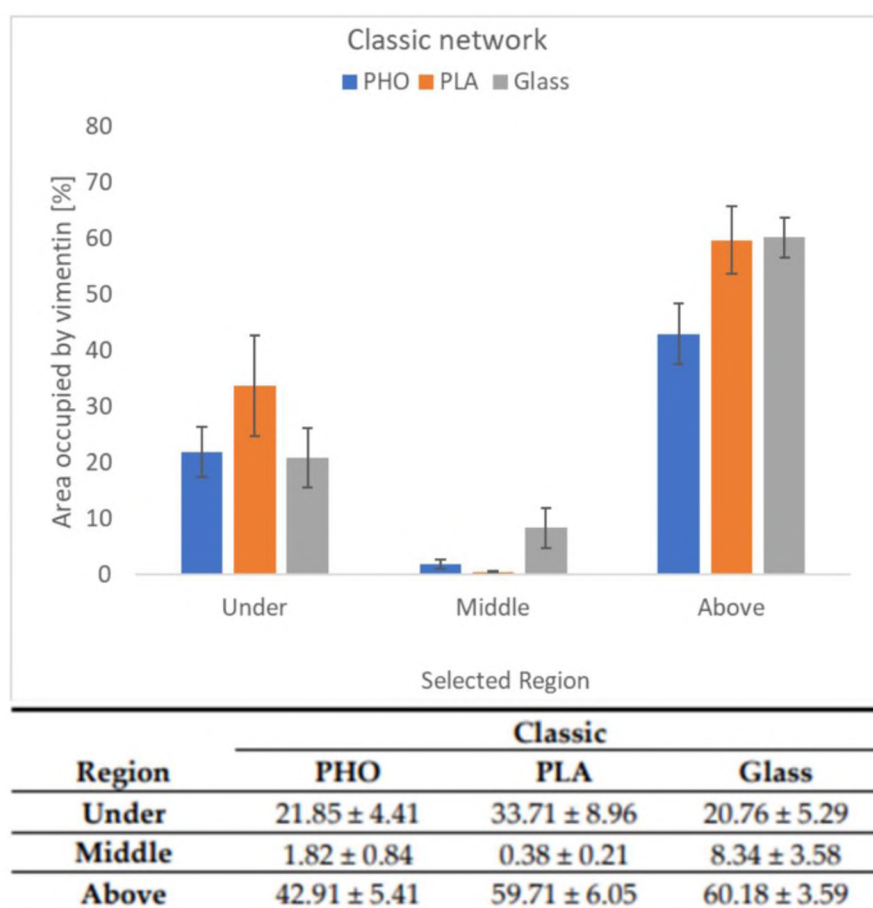


Figure 19 The amount of vimentin in selected $10 \times 10 \mu\text{m}$ squares regions of interest under the nucleus, in the middle of the cell height, and above the nucleus for “Classic” vimentin network. The table shows the calculated average values $\% \pm$ standard error value.

Calculations performed for the regions selected under the nucleus, in the middle of the cell height and above the nucleus, show significant differences between the two types of vimentin

network (confidence level = 95%). For the upper cell area, the classic network of cells cultured on P(3HO) has much less vimentin, than in cells cultured on PLA and glass. Nevertheless, the trend is similar for all areas – the presence of vimentin is confirmed both under the nucleus, and above the nucleus, and the values are quite high, in comparison to the same areas for the “nutshell” structure.

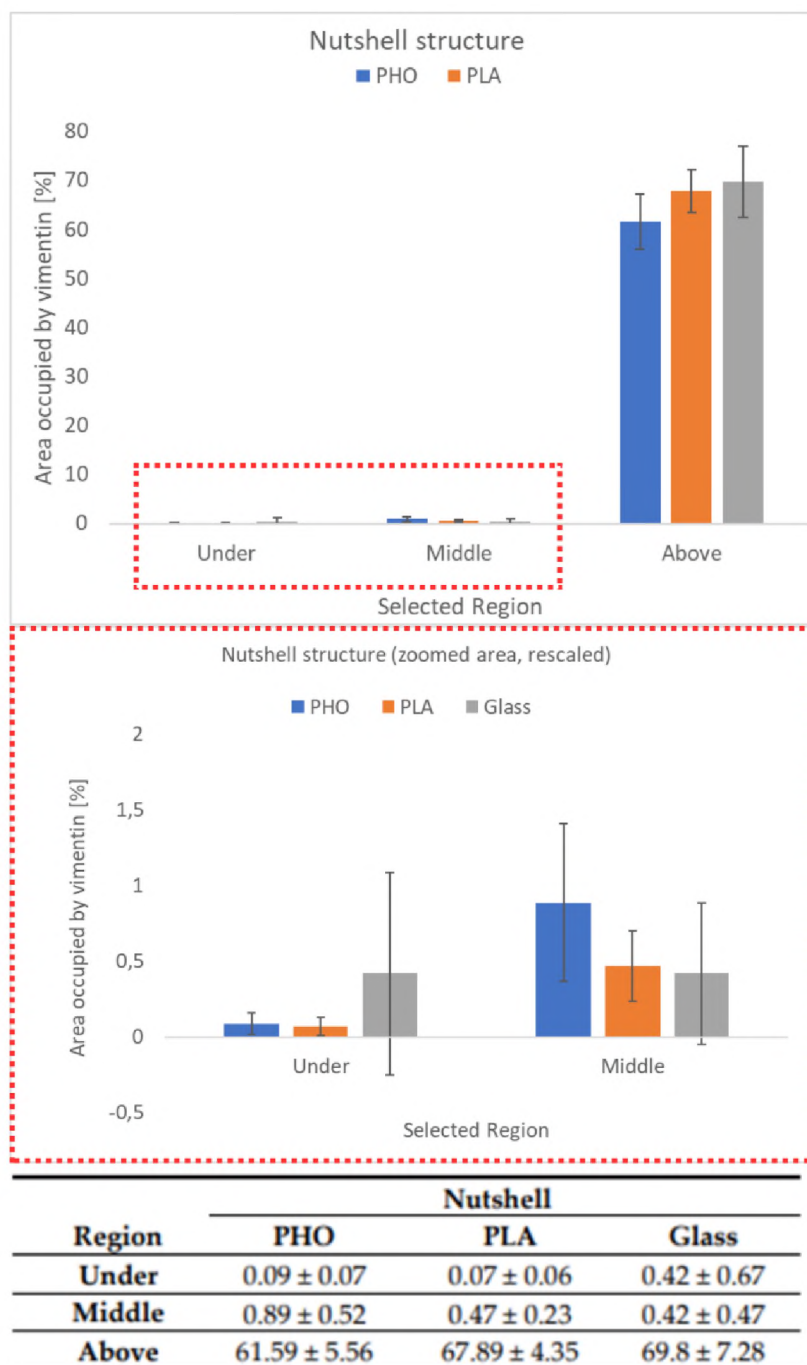


Figure 20 The amount of vimentin in selected 10 × 10 μm squares regions of interest under the nucleus, in the middle of the cell height, and above the nucleus for the “Nutshell” structure. The table shows the calculated average values % ± standard error value. Chart of “Nutshell structure” is rescaled for the “Under” and “Middle” region, to present the data more accurately for these areas, in comparison to “Above” region.

For all substrates, in “classic” network, there is also a presence of vimentin confirmed in the middle of the cell height. It is related to the presence of vimentin thicker fibres, creating bundles in “classic” network, that are associated to the grooves and invaginations of the nuclei of MEF cells. Considering only “nutshell” structure, for all substrates used (PLA, P(3HO) and glass), the amount of vimentin in the middle of the cell height, and under the nucleus is approximately 0. In the area above the nucleus – the amount of vimentin varies between 61% and 69%, but the difference between the samples is not statistically significant. The results, with a confidence level of $\alpha = 95\%$, support the hypothesis, that there are minimal amounts of vimentin in the middle of the cell height and under the nucleus for the “nutshell” structure, and also, that much lower amount of vimentin is present in the middle of the cell height for the “classic” network.

8.4 Vimentin association to nuclear grooves

Analysis of vimentin cytoskeleton subtypes, fruited with some other findings. The close proximity of VIFs to the nuclear area, and the differences in the arrangement of vimentin in relation to the nucleus for “nutshell” and “classic” network, led to more focused analysis of the perinuclear area of MEFs. In “classic” vimentin subtype, there could be noticed some slight, but clearly distinguishable thickenings on the top of the cell, right above the nucleus. The observations of the cells with these thickenings showed the association of vimentin with similar, thicker filaments of microtubules and actin.

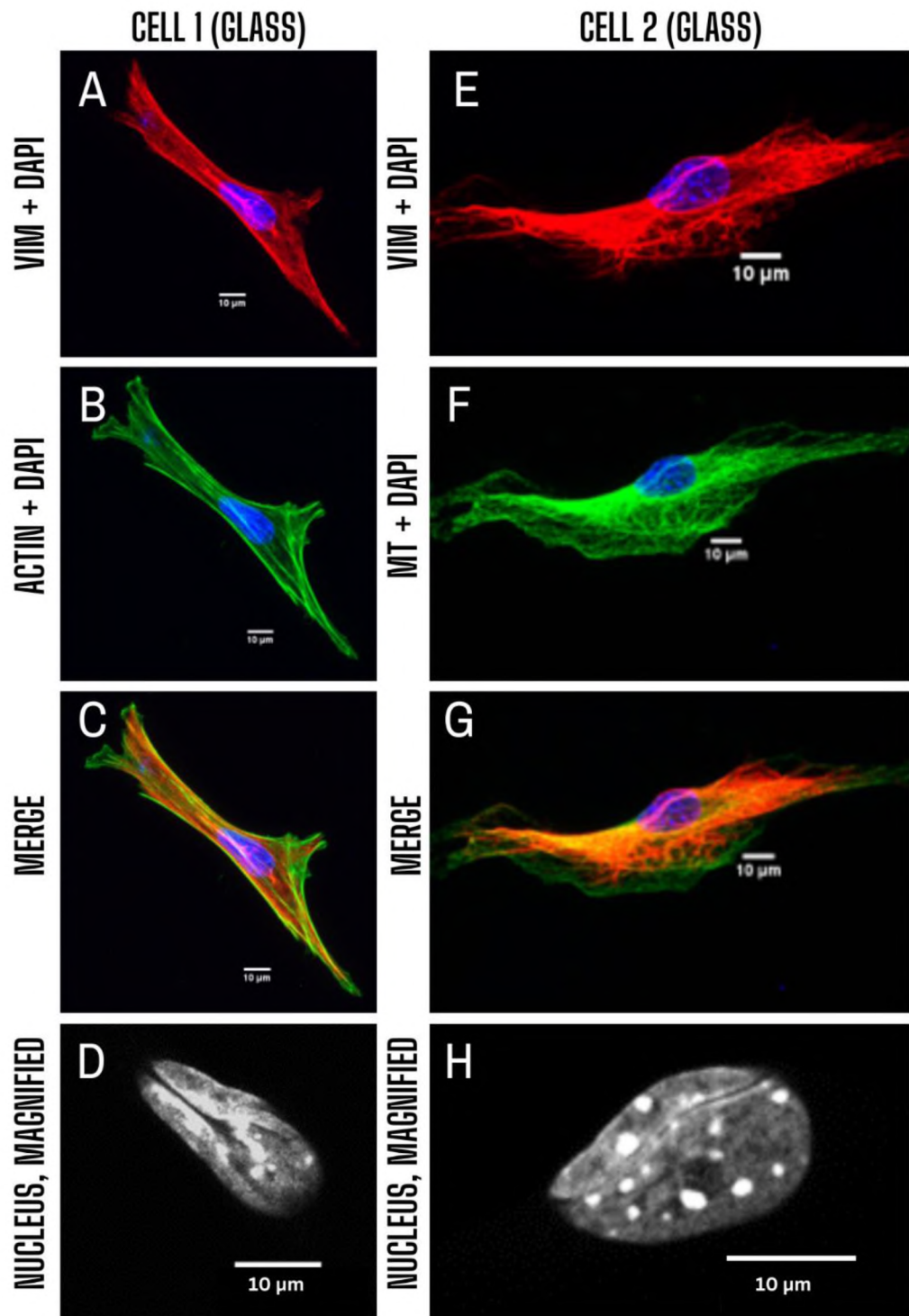


Figure 21 The visualization of two cells cultured on glass, stained for vimentin (Red), actin (Green), and nucleus (Blue) (A-D) and vimentin (Red), microtubules (Green) and nucleus (Blue) (E-H). Nucleus, magnified presented on pictures D and H. In both cell's nuclei, the thickened fibers of vimentin, actin and microtubules, align to the grooves visible in the nucleus.

Figure 21 shows the examples of two cells cultured on glass, with stained vimentin, nucleus, and actin or microtubules. Both, microtubules and actin, align with the thickened vimentin fibers, and also with the grooves presented in the D and H. The nuclei with these types of grooves, are called “coffee beans” nuclei, have the invagination in the central, upper area, resembling coffee seeds [105]. Deeper analysis of the data allowed identifying these invaginations not only on the top, but also on the sides and bottom of the nuclei. And another step was eventually observing vimentin association not only to the grooves, but also to different deformations of the nucleus.

To confirm these findings, some further analysis was conducted, and the examples are presented in Figure 22.

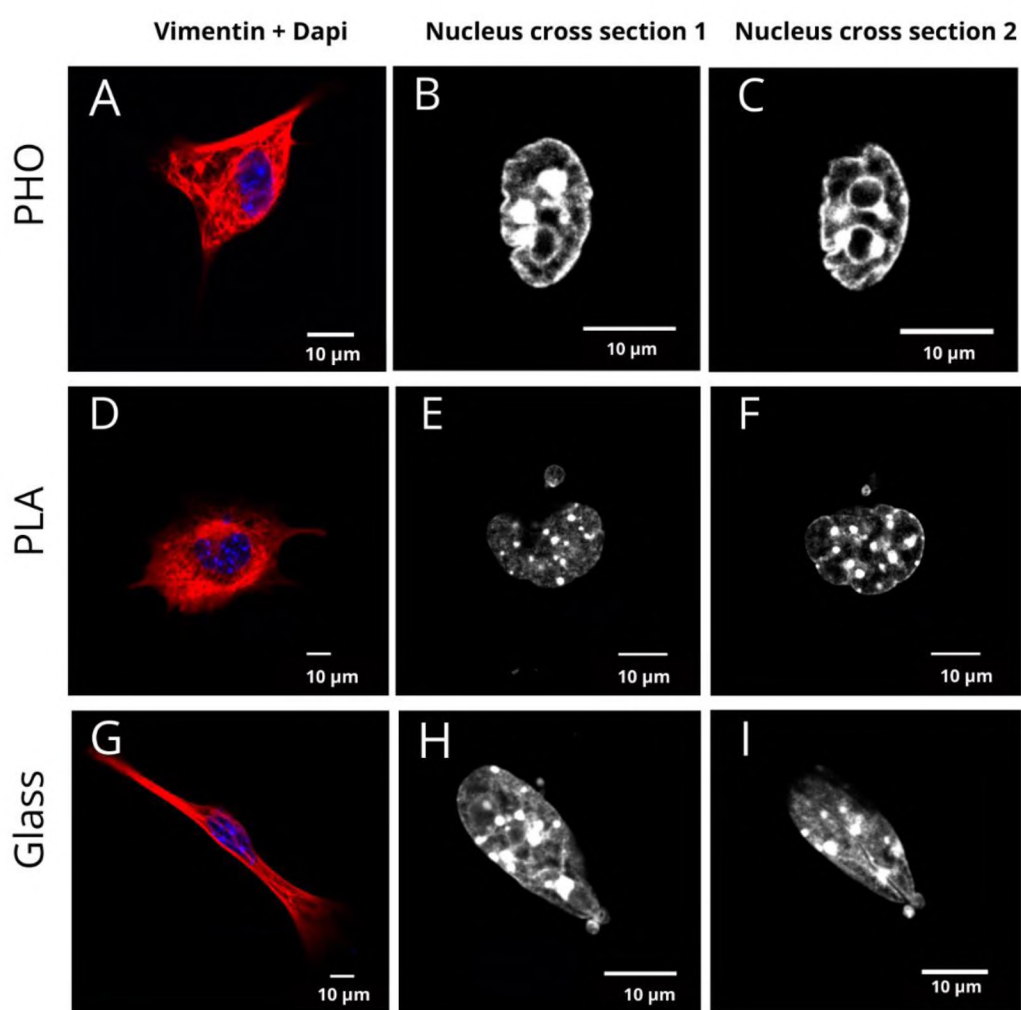


Figure 22 The examples of grooved and deformed nuclei of MEF cells cultured on P(3HO) (A-C), PLA (D-F), and glass (G-I). Two single images of cross section of the nucleus were selected for each cell.

The grooves, deformations and nuclear distortions observed in MEF cells, cultured on glass, PLA and P(3HO) are presented in Figure 22. For cells cultured on polymeric films, the grooves can be noticed not in the central top area, as in the “coffee beans” nuclei, but on the sides, and bottom of the nuclei. The invaginations are irregular, and the nuclei are significantly deformed. For the cell cultured on glass, one edge of the nucleus is narrower than the other, and also a nuclear blebbing can be noticed. For all the indicated grooves, there is a colocalization of vimentin fibers, running exactly along the invaginations.

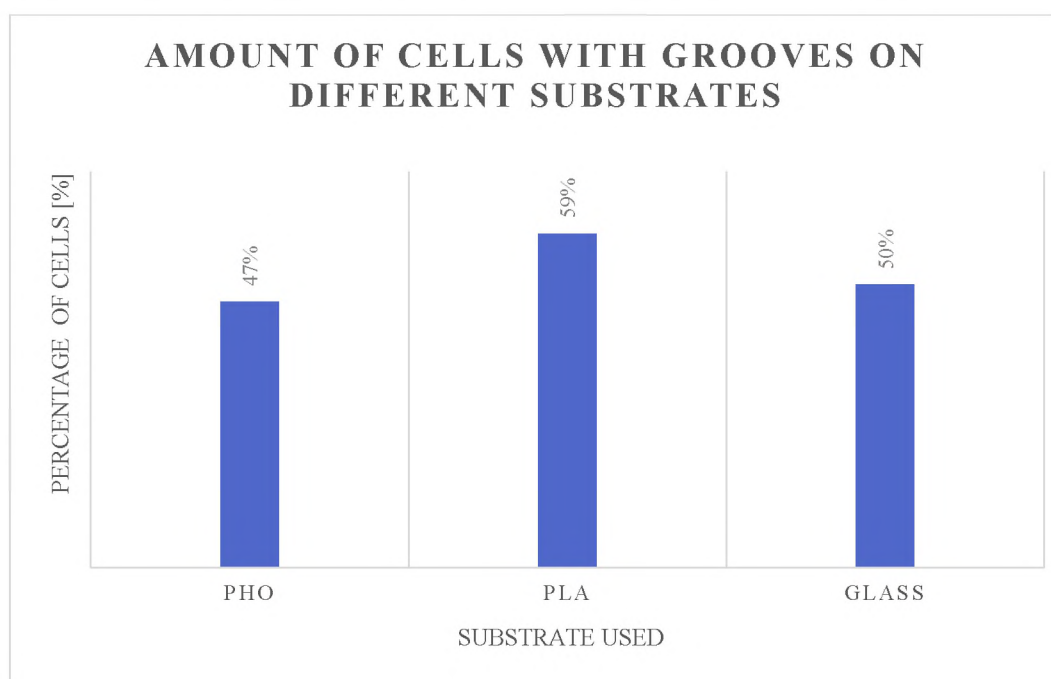


Figure 23 The amount of cells [%] with nuclear grooves, cultured on various substrates.

In Figure 23 the bar chart presents the percentage number of cells with grooved nuclei that were cultured on different substrates — P(3HO) - 47%, PLA—59%, and glass—50%. The of χ^2 test analysis indicated that this was not statistically significant.

To present the nuclear deformations in the clearer way, 3D reconstruction was prepared for an example of a cell cultured on P(3HO).

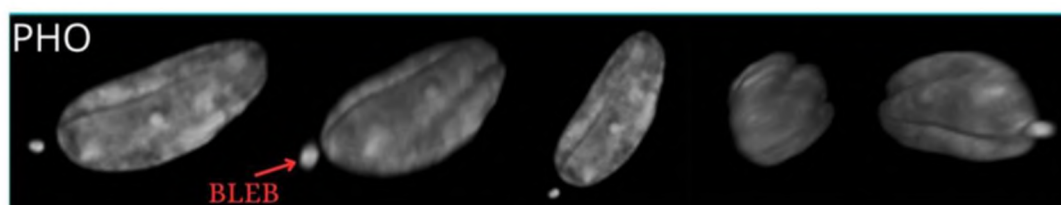


Figure 24 Example of 3D reconstruction of the nuclei of cell cultured on P(3HO).

In Figure 24 the presented nucleus has few longitudinal invaginations, that can be clearly visible under different angles. There is also a nuclear bleb present for that nucleus, and since blebs

were observed in many cells cultured on different substrates, an analysis was conducted, considering the dependency between blebbing and substrate used.

8.5 Occurrence of nuclear blebs in association to the substrate used for cell culturing

The invaginations and grooves of the nuclei are leading to changes in nuclear shapes. Cells, that are subjected to compressive stress, have to adapt their shape, and the nucleus is also forced to modification of its original figure. This often leads to formation of nuclear blebs, which might be appearing spontaneously, when cell migrate through narrow spaces. Another explanation of the presence of blebs, might be the expression of specific isoforms of lamins during migration [46]. For the analysed MEFs, nuclear blebs were identified in 23% of cells on PLA, 15% of cells on P(3HO), and 6% on glass. The χ^2 test was performed, in order to determine the relation between the substrates used, and the occurrence of nuclear blebs. The final results showed that the presence of blebs might be dependent on the matrix for culturing ($\chi^2_{0.01} = 10.810$), and the test was statistically significant. This can lead to another assumption, that the used substrate is influencing the nuclei deformations. Also, since the highest number of cells with blebs was cultured on PLA, the explanation for this might be the fact, that PLA influences the pH of the culture medium, as it easily hydrolyses in aqueous solutions.

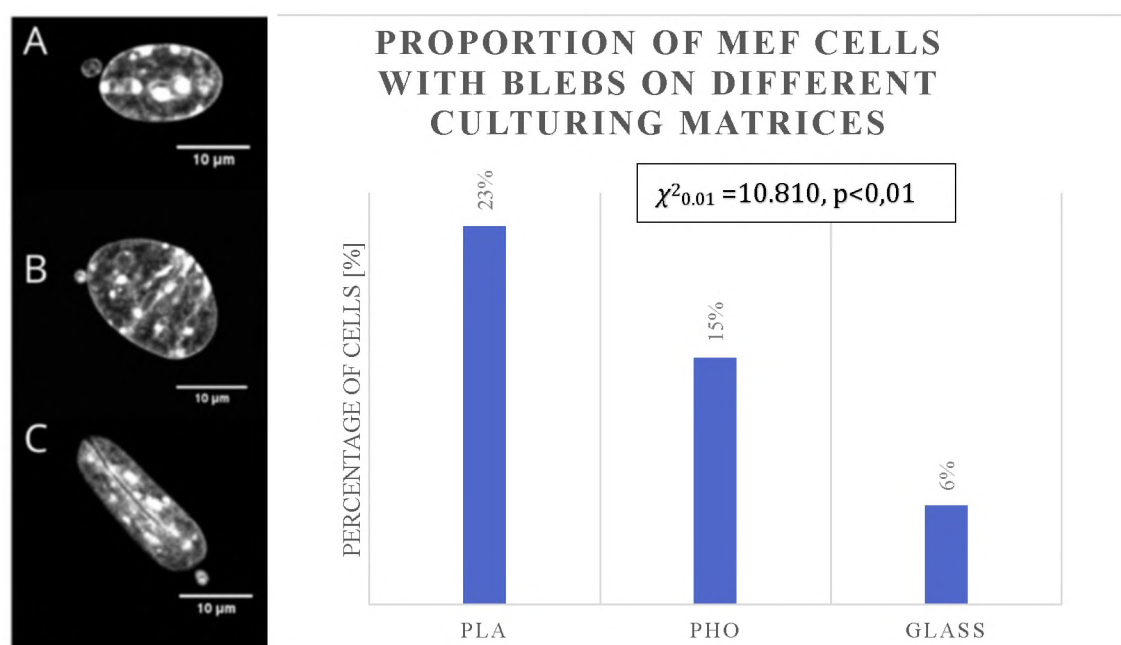


Figure 25 Nuclear blebs for selected single cells cultured on P(3HO) (A), PLA (B), and glass (C). The chart presents percentage of cells with blebs from the groups of cells cultured on PLA, P(3HO), and glass.

8.6 Vimentin – Nuclear Grooves coalignment review

Vimentin was observed to align to the nuclear grooves, and invaginations. To check correlation of vimentin with actin and microtubules in nuclear furrows, cells were stained for vimentin, and nucleus, and at this point – the third element that was stained was actin in the first group, and microtubules in the other. The images captured were then analysed as cross-sections, with special consideration of the grooved areas. The objective of those analysis was to observe the position of vimentin fibers in relation to both, nucleus, and at the same time to actin or microtubules. Cells cultured on all substrates (P(3HO), PLA and glass), were investigated, and for each case vimentin was closer to the nucleus, than actin or microtubules, although all fibers were accurately adhering to invaginations. The examples of cells are presented in Figure 26.

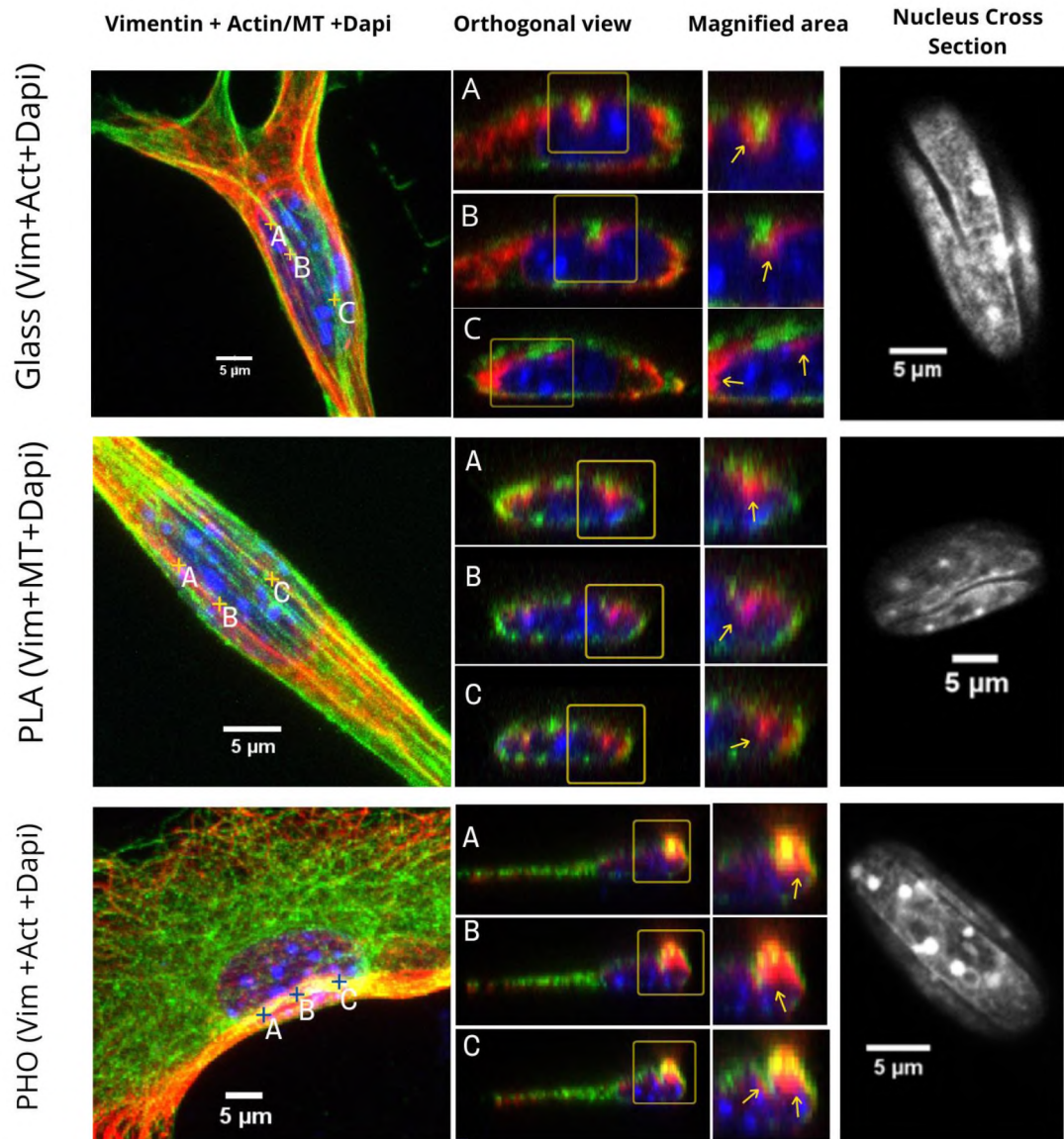


Figure 26 Examples of grooved nuclei for MEF cells cultured on Glass, P(3HO) and PLA. Actin or microtubules – green, vimentin – red, nucleus – blue. Points marked with “+” signs (red, yellow and blue) indicate the spots selected for cross-section pictures. The magnified areas are marked with yellow frames, yellow arrows show the regions of interest.

After thorough analysis of Figure 26, there can be noticed firm red and green fibers, that run above the nuclei. Three spots were randomly selected in each cell, and then magnified. In the regions of interest, marked with the yellow frame, there were spots indicated by arrows – showing the arrangement of fibers of vimentin and microfilaments or microtubules closest to the nucleus. In each case presented vimentin can be noticed nearest the nucleus, which opens questions on the potential role of vimentin in the appearance of the grooves in the nuclei.

9. Discussion

9.1 Influence of the substrate used for cell culturing on formation of classic vimentin vs “nutshell” structure

It is commonly established that material roughness influences cell adhesion and proliferation. Also, for the polymers with various Young's moduli, the distribution of the local changes in a material's stiffness is extremely likely to affect cell adhesion, motility, and spreading. Considering this, the first step taken was to evaluate the influence of the substrate on formation of vimentin cytoskeleton.

Vimentin's spatial architecture, as demonstrated in the current study, is diverse in MEF cells. It can be expressed as the classic vimentin network, which is tightly bound to the nucleus and present throughout the cell volume, or as a "nutshell" structure, which encloses the cell from above and is only connected to the top of the nucleus. At the beginning of the experiments, it was shown, that the amount of cells with classic vs. "nutshell" vimentin structure varied for various substrates, which led to the conclusion that the substrate affects the morphology of the vimentin cytoskeleton. However, because the Young's modulus seemed not to have an influence on the frequency of occurrence of various vimentin subtypes, other factors were considered.

It was noted that higher hydrophilicity of the substrate and reduced crystallinity may lead to an increase in the presence of vimentin's nutshell structure (Figure 14). The idea that more crystalline P(3HO) films induce better fibroblast adhesion might be supported by the fact that crystallinity also increases roughness. This could lead to formation of more focal contacts between the cell and the substrate, which in turn increases the presence of vimentin at the bottom of the cell and underneath the nuclear region. Since substrates with higher crystallinity often result in stronger adhesion [106], this may imply that the classic network form of the vimentin cytoskeleton is the favoured one.

Wettability and crystallinity of P(3HO) and PLA were further analysed, as these characteristics are known to have an effect on the biological performance of the materials. This, in turn, influences cellular behaviour, attachment, migration, and proliferation [107]. This allowed to perform a more thorough assessment of the potential impact of the PLA and PHO films' features on cellular behaviour. P(3HO) was more hydrophobic than PLA, according to the hydrophilicity tests carried out in the prior experiments [108].

For PLA, the measured contact angle was $68^\circ (\pm 2^\circ)$, while for P(3HO), it was $100^\circ (\pm 6^\circ)$. In terms of phase composition, which was examined in the earlier research, PLA films were less crystalline than semi-crystalline P(3HO) ($X_{C,DSC} = 4\%$ vs. 37% , respectively) [108]. Also, the migration process in a wound-healing model was investigated, as crystallinity and wettability are known to influence cell migration. Less hydrophobic and less crystalline PLA is associated with slower single cell migration rates than more hydrophobic and semi-crystalline P(3HO). According to the findings, wound-healing processes on P(3HO) and on glass occurs quickly and dynamically, whereas on PLA, the wound overgrowth is significantly slower [108].

Considering that materials of higher hydrophilicity have a positive impact on cellular adhesion and proliferation as well as a better capacity for protein adsorption, they might also have implications for single-cell behaviour in terms of vimentin structure. Therefore, based on these characteristics, it might be claimed, that the occurrence of the nutshell vimentin subtype in MEF cells is greatly influenced by the wettability and the crystalline structure of the substrate used.

9.2 Influence of the substrate for cell culturing on the occurrence of nuclear grooves, invaginations and blebs

Although grooved nuclei can be seen in a variety of healthy cell types, they are a hallmark of some tumours [109] and are used to diagnose several cancers. It is known that nuclear invaginations occur in some cells depending on dppa2 (developmental pluripotency associated factor 2) [110]. For the 288 MEF cell nuclei, that were examined for the purpose of this thesis, it was found, that vimentin not only fills the spaces created by the nuclear grooves, but also reaches deeper regions of the invaginations toward the interior of the nucleus. So far, the formation of nuclear grooves was mostly linked to microtubules [111]. The question is whether vimentin causes the grooves to form during cell movement and migration, or if the grooves are initially formed by microtubules, and vimentin serves only as a protection against nucleus rupture. It has been noted that vimentin serves as a protection from the external mechanical forces, and as a maintenance of nuclear integrity [46]. Vimentin bundles appear to extend farther into the nuclear region than either actin or microtubules, regardless of the size, depth, or position of the grooves that have been found. Although the creation of grooves is not affected by the lack of actin [112], this is not the case for microtubules. Microtubules have been demonstrated to affect the mechanical regulation of chromatin dynamics as well as the structure of the nuclear envelope [113]. Vimentin, however, fills the grooves and delves into the invaginations deeper, aligning with microtubule bundles, which raises the possibility that its presence may be necessary for the development of these structures. Since intermediate

filaments are known for their affinity to histones [114], and for their protective functions towards cell nucleus and cellular integrity [46]. Considering the above-mentioned facts, the question is whether VIFs work in with microtubules to deepen the fluctuations in the nuclear envelope and create the grooves, or do they act as a protective layer against microtubules or other factors that invaginate the nucleus? Does vimentin just show up in the deeper parts of the grooves to guard the nucleus from additional harm that is initiated by microtubules? After all, for the processes of transcription, initiation and elongation, it has been postulated that oligomers of intermediate filaments can remove histone octamers from nucleosomes and deposit them on their coiled-coil domains [115]. Vimentin role around the nuclear envelope is still to be investigated.

Another issue worth to discuss is to consider the occurrence of nuclear grooves in cells, as if they were in special circumstances: when the cells are exposed to certain conditions, the grooves appear. Nuclear grooves have been so far mostly connected to cancer cells. Nevertheless, the nuclei with different kinds of grooves were identified in almost a half of MEF cells (on glass and P(3HO) studied in this work, with a slightly higher rate for PLA substrate (58%). This could imply that "coffee-bean" nuclei aren't always a characteristic of cancer cells, but rather of cells with a strong mesenchymal phenotype. The existence of cells with nuclear grooves and blebs on PLA substrate also increases the possibility that the presence of these structures is influenced by the microenvironment. It may be assumed that the type of a substrate used might have an impact on the occurrence of the blebs as it is known that extracellular acidic pH is generally related to cancer cells [116]. The fact that PLA was hydrolysed into lactic acid ($pK_a = 3.86$) during cell growth might explain why cells grown on PLA displayed a larger frequency of nuclei with blebs [117]. As a result, the medium's pH might be impacted, potentially making it more acidic. PLA is well known for degrading quickly during the hydrolysis process. Although PHO is a bit more resistant to that process, it also degrades and affects the pH of the cellular medium [118–120]. However, because the presence of the grooves is statistically insignificant in relation to the type of substrate used, further research should concentrate on examining the grooves themselves as well as the relationship between their incidence and dependencies with cytoskeleton. The situation is quite the opposite in terms of blebbing. There were substantially more cells with nuclear blebs discovered on PLA than on the other substrates, thus the incidence of the blebs might be further explored in terms of the external, microenvironmental settings.

Cancer cells are frequently noticed in grooved nuclei and are typically linked to an acidic microenvironment. Because of this, additional investigation into that phenomenon could be a good place to start based on the identification of these structures on the PLA substrate. For PHO, the proportion of grooved cells was comparable to that of glass (reference), suggesting

that this substrate might be useful for medical equipment. It should be noted, though, that PHO experiences hydrolysis in similar environments. PHO degrades mostly to dimers of (R)-3-hydroxyacids and in a lesser amount to (R)-3-hydroxyoctanoic acid ($pK_a = 4.84$), which is significantly less acidogenic than lactic acid [121]. Consequently, the pH change has less of an impact on the cell's surroundings. The final question that can be asked, in terms of the substrate's impact on the occurrence of blebs and grooves is, whether the expression of vimentin is noticeably different on various substrates? Do the characteristics of the microenvironment cause vimentin to encourage the development of nuclear alterations? These, and many more questions still remain unanswered, but open to follow up on.

10. Summary of the results and comments

The results of this work were published in two publications, that support the thesis of this work. They both focus on the role of vimentin in the cytoskeleton, cell behaviour, migration, and morphology. They both describe the effects of sensing the external stimuli from the physicochemical features of the cellular substrates, on internal cellular structure, and the potential role of vimentin in transducing signals to the core of the cell.

The research presented in this thesis also explores the co-dependencies between vimentin fibers and nuclei, shedding light on their interaction and potential influence on nuclear morphology.

One of the important findings in the first publication is the presence of vimentin fibers in nuclei grooving, indicating a potential connection between intermediate filaments and nuclear deformations. The study suggests that these deformations in the nucleus may have implications on gene transcription, emphasizing the importance of understanding the precise mechanisms underlying nuclear invaginations.

The second publication refers to the observation of the two various types of vimentin assembly, which has not been observed before. This suggests that intermediate filaments may have more functionalities and importance in cell development than previously assumed. The study emphasizes the importance of understanding the diversity of vimentin structures and their potential roles in cellular processes. The publication also notes the clear expression of mechanotransduction signals from the external environment through the shape of vimentin in mouse embryonic fibroblast cells. Nevertheless, more research is needed to make definitive statements about the ultimate implications of these findings in the context of cell development and mechanotransduction.

10.1 Vimentin association with nuclear grooves in normal MEF 3T3 cells

Highlights:

- Vimentin role in transferring signals between the exterior of the cell and the nucleus is proven, yet not entirely studied and understood
- Cell nuclei are often with shape irregularities, invaginations, grooves, or blebs
- Nuclear grooves, also known as “coffee beans nuclei”, have been associated with multiple cancers, and serve as an important marker for recognition of the disease
- Confocal microscopy imaging showed the overlap of the nuclear grooves, invaginations, and distortions with the vimentin intermediate filaments arrangements
- Migration of cells, especially in a rough environment or through narrow spaces, causes nuclear distortions, and might lead to appearance of nuclear blebs, that are more frequent in vimentin depleted cells
- Vimentin is tightly associated with the nucleus, and is present in nuclear distortions, invaginations and grooves
- Nuclear blebs are frequent in MEFs, and the frequency of their occurrence is associated to the substrate used for cell culturing
- Vimentin function is protective against the external conditions, like for example: changes of pH in the culture medium, and VIFs goal might be to prevent the nuclei from the further damage.

Publication I describes the observed deformations of nuclei in MEF 3T3 cells, that were cultured on PLA and P(3HO) films, and glass for reference. The noticed invaginations, nuclear grooves and nuclear blebs were captured by confocal microscopy images, where vimentin fibers could be carefully studied, and presented in terms of their co-dependencies with the nuclei. The influence of the physicochemical characteristics of the substrates used was also considered, and the association of vimentin with nuclear grooves and blebs was found.

In Publication I intermediate filaments of MEF cells –vimentin fibres - were observed to have an important role in the nuclei grooving. The deformations of the nucleus might lead to the effects in transcription of genes, thus this is highly important to learn about the exact mechanisms causing the invaginations of the nucleus.

10.2 Vimentin cytoskeleton architecture analysis on polylactide and polyhydroxyoctanoate substrates for cell culturing

Highlights:

- Contemporary science and industry are leaning towards the use of biopolymers of eco-friendly background – either the well-known ones, or the novel materials
- The examples of these polymers are PLA, and P(3HO), which were carefully selected for the experiments, due to their valuable characteristics
- PLA and P(3HO) were dissolved in ethyl acetate, and formed as thin films, that served as substrates for cell culturing
- Physicochemical features of these polymeric films were studied in terms of their influence on the behaviour of MEF 3T3 cells, as the materials have the potential to be used for tissue engineering, for instance: wound healing plasters, skin regeneration scaffolds, bone plates, cardiovascular patches, nerve guides, surgical sutures or as drug release 3d platforms (carriers)
- The morphology and behaviour of the MEF 3T3 cells was thoroughly studied on both materials in multiple research, yet still vimentin, that is an IF crucial in wound healing procedure, has been omitted so far
- The influence of the P(3HO) and PLA on the morphology and assembly of vimentin cytoskeleton network is attentively described in this work
- Vimentin has two types of assembly – “classic network” and a “nutshell”, which has been identified during experiments conducted for this thesis; The external stimuli from the microenvironmental features of the substrate can influence the appearance of the mentioned subtypes of VIFs.

Publication II describes the influence of two materials of a high potential of use in tissue engineering and medicine, on behaviour of the intermediate filaments in normal mouse embryonic fibroblast cells. The focus on the IFs, namely: vimentin, was due to its significant engagement in wound healing process. Since the studied polymers P(3HO) and PLA, were considered as materials, that could potentially help in accelerating the treatment of injuries, observation of their influence on one of the crucial proteins in the process is extremely important. Experiments allowed observations of two subtypes of vimentin intermediate filaments – classic vimentin network, and “nutshell” structure. The frequency of occurrence of these subtypes was correlated with some characteristics of the substrates used.

In Publication II, vimentin was identified to form two various assemblies, which has not been observed before. This suggests that intermediate filaments might have much more functionalities and importance in cell development, than previously assumed. The mechanotransduction of signals from the external environment was clearly expressed by the vimentin shape in MEF cells, yet still more research is needed to provide the ultimate statements.

11. Final remarks

Nowadays, in the face of an ecological disaster, most industries are seeking for the effective solutions, that could allow further work, but with no harm for the environment. Medicine and related areas, that use huge amounts of single-use plastics, are truly balancing on the border of human welfare. On one hand the use of disposables is indispensable, due to the basic hygiene procedures. Whole world has been forced to appreciation of disposable masks during recent pandemic of Covid-19. On the other hand, the amount of single-use products, that are essential for our safety, turned into another huge problem of wastes on the environmental side. Only for those reasons, seeking new bioplastics is justified, and broadly encouraged. Given the fact, that some of the novel polymers have excellent properties in terms of the influence on the patient's health, the focus on finding the environmentally friendly materials constantly grows. The primary goal of using a material in patient's tissues is to provide more benefits by using the treatment, than harms that this treatment could potentially cause. One of these materials is PLA, that not only is non-toxic and biocompatible, but also biodegradable, and of natural origin. PLA has been broadly used in medicine, however as any material, it has its limitations. Therefore, the need of finding other candidates with improved characteristics is pursued, and fortunately achieved. The example of such material is P(3HO), that not only is of bacterial origin, but also characterizes with biodegradability, non-toxicity, and has a great potential of use in tissue engineering and medicine. Its physicochemical features and possibilities of modifications suggest that this material could be used for wound healing, thus it has been studied first as a film for culturing fibroblast cells. Fibroblasts as cells, that take part in wound healing in vivo, serve as a model culture for cytoskeleton observation regarding migration and motility. Both, microfilaments and microtubules are quite well studied, and their behaviour on different materials is broadly described. Intermediate filaments however are a challenging field for investigation, due to the abundance of their subtypes. Nevertheless, for the purpose of this work, the selected IF protein was vimentin, as it has been reported to be a crucial element of initiation and conduct of the wound healing process. The observations allowed identifying two types of the assembly of vimentin intermediate filaments and opened the

discussion on the external environment influence on the appearance of specific subtype of vimentin. The research also showed that more observations are needed to identify the exact role of intermediate filaments in mechanosensing and mechanotransduction, as well as its interplay with the core of the cell – the nucleus.

Cell nucleus is the core organelle, a command centre for the cell, responsible for crucial cellular processes. It manages cellular life, and determines key cellular actions, such as proliferation, homeostasis, nutrition and migration. For the appropriate functioning of all these activities, there is one, most important condition, that has to be fulfilled: communication. The nucleus is tightly connected with other cellular organelles, but the cytoskeleton provides a connectivity network with the external environment. Mammalian cells migration often requires movement through tight spaces, i.e. during wound healing or metastasis, which influences nuclear size and shape. It has been noticed, that at the very beginning of migration process, the expression of vimentin starts immediately. Vimentin is an intermediate filament protein, that spreads out from the juxtannuclear cage to the cell edges and plays an important role in cell protection.

Nuclei of multiple cells are imperfect, involving numerous malformations or blebs; grooved nuclei (aka “coffee beans nuclei”) are associated with multiple cancers, as markers for recognition of the presence of the tumours. The origin of these grooves is not fully understood, yet observations of MEF 3T3 cells under different microenvironmental conditions, allowed to claim, that vimentin intermediate filaments might be associated with shaping the nucleus to the extent, that has not been considered before. Observation of the same cells and their nuclear blebs is also suggesting an active involvement of vimentin in the formation of these distortions.

The presented thesis focuses on the interplay of the intermediate filaments, with the external environment signals, that induce cascades of changes, and processes influencing cell behaviour. Solving the mechanotransduction “puzzle” might be crucial for discovering the mechanisms of development of multiple diseases, hence could contribute to their treatment. Therefore, various studies on the smallest organelles of cells are needed, to enable understanding the process of cooperation of all organelles within the cell, as well as with the external environment.

Focusing on one of the key elements of the cell – cytoskeleton, allowed observation of cell morphology, behaviour, and dependencies between the external environment of the cell, and cooperation of the internal cytoskeletal elements. Finally, vimentin being one of a key protein for wound healing process, and a driving force of epithelial – mesenchymal transition, leaves us with many questions, that remain unresolved, but ready to be answered.

12. List of Figures

FIGURE 1 EXAMPLE OF DISTRIBUTION OF DIFFERENT ELEMENTS OF THE CYTOSKELETON IN THE CELL. FIGURE CREATED IN BIORENDER.	16
FIGURE 2 MICROFILAMENTS DISTRIBUTED IN A CELL. FIGURE CREATED IN BIORENDER.....	17
FIGURE 3 STRUCTURE OF VIMENTIN. A. BASIC CONSTRUCTION OF VIMENTIN – BLOCK CHAIN. B. COILED-COIL DIMER OF VIMENTIN FORMED BY A PAIR OF PARALLEL CHAINS COILED-COIL SEGMENTS 1A, 1B, 2A, AND 2B ARE CONNECTED BY A SHORT LINKERS (L1, L12, L2), C. CRYSTAL STRUCTURE OF VIMENTIN COIL 1B PACKED IN A HIGH-ORDER FILAMENTOUS FORM (SOURCE: – HTTPS://WWW.RCSB.ORG/STRUCTURE/5WHF).....	21
FIGURE 4 CELL NUCLEUS SCHEMA WITH THE CRUCIAL ELEMENTS INDICATED. FIGURE CREATED IN BIORENDER.	24
FIGURE 5 MATERIAL SHARE OF GLOBAL BIOPOLYMER PRODUCTION CAPACITY (SOURCE: EUROPEAN BIOPLASTICS, NOVA INSTITUTE (2023) [69] INNER DOUGHNUT – BIOBASED/ NON-BIODEGRADABLE MATERIALS. OUTER DOUGHNUT – BIODEGRADABLE MATERIALS.	30
FIGURE 6 THREE METHODS OF OBTAINING POLY(LACTIC ACID).....	32
FIGURE 7 CLASSIFICATION OF BIOPOLYMER TYPES IN DIFFERENT SUBGROUPS [89,90] .].	35
FIGURE 8 CHEMICAL FORMULA OF P(3HO) MONOMER. – POLYHYDROXYOCTANOATE [98].	37
FIGURE 9 SCHEMATIC PICTURE OF A STANDARD CONFOCAL MICROSCOPE.....	39
FIGURE 10 EXCITATION AND EMISSION SPECTRUM OF THE FLUOROPHORES USED IN THE EXPERIMENTS: BLUE – DAPI, GREEN - ALEXA FLUOR 488, RED – ALEXA FLUOR 633. THE DASHED LINES PRESENT EXCITATION SPECTRUM, WHILE THE SOLID LINE WITH THE BACKGROUND – PRESENTS THE SPECTRUM OF EMISSION OF THE GIVEN FLUOROPHORE.	42
FIGURE 11 POLARIZED LIGHT MICROSCOPIC IMAGES OF PLA (A–C) AND P(3HO) (D–F) FILMS IN 5× MAGNIFICATION (A, D), 10× MAGNIFICATION (B, E), AND 40X MAGNIFICATION (C, F).	44
FIGURE 12 BASIC ARCHITECTURE OF VIMENTIN FIBERS TYPES IN A SINGLE MEF CELL, CULTURED ON GLASS. (GREEN SQUARE) – FIBERS - LONG VIMENTIN FILAMENTS OCCURRING ABOVE THE NUCLEUS, AND IN CELL’S TAIL AND EDGE. SQUIGGLES (BLUE SQUARE) – SHORTER, THAN FIBERS, BUT LONGER THAN PARTICLE-LENGTH ULFs (PINK SQUARE). IMAGE CAPTURED WITH THE USE OF CONFOCAL MICROSCOPY.	45
FIGURE 13 THE COMPARISON BETWEEN THE “NUTSHELL” (A-E) AND “CLASSIC” (F-J) STRUCTURE OF VIMENTIN. VIMENTIN- RED, NUCLEUS – BLUE. B, G, - SLICE OF VIMENTIN IMAGE, IN THE NUCLEAR AREA. C, H- SLICE OF VIMENTIN IMAGE, WITHOUT DAPI CHANNEL.	46
FIGURE 14 THE PROPORTION OF CELLS WITH "CLASSIC" OR "NUTSHELL" VIMENTIN CYTOSKELETON, CULTURED ON GLASS, P(3HO), AND PLA.	47
FIGURE 15 EXAMPLE OF CROSS SECTION FOR “CLASSIC” VIMENTIN STRUCTURE. A - SINGLE Z-STACK IMAGE OF THE BOTTOM OF THE CELL. B- Z-STACK IMAGE OF THE CENTRAL PART OF THE CELL. C – Z-STACK IMAGE OF THE TOP OF THE CELL. D, D.1, D.2- ORTHOGONAL VIEW.	48
FIGURE 16 EXAMPLE OF CROSS SECTION FOR “NUTSHELL” VIMENTIN STRUCTURE. A - SINGLE Z-STACK IMAGE OF THE BOTTOM OF THE CELL. B- Z-STACK IMAGE OF THE CENTRAL PART OF THE CELL. C – Z-STACK IMAGE OF THE TOP OF THE CELL. D, D.1, D.2- ORTHOGONAL VIEW.	49
FIGURE 17 ADJUSTMENT OF THE REGION OF INTEREST FOR THE “WHOLE AREA OCCUPIED BY VIMENTIN” ANALYSIS. MEF CELL CULTURED ON PLA, WITH A ‘NUTSHELL’ VIMENTIN STRUCTURE. A) INITIAL ADJUSTMENT OF IMAGE PARAMETERS FOR CROPPED CELL IMAGE. B) SELECTED ROI (REGION OF INTEREST) – VIMENTIN CYTOSKELETON 16-BIT (B&W) IMAGE – OUTLINED WITH THE YELLOW LINE, BASED ON COLOUR THRESHOLDING.....	51
FIGURE 18 STEPS PERFORMED DURING THE ANALYSIS OF VIMENTIN CYTOSKELETON IN THE SELECTED 10 X 10 μM SQUARE ROIS. INDICATING REGIONS UNDER THE CELL NUCLEUS (A), IN THE MIDDLE OF THE CELL HEIGHT (B), AND BELOW THE NUCLEUS (C). A1, B1, C1 – MAGNIFIED	

REGIONS OF INTEREST, AFTER IMAGE THRESHOLDING, SHOWING THE FINAL AMOUNT OF VIMENTIN IN THE SELECTED REGIONS.....	51
FIGURE 19 THE AMOUNT OF VIMENTIN IN SELECTED 10 X 10 μm SQUARES REGIONS OF INTEREST UNDER THE NUCLEUS, IN THE MIDDLE OF THE CELL HEIGHT, AND ABOVE THE NUCLEUS FOR “CLASSIC” VIMENTIN NETWORK. THE TABLE SHOWS THE CALCULATED AVERAGE VALUES $\% \pm$ STANDARD ERROR VALUE.	52
FIGURE 20 THE AMOUNT OF VIMENTIN IN SELECTED 10 X 10 μm SQUARES REGIONS OF INTEREST UNDER THE NUCLEUS, IN THE MIDDLE OF THE CELL HEIGHT, AND ABOVE THE NUCLEUS FOR THE “NUTSHELL” STRUCTURE. THE TABLE SHOWS THE CALCULATED AVERAGE VALUES $\% \pm$ STANDARD ERROR VALUE. CHART OF “NUTSHELL STRUCTURE” IS RESCALED FOR THE “UNDER” AND “MIDDLE” REGION, TO PRESENT THE DATA MORE ACCURATELY FOR THESE AREAS, IN COMPARISON TO “ABOVE” REGION.	53
FIGURE 21 THE VISUALIZATION OF TWO CELLS CULTURED ON GLASS, STAINED FOR VIMENTIN (RED), ACTIN (GREEN), AND NUCLEUS (BLUE) (A-D) AND VIMENTIN (RED), MICROTUBULES (GREEN) AND NUCLEUS (BLUE) (E-H). NUCLEUS, MAGNIFIED PRESENTED ON PICTURES D AND H. IN BOTH CELL’S NUCLEI, THE THICKENED FIBERS OF VIMENTIN, ACTIN AND MICROTUBULES, ALIGN TO THE GROOVES VISIBLE IN THE NUCLEUS.	55
FIGURE 22 THE EXAMPLES OF GROOVED AND DEFORMED NUCLEI OF MEF CELLS CULTURED ON P(3HO) (A-C), PLA (D-F), AND GLASS (G-I). TWO SINGLE IMAGES OF CROSS SECTION WERE SELECTED FOR EACH CELL.....	56
FIGURE 23 THE AMOUNT OF CELLS [%] WITH NUCLEAR GROOVES, CULTURED ON VARIOUS SUBSTRATES.....	57
FIGURE 24 EXAMPLE OF 3D RECONSTRUCTION OF THE NUCLEI OF CELL CULTURED ON P(3HO).	57
FIGURE 25 NUCLEAR BLEBS FOR SELECTED SINGLE CELLS CULTURED ON P(3HO) (A), PLA (B), AND GLASS (C). THE CHART PRESENTS PERCENTAGE OF CELLS WITH BLEBS FROM THE GROUPS OF CELLS CULTURED ON PLA, P(3HO), AND GLASS.....	58
FIGURE 26 EXAMPLES OF GROOVED NUCLEI FOR MEF CELLS CULTURED ON GLASS, P(3HO) AND PLA. ACTIN OR MICROTUBULES – GREEN, VIMENTIN – RED, NUCLEUS – BLUE. POINTS MARKED WITH “+” SIGNS (RED, YELLOW AND BLUE) INDICATE THE SPOTS SELECTED FOR CROSS-SECTION PICTURES. THE MAGNIFIED AREAS ARE MARKED WITH YELLOW FRAMES, YELLOW ARROWS SHOW THE REGIONS OF INTEREST.	60

13. References

- [1] C.M. Kraning-Rush, C.A. Reinhart-King, Controlling matrix stiffness and topography for the study of tumor cell migration, *Cell Adh Migr* 6 (2012) 274–279.
<https://doi.org/10.4161/cam.21076>.
- [2] I. Hopp, A. Michelmore, L.E. Smith, D.E. Robinson, A. Bachhuka, A. Mierczynska, K. Vasilev, The influence of substrate stiffness gradients on primary human dermal fibroblasts, *Biomaterials* 34 (2013) 5070–5077.
<https://doi.org/10.1016/j.biomaterials.2013.03.075>.
- [3] K.N. Dahl, E.A. Booth-Gauthier, B. Ladoux, In the middle of it all: Mutual mechanical regulation between the nucleus and the cytoskeleton, *J Biomech* 43 (2010) 2–8.
<https://doi.org/10.1016/j.jbiomech.2009.09.002>.
- [4] O. Valiron, N. Caudron, D. Job, Cellular and Molecular Life Sciences Microtubule dynamics, 58 (2001) 2069–2084.

- [5] A.E. Patteson, A. Vahabikashi, R.D. Goldman, P.A. Janmey, Mechanical and Non-Mechanical Functions of Filamentous and Non-Filamentous Vimentin, *BioEssays* 42 (2020) 1–12. <https://doi.org/10.1002/bies.202000078>.
- [6] M. Schoumacher, R.D. Goldman, D. Louvard, D.M. Vignjevic, Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia, *Journal of Cell Biology* 189 (2010) 541–556. <https://doi.org/10.1083/jcb.200909113>.
- [7] Hohmann, Dehghani, The Cytoskeleton—A Complex Interacting Meshwork, *Cells* 8 (2019) 362. <https://doi.org/10.3390/cells8040362>.
- [8] D.A. Fletcher, R.D. Mullins, Cell mechanics and the cytoskeleton, *Nature* 463 (2010) 485–492. <https://doi.org/10.1038/nature08908>.
- [9] H.P. Erickson, Evolution of the cytoskeleton, *BioEssays* 29 (2007) 668–677. <https://doi.org/10.1002/bies.20601>.
- [10] A. Hall, The cytoskeleton and cancer, *Cancer and Metastasis Reviews* 28 (2009) 5–14. <https://doi.org/10.1007/s10555-008-9166-3>.
- [11] J. Bui, D.E. Conway, R.L. Heise, S.H. Weinberg, Mechanochemical Coupling and Junctional Forces during Collective Cell Migration, *Biophys J* 117 (2019) 170–183. <https://doi.org/10.1016/j.bpj.2019.05.020>.
- [12] Y. Shafir, D. ben-Avraham, G. Forgacs, Trafficking and signaling through the cytoskeleton: a specific mechanism, *Journal of Cell Biology* 113 (Pt 1 (2000) 2747–2757.
- [13] D. Nowak, Funkcjonalne zróżnicowanie cytoplazmatycznych izoform aktyny, *Postepy Biochem* 59 (2013) 285–294.
- [14] H. Herrmann, S. V. Strelkov, P. Burkhard, U. Aebi, Intermediate filaments: Primary determinants of cell architecture and plasticity, *Journal of Clinical Investigation* 119 (2009) 1772–1783. <https://doi.org/10.1172/JCI38214>.
- [15] J. Moraczewska, Filamenty cienkie i mikrofilamenty - funkcjonalne kompleksy aktyny z tropomiozyną, 1 (2018).
- [16] M. Izdebska, A. Grzanka, M. Ostrowski, Reorganizacja cytoszkieletu a różnicowanie się komórek linii ludzkich białaczek HL-60 i K-562, *Postepy Hig Med Dosw* 60 (2006) 64–70.
- [17] V. Dugina, I. Zwaenepoel, G. Gabbiani, S. Clément, C. Chaponnier, B- and Γ -Cytoplasmic Actins Display Distinct Distribution and Functional Diversity, *J Cell Sci* 122 (2009) 2980–2988. <https://doi.org/10.1242/jcs.041970>.
- [18] C.L. Hyder, K.O. Isoniemi, E.S. Torvaldson, J.E. Eriksson, Insights into intermediate filament regulation from development to ageing, *J Cell Sci* 124 (2011) 1363–1372. <https://doi.org/10.1242/jcs.041244>.
- [19] R. Sanghvi-Shah, G.F. Weber, Intermediate filaments at the junction of mechanotransduction, migration, and development, *Front Cell Dev Biol* 5 (2017) 1–19. <https://doi.org/10.3389/fcell.2017.00081>.
- [20] K. Ohashi, S. Fujiwara, K. Mizuno, Roles of the cytoskeleton, cell adhesion and rho signalling in mechanosensing and mechanotransduction, *J Biochem* 161 (2017) 245–254. <https://doi.org/10.1093/jb/mvw082>.

- [21] Y. Jiu, J. Lehtimäki, S. Tojkander, F. Cheng, H. Jäälinoja, X. Liu, M. Varjosalo, J.E. Eriksson, P. Lappalainen, Bidirectional Interplay between Vimentin Intermediate Filaments and Contractile Actin Stress Fibers, *Cell Rep* 11 (2015) 1511–1518. <https://doi.org/10.1016/j.celrep.2015.05.008>.
- [22] J. Lowery, E.R. Kuczmarski, H. Herrmann, R.D. Goldma, Intermediate filaments play a pivotal role in regulating cell architecture and function, *Journal of Biological Chemistry* 290 (2015) 17145–17153. <https://doi.org/10.1074/jbc.R115.640359>.
- [23] R.A. Battaglia, S. Delic, H. Herrmann, N.T. Snider, Vimentin on the move: New developments in cell migration [version 1; referees: 2 approved], *F1000Res* 7 (2018) 1–10. <https://doi.org/10.12688/f1000research.15967.1>.
- [24] M. Shigyo, C. Tohda, Extracellular vimentin is a novel axonal growth facilitator for functional recovery in spinal cord-injured mice, *Sci Rep* 6 (2016) 1–9. <https://doi.org/10.1038/srep28293>.
- [25] J.E. Eriksson, H. Pallari, D. Robert, J.E. Eriksson, T. Dechat, B. Grin, B. Helfand, M. Mendez, H. Pallari, R.D. Goldman, Introducing intermediate filaments : from discovery to disease Find the latest version : Review series Introducing intermediate filaments : from discovery to disease, *J. Clin. Invest.* 119 (2009) 1763–1771. <https://doi.org/10.1172/JCI38339.cells>.
- [26] T. Liu, O.E. Guevara, R.R. Warburton, N.S. Hill, M. Gaestel, U.S. Kayyali, Regulation of vimentin intermediate filaments in endothelial cells by hypoxia, *Am J Physiol Cell Physiol* 299 (2010). <https://doi.org/10.1152/ajpcell.00057.2010>.
- [27] N. Costigliola, L. Ding, C.J. Burckhardt, S.J. Han, E. Gutierrez, A. Mota, A. Groisman, T.J. Mitchison, G. Danuser, Vimentin fibers orient traction stress, *Proc Natl Acad Sci U S A* 114 (2017) 5195–5200. <https://doi.org/10.1073/pnas.1614610114>.
- [28] B.T. Helfand, Intermediate filaments are dynamic and motile elements of cellular architecture, *J Cell Sci* 117 (2004) 133–141. <https://doi.org/10.1242/jcs.00936>.
- [29] Y.H. Chou, J.R. Bischoff, D. Beach, R.D. Goldman, Intermediate filament reorganization during mitosis is mediated by p34cdc2 phosphorylation of vimentin, *Cell* 62 (1990) 1063–1071. [https://doi.org/10.1016/0092-8674\(90\)90384-Q](https://doi.org/10.1016/0092-8674(90)90384-Q).
- [30] M.E. Kidd, D.K. Shumaker, K.M. Ridge, The role of Vimentin intermediate filaments in the progression of lung cancer, *Am J Respir Cell Mol Biol* 50 (2014) 1–6. <https://doi.org/10.1165/rcmb.2013-0314TR>.
- [31] F. Danielsson, M. Peterson, H. Caldeira Araújo, F. Lautenschläger, A. Gad, Vimentin Diversity in Health and Disease, *Cells* 7 (2018) 147. <https://doi.org/10.3390/cells7100147>.
- [32] R. Boujemaa-Paterski, R. Galland, C. Suarez, C. Guérin, M. Théry, L. Blanchoin, Directed actin assembly and motility, in: *Methods Enzymol*, Academic Press Inc., 2014: pp. 283–300. <https://doi.org/10.1016/B978-0-12-397924-7.00016-9>.
- [33] E.A. Lepekhin, C. Eliasson, C.H. Berthold, V. Berezin, E. Bock, M. Pekny, Intermediate filaments regulate astrocyte motility, *J Neurochem* 79 (2001) 617–625. <https://doi.org/10.1046/j.1471-4159.2001.00595.x>.
- [34] C.M. Wells, M. Parsons, *Cell Migration: Developmental Methods and Protocols*, Second, Humana Press, London, 2011.

- [35] J.L. Walker, B.M. Bleaken, A.R. Romisher, A.A. Alnwibit, A.S. Menko, In wound repair vimentin mediates the transition of mesenchymal leader cells to a myofibroblast phenotype, *Mol Biol Cell* 29 (2018) 1555–1570. <https://doi.org/10.1091/mbc.E17-06-0364>.
- [36] F. Cheng, Y. Shen, P. Mohanasundaram, M. Lindström, J. Ivaska, T. Ny, J.E. Erikss, Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF- β -Slug signaling, *Proc Natl Acad Sci U S A* 113 (2016) E4320–E4327. <https://doi.org/10.1073/pnas.1519197113>.
- [37] J.L. Walker, N. Zhai, L. Zhang, B.M. Bleaken, I. Wolff, J. Gerhart, M. George-Weinstein, A.S. Menko, Unique precursors for the mesenchymal cells involved in injury response and fibrosis, *Proc Natl Acad Sci U S A* 107 (2010) 13730–13735. <https://doi.org/10.1073/pnas.0910382107>.
- [38] A. Satelli, S. Li, Vimentin as a potential molecular target in cancer therapy Or Vimentin, an overview and its potential as a molecular target for cancer therapy, *Cell Mol Life Sci*. 68 (2011) 3033–3046. <https://doi.org/10.1007/s00018-011-0735-1>.Vimentin.
- [39] B.M. Bleaken, A.S. Menko, J.L. Walker, Cells activated for wound repair have the potential to direct collective invasion of an epithelium, *Mol Biol Cell* 27 (2016) 451–465. <https://doi.org/10.1091/mbc.E15-09-0615>.
- [40] L. Liu, Q. Luo, J. Sun, G. Song, Nucleus and nucleus-cytoskeleton connections in 3D cell migration, *Exp Cell Res* 348 (2016) 56–65. <https://doi.org/10.1016/j.yexcr.2016.09.001>.
- [41] F. Martins, J. Sousa, C.D. Pereira, O.A.B. da Cruz e Silva, S. Rebelo, Nuclear envelope dysfunction and its contribution to the aging process, *Aging Cell* 19 (2020) 1–17. <https://doi.org/10.1111/accel.13143>.
- [42] Y. Turgay, M. Eibauer, A.E. Goldman, T. Shimi, M. Khayat, K. Ben-Harush, A. Dubrovsky-Gaupp, K.T. Sapra, R.D. Goldman, O. Medalia, The molecular architecture of lamins in somatic cells, *Nature* 543 (2017) 261–264. <https://doi.org/10.1038/nature21382>.
- [43] D.A. Starr, Communication between the cytoskeleton and the nuclear envelope to position the nucleus, *Mol Biosyst* 3 (2007) 583–589. <https://doi.org/10.1039/b703878j>.
- [44] C.S. Janota, F.J. Calero-Cuenca, E.R. Gomes, The role of the cell nucleus in mechanotransduction, *Curr Opin Cell Biol* 63 (2020) 204–211. <https://doi.org/10.1016/j.ceb.2020.03.001>.
- [45] P. Friedl, K. Wolf, J. Lammerding, Nuclear mechanics during cell migration, *Curr Opin Cell Biol* 23 (2011) 55–64. <https://doi.org/10.1016/j.ceb.2010.10.015>.
- [46] A.E. Patteson, A. Vahabikashi, K. Pogoda, S.A. Adam, Vimentin protects the structural integrity of the nucleus and suppresses nuclear damage caused by large deformations, (n.d.) 1–26.
- [47] D.J. Tschumperlin, Mechanotransduction, *Compr Physiol* 1 (2011) 1057–1073. <https://doi.org/10.1002/cphy.c100016>.
- [48] M. Maurer, J. Lammerding, The Driving Force: Nuclear Mechanotransduction in Cellular Function, Fate, and Disease, *Annu Rev Biomed Eng* 21 (2019) 443–468. <https://doi.org/10.1146/annurev-bioeng-060418-052139>.

- [49] D.E. Jaalouk, J. Lammerding, Mechanotransduction gone awry, *Nat Rev Mol Cell Biol* 10 (2009) 63–73. <https://doi.org/10.1038/nrm2597>.
- [50] J. Zhang, J. Uchiyama, K. Imami, Y. Ishihama, R. Kageyama, T. Kobayashi, Novel Roles of Small Extracellular Vesicles in Regulating the Quiescence and Proliferation of Neural Stem Cells, *Front Cell Dev Biol* 9 (2021) 1–11. <https://doi.org/10.3389/fcell.2021.762293>.
- [51] M.T. Ho Thanh, A. Grella, D. Kole, S. Ambady, Q. Wen, Vimentin intermediate filaments modulate cell traction force but not cell sensitivity to substrate stiffness, *Cytoskeleton* 78 (2021) 293–302. <https://doi.org/10.1002/cm.21675>.
- [52] M. Guo, A.J. Ehrlicher, S. Mahammad, H. Fabich, M.H. Jensen, J.R. Moore, J.J. Fredberg, R.D. Goldman, D.A. Weitz, The role of vimentin intermediate filaments in cortical and cytoplasmic mechanics, *Biophys J* 105 (2013) 1562–1568. <https://doi.org/10.1016/j.bpj.2013.08.037>.
- [53] Z. Qin, L. Kreplak, M.J. Buehler, Nanomechanical properties of vimentin intermediate filament dimers, *Nanotechnology* 20 (2009). <https://doi.org/10.1088/0957-4484/20/42/425101>.
- [54] L. Kreplak, H. Herrmann, U. Aebi, Tensile properties of single desmin intermediate filaments, *Biophys J* 94 (2008) 2790–2799. <https://doi.org/10.1529/biophysj.107.119826>.
- [55] F. Fleissner, S. Kumar, N. Klein, D. Wirth, R. Dhiman, D. Schneider, M. Bonn, S.H. Parekh, Tension Causes Unfolding of Intracellular Vimentin Intermediate Filaments, *Adv Biosyst* 4 (2020) 1–12. <https://doi.org/10.1002/adbi.202000111>.
- [56] Y. Jiu, J. Peränen, N. Schaible, F. Cheng, J.E. Eriksson, R. Krishnan, P. Lappalainen, Vimentin intermediate filaments control actin stress fiber assembly through GEF-H1 and RhoA, *J Cell Sci* 130 (2017) 892–902. <https://doi.org/10.1242/jcs.196881>.
- [57] F. Danielsson, M. Peterson, H. Caldeira Araújo, F. Lautenschläger, A. Gad, Vimentin Diversity in Health and Disease, *Cells* 7 (2018) 147. <https://doi.org/10.3390/cells7100147>.
- [58] F. V. Almeida, G. Walko, J.R. McMillan, J.A. McGrath, G. Wiche, A.H. Barber, J.T. Connelly, The cytolinker plectin regulates nuclear mechanotransduction in keratinocytes, *J Cell Sci* 128 (2015) 4475–4486. <https://doi.org/10.1242/jcs.173435>.
- [59] M.E. Murray, M.G. Mendez, P.A. Janmey, Substrate stiffness regulates solubility of cellular vimentin, *Mol Biol Cell* 25 (2014) 87–94. <https://doi.org/10.1091/mbc.E13-06-0326>.
- [60] S. Cho, M. Vashisth, A. Abbas, S. Majkut, K. Vogel, Y. Xia, I.L. Ivanovska, J. Irianto, M. Tewari, K. Zhu, E.D. Tichy, F. Mourkoti, H.Y. Tang, R.A. Greenberg, B.L. Prosser, D.E. Discher, Mechanosensing by the Lamina Protects against Nuclear Rupture, DNA Damage, and Cell-Cycle Arrest, *Dev Cell* 49 (2019) 920–935.e5. <https://doi.org/10.1016/j.devcel.2019.04.020>.
- [61] S.B. Khatau, C.M. Hale, P.J. Stewart-Hutchinson, M.S. Patel, C.L. Stewart, P.C. Searson, D. Hodzic, D. Wirtz, A perinuclear actin cap regulates nuclear shape, *Proc Natl Acad Sci U S A* 106 (2009) 19017–19022. <https://doi.org/10.1073/pnas.0908686106>.
- [62] M. Maninova, J. Caslavsky, T. Vomastek, The assembly and function of perinuclear actin cap in migrating cells, *Protoplasma* 254 (2017) 1207–1218. <https://doi.org/10.1007/s00709-017-1077-0>.

- [63] S.J. Paluck, T.H. Nguyen, H.D. Maynard, Heparin-Mimicking Polymers: Synthesis and Biological Applications, *Biomacromolecules* 17 (2016) 3417–3440. <https://doi.org/10.1021/acs.biomac.6b01147>.
- [64] J.J. Green, J.H. Elisseeff, Mimicking biological functionality with polymers for biomedical applications, *Nature* 540 (2016) 386–394. <https://doi.org/10.1038/nature21005>.
- [65] R. Aksakal, C. Mertens, M. Soete, N. Badi, F. Du Prez, Applications of Discrete Synthetic Macromolecules in Life and Materials Science: Recent and Future Trends, *Advanced Science* 8 (2021) 1–22. <https://doi.org/10.1002/advs.202004038>.
- [66] P. Yadav, H. Yadav, V.G. Shah, G. Shah, G. Dhaka, Biomedical biopolymers, their origin and evolution in biomedical sciences: A systematic review, *Journal of Clinical and Diagnostic Research* 9 (2015) 21–25. <https://doi.org/10.7860/JCDR/2015/13907.6565>.
- [67] T. Witko, D. Solarz, K. Feliksiak, Z. Rajfur, M. Guzik, Cellular architecture and migration behavior of fibroblast cells on polyhydroxyoctanoate (PHO): A natural polymer of bacterial origin, *Biopolymers* 110 (2019) e23324. <https://doi.org/10.1002/bip.23324>.
- [68] K. Sofińska, J. Barbasz, T. Witko, J. Dryzek, K. Haraźna, M. Witko, J. Kryściak-Czerwenka, M. Guzik, Structural, topographical, and mechanical characteristics of purified polyhydroxyoctanoate polymer, *J Appl Polym Sci* 136 (2019). <https://doi.org/10.1002/app.47192>.
- [69] Global Production capacities of bioplastics 2023-2028 Global production capacities of bioplastics 2023 by material type Global production capacities of bioplastics 2028 by material type D E C E M B E R 2 0 2 3, n.d. <http://www.european-bioplastics.org/news/publica->
- [70] E. de Jong, H.A. Visser, A.S. Dias, C. Harvey, G.J.M. Gruter, The Road to Bring FDCA and PEF to the Market, *Polymers (Basel)* 14 (2022) 1–32. <https://doi.org/10.3390/polym14050943>.
- [71] H. RISK, O.F. EXPOSURE, T.O. BISPHENOL, A. (BPA), *Rocz Panstw Zakl Hig* 2015;66(1), 66 (2015) 5–11.
- [72] T.P. Ahern, L.G. Spector, P. Damkier, B. Öztürk Esen, S.P. Ulrichsen, K. Eriksen, T.L. Lash, H.T. Sorensen, D.P. Cronin-Fenton, Medication–Associated Phthalate Exposure and Childhood Cancer Incidence, *JNCI: Journal of the National Cancer Institute* (2022). <https://doi.org/10.1093/JNCI/DJAC045>.
- [73] Market – European Bioplastics e.V., (n.d.). <https://www.european-bioplastics.org/market/> (accessed April 25, 2022).
- [74] Guidance on the application of Single-Use Plastic rules, (n.d.). https://ec.europa.eu/commission/presscorner/detail/en/qanda_21_2709 (accessed April 25, 2022).
- [75] Branża tworzyw sztucznych w pandemii, (n.d.). [Raport_Roczny_PEP_2020-2021_PL.pdf](https://raport.pep.pl/) (plasticseurope.org)
- [76] S. Farah, D.G. Anderson, R. Langer, Physical and mechanical properties of PLA, and their functions in widespread applications — A comprehensive review, *Adv Drug Deliv Rev* 107 (2016) 367–392. <https://doi.org/10.1016/j.addr.2016.06.012>.

- [77] E. Castro-Aguirre, F. Iñiguez-Franco, H. Samsudin, X. Fang, R. Auras, Poly(lactic acid)—Mass production, processing, industrial applications, and end of life, *Adv Drug Deliv Rev* 107 (2016) 333–366. <https://doi.org/10.1016/j.addr.2016.03.010>.
- [78] K. Madhavan Nampoothiri, N.R. Nair, R.P. John, An overview of the recent developments in polylactide (PLA) research, *Bioresour Technol* 101 (2010) 8493–8501. <https://doi.org/10.1016/j.biortech.2010.05.092>.
- [79] “Global Bioplastics and Biopolymer Market size, (2024). <https://www.precedenceresearch.com/biopolymers-market>,” (n.d.).
- [80] R. Auras, B. Harte, S. Selke, An overview of polylactides as packaging materials, *Macromol Biosci* 4 (2004) 835–864. <https://doi.org/10.1002/mabi.200400043>.
- [81] T.R. Ahammad, S. Z.; Gomes, J.; Sreekrishnan, Wastewater treatment for production of H₂S-free biogas, *Journal of Chemical Technology & Biotechnology* 83 (2008) 1163–1169. <https://doi.org/10.1002/jctb>.
- [82] S.M. Davachi, B. Kaffashi, Polylactic Acid in Medicine, *Polymer - Plastics Technology and Engineering* 54 (2015) 944–967. <https://doi.org/10.1080/03602559.2014.979507>.
- [83] C. Dartailh, W. Blunt, P.K. Sharma, S. Liu, N. Cicek, The Thermal and Mechanical Properties of Medium Chain-Length Polyhydroxyalkanoates Produced by *Pseudomonas putida* LS46 on Various Substrates, *Front Bioeng Biotechnol* 8 (2021) 1–9. <https://doi.org/10.3389/fbioe.2020.617489>.
- [84] Polymers - Physical Properties, (n.d.). https://www.engineeringtoolbox.com/polymer-properties-d_1222.html (accessed March 17, 2022).
- [85] S. Mohamed, A.A.A. Amirul, A.W.M. Effendy, K. Bhupalan, Characterization and cytotoxicity of Polyhydroxyalkanoate microparticles as adjuvant matrix for the immobilization of *Pasteurella multocida* whole-cell vaccine, *J Sustain Sci Manag* 12 (2017) 89–95.
- [86] Y. Wang, J. Yin, G.Q. Chen, Polyhydroxyalkanoates, challenges and opportunities, *Curr Opin Biotechnol* 30 (2014) 59–65. <https://doi.org/10.1016/j.copbio.2014.06.001>.
- [87] T. Nitkiewicz, M. Wojnarowska, M. Sołtysik, A. Kaczmarek, T. Witko, C. Ingrao, M. Guzik, How sustainable are biopolymers? Findings from a life cycle assessment of polyhydroxyalkanoate production from rapeseed-oil derivatives, *Science of the Total Environment* 749 (2020) 141279. <https://doi.org/10.1016/j.scitotenv.2020.141279>.
- [88] R.A.J. Verlinden, D.J. Hill, M.A. Kenward, C.D. Williams, I. Radecka, Bacterial synthesis of biodegradable polyhydroxyalkanoates, *J Appl Microbiol* 102 (2007) 1437–1449. <https://doi.org/10.1111/j.1365-2672.2007.03335.x>.
- [89] R. Singh, S. Gautam, B. Sharma, P. Jain, K.D. Chauhan, Biopolymers and their classifications, in: *Biopolymers and Their Industrial Applications*, Elsevier, 2021: pp. 21–44. <https://doi.org/10.1016/b978-0-12-819240-5.00002-x>.
- [90] S. Ibrahim, O. Riahi, S.M. Said, M.F.M. Sabri, S. Rozali, Biopolymers From Crop Plants, in: *Reference Module in Materials Science and Materials Engineering*, Elsevier, 2019. <https://doi.org/10.1016/b978-0-12-803581-8.11573-5>.

- [91] A. Steinbüchel, H.E. Valentin, A. Steinbüchel, H.E. Valentin, Diversity of bacterial polyhydroxyalkanoic acids, *FEMS Microbiol Lett* 128 (1995) 219–228. [https://doi.org/10.1016/0378-1097\(95\)00125-O](https://doi.org/10.1016/0378-1097(95)00125-O).
- [92] T. Pulingam, J.N. Appaturi, T. Parumasivam, A. Ahmad, K. Sudesh, Biomedical Applications of Polyhydroxyalkanoate in Tissue Engineering, *Polymers (Basel)* 14 (2022) 2141. <https://doi.org/10.3390/polym14112141>.
- [93] Y. min Han, T. Bedarida, Y. Ding, B.K. Somba, Q. Lu, Q. Wang, P. Song, M.H. Zou, β -Hydroxybutyrate Prevents Vascular Senescence through hnRNP A1-Mediated Upregulation of Oct4, *Mol Cell* 71 (2018) 1064-1078.e5. <https://doi.org/10.1016/j.molcel.2018.07.036>.
- [94] R. Rai, T. Keshavarz, J.A. Roether, A.R. Boccaccini, I. Roy, Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future, *Materials Science and Engineering R: Reports* 72 (2011) 29–47. <https://doi.org/10.1016/j.mser.2010.11.002>.
- [95] Z. Sun, J.A. Ramsay, M. Guay, B.A. Ramsay, Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440, *Appl Microbiol Biotechnol* 74 (2007) 69–77. <https://doi.org/10.1007/s00253-006-0655-4>.
- [96] G.D. Christian, A. Díaz-Barrera, R. Andler, I. Martínez, C. Peña, K.L. Lee, C.C. Chong, B. Kessler, R. Weusthuis, B. Witholt, G. Eggink, Production of Microbial Polyesters :, *Managing* 71 (2001) 10.
- [97] S. Philip, T. Keshavarz, I. Roy, Polyhydroxyalkanoates: Biodegradable polymers with a range of applications, *Journal of Chemical Technology and Biotechnology* 82 (2007) 233–247. <https://doi.org/10.1002/jctb.1667>.
- [98] Z.A. Raza, S. Abid, I.M. Banat, Polyhydroxyalkanoates: Characteristics, production, recent developments and applications, *Int Biodeterior Biodegradation* 126 (2018) 45–56. <https://doi.org/10.1016/j.ibiod.2017.10.001>.
- [99] W. Justyna, Confocal Microscopy: Principles and Modern Practices, *Curr Protoc Cytom* 176 (2017) 139–148. <https://doi.org/10.1002/cpcy.68>.
- [100] A. Nwaneshiudu, C. Kuschal, F.H. Sakamoto, R. Rox Anderson, K. Schwarzenberger, R.C. Young, Introduction to confocal microscopy, *Journal of Investigative Dermatology* 132 (2012) 1–5. <https://doi.org/10.1038/jid.2012.429>.
- [101] C.L. Smith, Basic confocal microscopy, *Curr Protoc Neurosci* (2011) 1–13. <https://doi.org/10.1002/0471142301.ns0202s56>.
- [102] P.O. Bayguinov, D.M. Oakley, C.C. Shih, D.J. Geanon, M.S. Joens, J.A.J. Fitzpatrick, Modern Laser Scanning Confocal Microscopy, *Curr Protoc Cytom* 85 (2018) 1–17. <https://doi.org/10.1002/cpcy.39>.
- [103] T. Nitkiewicz, M. Wojnarowska, M. Soltysik, A. Kaczmarek, T. Witko, C. Ingrao, M. Guzik, How sustainable are biopolymers? Findings from a life cycle assessment of polyhydroxyalkanoate production from rapeseed-oil derivatives, *Science of the Total Environment* 749 (2020) 141279. <https://doi.org/10.1016/j.scitotenv.2020.141279>.

- [104] H. Maclean, Z. Sun, J. Ramsay, B. Ramsay, Decaying exponential feeding of nonanoic acid for the production of medium-chain-length poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2440, *Can J Chem* 86 (2008) 564–569. <https://doi.org/10.1139/V08-062>.
- [105] M. Hirokawa, D. Ph, Y. Miyake, Nuclear Findings of Ovarian Surface Epithelial Tumors, 22 (2000) 27–29.
- [106] N. Oleszko, W. Walach, A. Utrata-Wesołek, A. Kowalczyk, B. Trzebicka, A. Kłama-Baryła, D. Hoff-Lenczewska, M. Kawecki, M. Lesiak, A.L. Sieroń, A. Dworak, Controlling the crystallinity of thermoresponsive poly(2-oxazoline)-based nanolayers to cell adhesion and detachment, *Biomacromolecules* 16 (2015) 2805–2813. <https://doi.org/10.1021/acs.biomac.5b00745>.
- [107] O. Bretcanu, S. Misra, I. Roy, C. Renghini, F. Fiori, A.R. Boccaccini, V. Salih, In vitro biocompatibility of 45S5 Bioglass®-derived glass-ceramic scaffolds coated with poly(3-hydroxybutyrate), *J Tissue Eng Regen Med* 3 (2009) 139–148. <https://doi.org/10.1002/term.150>.
- [108] T. Witko, D. Solarz, K. Feliksiak, K. Haraźna, Z. Rajfur, M. Guzik, Insights into In Vitro Wound Closure on Two Biopolyesters—Polylactide and Polyhydroxyoctanoate, *Materials* 13 (2020) 2793. <https://doi.org/10.3390/ma13122793>.
- [109] B. V Vtyurin, I.A. Chekmaryova, E.A. Dubova, M.N. Podgornova, A.I. Shchegolev, Ultrastructural Characteristics of Solid Pseudopapillary Tumors of the Pancreas, 151 (2011) 230–233.
- [110] Y. Guo, Y. Zheng, Sculpting the Nucleus with Dynamic Microtubules, *Dev Cell* 27 (2013) 1–2. <https://doi.org/10.1016/j.devcel.2013.09.027>.
- [111] T. Abe, K. Takano, A. Suzuki, Y. Shimada, M. Inagaki, N. Sato, T. Obinata, T. Endo, Myocyte differentiation generates nuclear invaginations traversed by myofibrils associating with sarcomeric protein mRNAs, *J Cell Sci* 117 (2004) 6523–6534. <https://doi.org/10.1242/jcs.01574>.
- [112] D.A. Collings, C.N. Carter, J.C. Rink, A.C. Scott, S.E. Wyatt, N.S. Allen, Plant Nuclei Can Contain Extensive Grooves and Invaginations Plant Nuclei Can Contain Extensive Grooves and Invaginations, (2001). <https://doi.org/10.2307/3871239>.
- [113] B. Hampoelz, Y. Azou-gros, R. Fabre, O. Markova, P. Puech, T. Lecuit, Microtubule-induced nuclear envelope fluctuations control chromatin dynamics in *Drosophila* embryos, 3386 (2011) 3377–3386. <https://doi.org/10.1242/dev.065706>.
- [114] R. Hartig, R.L. Shoeman, A. Janetzko, G. Tolstonog, P. Traub, DNA-mediated transport of the intermediate filament protein vimentin into the nucleus of cultured cells, 3584 (1998) 3573–3584.
- [115] P. Traub, R.L. Shoeman, Hypothesis: Intermediate filament and related proteins: Potential activators of nucleosomes during transcription initiation and elongation?, *BioEssays* 16 (1994) 349–355. <https://doi.org/10.1002/bies.950160510>.
- [116] Y. Kato, S. Ozawa, C. Miyamoto, Y. Maehata, A. Suzuki, T. Maeda, Y. Baba, Acidic extracellular microenvironment and cancer, *Cancer Cell Int* 13 (2013) 89. <https://doi.org/10.1186/1475-2867-13-89>.

- [117] M. Jamshidian, E.A. Tehrany, M. Imran, M. Jacquot, S. Desobry, Poly-Lactic Acid: Production, applications, nanocomposites, and release studies, *Compr Rev Food Sci Food Saf* 9 (2010) 552–571. <https://doi.org/10.1111/j.1541-4337.2010.00126.x>.
- [118] M.A. Elsayy, K.H. Kim, J.W. Park, A. Deep, Hydrolytic degradation of polylactic acid (PLA) and its composites, *Renewable and Sustainable Energy Reviews* 79 (2017) 1346–1352. <https://doi.org/10.1016/j.rser.2017.05.143>.
- [119] D. Mallardé, M. Valière, C. David, M. Menet, P. Guérin, Hydrolytic degradability of poly(3-hydroxyoctanoate) and of a poly(3-hydroxyoctanoate)/poly(R,S-lactic acid) blend, *Polymer (Guildf)* 39 (1998) 3387–3392. [https://doi.org/10.1016/S0032-3861\(97\)10172-0](https://doi.org/10.1016/S0032-3861(97)10172-0).
- [120] Y. Chen, Y.H. Tsai, I.N. Chou, S.H. Tseng, H.S. Wu, Application of biodegradable polyhydroxyalkanoates as surgical films for ventral hernia repair in mice, *Int J Polym Sci* 2014 (2014). <https://doi.org/10.1155/2014/789681>.
- [121] E. Cichoń, K. Harażna, S. Skibiński, T. Witko, A. Zima, A. Ślósarczyk, M. Zimowska, M. Witko, B. Leszczyński, A. Wróbel, M. Guzik, Novel bioresorbable tricalcium phosphate/polyhydroxyoctanoate (TCP/PHO) composites as scaffolds for bone tissue engineering applications, *J Mech Behav Biomed Mater* 98 (2019) 235–245. <https://doi.org/10.1016/j.jmbbm.2019.06.028>.