



JAGIELLONIAN UNIVERSITY
IN KRAKÓW

PHD THESIS

The effects of cell – microspheres
interaction on the cytoskeletal
architecture in fibroblasts

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KRAKÓW 2024

*Chciałabym serdecznie podziękować mojemu Promotorowi
Doktorowi habilitowanemu Zenonowi Rajfurowi, profesorowi UJ,
za opiekę naukową, wieloletnią współpracę oraz cierpliwość.*

*Dziękuję mojej rodzinie,
Rodzicom Janinie i Lechosławowi Adamczyk,
za umożliwienie mi nauki, mobilizację do niej i ogromne wsparcie.*

*Siostrze Marcie,
za dobre rady i zachowanie rozsądku, kiedy mi go brakowało.*

*Oraz Babciom i Dziadkowi,
za wiarę we mnie i wsparcie.*

*Dziękuję Przyjaciółom,
w szczególności Marcie, która od lat wspiera mnie rozmową i radą,
oraz moim koleżankom i kolegom
z Zakładu Biofizyki Molekularnej i Międzyfazowej Instytutu Fizyki UJ
za wszelką pomoc i nieocenioną współpracę.*

Bez Was nie byłabym w miejscu, w którym teraz jestem.

Wydział Fizyki, Astronomii i Informatyki Stosowanej
Uniwersytet Jagielloński

OŚWIADCZENIE

Ja niżej podpisana Olga Adamczyk (nr albumu: 1079546), doktorantka Wydziału Fizyki,
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Kraków, dnia

.....

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ACRONYMS AND ABBREVIATIONS LIST

ABP – Actin Binding Proteins

ADP – Adenosine Diphosphate

AF555 – Alexa Fluor 555 Fluorophore

AF633 – Alexa Fluor 633 Fluorophore

ATP – Adenosine Triphosphate

CLASP - Cytoplasmic Linker Associated Protein

CLIP – Cytoplasmic Linker Proteins

CLIP1 - CAP-GLY domain containing linker protein 1

EB1 - Microtubules End-binding proteins 1

FC – Fibronectin- coated beads

FtsZ - Filamenting Temperature Sensitive Mutant Z

GDP – Guanosine Diphosphate

GTP – Guanosine Triphosphate

IF – Intermediate filaments

IFAP – Intermediate Filaments Associated Proteins

IQGAP1 - Ras GTPase-activating-like protein

MAP – Microtubule Associated Protein

MARK2 - Microtubule Affinity-Regulating Kinase 2

MEF 3T3 – Mouse Embryonic Fibroblast Cells

MF – Microfilaments/ Actin filaments

MT – Microtubule filaments

MTOC – Microtubule Organizing Center

NFTs - Neurofibrillary Tangles

OT – Optical Tweezers

PA Gel Substrates – Polyacrylamide Gel Substrates

SKAP - Src Kinase-associated Phosphoproteins

UC – Uncoated beads

YG fluorophore – Yellow Green Fluorophore

ABSTRACT

Living cells interact mechanically with their microenvironment, as it is widely known. External factors, acting on the cell, have an impact on many fundamental cell processes, including migration, proliferation, gene expression, and differentiation. Therefore, it is crucial to study how the mechanical properties of the culture substrate (for example its different stiffness) affect the cellular and molecular regulatory pathways.

Optical microscopy is one of the most popular experimental techniques to observe and study biological activities. Confocal microscopy gives us the ability to observe how the three-dimensional organisation of the cell components like the cytoskeleton changes in response to a specific mechanical stimulation.

A concentrated laser beam and polystyrene or latex beads are used in the optical tweezers-based approach (OT), a microrheological technique, employed to examine the mechanical characteristics of biological systems. Latex beads, functionalized with a particular protein, can interact with proteins found on the surface of cellular membranes. The behavior of the cell may be dramatically impacted by such interactions.

Here, we show that the architecture of actin, microtubules, and intermediate filaments significantly changed by the simple placement of latex beads of 2 μm diameter on the cell surface. It is also demonstrated that the duration of the cell-bead interaction affects the reaction of the cytoskeleton to the stimulation. Application of the cytoskeletal drug like cytochalasin D eliminates the actin cytoskeleton's remodelling effects.

More importantly, when cells are plated on elastic substrates that mimic the mechanical characteristics of the physiological cellular environment, we see that the microtubule cytoskeleton interacts with latex beads to form novel, "cup-like" structures around those beads. These findings offer new perspectives on the role of microtubule cytoskeleton in the process of endocytosis. It leads us to the conclusion that the stiffness of the substrate can have an impact on cellular activities involving every cytoskeleton component, especially on their architecture.

To explain this effect, the behavior of three proteins from the MAP (Microtubule Associated Proteins) family, which interact directly or indirectly with the microtubule cytoskeleton - CLIP1, IQGAP1, and MARK2, was examined. It showed that the presence of external stimuli like microspheres or elastic substrates had an impact on the behavior of these proteins. These experiments brought us closer to answering the question of which of the MAP proteins may also take part in the forming a new, specific structure.

STRESZCZENIE

Powszechnie wiadomo, że żywe komórki oddziałują mechanicznie ze swoim mikrośrodowiskiem. Czynniki zewnętrzne działające na komórkę mają wpływ na wiele podstawowych procesów komórkowych, w tym migrację, proliferację, ekspresję genów i różnicowanie. Z tego powodu kluczowe znaczenie ma badanie wpływu właściwości mechanicznych podłoża hodowlanego na komórkowe szlaki regulacyjne. Jedną z najpopularniejszych eksperymentalnych technik obserwacji i badania aktywności biologicznej jest mikroskopia optyczna. Mikroskopia konfokalna umożliwia monitorowanie zmian w trójwymiarowej organizacji cytoszkieletu w odpowiedzi na precyzyjny bodziec mechaniczny, taki jak mikrometrowa kulka uwięziona we wiązce lasera.

Skoncentrowana wiązka lasera i kulki polistyrenowe lub lateksowe są wykorzystywane w metodzie zwanej szczypcami optycznymi (ang. *Optical Tweezers*; OT), technice mikroeologicznej, która służy do badania właściwości mechanicznych układów biologicznych. Kulki lateksowe mogą być funkcjonalizowane przez konkretne białko, a więc mogą wchodzić w interakcje z białkami znajdującymi się na powierzchni błon komórkowych.

W tej pracy wykazano, że architektura aktyny, mikrotubul i włókien pośrednich została znacząco zmieniona przez umieszczenie lateksowych kulek na powierzchni komórki. Wykazano również, że długość interakcji komórka-kulka wpływa na oddziaływanie. Zastosowanie toksyny cytoszkieletowej, takiej jak cytochalazyna D zahamowało efekt przebudowy cytoszkieletu.

Co ważniejsze, gdy komórki są umieszczane na elastycznych podłożach, które odtwarzają mechaniczne właściwości fizjologicznego środowiska komórkowego, widzimy, jak cytoszkielet mikrotubul wchodzi w interakcję z kulkami lateksowymi, tworząc niezwykle struktury przypominające miseczki. Odkrycia te oferują nowe spojrzenie na działanie cytoszkieletu mikrotubul oraz prowadzą do wniosku, że sztywność podłoża ma duży wpływ na aktywność komórkową.

Aby wyjaśnić ten efekt, zbadano zachowanie się trzech białek z rodziny MAP (ang. *Microtubule Associated Proteins*), które oddziałują bezpośrednio lub pośrednio z

cytoszkieletem mikrotubul – CLIP1, IQGAP1 i MARK2. Okazało się, że każde z tych białek zachowuje się inaczej, gdy komórki są na elastycznych podłożach i obecne są mikrosfery. Eksperymenty te przybliżyły nas nieco do odpowiedzi na pytanie, które z białek MAP może również pomóc mikrotubulom w tworzeniu nowo zaobserwowanej, specyficznej struktury.

1. INTRODUCTION

1.1 CELL STRUCTURE

It is not easy to say what a cell is since it is a very complex creation. We are talking here not only about the structure, which is extremely complex but also about the multitude of its functions. Any scientist who has worked with live cells realises how deep and uncharted this topic is. One of the most classic definitions given by the Encyclopedia Britannica is:

A cell is a mass of cytoplasm that is bound externally by a cell membrane. Usually microscopic in size, cells are the smallest structural units of living matter and compose all living things. Most cells have one or more nuclei and other organelles that carry out a variety of tasks. Some single cells are complete organisms, such as a bacterium or yeast. Others are specialised building blocks of multicellular organisms, such as plants and animals. [1]

This describes the structure of the cell, but it is important to note that it is also a perfectly integrated physicochemical entity and an elementary unit of life.

Cells differ in morphological properties which may be dependent on their origin or location. It is worth starting with the basic classification of the cells into prokaryotic cells (i.e., unicellular, without a cell nucleus) and eukaryotic ones [2]. Going deeper, eukaryotes can be classified into plants, fungi, or animals. Focusing on the latter group, here you can also find various types of cells that are radically different from each other. Cancer cells are a creature that behaves in a way completely incomparable to normal cells [3].

Structural variations can be seen between different types of cells. It is difficult to say which organelle is the most crucial for cell living, because each is necessary for the proper functioning of the cell and its life, and there are many of them. Below is a brief presentation of a few chosen organelles based on widely accessible rankings.

To mention the most significant of them, it is worth starting with the cell nucleus. They can be found in almost every eukaryotic cell (the exception is red blood cells [4]), and sometimes even some have more than one (e.g., osteoclasts [5]). The nucleus contains

genetic material that is separated from the cytoplasm by the nuclear membrane, which maintains its integrity and facilitates transcription and replication [6].

The second one is the mitochondrion, which is present in most eukaryotes. Like the nucleus, mitochondrion can also contain genetic material [7], but most importantly, it is a source of chemical energy by generating adenosine triphosphate (ATP) using aerobic respiration [8].

Ribosomes are found in all cells that carry out protein synthesis. They use mRNA as a template that enables them to create a protein [9]. Another important organelle can also be associated with ribosomes, the endoplasmic reticulum (the rough endoplasmic reticulum in the case of ribosomes), which divides the cell into compartments and is responsible for protein folding [10], [11].

Penultimate on the list is the Golgi apparatus, which looks like a stack of flattened disks [12]. It is responsible for the synthesis and packaging of molecules intended for secretion outside the cell and directs the newly synthesised proteins to the appropriate cell compartments [13].

And finally - the cytoskeleton, which will be the main topic of this dissertation.

All organelles described above are shown in the schematic drawing [Figure 1].

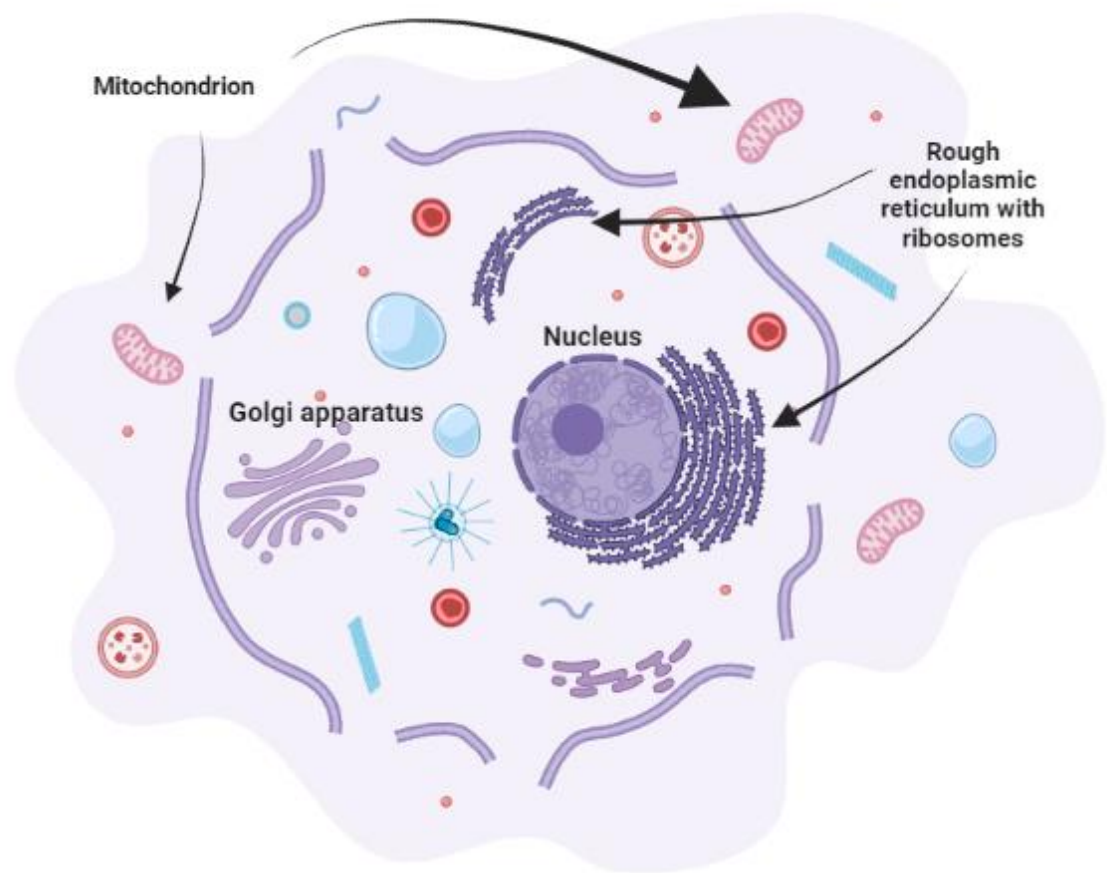


Figure 1. Schematic drawing showing the organelles described above as the most important in the cell (the cytoskeleton has not been signed here).

1.2 CYTOSKELETON

The subject of the cytoplasmic matrix began to be discussed for the first time in the world of science, in the nineteenth century. In the meantime, there were many theories to explain how the cytoplasm is structured. However, at the end of the 1960s, the first electron microscopy images began to appear, which revolutionised the approach of researchers to the structure of the inside of a cell. Previously, there were voices that there is a structure resembling a three-dimensional mesh in the cytoplasm, but this was widely regarded as an artefact resulting from the fixation of cells [14].

In 1983, an international conference on the cytoplasmic matrix was held in the USA. It is worth quoting the words of Prof. Walter Birmeier: *It is reasonable to assume that in 1976 around 95% of experimental biologists were not aware of the facts that living cells have cytoskeletons (...). In 1984, however, 95% seem to know, and many of them now even consider the cytoskeleton to play a fundamental role in quite a wide variety of biological phenomena..* [15]

As the name suggests, the cytoskeleton plays the role of the cellular skeleton. It is recognized that the cytoskeleton consists of three types of filaments: microfilaments (also called actin; MF), intermediate filaments (IF), and microtubules (MT). The order of their listing is not accidental here, they have been ranked from those with the smallest single filament diameter to the largest one. They differ in the type of proteins that create them and, to a large extent, in the functions they perform. Similarities can be found between microfilaments and microtubules, which are made of globular protein subunits and which facilitate rapid polymerization and depolymerization of the fibers. This may suggest that these filaments are built to perform a specific short-term function and then depolymerize [16].

Below [Figure 2] a distribution of individual filaments in the cell body is shown.

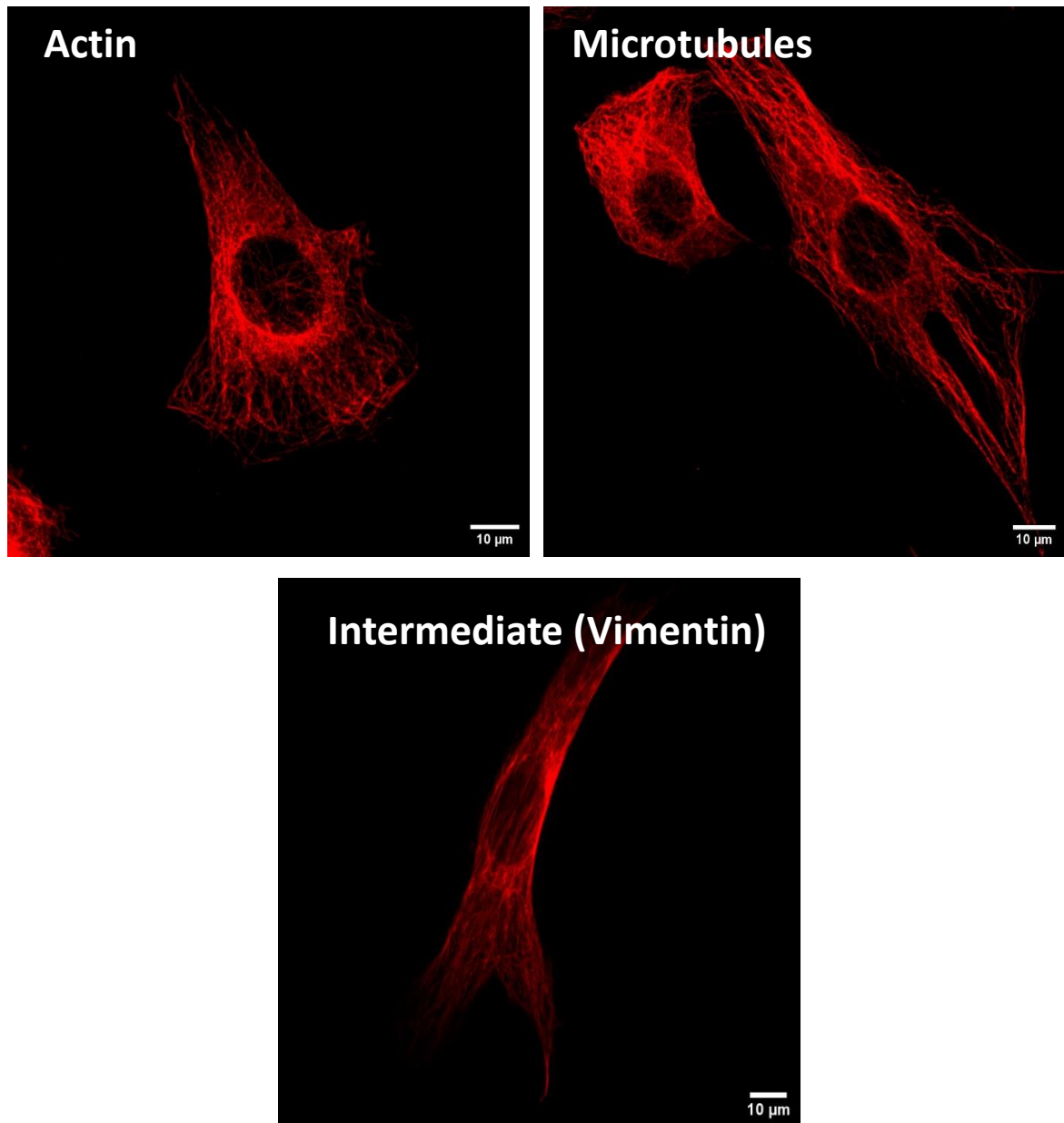


Figure 2. Confocal picture of the distribution of filaments in the cell body, respectively actin, microtubule and intermediate filaments in fibroblast cells.

In 1970 a group of small membrane GTP-binding proteins, septins, was discovered [17]. For a little over 10 years, these proteins have been recognized by some scientists as the 4th component of the cytoskeleton [18]. These proteins are still not fully understood, but in the future, they may be considered one of the components of cytoskeleton.

1.2.1 MICROFILAMENTS – ACTIN FILAMENTS

In 1942, one of the two major muscle proteins, besides myosin, was identified and this protein was named actin [19]. Exactly 10 years later, the hypothesis that a substance similar to actomyosin was found in *Physarium polycephalum* [20] appears. This was the beginning of the interest in non-muscle actin because 4 years later actin was isolated from the same organism [21], and research on it began. The fact that the primary structure is extremely conserved in eukaryotes is unusual. The amino acid structure of actin is typically at least 90% identical. It is also a very important part of the cell, which is indicated by the fact that it constitutes 15-20% of all proteins in an eukaryotic cell [22]. In response to these findings, questions about the consistency and morphology of the cell were clarified. Some gaps in knowledge had been filled in, allowing the definition of hypotheses and conclusions about the cell structure.

In the cell, actin is present in two basic forms. The first is called G-actin, which comes from the word "globular". It is a single molecule made of a chain of 375 amino acids with the associated molecule ATP. However, they are not perfectly spherical particles, as their size is 5.5 nm by 3.5 nm.

The second one, F-actin, from the word "filamentous", is polymerized fibrous form and looks like two strings of "beads" helically wrapped around each other. One twist is repeating every 37 nm. Ends of filaments are structurally different from each other. There is a more stable minus end and a dynamic plus end. The thickness of a single filament is about 7 nm and it is the thinnest of the cytoskeletal filaments [20] [23] [Figure 3]. There is also the third type of structure in living cells – a bundle of the actin filament, which is more stable and rigid than an individual filament [22].

The secondary structure is very similar in the G-actin monomer (26% alpha-helix and 26% beta-sheet) and in the F-actin filament (29% alpha-helix and 23% beta-sheet) [23] [Figure 4].

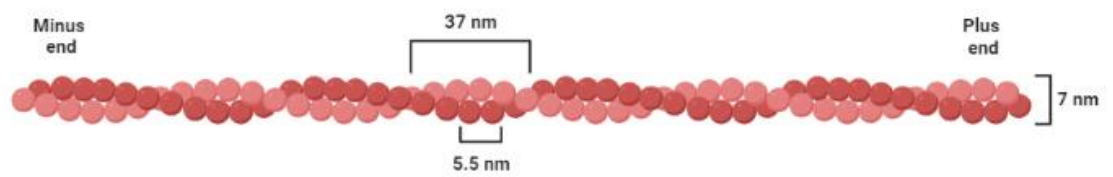


Figure 3. The structure of an F- actin filament in the form of a helix.

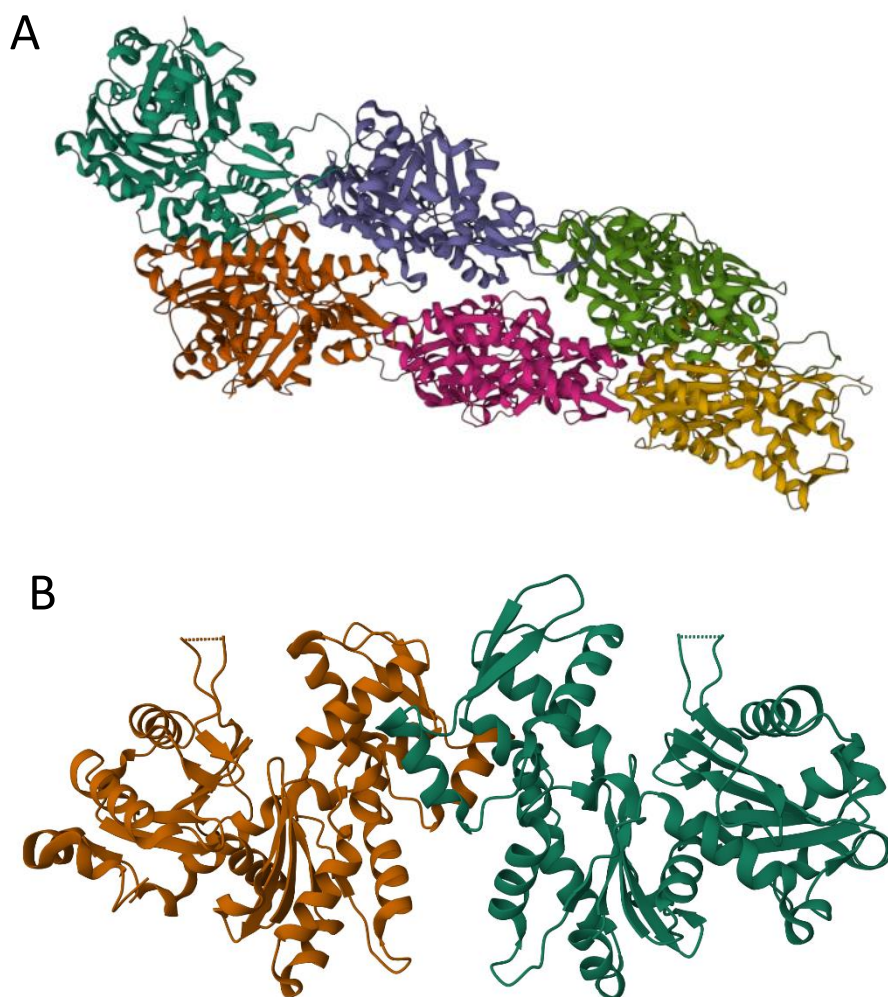


Figure 4. (A) Cryogenic electron microscopy 3D map of F-actin. Structure PDB code: 7UXF. (B) Crystal structure of a Cross-linked Actin Dimer. Structure PDB code: 2A5X. Visualizations made with the toolkit for visualization and analysis of a large-scale.

Before discussing the variety of actin functions, it is important to make a connection with this protein isoform diversity. In total, six actin isoforms can be found in cells. Four of them: α_{skeletal} -actin, α_{cardiac} -actin, α_{smooth} -actin, and γ_{smooth} -actin are expressed in muscles (skeletal, cardiac, and smooth muscles, as the names indicate) [24]. The other two isoforms, β_{cyto} -actin, and γ_{cyto} -actin, are common in non-muscle cells [25] [26]. The sequence of amino acids in all forms is remarkably similar [27] [28], but the diversity of actin filament isoforms may be related to the variable rates of polymerization and depolymerization of the filaments. Actin is thus able to adapt to the specific type of cell in which it is situated [29].

Microfilament architecture can be divided into several types. In cells cultured *in vitro* and in the intestinal epithelium, they form bundles of parallel filaments that also show structural polarity. Looser bundles can be seen in the cells lamellipodia, and they can also form stress fibers in adherent cells. Actin fibers are also localised near the periphery of the cell, and this is related to nuclear mechanotransduction. However, most often there is simply a mesh of microfilaments, where short fibers intersect at different angles. In addition to monomers and polymers, it is also possible that other small filament pieces, such as oligomers, are also present in the cytoplasm, but this is challenging to observe with the tools available today [30].

Actin is perhaps the best-known cytoskeletal protein. It plays an extremely important role in muscle cells, where it occurs in the form of aligned bundles. Together with myosin II, they form a structure called the sarcomere, which is responsible for muscle contractility [31]. This process is powered by the energy released during ATP hydrolysis, and it is supported by two proteins known as ABPs (Actin Binding Proteins)- tropomyosin and troponin. ABPs bind to the actin filament at rest and prevent myosin binding [32].

In non-muscle cells, actin filaments also play many key functions for cell life. For example, they could provide structural support for the cell [33] and connect its interior with the environment by receiving signals from the outside [34]. Actin filaments also enable cellular movement through the formation of lamellipodia and filopodia [35] and are involved in the process of mitosis [31]. Participation of microfilaments in endocytosis is also important [32], and this topic will be described in more detail later in this paper.

It is important to mention ABPs and cytoskeletal drugs that alter filament dynamics, their stability or decomposition. The functions mentioned above would not be possible without a set of proteins that also participate in them. Some examples of proteins belonging to the ABP family are: profilin, which promotes loading of the actin molecule with ATP and supports polymerization [33]. In opposition to this are cofilin and gelsolin, which support depolymerization of actin filaments [34]. Spectrins help actin to form a network beneath the cell membrane [35]. There are many more proteins in the ABP family than are listed here, and they constitute a powerful research branch. However, behavior of actin fibers can also be controlled by other factors. Actin concentration, pH [36], salt concentration, and ATP [37] all influence polymerization. Chemical substances are another option, and Table 1 lists them [38]–[41] They are commonly used in laboratory work for cytoskeletal research.

Table 1. Chemical inhibitors of Actin

DRUG NAME	EFFECT
Cytochalasin	Prevents polymerization
Latrunculin	Prevents polymerization, enhance depolymerisation
Jasplakinolide	Enhance polymerization
Phalloidin	Stabilises filaments
Swinholide	Sequesters actin dimers

1.2.2 INTERMEDIATE FILAMENTS

Intermediate filaments were discovered relatively early in the history of cell research. An article describing novel cytoplasmic filaments with a diameter of 8–13 nm was already published in the 1960s. The term for them was cytofilaments. In subsequent studies, they were named "intermediate" or "10 nm filaments" because of their average size. It was noteworthy that they were located around the nucleus in a characteristic way, the structure formed by the filaments is similar to a cage and encircles the nucleus [42], [43].

One of the features that distinguishes them is their very high mechanical resistance. They are very hard to break and can stretch up to three times their length [44]. Therefore, there was a hypothesis that intermediate filaments are present in cells exposed to mechanical stress and cannot be found in the cells of organisms with a rigid exoskeleton, such as arthropods. However, a novel cytoplasmic intermediate filament protein, isomin, was discovered there as well [45].

IF also offer structural support to numerous non-cellular tissues, including hair, hooves, and nails, which is another amazing aspect of IF [46]. This was probably the beginning of the interest and work that influenced the discovery of the IF structure. Since the 1930s, scientists have been fascinated by the structure of sheep's wool or hedgehog spines [47]. Less than 40 years later, the amino acid structure of hard keratin from sheep's wool was determined [48], [49].

The question is how one kind of protein can have such wide applications. So here it must be clarified that, unlike the other elements of the cytoskeleton, IFs do not consist of one basic protein. The proteins that make up IFs are biochemically and immunologically distinct, encoded by over 70 different genes with distinct, cell-type-specific functions [50], [51].

The IFs proteins were categorised into five or six groups (depending on whether type I and type II keratin are divided into two different categories) based on their structure and amino acid sequence, which are shown in Table 2 [52], [53], [54], [55], [56], [57], [58], [59], [60], [61], [62], [63].

Table 2. Major types of intermediate filaments proteins

Type of IF	Component polypeptides	Location
Epithelial	Type I keratins (acidic)	Epithelial cells and their derivatives (e.g., hair and nails)
	Type II keratins (neutral/basic)	
Vimentin- like	Vimentin	Many cells of mesenchymal origin
	Desmin	Muscles
	Glial fibrillary acidic protein	Glial cells (astrocytes and some Schwann cells)
	Syncoilin	Skeletal and cardiac muscle
	Peripherin	Some neurons
Axonal	Neurofilaments (NF-L, NF-M and NF-H)	Neurons
	Alpha-internexin	Brain and central nervous system
	Synemin	Non-junctional membrane locations in astrocytoma cells and cardiac myocytes
Nuclear	Lamins A, B and C	Nuclear lamina (inner lining of nuclear envelope)
Newly discovered and unclassified	Beaded filaments: Filensin, Phakinin	Lenses
	Nestin	Central nervous system
	Isomin	Component of the Isotomurus midgut terminal web

Amino- and carboxy-terminal domains are present in individual intermediate filaments. All filaments have an elongated alpha-helical structure that contains about 40 repeat motifs. Single monomers combine to generate an extended coiled-coil structure with another monomer. With an antiparallel dimer, having a parallel structure becomes a tetramer. These tetramers group together to form an eight-parallel protofilament filament, or 32 distinct alpha-helical coils, that is made up of these tetramers. This filament is incredibly robust thanks to its amazing design [64]. This process is visualised on Figure 5.

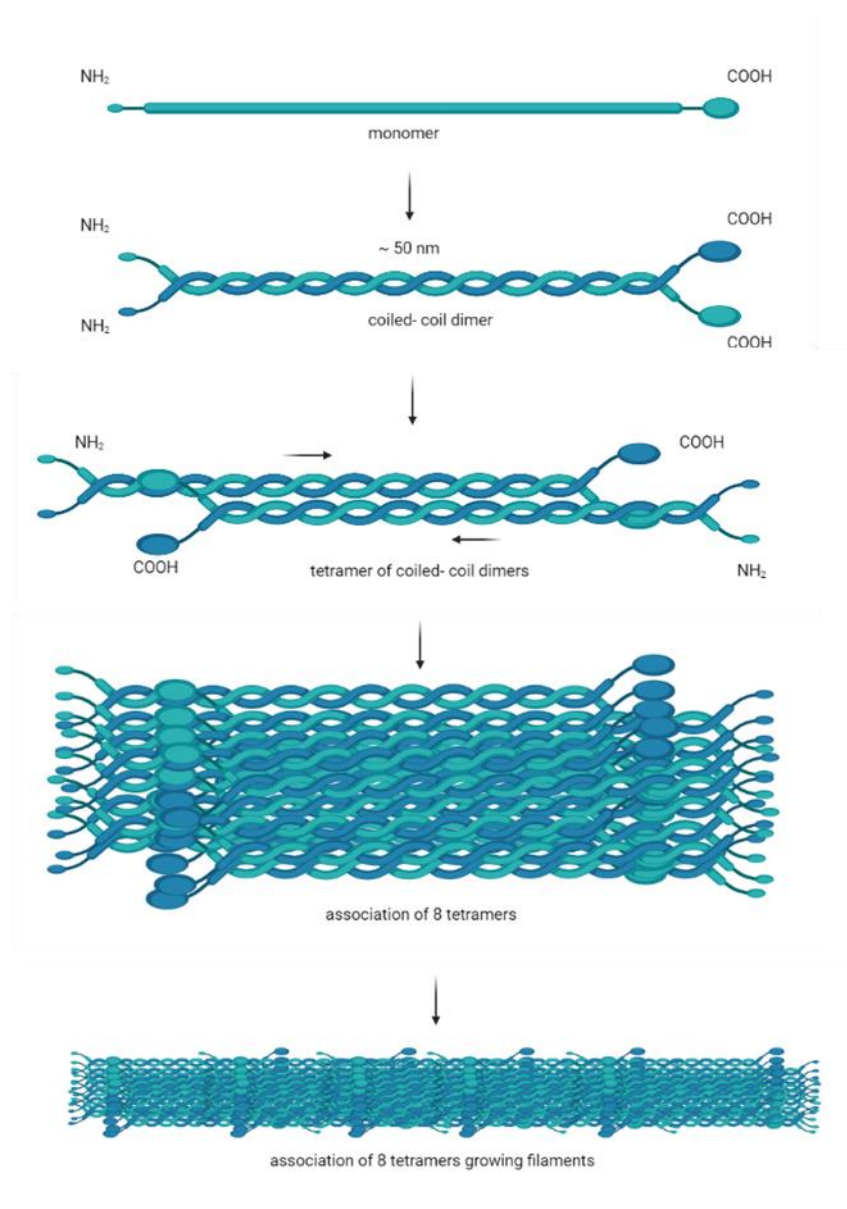


Figure 5. A model of intermediate filament construction (inspired by: Molecular biology of the cell/ Bruce Alberts [et al.]. 6th edition).

Intermediate filaments are still the least understood filaments of the cytoskeleton. Therefore, it is difficult at this point to have a broader view of IF associated proteins. However, more and more data indicate that they exist and, similarly to other structures of the cytoskeleton, they support the functions of intermediate filaments. The family of these proteins is called IFAP (Intermediate Filaments Associated Proteins).

This family includes proteins that show direct or indirect interactions with IFs [65]. Four criteria have been established that can qualify a protein as IFAP in terms of morphology or biochemistry, such as direct co-localization with IF or participation in their assembly and disassembly [66].

In the context of this work, the most important IF building protein is vimentin. Its name comes from the Latin word *vimen*, which means an array of flexible twigs. It can be found not only in non-muscle cells derived from mesoderm but also in bacterial cells.

It is a monomeric protein with an alpha-helical structure and amino and carboxyl domains [Figure 6] [67], [68]. The formation of a filament from a single monomer is shown previously in Figure 5.

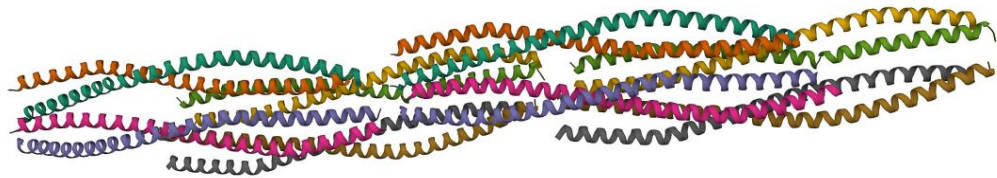


Figure 6. Crystal structure of vimentin coil packed as a filamentous form. Structure PDB code: 5WHF. Visualizations made with the toolkit for visualization and analysis of large-scale molecular data – Molstar.

Vimentin filaments help anchor the organelles in the cytoplasm. Thanks to their strong mechanical properties, they support the cell's resistance to external factors [69]. It has been proven that a cell with damaged vimentin cytoskeleton is extremely delicate and vulnerable to micropunctures [70]. A study was carried out on mice whose vimentin-expressing gene was silenced. Superficially, the mice appeared healthy, but when injured, their wounds healed more slowly than in the control mice [71].

Intermediate filaments extend from the nuclear envelope to the cell membrane. At the same time, they join together in bundles, which may later separate. Microscopy images show a significant density of filaments around the nucleus, probably holding it in a specific position in the cell [72].

It is interesting to note that the first identified biochemical function for IF was to regulate LDL lipoproteins transport to the site of esterification. In other words, they have an impact on how much lipoprotein is kept in reserve by the cell [73].

It is still a protein that has not been fully studied and is therefore the subject of research for many scientists.

1.2.3 MICROTUBULES

The 1950s can be considered the beginning of research on microtubules. At that time, the first electron microscopy images suggesting the existence of such a structure appeared [74]. However, 1963 was a breakthrough year because glutaraldehyde was introduced as a fixing agent in cell sample preparations, which prevented the disintegration of protein polymers [75]. This made it possible to observe and describe the filaments with hollow cylindrical structure in hydra cells [76] and in the cytoplasm of plant cells. It was then that the name "microtubule" was first used [77]. Exactly 34 years later, William Brinkley mentions in his work about three decades of work on the morphology and molecular structure of microtubules, which allowed us to enter "an exciting new biophysical era" [78]

Microtubules are very thin, unbranched tubes. They are the largest component of the cytoskeleton. Their outer diameter is about 25 nm, the inner diameter is 15 nm, and the wall thickness is 5 nm [79] [Figure 7]. However, these parameters can change, and depending on the location of the microtubules, their outer diameter can range from 18 to even 34 nm [76]. Along with this, the inner diameter changes accordingly. The length of the filament is usually from 1 μm to several microns, but in some neurons it can be from 25 μm to several millimetres [80]. In the lumen of microtubules is a transparent substance with small particles [81].

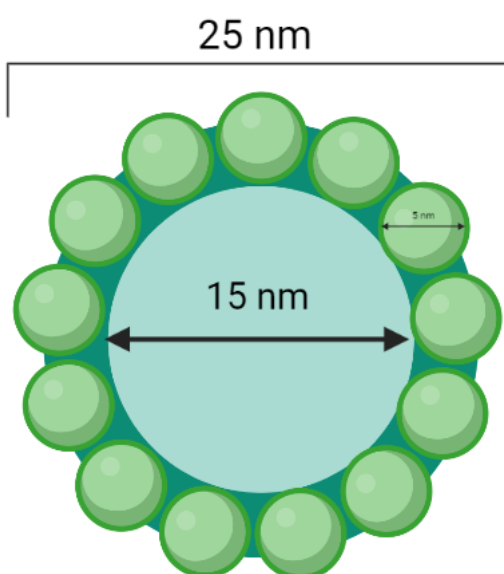


Figure 7. Cross-section through the microtubule structure.

A single filament is made of 13 adjacent parallel protofilaments arranged along the MT parallel axis [82]. They are made of a protein called tubulin. The heterodimer of the linear assembly of two single monomers of alpha and beta tubulin is the fundamental building element of the chain [83]. Monomers differ slightly in molecular weight; alpha tubulin has a slightly larger one [84].

Each filament consists of 13 protofilaments, but there are exceptions. Microtubules found in cilia, flagella, basal bodies, and centrioles have 10 or 11 protofilaments. Some nematode filaments have 9–11 protofilaments [85]. Additionally, there are filaments with higher amounts of protofilaments. In the cockroach's epidermal cells there are 15 protofilaments [86].

A schematic drawing showing the structure of a single microtubule filament is shown below - Figure 8.

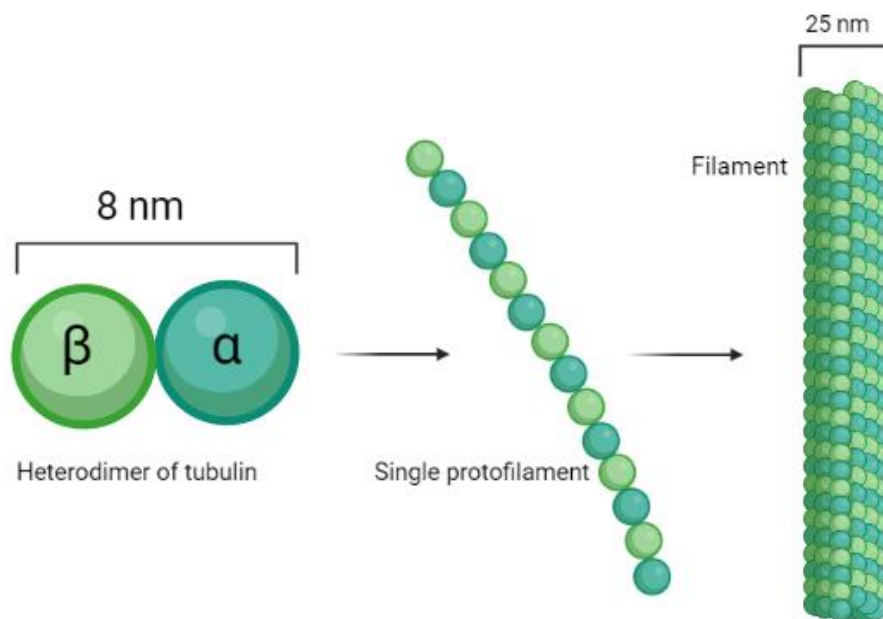


Figure 8. The structure of microtubules and its subunits.

Alpha- and beta-tubulin are 40% amino acid identical, and both have several isoforms. However, their secondary structure is the same. The monomer consists of two beta-sheets, which are surrounded by twelve alpha-helices [Figure 9] [87].

The highly compact monomer structure can be categorised into three domains: the carboxy-terminal domain, which most likely acts as the binding surface for motor proteins, the middle domain, which contains the Taxol-binding site, and the amino-terminal domain, which includes the nucleotide-binding region [87].

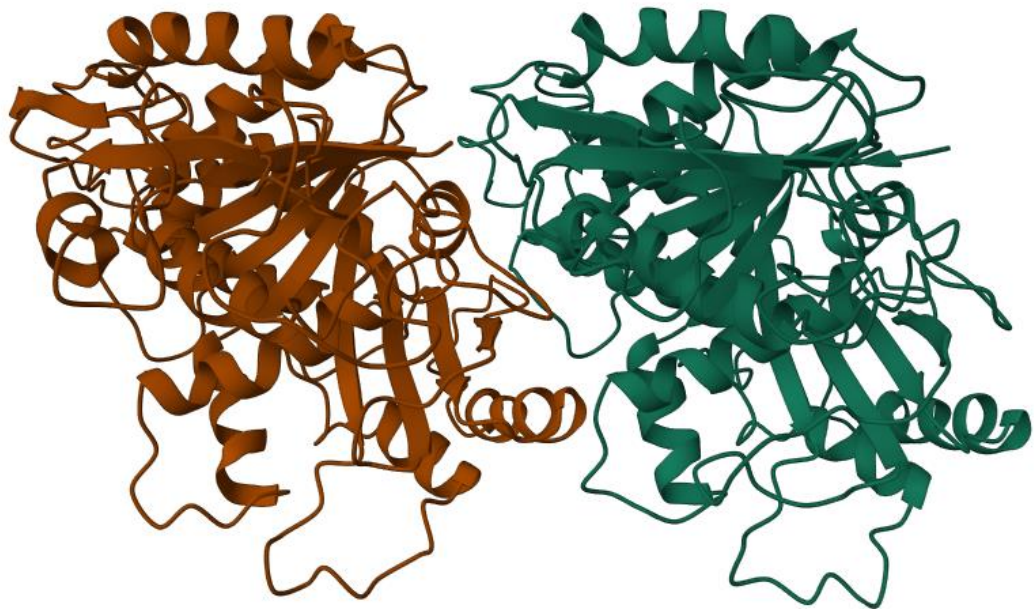


Figure 9. Crystal structure of alpha-, beta-tubulin heterodimer. Structure PDB code: 1TUB. Visualizations made with the toolkit for visualization and analysis of large-scale molecular data – Molstar.

The tubulin dimer has two sites called N-site and E-site. The N-site is situated on the side of beta-tubulin that interacts with alpha-tubulin. The E-site is situated on the beta-tubulin's outer surface. E-site exhibits GTP (guanosine-5'-triphosphate) activity, whereas N-site does not [88].

Microtubules are closely influenced by the nucleotide hydrolysis, and GTP is necessary for this. Hydrolysis occurs only in the beta subunit and the process of hydrolysis is very slow in loose subunits but accelerates significantly when the dimer is attached to the filament. After hydrolysis, the phosphate group is released, and the GDP remains in the beta subunit [89] [90]. This is extremely important because the highly dynamic nature of microtubules is crucial to their function. Heterodimers mainly polymerize and depolymerize at one end of the microtubule, called the "plus" end, which is more unstable than the minus end. [Figure 10]. This feature makes microtubules polarised. Typically the plus end is distal, away from the centre of the cell. In contrast, the „minus“ end is proximal and located closer to the centre of the cell [91].

An interesting and still not understood issue is the formation of "GTP caps", which have a protective function against MT depolymerization. In other words, when the heterodimer is added to the filament, the hydrolysis of GTP to GDP automatically occurs. The "GTP cap" is a structure at the „plus“ end of the filament consisting exclusively of GTP tubulin because hydrolysis has been blocked. Such a filament is more durable and does not depolymerize so quickly, while deprivation of the "GTP cap" leads to automatic destabilisation of the filament. This phenomenon is still waiting to be explained in terms of biochemistry [90], [92], [93], [94], [95], [96].

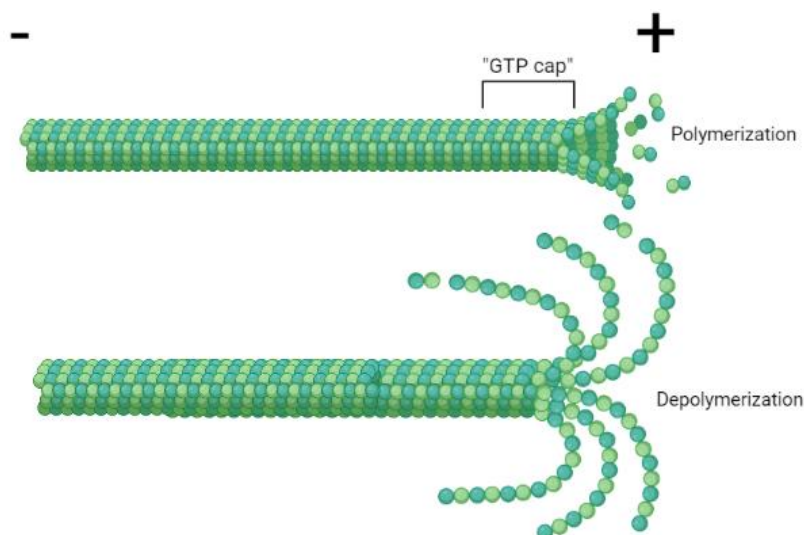


Figure 10. Dynamic difference due to structural variation between plus and minus end of microtubule.

Contrary to the previously described IFs, microtubules are structures that are highly sensitive to pharmacological drugs and physical environmental factors. If there is even a small change in hydrostatic pressure or temperature, the filament can alter its rate of depolymerization [97] [98].

Due to its important role in the process of cell division (described in detail below), tubulin is the target protein for many drugs and spindle fibers poisons (listed in Table 3) [99], [100], [101], [102].

Table 3. Chemical inhibitors of Tubulin

DRUG NAME	EFFECT
Colchicine	Prevents polymerization
Demecolcine	Depolymerised MTs
Nocodazole	Prevents polymerization
Paclitaxel (taxol)	Stabilises filaments and therefore prevents mitosis
Vinblastine	Prevents polymerization
Rotenone	Prevents polymerization

Some of them, such as demecolcine, paclitaxel, and vinblastine, are commonly used in chemotherapy.

However, microtubules do not respond similarly to the various toxins. There are also stable microtubules that are less sensitive to toxins and temperature changes. The microtubules found in cilia and flagella are much more tolerant than the fragile cytoplasmic and spindle microtubules [103], [104], [105]. Eukaryotic animals and some plant cells have a structure called the MTOC (Microtubule Organizing Center), also commonly known as the centrosome. Usually they can be observed in the vicinity of the cell nucleus. The centrosome is made up of structures called centrioles [106]. A single centriole usually consists of nine microtubule triplets. Two perpendicular centrioles form a centrosome [Figure 11] [107], [108].

MTOC plays an important role in regulating the size, shape, and dynamics of microtubules. The minus end comes from the centromere, and the section closest to it is more resistant to factors destabilising the structure. It is not fully known how MTOC is formed, how it combines with filaments, or how it is produced [109], [110].

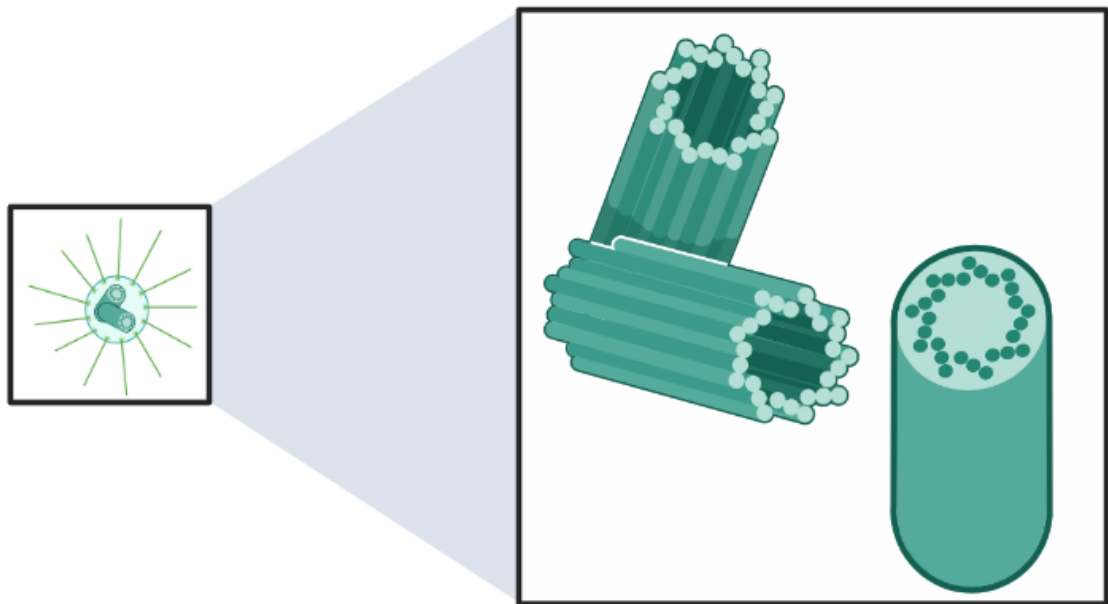


Figure 11. The centrosome and zoom on centrioles structures.

Microtubules are not as well understood as actin filaments. Research continues not only on the issues mentioned above but also on the functions of microtubules. Below, the most important functions of microtubule filaments will be briefly described.

Microtubules are a key organelle for eukaryotic cilia and flagella. These structures can be regarded as enveloped cytoplasmic projections with a diameter of about 0.2 μm . Cilia are numerous and short (5–10 μm) while flagella are much longer (15–70 μm), both of them are present in epithelial cells and free-living single-celled eukaryotic organisms. Epithelial cells in the respiratory tract have a large number of protrusions, and their role is to support surface transport. In single-celled organisms, protrusions have a slightly different role because they use cilia or flagella as a means of locomotion.

The electron micrographs showed that there were nine doublets of microtubules around the periphery of the cilium, or flagella [111]. Each pair consists of a complete microtubule consisting of 13 protofilaments and one incomplete microtubule consisting of 10–11 protofilaments. There are also two central microtubules, consisting of 13 protofilaments each [112], [113], [114].

Microtubules are also widespread in the cytoplasm [115]. It is worth noting that together with actin and the IF-forming protein, they constitute as much as 85% of all cytoplasmic proteins [116]. However, cell shaping and stabilisation are not the only functions performed by microtubules; they are also responsible for intracellular transport. Microtubules are the pathway along which exo- and endocytic vesicles and organelles, such as the mitochondrion, are transported [117].

At this point, it is necessary to describe how internal transport takes place on microtubules. As previously mentioned, the typical arrangement of microtubules in a cell is that the negative ends are closer to the centre of the cell (in MTOC) and the unstable positive ends are closer to the edge of the cell. The whole process requires the presence of two motor proteins - dynein and kinesin.

Dynein is the largest and fastest known motor protein, with a speed of approximately 14 $\mu\text{m}/\text{sec}$. It does this by converting the chemical energy contained in ATP into mechanical energy. It moves towards the negative end of the microtubules (i.e., towards the MTOC). Dyneins can be divided into two groups: axonemal (also called ciliary) and cytoplasmic. Axonemal ones are multi-subunit ATPases that are specialised for the rapid sliding motion of microtubules as in cilia and flagella. Cytoplasmic dynein is found in almost all eukaryotic cells. Its main functions are the positioning of the centrosome and the nucleus during cell migration and participation in the formation of the division spindle [118], [119] Dynein can be associated with microtubules of various origins, and this also distinguishes it from other ATPases, such as myosin [120].

Dynein is not the only ATPase responsible for intracellular movement. The discovery of kinesin was not surprising, because since dynein moves towards the minus end, there were suspicions that another motor protein would be responsible for the movement in the other direction [Figure 12]. Kinesin is a protein smaller than dynein and responsible

for the opposite movement: towards the plus end (periphery of the cell) [121]. Kinesin differs from other ATPases in that it has a high affinity for the ATP analogue, AMP-PNP (adenyl-5'-imidodiphosphate) [122]. This causes the affinity of kinesin to microtubules to increase rapidly and dynein to microtubules to decrease [122].

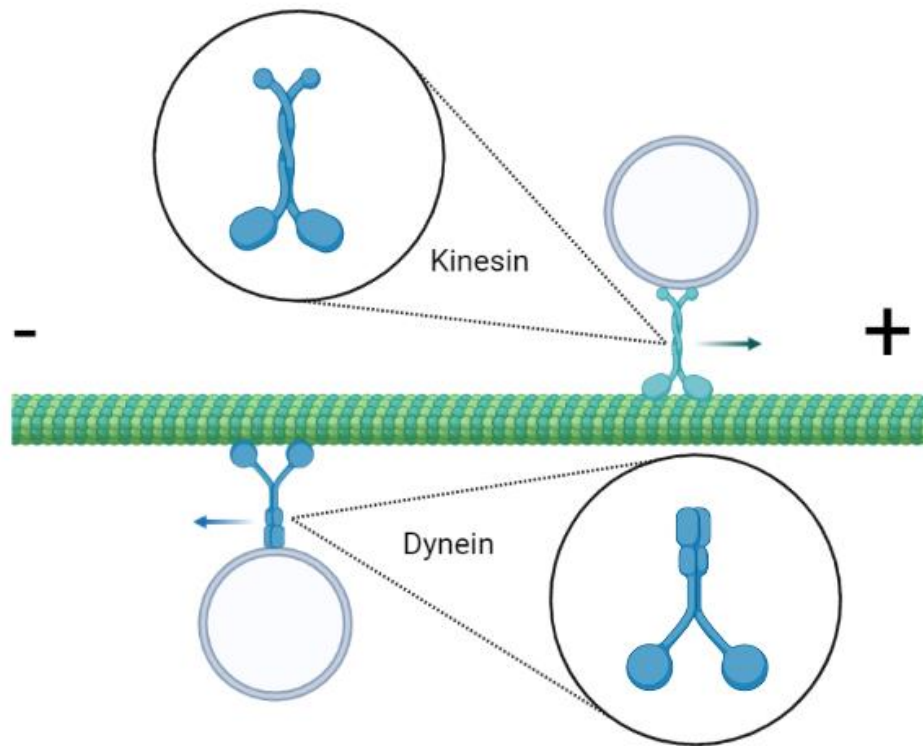


Figure 12. Schematic drawing illustrating the movement and structure of motor proteins - dynein and kinesin.

Another very important role of microtubules is their participation in cell division. In nature, there are two types of cell division: meiosis and mitosis. Meiosis occurs in germ cells and divides the parent cell into four daughter cells, each having only half of the chromosomes. In contrast, in the process of mitosis, the daughter cell is the same as the parent cell [124].

The cell cycle consists of four phases – M, G₁, S, and G₂ - which together are called interphase [Figure 13]. Each phase is briefly described below.

- **G₁ phase:** In this phase, compounds needed for DNA replication are synthesised. The duration of the G₁ phase is undefined and cell type dependent.
- **S phase:** The amount of genetic material is doubled. The duration is very similar for cells of the same species.
- **G₂ phase:** Protein synthesis is significantly increased, in particular tubulin, which will be responsible for the creation of the karyokinetic spindle. This phase lasts until mitosis begins.
- **M phase:** karyokinesis (nucleus division) and cytokinesis (cytoplasm division) occur. This is where cell division occurs.

There is another phase, **G₀**, which is the resting phase. Non-dividing cells go from G₁ to G₀. Often, cells that are damaged in a way that could make daughter cells defective enter the G₀ phase. However, it is also possible for the cell to exit the G₀ phase and take part in the division again [125].

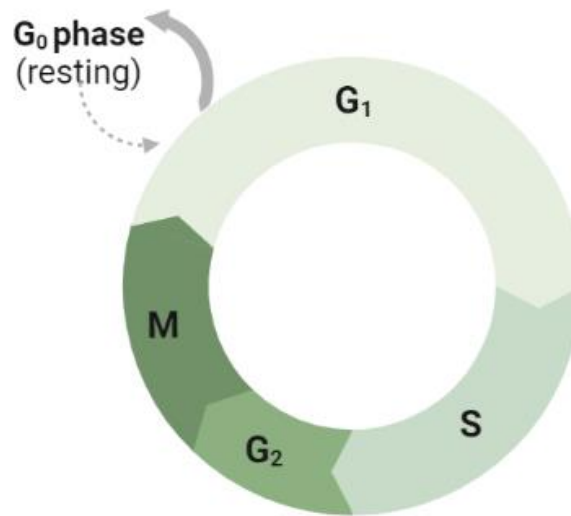


Figure 13. The cell cycle scheme.

In order to separate genetic material (sister chromatids) between two cells, a karyokinetic spindle must be formed. This spindle is made of microtubule fibers. Two centromeres are necessary for the formation of a spindle; therefore, immediately before the division, the centrosome is duplicated and, along with the course of karyokinesis, it gradually moves to the opposite poles of the cell. They control the rearrangement of cytoplasmic microtubules into spindle microtubules. In addition to tubulin, the spindle also includes other proteins, such as motor proteins - dynein and kinesin, and a complex of proteins, called kinetochore. Kinetochore is extremely important in enabling the connection of microtubules with chromosomes.

The M phase can be divided into stages, which will be discussed and visualised below in the context of what happens to microtubules and the spindle [126], [127].

The four stages of mitosis are described by such a simple separation into stages [Figure 14] [128].

Prophase: At this stage, chromatin condenses, and chromosomes are formed. This is also where the karyokinetic spindle begins to form. Centrioles diverge to opposite ends of the cell and add a new centriole there, creating two separate centrosomes. These centrosomes are responsible for disassembling the microtubules in the cytoplasm and forming a spindle for division.

Metaphase: It begins with the breakdown of the nuclear membrane, allowing microtubules to attach to the chromosomes. The place where the microtubule attaches is called the centromere and is located approximately in the centre of the chromosome. The protein structure, kinetochore, is responsible for the attachment. A metaphase plate is formed, i.e., the chromosomes line up across the cell.

Anaphase: By separating sister chromatids, daughter chromosomes are formed that migrate to the opposite ends of the cell. This is possible due to the very strong binding of the kinetochore with microtubules and the ability of microtubule filaments to efficiently depolymerize. The filament depolymerizes and thus shortens, so the daughter chromosomes can be "pulled" towards the periphery of the cell.

Telophase: The nuclear membrane begins to form, which separates the two chromosome slips. After the formation of two nuclei, the chromosomes decondense into chromatin and then the cell enters the cytokinesis, i.e., the division of the cytoplasm. This produces two daughter cells, each with a separate, identical nucleus. The indexing spindle is disassembled.

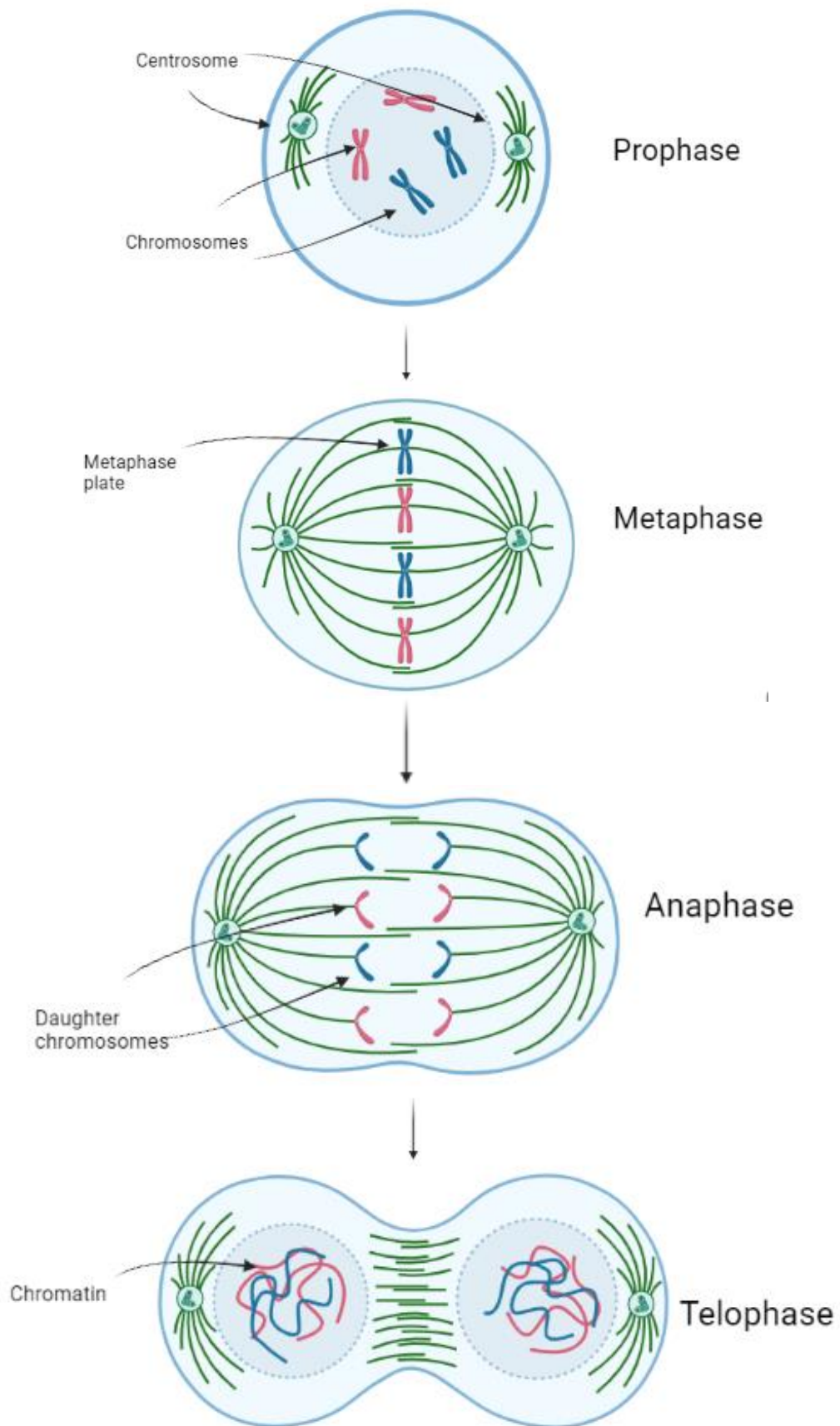


Figure 14. The cell cycle stages.

1.2.4 MAPs – MICROTUBULE ASSOCIATED PROTEINS

MAPs are the proteins that interact with the microtubule network. They were first described in the mid-1970s as non-tubulin proteins of relatively high molecular weight [129].

From that moment on, articles describing newer and newer microtubule-related proteins began to appear. The problem, however, is that it is not so easy to clearly describe the function of each protein. Therefore, the classification of proteins based on their molecular weight is often used [130]. These proteins can be divided into:

High-molecular-weight MAPs: molecular weight > 250,000 Da; these are proteins that help to assemble microtubules and stabilise them. Electrophoretic separation showed that they can be divided into two basic groups: MAP1 (MAP-1A, MAP-1B, and MAP-1S) and MAP2 (MAP-2A and MAP-2B), which slightly differ in molecular weight [131].

MAP1 is a group of proteins, mainly expressed in neurons and plays a significant role in the microtubules structures in axons and dendrites. They stabilise the microtubules and ensure the proper distance between the filaments. They can also interact with actin filaments and signalling proteins [132].

MAP2 is involved in the development of neuronal microtubules and their stabilisation. Tau protein (MAPT) is important in the context of stabilising neurons. This protein is also responsible for maintaining the appropriate distance between microtubules and, thus, the appropriate diameter of the axon. It is also involved in processes such as apoptosis, cell signalling, and mRNA translation. The degree of its activity strongly depends on the degree of phosphorylation [133], [134].

Medium-molecular-weight MAPs: molecular weight $\sim 200,000$ Da; these proteins are also found in neurons, but most often in non-neuronal cells. They are thermostable proteins that can be found in normal and cancer cells. Medium-molecular-weight MAPs, unlike other groups, have no affinity for actin and are distinguished by their immunological reactivity and amino acid composition [135]. The best-studied subgroup is MAP4, which is responsible for the organisation of filaments into antiparallel bundles and prevents the destabilisation of microtubules [136].

Low-molecular-weight MAPs: molecular weight $< 150,000$ Da; family of STOP proteins (stable tubule only peptides). Initially, these proteins were found in the rat brain, but later they were also found in fibroblasts and were named F-STOP. They play a very important role, namely, they are responsible for the stability of microtubules and make them resistant to factors that can induce depolymerization, e.g., temperature [137].

It is almost impossible to list and characterise all known MAP proteins here because, according to the latest source, there are 889 of them [138]. Usually, these proteins are poorly described at the moment and not fully understood. The best-known MAP protein is the aforementioned Tau. The great interest in this protein is due to the fact that it has a significant effect on Alzheimer's disease by forming insoluble fibers [139]. Other proteins that also belong to MAP are, for example, dynein and kinesin, i.e., motor proteins, which have been described above.

When it comes to the classification of MAP proteins, it is also difficult to find a clear classification, and the assignment of proteins to existing divisions is not obvious. This proves how wide this field of study is and how much remains to be explored.

Three MAP proteins - CLIP1, IQGAP1 and MARK2 - will be relevant in the remainder of this work. The function of each of these proteins is still not fully understood, so they will be described as far as the current state of knowledge allows.

1.2.4.1 CAP- Gly DOMAIN CONTAINING LINKER PROTEIN 1 – CLIP1

Proteins from the CLIP family (cytoplasmic linker proteins) were first observed in HeLa cells, they mediate the interactions of organelles with microtubules. Their accumulation is observed at the plus end of microtubules, suggesting that they may be responsible for endosome binding and intracellular transport.

The **CLIP1** protein (CAP-Gly domain containing linker protein 1), also known in the literature as CLIP170 (molecular weight: 170,000 Da), is a protein located at the “plus” ends of microtubules and additionally in the cytosol [Figure 15] [140]. In addition to attaching endosomes to microtubules [141], it also regulates their dynamics. It is probably also responsible for the formation of the sperm head and sperm differentiation [142].

In an experiment to link CLIP1 to endocytic vesicles, the transferrin receptor (TRF) was used as an immunofluorescent marker for endosomes. This receptor is present in early stages of endocytosis and early endosomes [143]. However, it turned out that the correlation of CLIP1 with TRF was weak [144].

CLIP1 has the ability to recognize the plus end, but the mechanism by which this happens is not fully understood. Here, a protein from the EB1 (Microtubules End-binding proteins 1) family is also necessary, which binds the plus end of microtubules. CLIP1 binds strongly to EB1 and thus to alpha-tubulin. This whole process leads to the formation of a structural basis that allows the attachment of dynein, a motor protein [140].



Figure 15. Crystal structure of the CLIP-170 CAP-Gly domain 1 Structure PDB code: 2E3I. Visualizations made with the toolkit for visualization and analysis of large-scale molecular data – Molstar.

CLIP1 interacts with the **IQGAP1** protein (Ras GTPase-activating-like Protein). This protein is very important in many signalling pathways because it interacts with many other proteins and has a control function in the cell cycle or actin dynamics. It has been recognized that it is the interaction (direct and indirect) with the cytoskeleton that is the most important function of IQGAP1 (Ras GTPase-activating-like protein IQGAP1) [145].

1.2.4.2 Ras GTPase ACTIVATING- LIKE PROTEIN IQGAP1

It has been proven that the activation of GTPases from the Rho family of proteins indirectly affects the rearrangement of the actin cytoskeleton [146]. IQGAP1 acts as an effector of Rac1 and Cdc42 proteins, which are the regulators of actin dynamics, e.g., by stabilising Cdc42, which then induces the formation of filopodia and cell migration [147]. IQGAP1 can also cross-link actin [148] and participate in cytokinesis [149].

Much can be said about the role of IQGAP1 in actin processes or its other functions. However, in the context of this work, it is important to link, directly or indirectly, this protein to microtubules.

IQGAP1 can be included in the CLASP (cytoplasmic linker-associated protein) family as a protein that interacts with the CLIP1 protein. CLASP's mission is to support polarised MT growth. The CLIP1, IQGAP1, and Rho family GTPase proteins form a complex and act as linkers between the plus end of MT and the cortical actin meshwork [150].

IQGAP1 can also associate with microtubules through the SKAP (Src Kinase-associated Phosphoproteins) protein. It is one of the proteins associated with the kinetochore (occurring at the microtubule and centromere binding sites) during cell division [151]. It is also postulated that SKAP may play a role in cell migration, and the formation of a complex with IQGAP1 is very important in this process [152].

1.2.4.3 MICROTUBULE AFFINITY- REGULATING KINASE – MARK2

Proteins that cannot be fully included in the MAP family but are extremely important in the context of the interaction of MAP and MT are proteins from the MARK (microtubule affinity-regulating kinase) family.

These are serine-threonine kinases that have the ability to phosphorylate MAP and thus regulate the affinity of MAP to MT.

The **MARK2** protein (also known as Par1b) is a protein found in many types of cells [Figure 16] [153]. It is involved in the regulation of cell polarity and the control of microtubule stability. By phosphorylating MAP proteins (e.g., MAP2, MAP4, and tau), it dissociates them from microtubules and thus increases the activity of the filament [154].

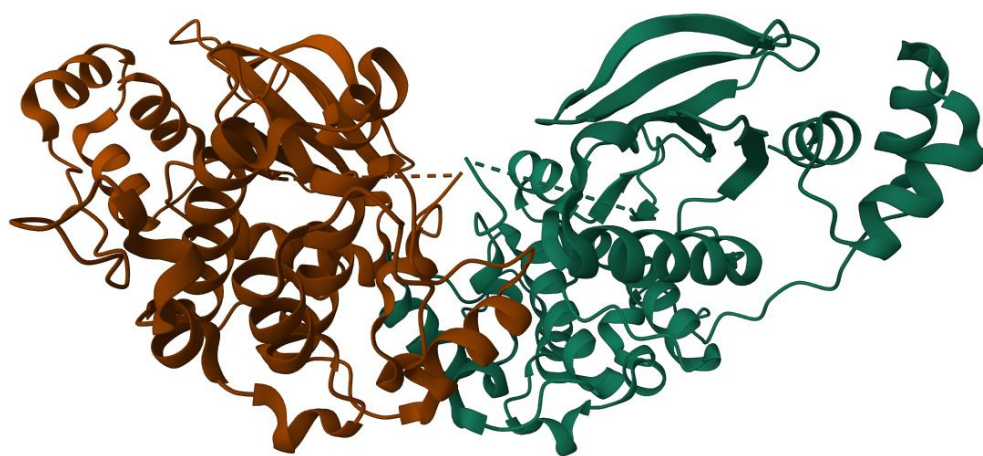


Figure 16. Structure of the catalytic and ubiquitin-associated domains of the protein kinase MARK/Par-1. Structure PDB code: 1Y8G. Visualizations made with the toolkit for visualization and analysis of large-scale molecular data – Molstar.

The tau protein plays a role in the pathogenesis of Alzheimer's disease. Its function is to stabilise microtubules. When the protein is unphosphorylated, it forms a complex with the microtubule and prevents it from disintegrating. However, when it is phosphorylated by a protein from the MARK family, it detaches from the microtubule, allowing it to depolymerize [Figure 17] [155]. Tau is found almost exclusively in nerve cells, which is why any anomalies are so dangerous because they lead to neurodegenerative diseases. It has been observed that excessive Tau phosphorylation leads to changes called neurofibrillary tangles (NFTs). These are bundles of aggregated Tau protein located in the brain, the amount of which correlates with the development of the disease [156].

Hyperphosphorylation of MAP proteins is associated with MARK overexpression. In addition to the formation of aggregated protein deposits, this often results in disruption of the microtubule system and leads to cell death [154].

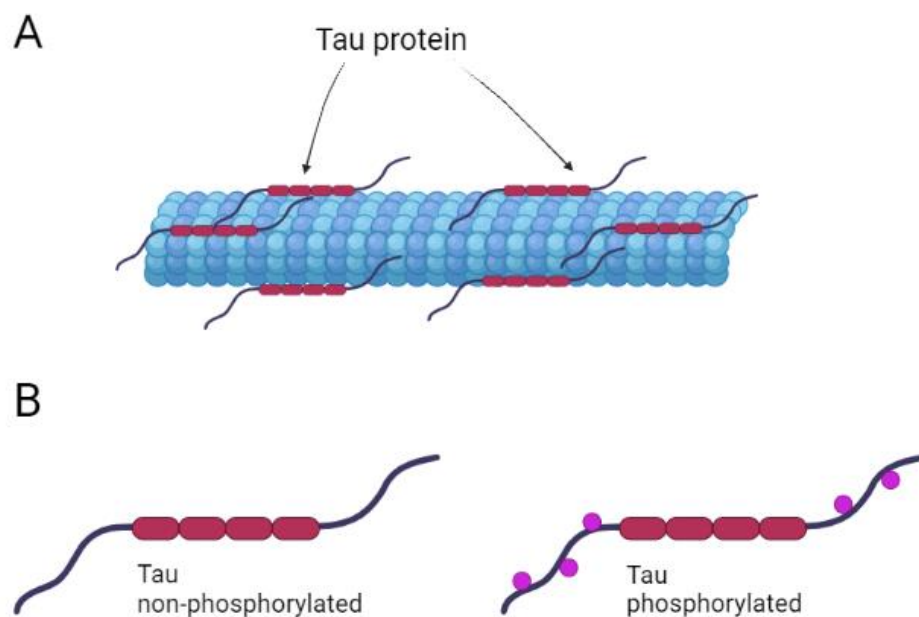


Figure 17. (A) Microtubule filament stabilised by non-phosphorylated tau proteins. (B) A schematic difference between tau phosphorylated and non-phosphorylated.

According to the knowledge at the time, the MARK2 protein has no direct effect on microtubule dynamics but only affects other proteins directly related to MT. However, research on it is still ongoing, so it is possible that there may be more functions for this protein.

1.2.5 CYTOSKELETAL SYSTEMS IN PROKARYOTES

Cytoskeletal system is very interesting in prokaryotic cells. They also have some kind of cytoskeleton. They are much smaller and less morphologically developed than eukaryotic cells, but contain homologues of all three filaments described above. Paradoxically, bacterial actin and tubulin are more diverse than in eukaryotic cells [157].

The tubulin homolog is a protein with the symbol FtsZ (filamenting temperature sensitive mutant Z). It is a universal cytoskeletal protein in prokaryotes that can polymerize and form protofilaments, which then organise into a ring (Z-ring). This ring is formed at the site of the future division and supports cell division by contracting [158]. It serves as a scaffold for other proteins involved in division and generates a force that constricts the cell [159].

Many bacteria also contain an actin equivalent. Two of these proteins - MreB and Mbl - are (like FtsZ) very dynamic and a disorder in their expression can cause problems with chromosome segregation or an undesirable change in the shape of the cell [160].

Another homolog of actin is the ParM protein, which is involved in chromosome segregation in many bacteria [161]. Chromosome segregation in eukaryotes is accomplished via tubulin rather than actin. From an evolutionary point of view, this is very logical, because actin and tubulin filaments are similar in basic features [162].

The equivalent of intermediate filaments is the crescentin, a protein discovered in 2009, which is found in *Caulobacter* bacteria. It is involved in cell shape determination (when the crescentin gene is silenced, the cell has a different shape [163]). Similar to IF, this protein is very flexible, but it does not stiffen the cell structure [164].

1.3 ENDOCYTOSIS PROCESSES

Endocytosis is a very broad term that describes ways of transporting molecules into the cell. Many proteins are involved in the whole process, which consists of the absorption of molecules by surrounding them with a cell membrane, which then unbundles inside the cell. The basic classification distinguishes two basic types of endocytosis: pinocytosis and phagocytosis [165].

These types of endocytosis are in some way intertwined with each other and are not unequivocal, which is why in more recent studies there has been talk of fluid-phase endocytosis and adsorptive endocytosis [166].

Fluid-phase (non-specific) endocytosis occurs when random particles, present in the fluid surrounding the cell, enter the endocytic vesicle. It is performed by folding random areas of the cell membrane and enclosing molecules and fluid in a vesicle surrounded by a single membrane. In the next step, these vesicles fuse with lysosomes (forming a complex called an endolysosome), which contain as many as 40 types of hydrolytic enzymes. Digested content is a source of elementary components necessary for the cell to carry out syntheses [167].

Adsorbent (specific) endocytosis proceeds with the participation of receptors, mainly affects macromolecules, and proceeds in two stages. First, molecules are captured from the environment in a selective manner, i.e., adsorption takes place on specific, immobile receptors located on the surface of the cell. Next, such a complex is encapsulated in a vesicle called an endosome and then either combines with the lysosome or delivers its contents to the Golgi apparatus, which transports the material directly to the cellular organelles [168].

1.3.1 PHAGOCYTOSIS

First type of endocytosis is **phagocytosis**, which means "eating by the cell" and involves the absorption of large particles, such as microorganisms or cellular debris. Unlike different kinds of endocytosis- pinocytosis (Chapter 1.3.2), phagocytosis is carried out only by large, specialised cells called phagocytic cells. The taken organic material is encapsulated in a vesicle with a diameter greater than 250 nm called a phagosome, which then combines with lysosomes and is finally digested. Complex of phagosome and lysosome is named phagolysosome (Figure 18, B).

Phagocytosis is common in single-celled protozoa because it is through it that they take in food. However, in multicellular organisms, only a few cell types are capable of phagocytosis, e.g., macrophages or some leukocytes, which absorb pathogenic microorganisms through phagocytosis. Particles are first opsonized with proteins (such as immunoglobulins) so that phagocytes are able to identify them, which is crucial for the mechanism of particle capture [169]. For example, the pathogen is absorbed into the macrophage after activation of one of the surface receptors, which will be recognized by antibodies [170]. All of this induces membrane extensions in the phagocytic cells that form the phagosome. In addition, phagocytic cells are responsible for getting rid of dead cells or parts of them [171].

1.3.2 PINOCYTOSIS

Pinocytosis, which means "cell drinking", was first observed in 1929 (Pinocytosis. Bull. Johns Hopkins Hosp., 49:17-26) and all eukaryotic cells are capable of pinocytosis.

It is a process in which fluid and small particles are absorbed by vesicles measuring approximately 150 nm in diameter. These vesicles fuse with the lysosome to allow the contents of the vesicles to be digested [Figure 18, A]. This is a process that requires a lot of energy, which is derived from ATP [172]. Usually, a part of the membrane is also "swallowed", which is quickly rebuilt in the process of exocytosis (a process opposite to endocytosis, the release of metabolites formed inside the cell).

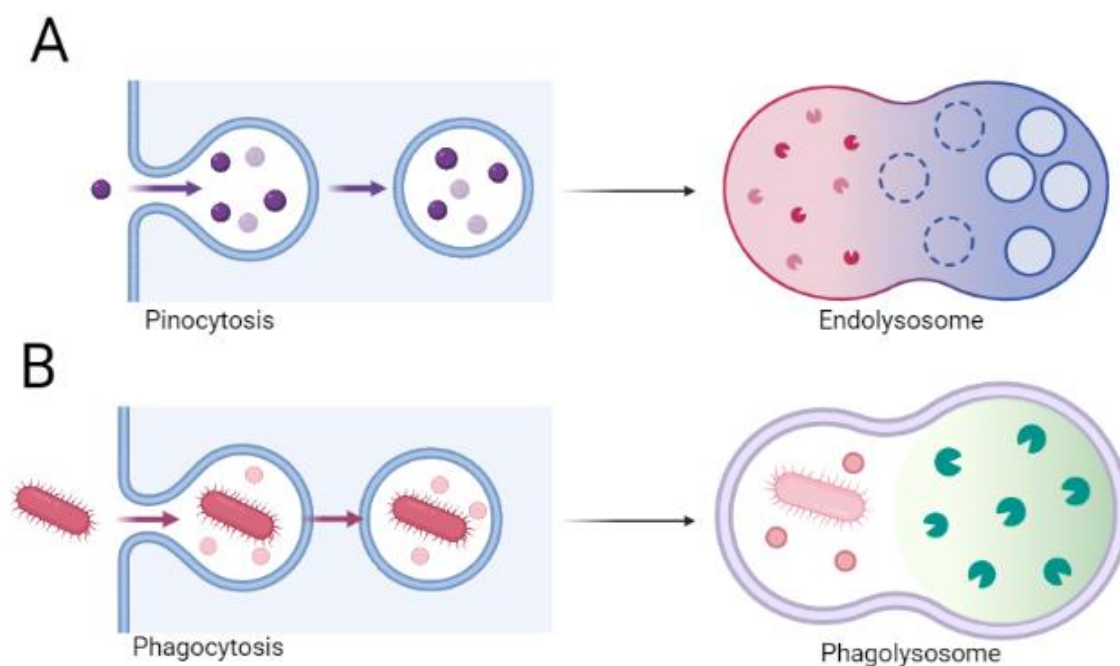


Figure 18. (A) Schematic representation of pinocytosis (B) Schematic representation of phagocytosis. Size ratio is not maintained.

Pinocytosis itself can be divided according to the size of the vesicles formed into macropinocytosis and micropinocytosis [173],[174]. Additionally, micropinocytosis, due to its mechanism, can be classified into two basic types: clathrin-mediated endocytosis and caveolae-mediated endocytosis [175].

1.3.2.1 MACROPINOCYTOSIS

Macropinocytosis is characterised by a lack of selectivity in the absorption of extracellular substances. They are closed in the so-called macropinosomes that have a diameter greater than 1 μm [176]. These vesicles are very sensitive to changes in cytoplasmic pH, and this may affect the macropinosome endocytic pathway. They can, for example, return to the cell surface and release their content there or combine with the lysosome [177]. Due to the above-mentioned lack of selectivity when capturing absorbed particles, macropinocytosis is a pathway through which fragments of apoptotic cells [178], as well as viruses [179] and bacteria [180], enter the cell.

Actin takes part in the formation of macropinosomes. Absorbed substances initially interact with proteoglycans associated with the cell membrane, and this affects the activation of Rho family proteins, which are involved in the control of the activity of the actin cytoskeleton [181]

During the process of macropinocytosis, the formation of specific actin structures has been observed and the word "cup" is commonly used to describe this structure in the literature. The molecular mechanism of the formation of cups from actin is known, but it is not fully known how these structures evolve over time and how the closure of the cup and the formation of a vesicle occur. Recent research presents two possible ways of closing the cups and thus encasing the absorbed molecule. The first one takes place at the cell edge through inward polymerization of actin. The second one takes place at the base of the cup by stretching and delaminating the membrane. According to this concept, the closure of the molecule in the cup occurs as a result of a combination of actin polymerization, membrane tension, and stalled cup expansion. These models can provide a basis for describing the evolution of cups over time [182].

Macropinocytosis is currently being investigated as a tool for intracellular drug delivery. This is an ideal route for bioactive therapeutic molecules to enter the cytoplasm [181].

1.3.2.2 CLATHRIN- MEDIATED ENDOCYTOSIS

Another type of endocytosis (micropinocytosis) is **clathrin-mediated endocytosis**. It occurs in all known eukaryotic cells and involves the formation of vesicles that are surrounded by a clathrin coat. A specific receptor must take part in this process, which will attach the absorbed material, e.g., viruses, proteins, metabolites, and hormones, to the outer side of the cell membrane.

Due to its coating function, clathrin is a protein with a characteristic triskelion shape. Its structure consists of three heavy and three light chains [Figure 19] [183].

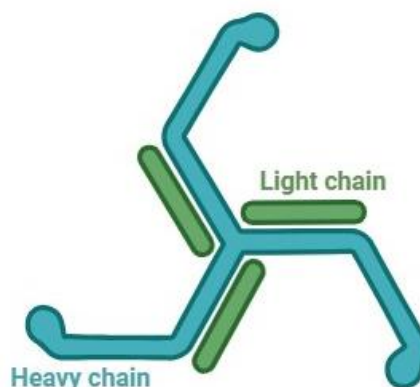


Figure 19. Triskelion shape of clathrin molecule.

The characteristic structure is due to the whole range of adapter proteins, which are additionally responsible for the incorporation of clathrin into the cell membrane and the unbudding of the vesicles.

The entire process of clathrin-mediated endocytosis is divided into five steps. The first step is nucleation, where a pit is formed and the clathrin is organised on the appropriate side of the membrane and in the appropriate location. It is starting to create a cargo-loaded vesicle. Then there is the mantle formation; the clathrin triskelions are organised into pentagons and hexagons and surround the vesicle. In the next step, the vesicle must be disconnected. The dynamin protein accumulates and tightens around the neck of the vesicle, causing the neck to rupture and detach from the membrane. In the last step, the clathrin is removed from the vesicle (and reused in the next process), and the vesicle attaches to the endosome [184].

1.3.2.3 CAVEOLAE- MEDIATED ENDOCYTOSIS

Caveolae are 50–100 nm-sized cavities in the membrane. Their function is, for example, signal transduction, but their participation in endocytosis has also been confirmed. This endocytosis process is highly regulated and involves complex signalling pathways [185].

A crucial component in the development of caveolae is the protein caveolin. The C- and N-termini of this protein are located in the cytoplasm, and it is inserted into the cell membrane from the cytoplasmic side in hairpin loop formation [Figure 20] [186].

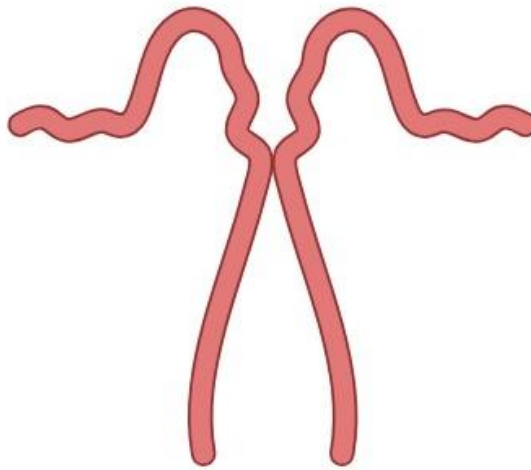


Figure 20. Hairpin shape of caveolin dimer.

The budding vesicle's neck contains the protein dynamin II (GTPase), which is responsible for detaching the vesicle (caveosome) from the membrane [187].

Caveolae-dependent endocytosis occurs, for example, during albumin endocytosis in the endothelium or during internalisation of the insulin receptor and it usually occurs in adherent cells [188].

The diagram below shows the schematic pathways of endocytosis that have been introduced in this chapter [Figure 21].

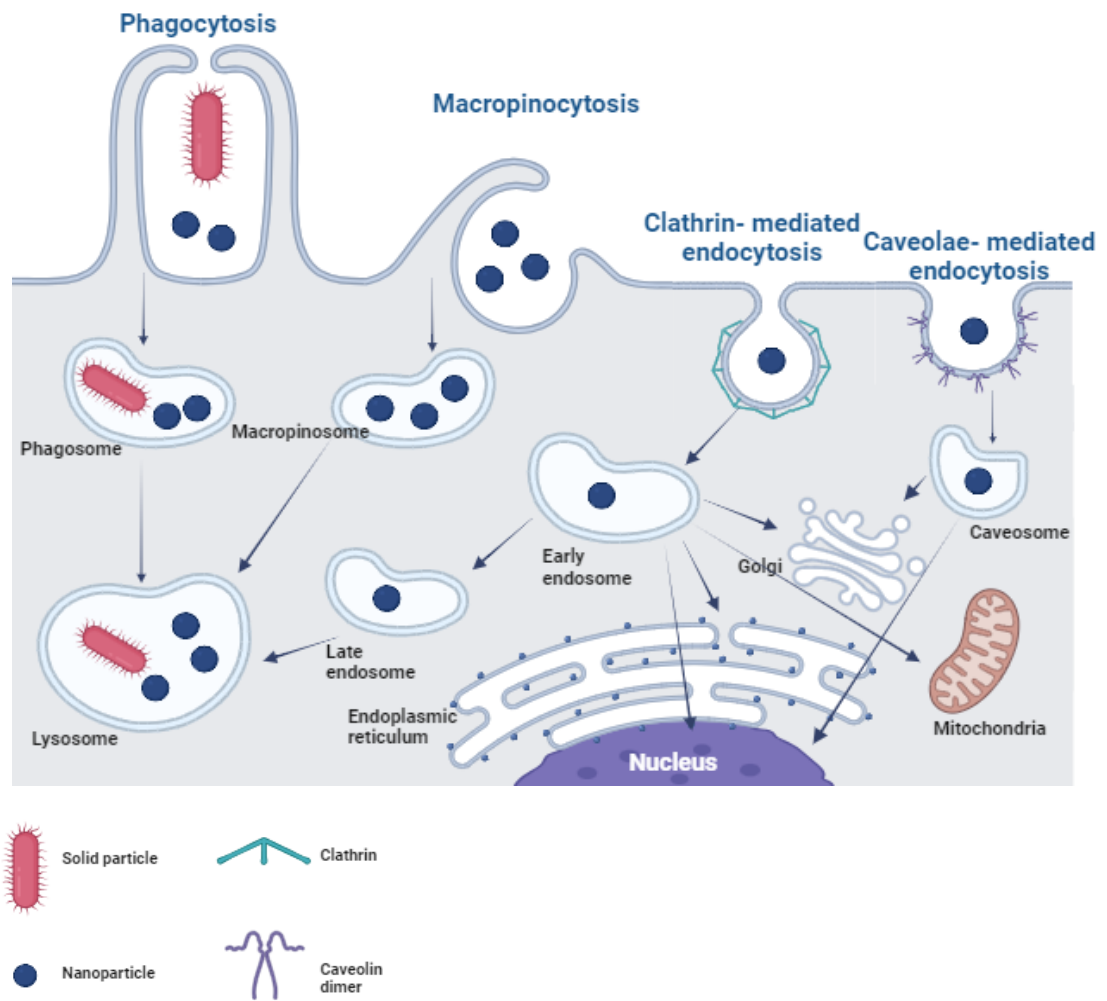


Figure 21. Illustration of endocytosis pathways (phagocytosis, micropinocytosis, clathrin-mediated and caveolae-mediated endocytosis). Inspired by [175].

1.3.3 PARTICIPATION OF THE CYTOSKELETON IN THE PROCESS OF ENDOCYTOSIS

It has been proven that the cytoskeleton also plays a significant role in endocytic processes. Actin filaments are involved in liquid-phase pinocytosis. Actin takes part in membrane folding [189], and membrane folding causes pinocytotic activity to increase [190]. To check the influence of the cytoskeleton on endocytic processes, the effectiveness of the process after the use of cytoskeletal drugs was investigated. Application of Cytochalasin D, which inhibits actin polymerization, had an effect on liquid-phase endocytosis - depending on the type of cell, endocytosis was stimulated or inhibited. It was also checked whether colchicine, which inhibits microtubule polymerization, somehow affects liquid-phase endocytosis. No changes were observed, and thus the involvement of microtubules was excluded [191].

Actin is also involved in phagocytosis, which is not spontaneous but requires ligand-receptor interaction. After activation of the receptor, there is a local increase in actin polymerization, which, together with associated proteins, is evenly distributed under the membrane around the phagosome. Interestingly, at some point, the contact between the cell surface and the cytoskeleton is broken, and actin condenses at the end of the retractable pseudopodia. Actin cytoskeleton proteins rearrangement occurs immediately after phagosome closure, which allows the vesicle to enter further stages of phagocytosis [192].

It is important to emphasise that the process of phagocytosis itself may differ depending on, for example, the type of cell or receptor interacting with the ligand.

A complement receptor (CR) is a membrane-bound receptor that is a component of the immune system. Effector protein fragments that are generated in reaction to damage-associated molecules or antigen-antibody complexes are bound by complement receptors. Complement receptor activation plays a role in the adaptive immune response and in the control of inflammation, leukocyte extravasation, and phagocytosis [193], [194]. CR-mediated phagocytosis is microtubule-dependent as well. After using colchicine, phagocytosis is blocked. However, in the case of the Fc-gamma receptor, microtubule degradation did not affect the course of the process [195].

Instead, microtubules are known to play a role in targeting and facilitating the transport of endocytic vesicles [196].

In the last type of endocytosis discussed above, clathrin-mediated, the actin cytoskeleton also plays a significant role. As with pinocytosis or phagocytosis, actin accumulates at the site of vesicle formation, and its polymerization is a source of force at multiple stages of the process. It is believed that the main role of actin is to assist in the neck constriction and subsequent budding of the clathrin-encased vesicle [197], [198].

The endocytic processes described in this section are, in fact, a topic that is studied by many scientists. A whole range of proteins is involved in these processes, which it would be impossible to list and describe here. Activities of actin filaments are also controlled by many proteins. However, the molecular mechanisms of microfilament participation in endocytosis are not yet fully understood.

According to current knowledge, it is postulated that intermediate filaments or microtubules do not play an active and necessary role in any type of endocytosis. However, as previously mentioned, microtubules are important in intracellular trafficking but play no significant role in endosome formation.

In the case of intermediate filaments, there is no mention of their potential involvement in endocytosis. It has been shown that they can interact with proteins involved in endocytosis, It has been shown that they can interact with proteins involved in endocytosis, but it cannot be said that IFs are directly involved [199]. This could just be the result of current insufficient knowledge of intermediate filaments functions as of today.

To sum up, at the moment there is talk of a significant participation of the cytoskeleton in endocytosis, but only in the context of actin filaments.

1.4 CYTOSKELETON RESEARCH TECHNIQUES

The invention of electron microscopy contributed significantly to the development of knowledge about the cytoskeleton. It is still one of the most important methods for studying the *in vitro* cytoskeleton and isolated filaments, mainly due to its resolution, as scanning electron microscopy can even reach a resolution of 1 nm.

Unfortunately, certain conditions must also be met, e.g., the sample must be in a vacuum, which is somewhat problematic when imaging biological samples. Another condition is the electrical conductivity of the sample, which is why metal is sputtered onto its surface. It is undoubtedly a major interference in the biological system [200].

In addition to electron microscopy, light microscopy also plays an important role.

1.4.1 OPTICAL MICROSCOPY

The first optical microscope was a scientific milestone in the studies of biological objects. It was invented around the turn of the 16th and 17th century and had a 10x magnification (the maximum magnification of a modern, basic optical microscope is 1500x). Back then, nobody considered it a scientific revolution. However, Antoni Van Leeuwenhoek, a Dutchman, improved the original microscope and obtained approximately 270x magnification. This determined the beginning of several breakthrough biological discoveries, including the identification and naming of the cell [201].

The microscopy technique was constantly being improved, e.g., by carefully polishing the lenses, eliminating the phenomenon of chromatic aberration, and adding an artificial light source. Modern microscopes are able to overcome the diffraction barrier and obtain nanometre resolution [202], [203].

Fluorescence is the phenomenon of light being emitted by a substance previously excited by light absorption. Light emission usually occurs very quickly, i.e., after time less than 10^{-8} seconds after absorption [204], [205].

In short, the whole phenomenon can be described as follows: the incident photon excites the electron, forcing the electron to transition to the singlet excited state. The emission of energy occurs when that electron returns from an excited state to a singlet ground state. The emitted photons usually have lower energy than the absorbed photons; therefore, the emitted light has a longer wavelength than the absorbed light. The loss of energy is due to the fact that the emission transition can only occur after transferring some energy to the environment in non-radiative processes. The difference in the spectral position of the excitation and emission bands is called the Stokes shift. This phenomenon is illustrated in the Jablonski diagram [Figure 22] [206].

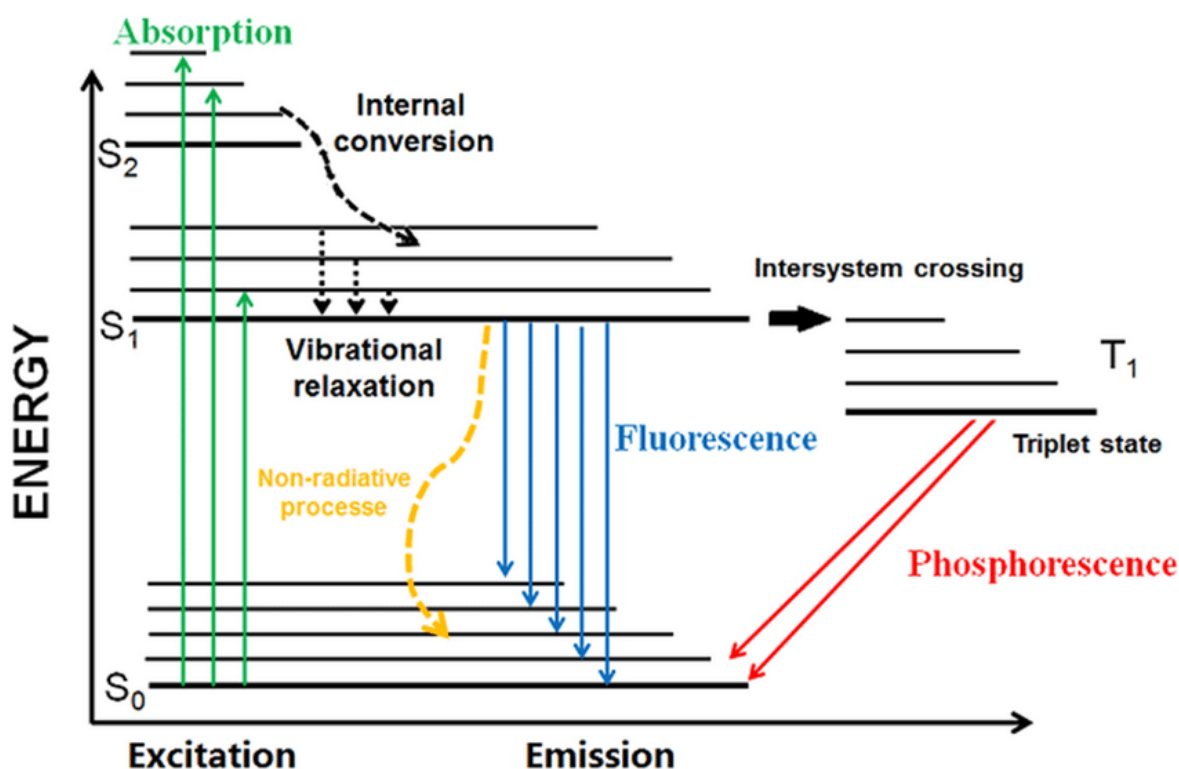


Figure 22. Perrin Jablonski diagram of fluorescence and phosphorescence. Picture taken from [191].

The phenomenon of fluorescence is also used in light microscopes. Most commonly it is used in the wide-field microscope, which evenly illuminates the entire sample, so one can see the fluorescence signal throughout the entire field of view.

Confocal microscopy is one of the most widely used types of light microscopy. Its great advantage is that you can get a three dimensional image of the sample.

One can distinguish, for example, spinning-disk confocal microscopy. The image is generated by the simultaneous illumination of many points on the object plane. For this purpose, a special, rapidly spinning disk (Nipkow disk) is used, which contains numerous pinholes to scan spots of light. Thanks to this, the area is scanned in parallel, and the energy needed for excitation is low. This provides fast imaging, but at the cost of image quality [207].

Scanning laser confocal microscopes (SLCM), the second and more common form of confocal microscopy, employ a precisely focused laser spot to scan the specimen. The three-dimensional representation is created by using a pinhole to specifically capture light coming from the selected plane and eliminate the out-of-focus signal. This removes light that is outside of the focal plane, providing images that are higher in contrast than those that come from a wide-field fluorescent microscope. Therefore, behind the pinhole, there is usually a photomultiplier that converts the light signal into an electrical signal [208].

The two primary modes of sample image acquisition are scanning and wide-field. Wide-field mode simultaneously collects the signal from the entirety of the image. However, scanning is the acquisition mode applied by SLCM. Due to the fact that one point of the sample is illuminated at a time, in order to obtain the image of the sample, linear scanning is necessary. The image is generated through the sequential, pixel-by-pixel manner. In this way, a single slice is created, which is then added to the remaining patches, and together they form the so-called Z stack. From a set of slices, you can create a 3D or 2D image that gives an overview of what is in the entire volume of the sample [209], [210].

However, scanning laser confocal microscopy is not without limitations. In order to prevent the loss of the valuable signal due to out-of-focus light rejection, it is necessary to employ a higher excitation beam intensity. However, this increases the risk of photodamage to biological samples and photobleaching. Additionally, damage and bleaching are caused by multiple scans of a single location during 3D imaging [211].

The source of light is lasers usually, which can provide different wavelengths in the range from UV to infrared. Laser beams pass through collimating optics to a dichroic mirror and through a lens that focuses the beam on the sample. The lenses used in confocal microscopy can be immersive but also dry [Figure 23] [212].

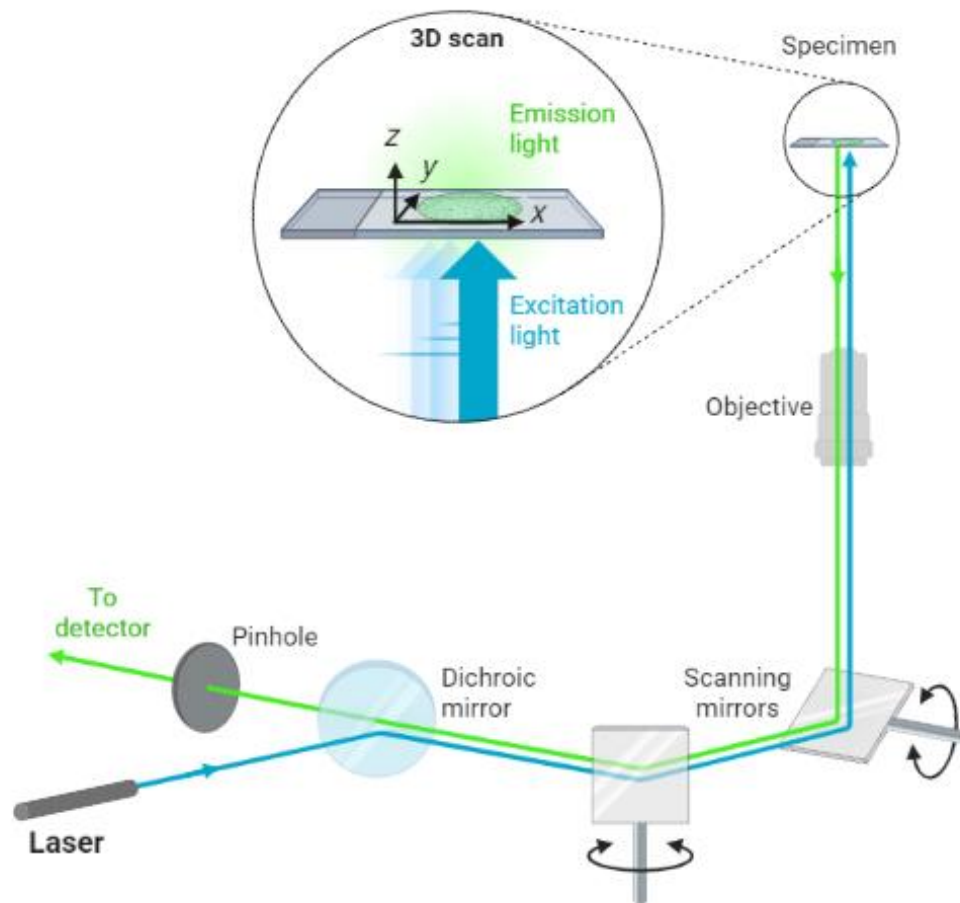


Figure 23. Scheme of confocal microscope light path.

In order to observe the samples under a confocal microscope, certain preparations must be performed. Some of the structures may show autofluorescence, but usually most samples do not and they need to be prepared by adding specific fluorophores. As an example, commonly used phalloidin, which attaches to actin filaments and, appropriately labelled with a fluorophore, makes these filaments visible to the researcher [213].

However, another staining method, called **immunofluorescence**, is very often used in the preparation. Briefly, it is a technique that permits specific visualization of many components in any given tissue or cell type using antibodies. The antibodies can be monoclonal (one antibody with a fluorophore) or polyclonal (the primary antibody is structure-specific, and the secondary antibody interacts with the primary and is fluorescently labelled) [Figure 24] [214].

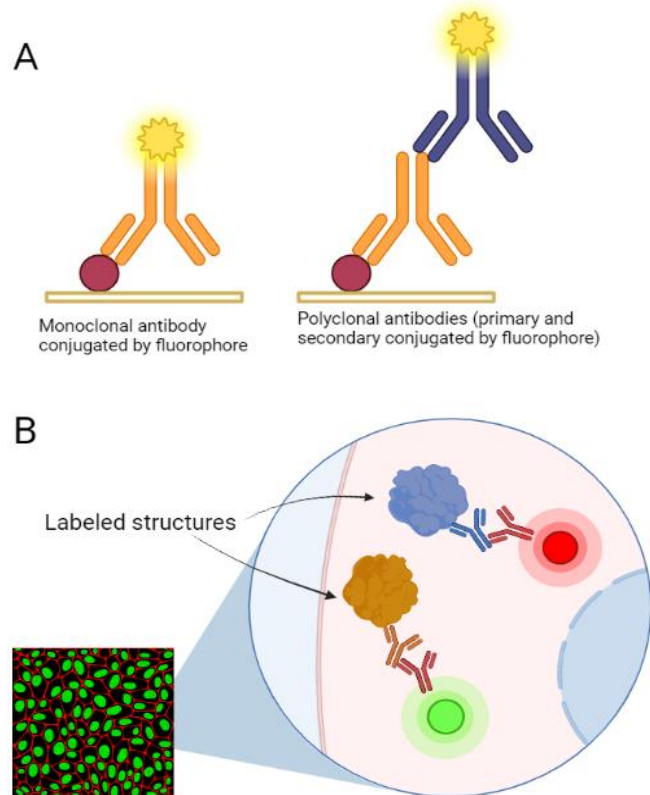


Figure 24. (A) Difference between monoclonal and polyclonal antibodies labelling. (B) Interaction of polyclonal antibodies labels with labelled structures.

Another frequently used labelling method is the **transfection** of eukaryotic cells (in prokaryotes, this is called transformation). It involves the introduction of a fragment of foreign DNA or RNA into the test cells. DNA can be transported to the host cell using a viral or non-viral vector (plasmid) [215]. It is important that the introduced fragment of genetic material encodes the so-called fluorescence protein fusion (FPF), which allows for fluorescent labelling of the examined organelle [216].

Transfection allows the cell to be “forced” to express fluorescent proteins [Figure 25]. The result of this procedure may be the permanent transfection when the introduced DNA integrates into the genome of the cell or transient transfection, where during cell division the expression of the inserted genome decreases. Transient transfection is useful in short-term studies, most often to investigate the effects of knock-in/-down of a particular gene [217]. Stable transfection is used in long-term genetic and pharmacological research [218]. This method allows for live imaging, i.e., cells are still alive during the measurement and changes can be observed in real time and precise cellular localization [219].

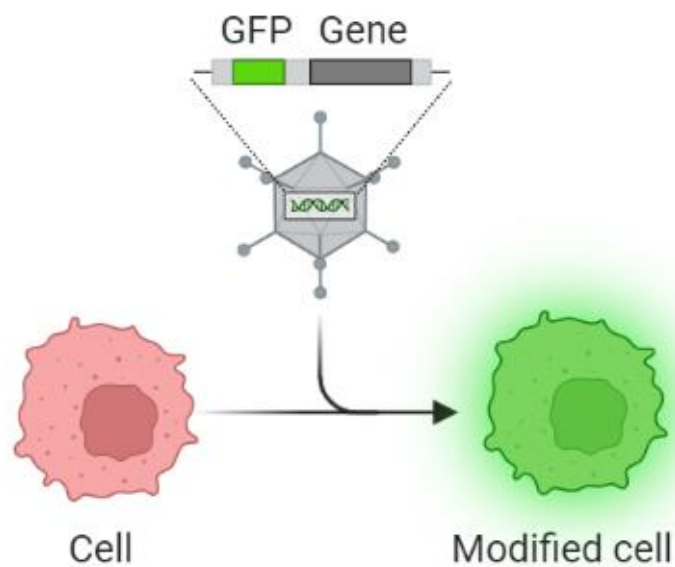


Figure 25. Cell transfection with the introduction of the GFP fluorophore.

2. SCOPE OF THE STUDY

The aim of the work was:

- 1) To describe the results of cell – latex beads interaction on the cytoskeletal architecture of fibroblasts grown on substrates with different elasticity.
- 2) To investigate the role of selected proteins from the MAPs family - CLIP1, IQGAP1, MARK2 - in the process of endocytosis of latex microspheres.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

MEF3T3 Swiss mouse embryonic fibroblast cell line (CRL-2752, ATCC, Manassas, Virginia, USA) cells were used in this study. Cells were cultured in DMEM Low Glucose medium (L0066, Biowest, Nuaille, France) supplemented with 10% Gibco FBS (10270106, Thermo Fisher Scientific, Waltham, MA, USA), and 100 I.U./mL Penicillin and 100 µg/mL Streptomycin Solution (L0022, Biowest, Nuaille, France) at 37 °C, 5% CO₂, and 100% humidity. Before experiment, cells were cultured overnight in a fresh medium and plated in a 35 mm glass bottom Petri dishes with thickness #1 (0.13–0.16 mm) or #1.5 (0.16–0.19 mm) designed for high resolution imaging (Cellvis, Mountain View, CA, USA).

3.2 MICROSPHERES AND MICROSPHERES COATING

Fluoresbrite YG Carboxylate Microspheres with diameter 2 µm were obtained from Polysciences (09847, Polysciences Inc., Warrington, PA, USA), are made of latex and can be visualised by fluorescent microscopy (441 nm absorption and 486 nm emission, Figure 26). Beads were coated with fibronectin from bovine plasma (341631, Merck, Darmstadt, Germany) in concentration of 0.7 mg/mL using PolyLink Protein Coupling Kit (24350-1, Polysciences Inc., Warrington, PA, USA). In short, a pellet of microparticles was suspended in PolyLink Coupling Buffer twice and the whole suspension was mixed with PolyLink EDAC solution. After these steps, fibronectin was added, the bead suspension was incubated in room temperature with gentle mixing for 1 h, centrifuged twice, and resuspended in PolyLink Wash/Storage Buffer. The mixture was stored in 4 °C. In the next step, the binding of the protein to the beads has been verified spectrophotometrically by measuring absorbance of suspension at 280 nm.

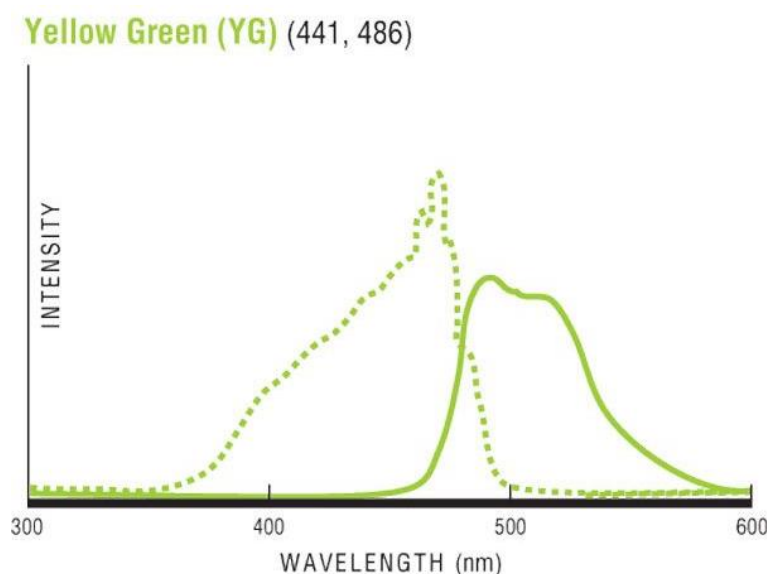


Figure 26. The excitation and emission spectra of fluorophore Yellow Green (YG) latex microspheres used in experiments; plot taken from the website of manufacturer (<https://www.polysciences.com/india/fluoresbrite-yg-carboxylate-microspheres-200m>)

3.3 PREPARATION OF POLYACRYLAMIDE SUBSTRATES

To manufacture substrates with varying stiffnesses, polyacrylamide (PA) gel substrates were fabricated based on established procedures [220]. In short, glass-bottom dishes were treated with a solution of 3-(Trimethoxy silyl) propyl methacrylate (440159, Sigma-Aldrich, St. Louis, MO, USA), 99.5% acetic acid, and 96% ethanol (1:1:14 ratio) for 30 min, washed twice with 96% ethanol, and dried.

Polymerization mixtures for selected elasticities were prepared by mixing acrylamide and bis-acrylamide (Bio-Rad, Herkules, CA, USA) in various proportions in the 10 mM HEPES buffer solution. The proportions for preparation of the substrates with elasticity of 40 kPa, 16.7 kPa, 8 kPa and 2 kPa were taken from protocol [221]. Each type of polymerase mixture was mixed with polymerizing agents: 0.05% ammonium persulfate and 0.05% tetramethylethylenediamine (Bio-Rad, Herkules, CA, USA). The resulting mixture was applied to silanized slides in an amount of approximately 23 μ l. The mixture was covered with an 18 mm thick coverslip and incubated for 1 h in room temperature to polymerize. Then the slide was removed and the prepared substrates

were stored in a PBS solution at 4° C overnight. After it, polyacrylamide substrates were incubated with 2 µg/mL Sulfo-SANPAH (22589, Thermo Fisher Scientific, Waltham, MA, USA) under ultraviolet light for 5 min. PA gels were rinsed with a sterile PBS solution and incubated with 10 µg/mL fibronectin at 4° C overnight.

3.4 ELASTICITY OF PA SUBSTRATES

The elasticity of the selected prepared PA substrates was checked using atomic force microscopy. The device used for this purpose was a JPK Nanowizard 3 microscope, and the measurements were carried out in force mapping mode.

A minimum of four preparations were produced for each of the substrates with the specified, according to the protocol, elasticity values of 40 kPa, 16.7 kPa, and 8 kPa. Measurements were done in at least two independent series. The elasticity of every substrate has been measured in at least five independent locations. In general, substrates that were specified with elasticity values of 40 kPa and 8 kPa, their elasticity remained within the error bars of 1 kPa. However, in all cases, the elasticity of a substrate of nominal value of 16.7 kPa according to the protocol, was closer to 22 kPa and subsequently this value was used in this work.

3.5 CELL LABELLING AND IMMUNOFLUORESCENCE

Cells were fixed with 4% formaldehyde solutions (47608, Sigma-Aldrich, St. Louis, MO, USA) for 10 min and permeabilized for 7 min in 0.1% Triton X-100 (T8787, Sigma-Aldrich, St. Louis, MO, USA) to allow antibodies or toxins to enter the cell. In the cases of antibody staining, the non-specific sites were blocked with 3% BSA PBS buffer (A7906, Sigma-Aldrich, St. Louis, MO, USA) for 30 min, then stained for α -tubulin (1:500 in 3% BSA PBS buffer; T9026, Sigma-Aldrich, St. Louis, MO, USA) or vimentin (1:100 in 3% BSA PBS buffer; EPR3776, Abcam, Cambridge, UK) overnight.

When additional proteins (MAPs family) related to organisation of microtubules were investigated, the antibodies against three proteins were used and incubated overnight

also: **MARK2** (1:100 in 3% BSA PBS buffer; SAB4500761, Sigma-Aldrich, St. Louis, MO, USA), **IQGAP1** (1:100 in 3% BSA PBS buffer; SAB4200079, Sigma-Aldrich, St. Louis, MO, USA) and **CLIP1** (1:100 in 3% BSA PBS buffer; HPA026678, Sigma-Aldrich, St. Louis, MO, USA).

Alexa Fluor 555-labelled goat anti-mouse IgG (H+L) antibody (1:100 in 3% BSA PBS buffer; A21422, Thermo Fisher Scientific, Waltham, MA, USA) or Alexa Fluor 633-labelled goat anti-rabbit IgG (H+L) antibody (1:50 in 3% BSA PBS buffer; A21070, Thermo Fisher Scientific, Waltham, MA, USA) were used as fluorophore conjugated secondary antibodies; [Figure 27].

For actin staining, cells were fixed, permeabilized and incubated with phalloidin conjugated with DyLight 633 (1:100 in PBS buffer; 21840, Thermo Fisher Scientific, Waltham, MA, USA) for 45 min. A scheme showing indirect immunofluorescence labelling shown below [Figure 28].

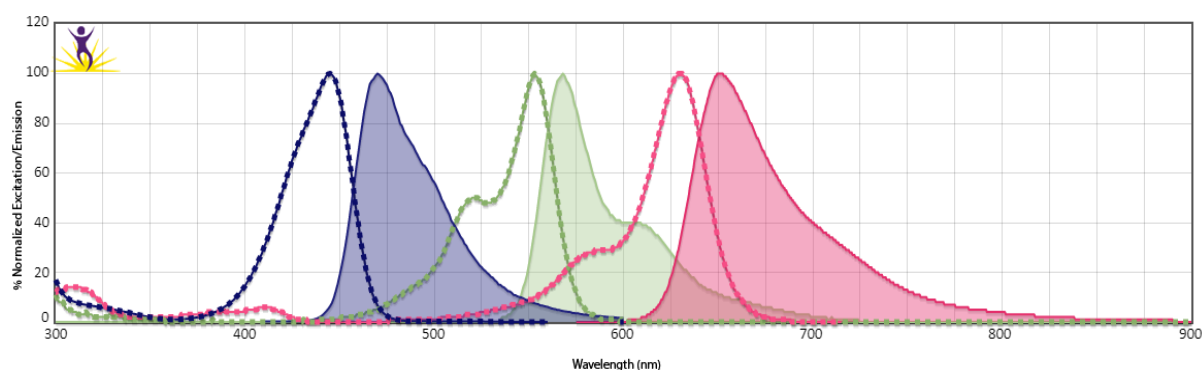


Figure 27. The excitation and emission curves of fluorophore Yellow Green (YG) for latex microspheres (blue); Alexa Fluor 555 (green) and Alexa Fluor 633 (red); the dotted line shows the excitation spectrum of the fluorophore, while the solid line shows the emission spectrum (spectrum generated on <https://www.biolegend.com/en-us/spectra-analyzer>)

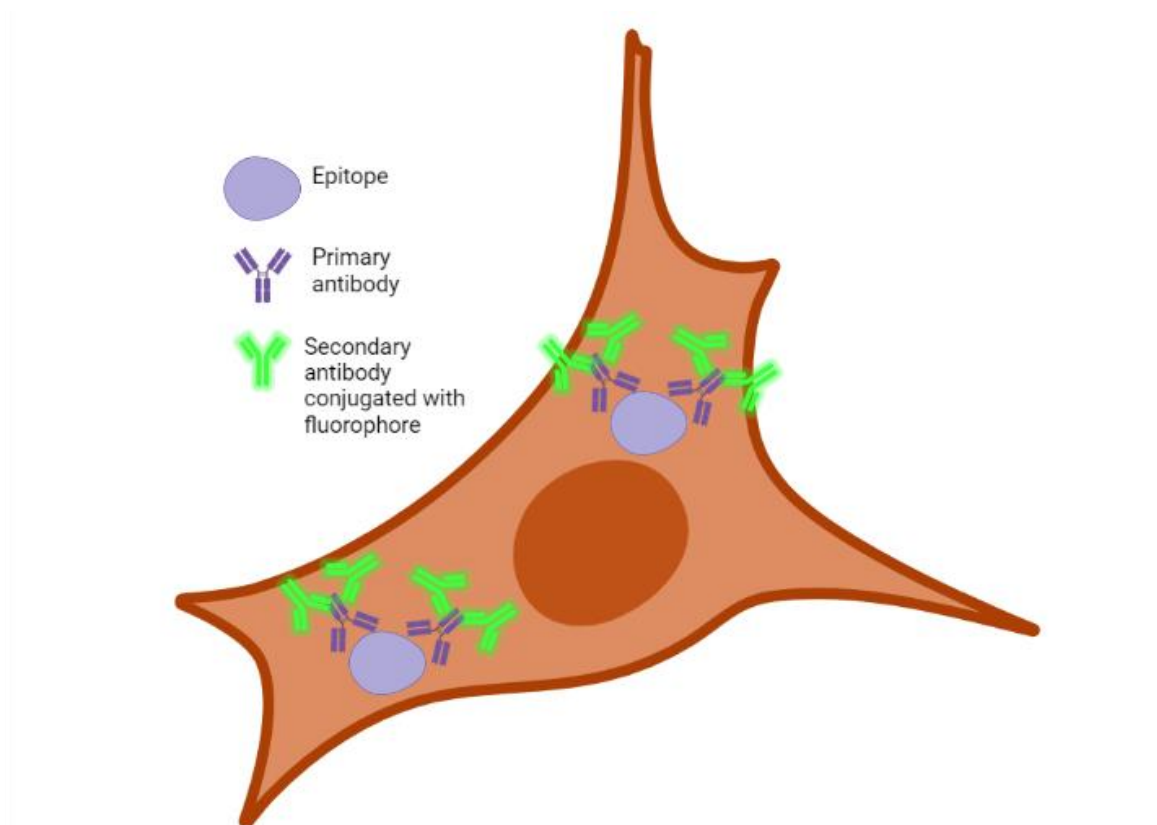


Figure 28. Schematic drawing showing indirect immunofluorescence labelling with primary and secondary antibodies conjugated with a fluorophore.

3.6 CYTOSKELETAL DRUGS

Cytoskeletal drug used in the experiments was Cytochalasin D solution (C8273, Sigma-Aldrich, St. Louis, MO, USA), which prevents polymerization of actin, 20 μ M cytochalasin D solution was applied for 1 hour.

3.7 CONFOCAL MICROSCOPY

Confocal images were acquired using Zeiss LSM 710 confocal module set on Zeiss Axio Observer.Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with an oil immersion 40x 1.4 NA Plan - Apochromat objective.

3.8 DATA ANALYSIS TOOLS

The images were subjected to individual evaluation and some of them were deconvoluted using Batch Deconvolution Fiji v0.49 plugin [222]. All calculations were made using the Fiji ImageJ environment [223].

3D rendering was made using FluoRender 2.24 software (<https://www.sci.utah.edu/software/fluorender.html>) [224].

A custom written macro was applied to analyse and prepare graphs showing the proportion of microspheres that were entirely enveloped and those that were not enveloped by cytoskeletal filaments. The program had two tasks. First, it cropped- out the cellular part containing the nucleus, leaving cytoplasmic region needed for analysis. Second, it present images in a sideview format. Then, it was manually determined whether the signal coming from the cytoskeleton filaments was located above the bead or not.

3.9 EXPERIMENTAL PROCEDURE

Prior to each experiment, cells were cultured overnight in glass bottom dishes with a thickness of #1.5 (0.16–0.19 mm) if the experiment was conducted on a glass substrate, or with a thickness of #1 (0.13–0.16 mm) if the bottom dishes were coated with polyacrylamide gel and fibronectin (described in Chapter 3.3). On the next day, uncoated or fibronectin-coated latex beads were introduced into the cell culture under sterile conditions and incubated at 37° C for 15, 1, or 3 hours, depending on the requirements of the experiment. This time was used alternately as a time of cell-bead interaction in subsequent paragraphs of this thesis. Immediately after this, the cells were fixed and stained in accordance with the immunofluorescence procedures described above (Chapter 3.5). The Z-stack projection was used to measure the prepared samples, with parameters being set for each sample individually.

4. RESULTS

4.1 CELL - LATEX BEAD INTERACTION

This chapter presents the results and their description for the cytoskeleton staining. All images were taken with a confocal microscope (more in Chapter 3, Materials and Methods).

The results contained in subsections 4.1–4.3 have been published in [225] (Adamczyk, O.; Baster, Z.; Szczypior, M.; Rajfur, Z. Substrate Stiffness Mediates Formation of Novel Cytoskeletal Structures in Fibroblasts during Cell–Microspheres Interaction. *Int. J. Mol Sci.* 2021, 22, 960. <https://doi.org/10.3390/ijms22020960>).

4.1.1 THE EFFECTS OF LATEX BEADS - FIBROBLAST INTERACTION ON CYTOSKELETON ARCHITECTURE

Nano- or micrometre-diameter spheres are commonly used in biological research. They are used, for example, to study endocytosis processes and are a crucial element in one of the more advanced microscopic methods, optical tweezers (OT).

In addition to the size, they may also differ in the material from which they are made, e.g., polystyrene, latex, or glass. They can also be fluorescent or non-fluorescent and their surface can be often covered with various proteins or antibodies.

OT is a microrheological technique which employs a focused laser beam and polystyrene or latex beads to study mechanical properties of biological systems. Latex beads, functionalized with a specific protein, can interact with proteins located on the surface of the cellular membrane. The beads are also used to study cell elasticity with OT technique. In this case, it is obvious that they should be completely neutral for the cell and biocompatible. However, the interaction has the potential to significantly impact cellular behavior, and changes in cellular morphology or behavior are frequently undesirable.

The effect of cell-bead interaction on cytoskeleton and its time dependence are shown in the results below.

The experimental findings are presented in four pictures below **[Figures 29- 32]**. Two figures represent the results achieved with uncoated latex beads: one represents the results after 15 minutes of bead-cell interaction and the other represents the results after 3 hours. The remaining two are for beads coated with fibronectin, as well as after an exposure time of 15 minutes and 3 hours.

An image of each of the three cytoskeletal components— actin filaments, microtubules, and vimentin (intermediate filaments)—is shown in each figure.

Summaries and conclusions are provided subsequent to the figures.

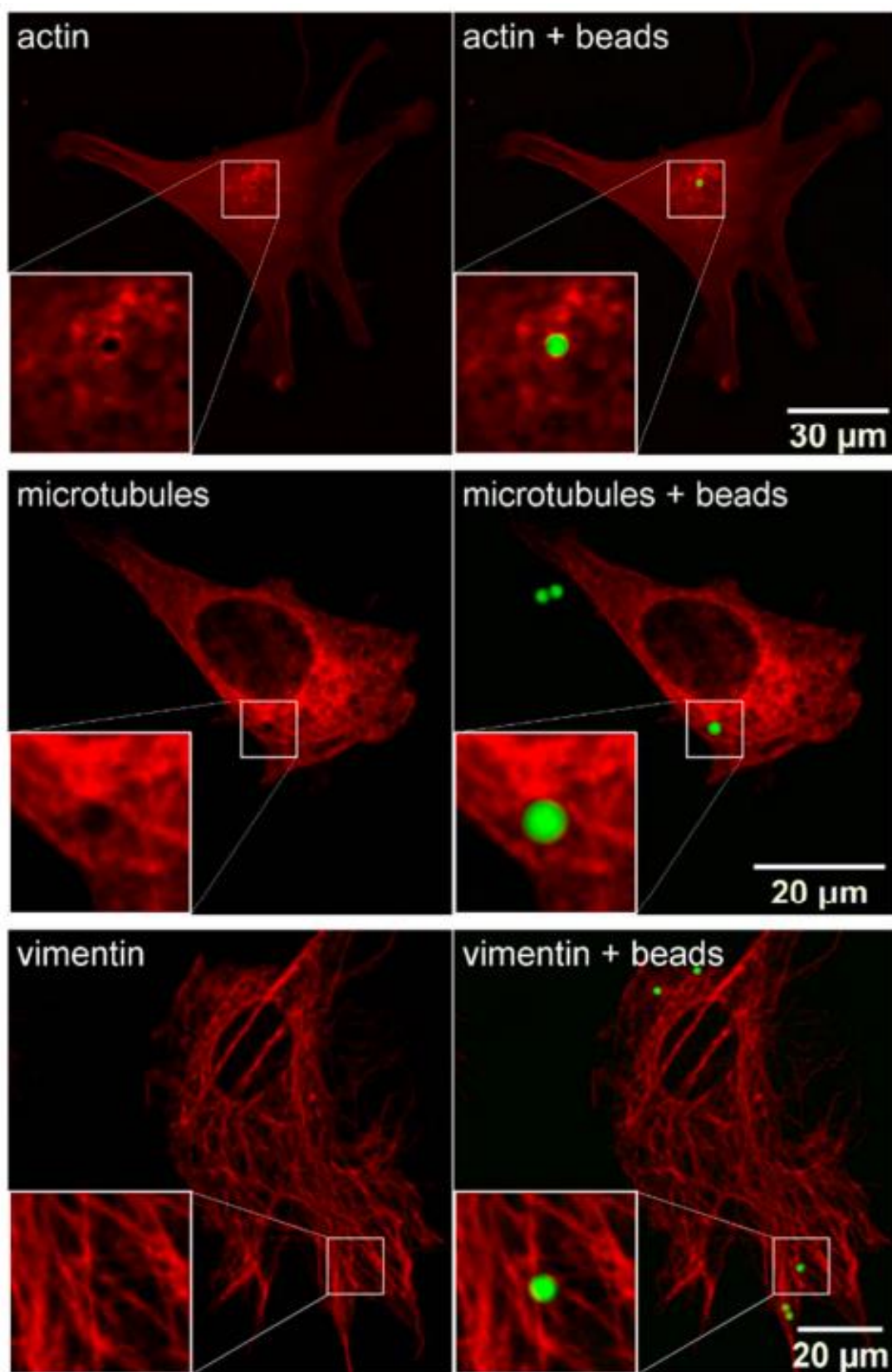


Figure 29. Images of actin, microtubules and vimentin structures in experiments with **uncoated latex beads** and time of interaction of **15 minutes**. Images are presented as sums of stacks with zoom on the immediate surroundings of the latex bead. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

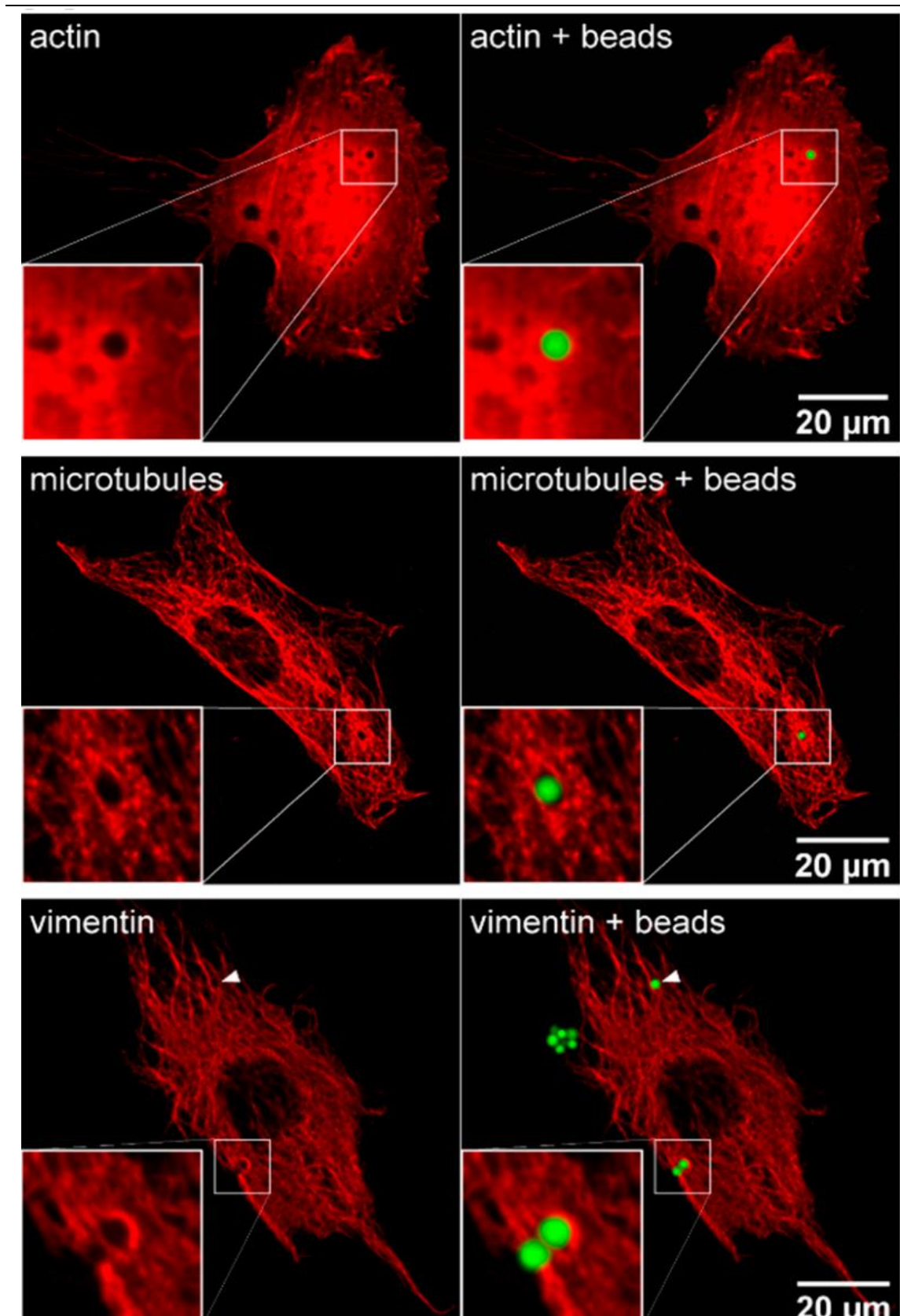


Figure 30. Images of actin, microtubules and vimentin structures in experiments with **fibronectin- coated latex beads** and time of interaction of **15 minutes**. Images are presented as sums of stacks with zoom on the immediate surroundings of the latex bead arrows point at spots where beads were enveloped by the cytoskeleton and visible changes could be observed.. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

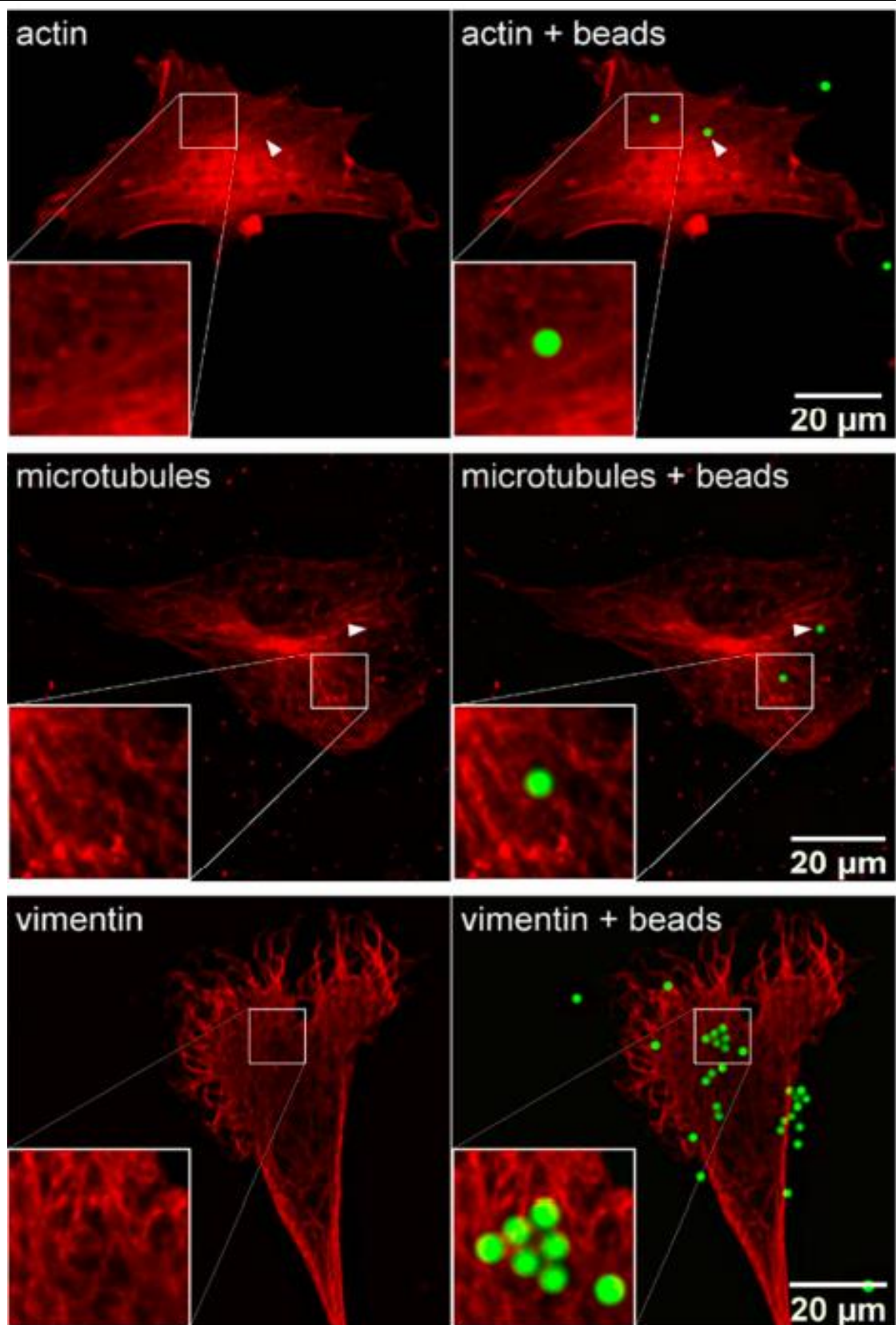


Figure 31. Images of actin, microtubules and vimentin structures in experiments with **uncoated latex beads** and time of interaction of **3 hours**. Images are presented as sums of stacks with zoom on the immediate surroundings of the latex bead arrows point at spots where beads were enveloped by the cytoskeleton and visible changes could be observed.. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

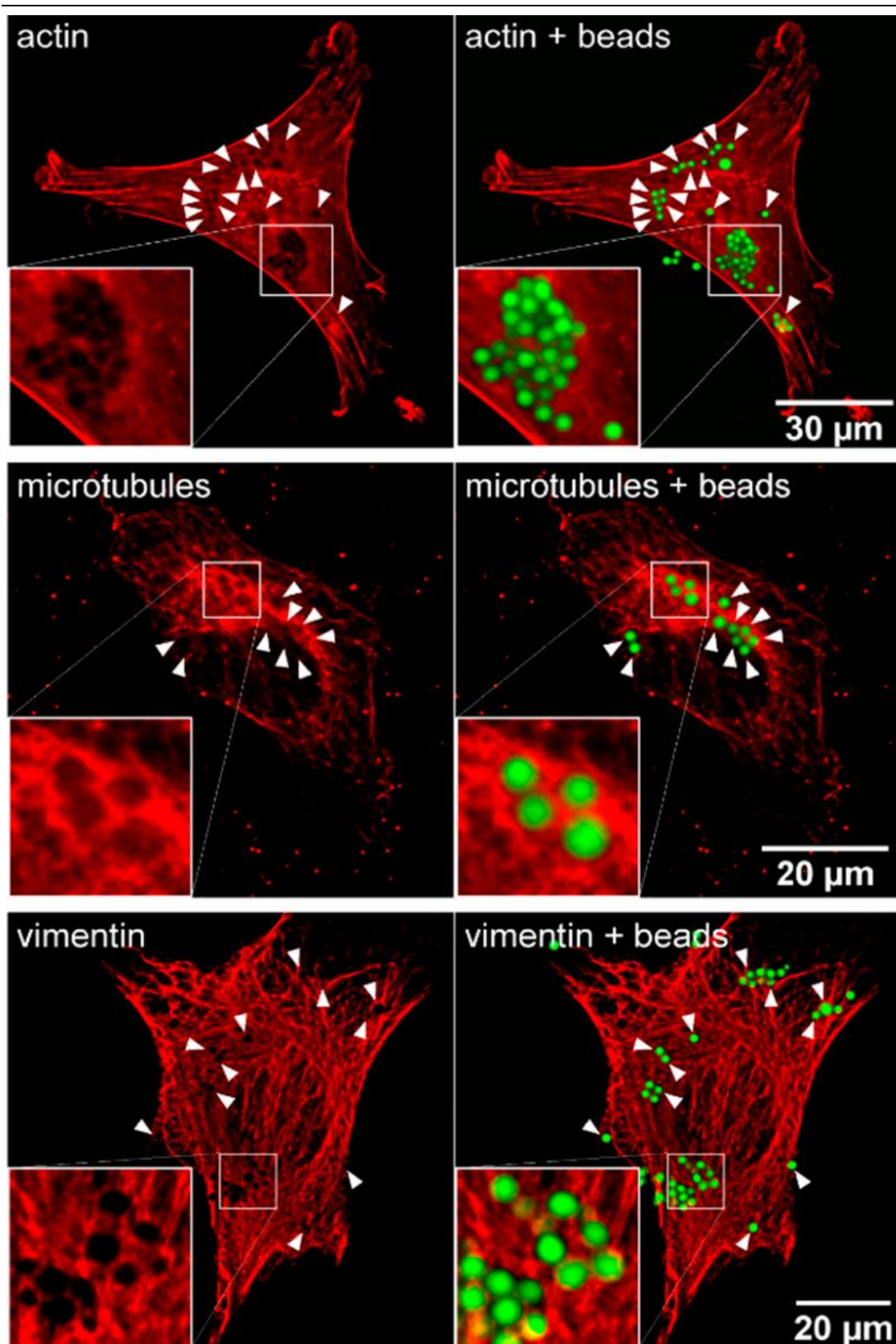


Figure 32. Images of actin, microtubules and vimentin structures in experiments with **fibronectin- coated latex beads** and time of interaction of **3 hours**. Images are presented as sums of stacks with zoom on the immediate surroundings of the latex bead and arrows point at spots where beads were enveloped by the cytoskeleton and visible changes could be observed. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

Figures 29–32 show images of stained for cytoskeletal filaments MEF 3T3 cells taken with a confocal microscope. Cytoskeleton filaments are marked in red, and fluorescent latex beads are marked in green. In each of the images, there is one area where, on the one hand, we have both channels visible, and on the other image, only the channel with the signal from the cytoskeleton is visible. In this experiment, all three components of cytoskeleton were stained and imaged. Cells were grown on glass- bottomed plates as described in Materials and Methods.

The experiments differed in regard of two external conditions:

1) Incubation time: the architecture of the cytoskeleton was checked after short (15 minutes) and long (3 hours) exposure to beads suspension.

2) Bead functionalization: two types of beads were used, one uncoated and the other coated with fibronectin protein. Fibronectin is an extracellular matrix protein that interacts with integrin proteins that are found in the cell membrane. The aim was to check the influence of those factors on the microsphere - cell interaction.

The pictures presented above prove that each type of cytoskeletal filament is rearranged under the influence of beads. Changes can be observed after both 15 minutes and 3 hours of cell-bead interaction.

One important research question was whether the bead was enveloped by filaments, that is whether elements of the cytoskeleton could be observed above the bead.

Therefore, in the next step, it was checked what fraction of the beads was enveloped by filaments for each parameter [**Figure 33**].

Numbers of analyzed beads for **Figure 33**: actin, 15 min, uncoated, $n = 16$; actin, 3 h, uncoated, $n = 71$; actin, 15 min, coated, $n = 129$; actin, 3 h, coated, $n = 523$; microtubules, 15 min, uncoated, $n = 19$; microtubules, 3 h, uncoated, $n = 135$; microtubules, 15 min, coated, $n = 285$; microtubules, 3 h, coated, $n = 905$; vimentin, 15 min, uncoated, $n = 39$; vimentin, 3 h, uncoated, $n = 100$; vimentin, 15 min, coated, $n = 192$; vimentin, 3 h, coated, $n = 581$.

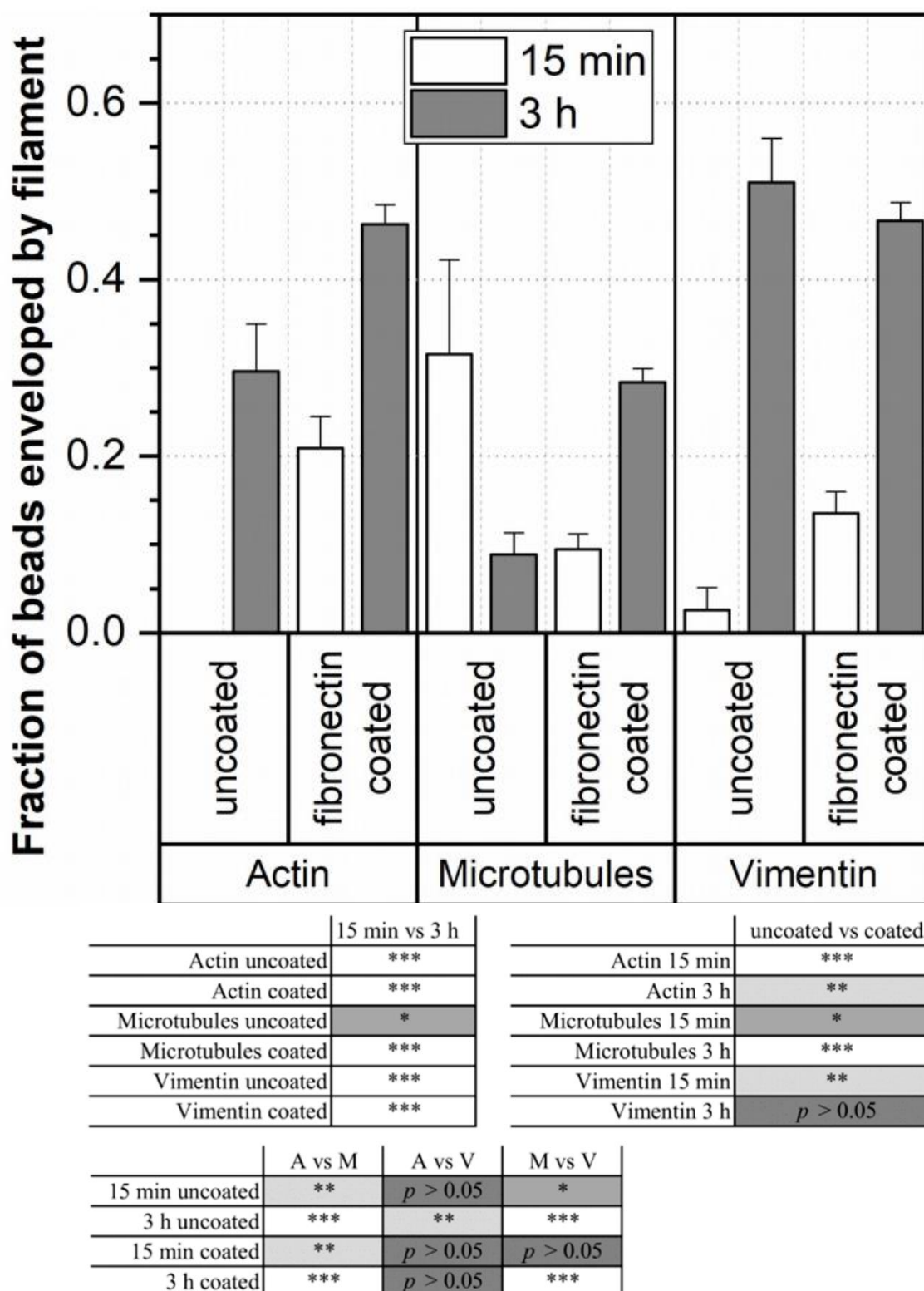


Figure 33. Histogram displaying the percentage of beads enveloped by an exact cytoskeleton component. Two proportion Z-test was used to determine statistical significances between specific samples: (upper left) bead incubation duration: 15 min versus 3 h; (upper right) uncoated versus coated beads; (lower left) comparison between cytoskeletal components: (A)ctin, (M)icrotubules, and (V)imentin. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Plot posted with permission from *International Journal of Molecular Sciences MDPI*.

The fraction of beads that were fully enveloped by cytoskeletal components is represented as a fraction of all beads interacting with cells [**Figure 33**]. The results of the calculation are presented as a percentage fraction $\pm$ SEM.

The statistical significance of obtained results was checked using the Z-test. A p-value was calculated where $p < 0.05$ and $p < 0.005$ are statistically significant, and $p < 0.001$ is statistically very significant (less than one chance in a thousand to be wrong). Results with a p-value greater than 0.05 are not considered statistically significant.

The data are representative of at least two independent experiments.

In the case of actin, it can be seen [**Figure 33**] that there are more fibronectin-coated spheres enveloped by actin filaments. For both coated and uncoated beads, we see an increase in the actin-enveloped bead fraction after 3 hours of bead-cell interaction. The number of enveloped beads after 15-minutes with uncoated beads was extremely low and that is not visible on the chart.

For intermediate filaments (vimentin), the disproportion between the number of enveloped beads after 15 minutes and 3 hours is very clear - more beads are enveloped after 3 hours. However, there is no significant difference in the number of enveloped beads when it comes to uncoated and fibronectin beads after 3 hours of interaction.

Microtubules behave differently. There are a greater number of enveloped uncoated beads after 15 minutes than after 3 hours. In contrast, fibronectin-coated beads have a higher amount of fully enveloped beads after a period of 3 hours.

4.1.2 INFLUENCE OF CYTOCHALASIN D ON CELL - BEAD INTERACTION

The next experiments investigated the influence of the cytoskeletal toxin on the previously observed effect. It was intended to show whether the bead-cytoskeleton interaction was based on the dynamic structure of the filaments.

Cytochalasin D, an actin polymerization inhibitor, was used to treat MEF 3T3 cells to investigate the effect of cytoskeleton-targeting drugs on the cytoskeleton-bead interaction. Cells were incubated for 1 hour with carboxylated fluorescent latex beads (fibronectin-coated or uncoated) and 0.1% cytochalasin D.

One can see the effect of cytochalasin D on the bead-cell interaction in **Figure 34** (number of analyzed beads: uncoated, $n = 34$; coated, $n = 145$). This toxin blocks actin polymerization, and as it could be observed in the picture, the number of polymerized filaments is lower than that in untreated cells. In some cases, the rearrangement and organization around the bead in the actin cytoskeleton was visible, but in most cases it was not. The histogram shows the fraction of cells enveloped by actin filaments. The data are representative of at least two independent experiments.

Fibronectin-coated beads display a larger amount of enveloped beads. However, it is important to highlight that the number of fully enveloped beads is definitely lower in cells that were treated with cytochalasin D.

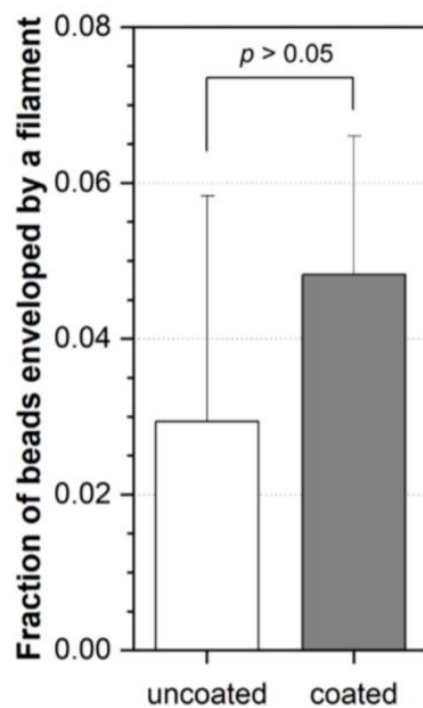
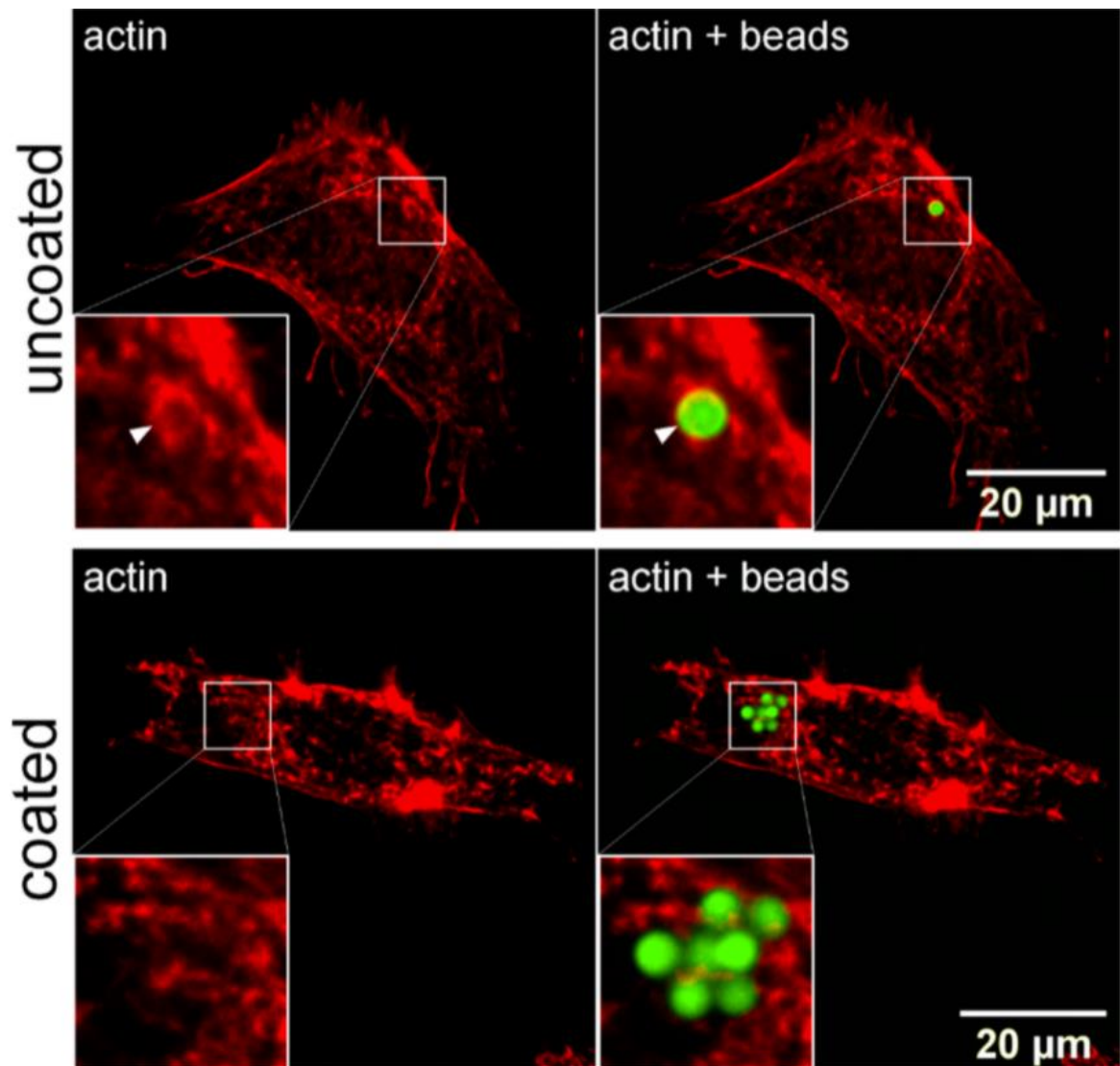


Figure 34. Absence of actin filament envelope of beads after cytochalasin D treatment. Actin-enveloped beads were quantified and reported as percentages of the total number of beads that interacted with cells. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

4.2 THE ROLE OF ELASTIC SUBSTRATE IN LATEX BEAD MEDIATED CYTOSKELETON REMODELLING: FORMATION OF NOVEL MICROTUBULE STRUCTURE

The goal of this part of the research was to examine the influence of elastic substrates on the architecture of microtubules during the cell – bead interaction.

It is obvious that glass does not have a similar stiffness to tissues. Its Young's modulus is defined in tens of GPa (10^9 Pa), while for tissues, these values are defined in kPa (10^3 Pa). To give an example, adipose tissue has the lowest stiffness (0.5 - 1 kPa), bone marrow (0.5 – 1.5 kPa) and brain tissue (1 – 4 kPa), while bone tissue has the highest stiffness (15,000-20,000 kPa) [226].

Therefore, elastic substrates are increasingly used in research (more on preparation in Chapter 3, Materials and Methods), because they can mimic the mechanical properties of the natural environment of cells as much as possible.

In order to explain how elastic substrates affect the bead- cell (mainly microtubule cytoskeleton) interaction, polyacrylamide substrates with the elasticities of 2 kPa, 22 kPa, and 40 kPa were prepared and top coated with fibronectin.

MEF 3T3 cells were plated and cultured overnight on those substrates as described in the Materials and Methods section. Cells were then incubated with carboxylated YG fluorescent latex beads, uncoated or coated with fibronectin, for 1 hour, then fixed, stained against tubulin, imaged, and analyzed as described above. The obtained data were compared with the respective data for cells plated on glass substrates.

During these experiments, emphasis was placed on the study of the microtubule cytoskeleton organization [**Figure 35**].

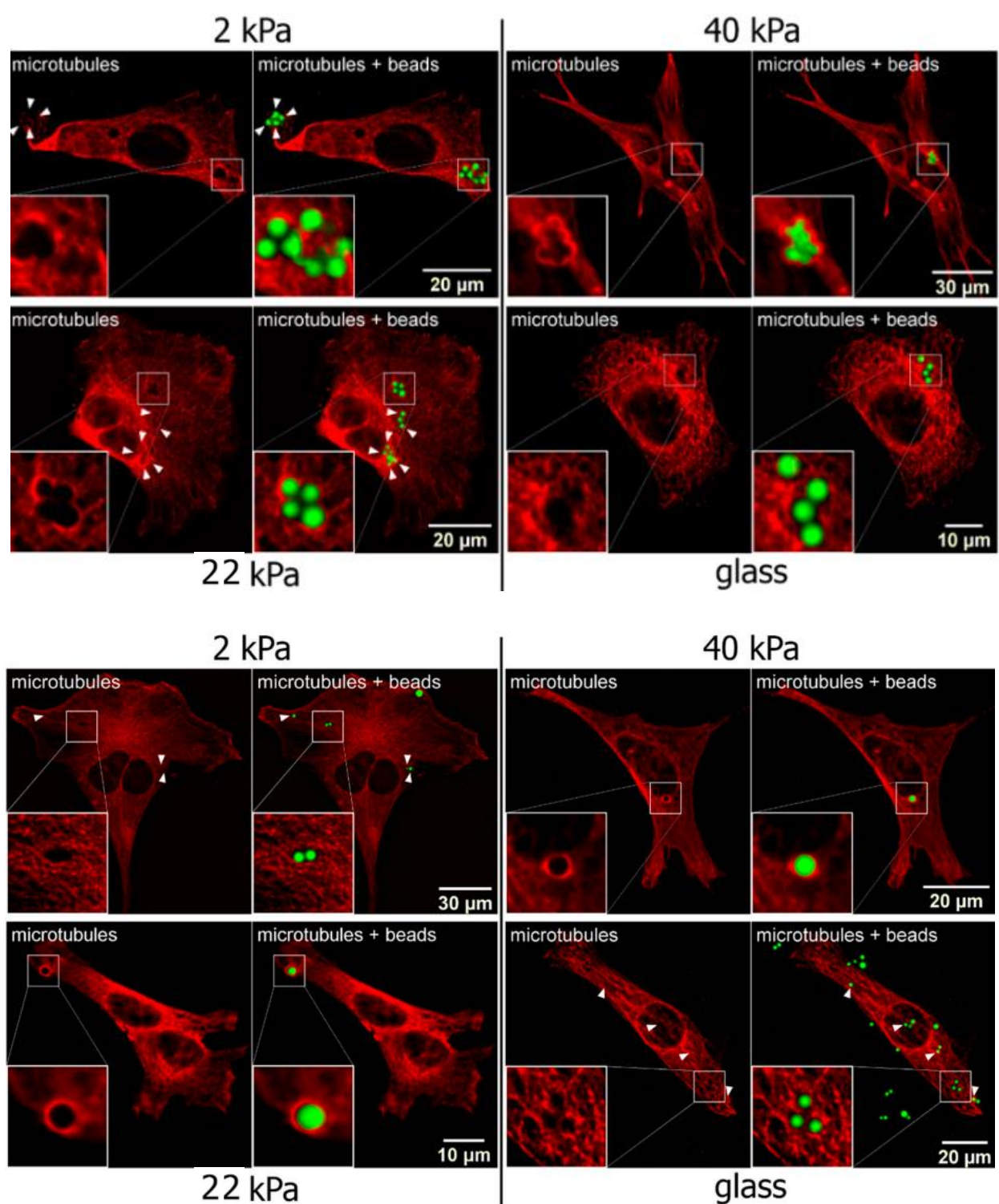


Figure 35. Microtubule cytoskeleton organization influenced by substrate elasticity and latex beads. Arrows point at spots where beads were enveloped by the cytoskeleton and visible changes could be observed. Images are presented as sums of stacks. In order to illustrate potential variations in flexibility, two sets of images were gathered. Image posted with permission from *International Journal of Molecular Sciences MDPI*.

In **Figure 35** one can see two sets of images for each substrate's elasticity. It can be observed that on flexible substrates, the result of bead-microtubule interaction looks different than on glass substrates. We can observe cases where microtubules organize themselves around beads, creating a rather unusual structure for them, similar to actin structures, called “phagocytic cups”, formed during endocytic processes. Until now, endocytic processes had been linked to the cytoskeleton only in the context of actin involvement [227]. An example of such structure formed by actin in cells grown on glass is shown on **Figure 36**.

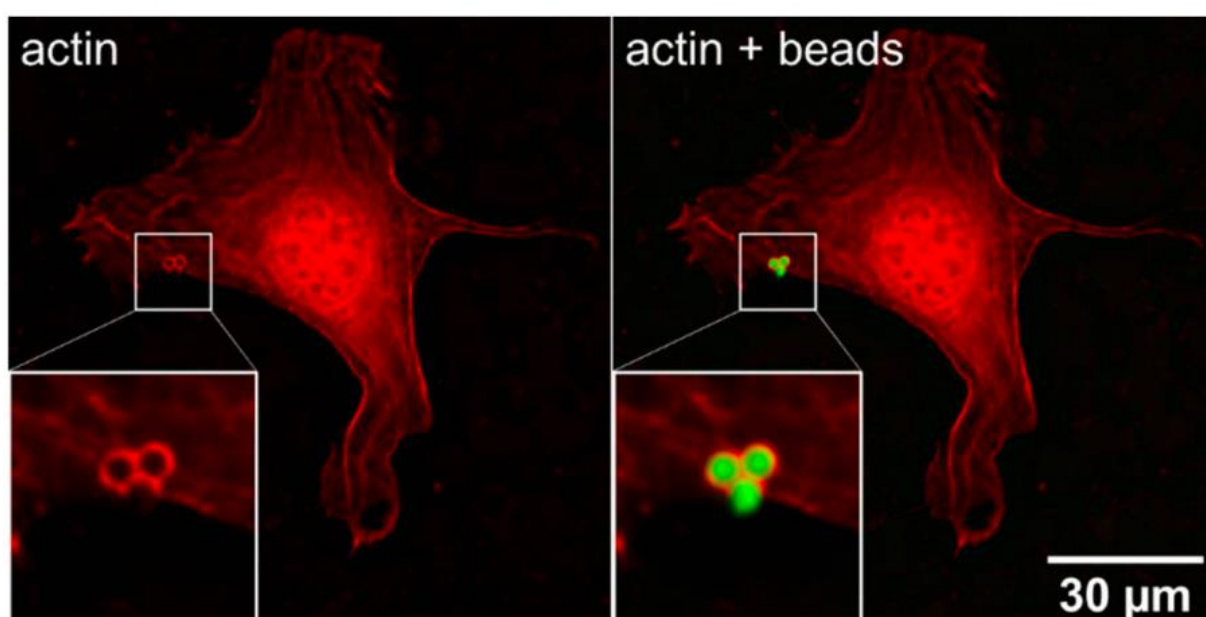
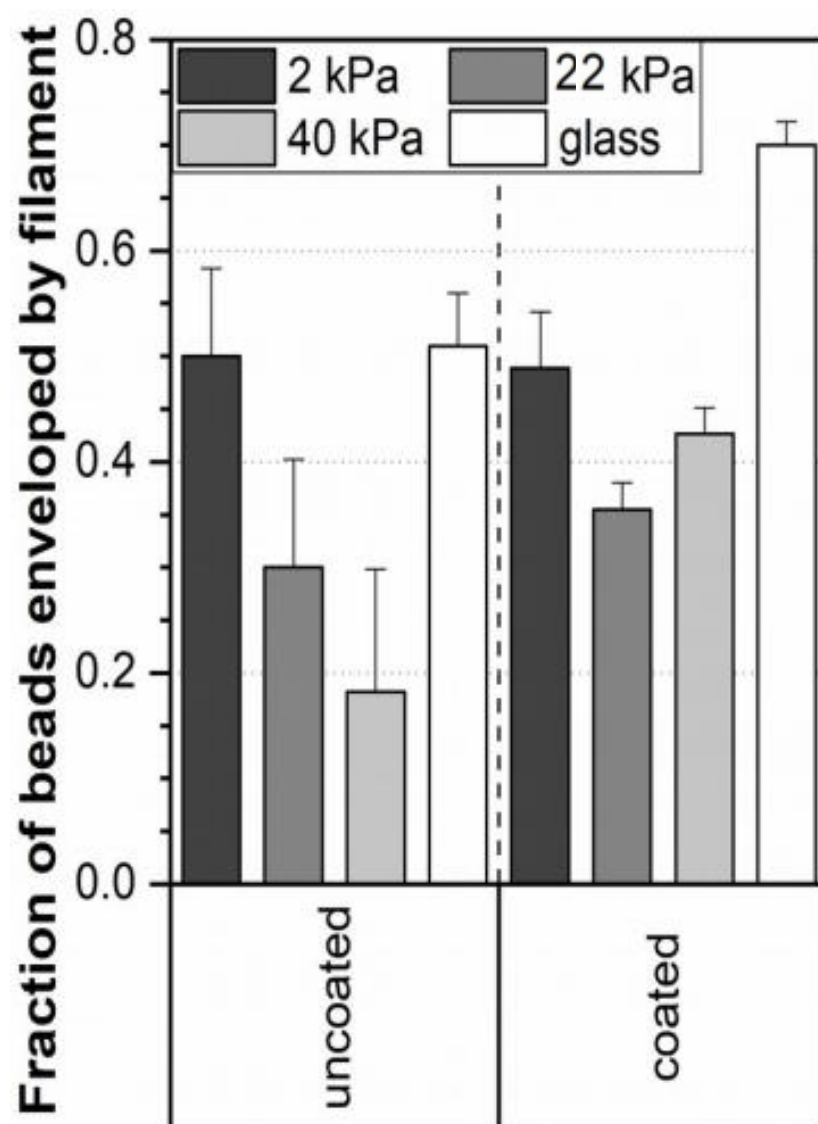


Figure 36. A cup-like structure formed with actin filaments in cell grown on glass. Images presented as sum of stack. Image posted with permission from *International Journal of Molecular*

As with the previous data, we checked the percentage of beads enveloped by microtubule filaments. These results are shown in **Figure 37**.

Numbers of analyzed beads for **Figure 37**: 2 kPa, uncoated, $n = 36$; 2 kPa, coated, $n = 88$; 17 kPa, uncoated, $n = 20$; 17 kPa, coated, $n = 369$; 40 kPa, uncoated, $n = 11$; 40 kPa, coated, $n = 394$; glass, uncoated, $n = 100$; glass, coated, $n = 434$.



	coated vs uncoated
2 kPa	$p > 0.05$
22 kPa	$p > 0.05$
40 kPa	*
glass	***

uncoated	22 kPa	40 kPa	glass
2 kPa	$p > 0.05$	*	$p > 0.05$
22 kPa		$p > 0.05$	$p > 0.05$
40 kPa			*

coated	22 kPa	40 kPa	glass
2 kPa	*	$p > 0.05$	***
22 kPa		*	***
40 kPa			***

Figure 37. Histogram showing the fraction of beads enveloped by microtubules in cells grown on elastic substrates. Two proportion Z-test was used to determine statistical significance between specific samples: (left) uncoated versus coated beads; comparison between different substrate elasticities using uncoated (upper right) and coated beads (lower right). * $p < 0.05$, *** $p < 0.001$. Plot posted with permission from International Journal of Molecular Sciences MDPI.

On stiffer substrates (glass and 40 kPa), there are more coated beads enveloped than uncoated ones **[Figure 37]**. When observing cells plated on elastic substrates at 2 and 22 kPa, one can see that the fraction of enveloped beads is not changed by bead coating. It is worth mentioning that the presence of “cup-like” microtubule structures can be observed in regions where the beads were positioned on the cells grown on elastic substrates with Young moduli of 22 kPa and 40 kPa **[Figures 38, 39]**.

This research into this phenomenon revealed that microtubule cup-like structures formed mostly on elastic substrates. A partial and very indistinctly cup shape was occasionally visible on the glass, but this occurrence is rather rare.

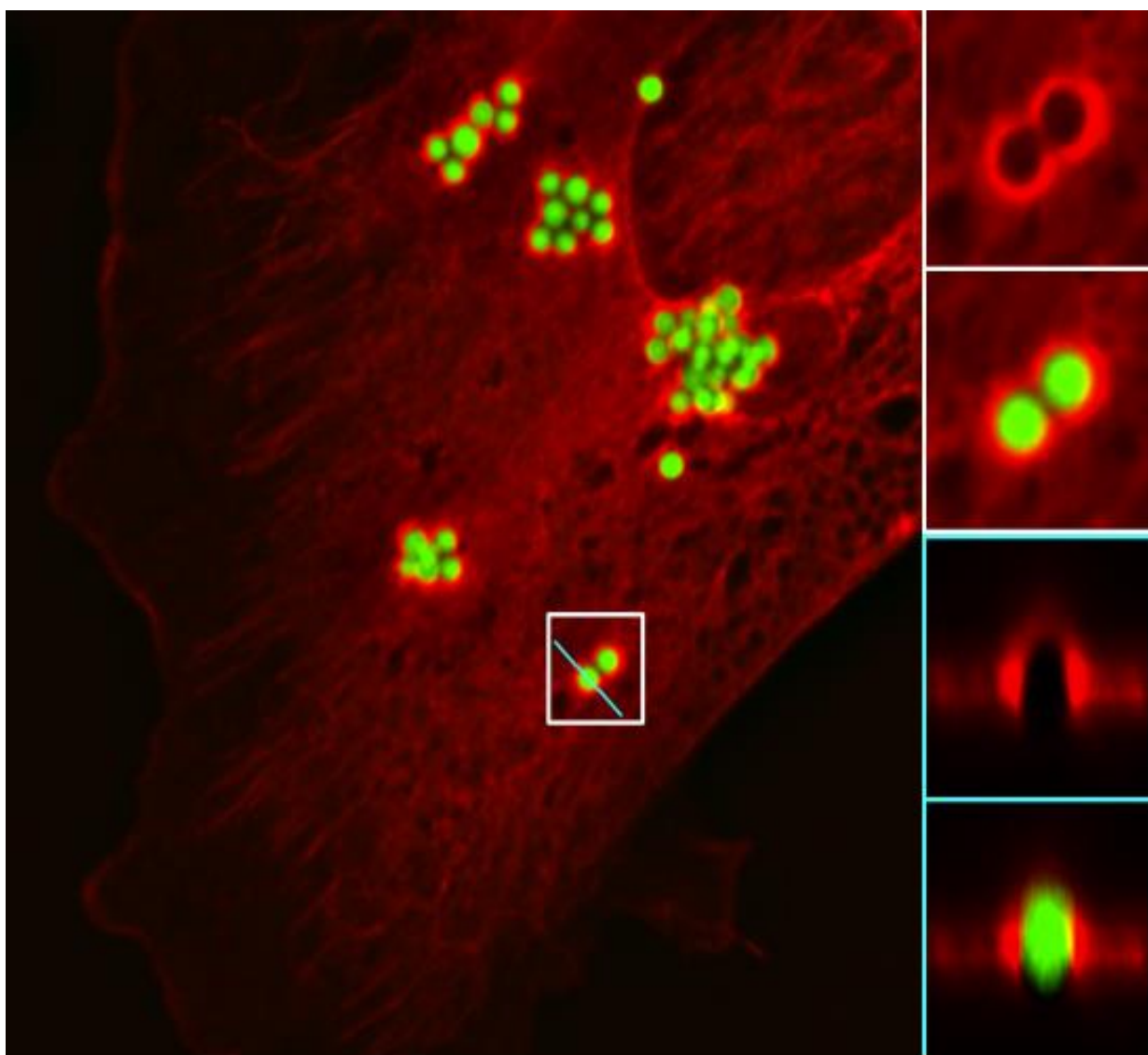


Figure 38. Plated MEF cells on a substrate with an elasticity of 22 kPa were scanned as three-dimensional images and displayed as a stack sum. Green indicates beads, while red indicates microtubules. An axial projection and an enlarged Z- section of the three-dimensional image are displayed on the right (white and cyan frames). Image posted with permission from *International Journal of Molecular Sciences MDPI*.

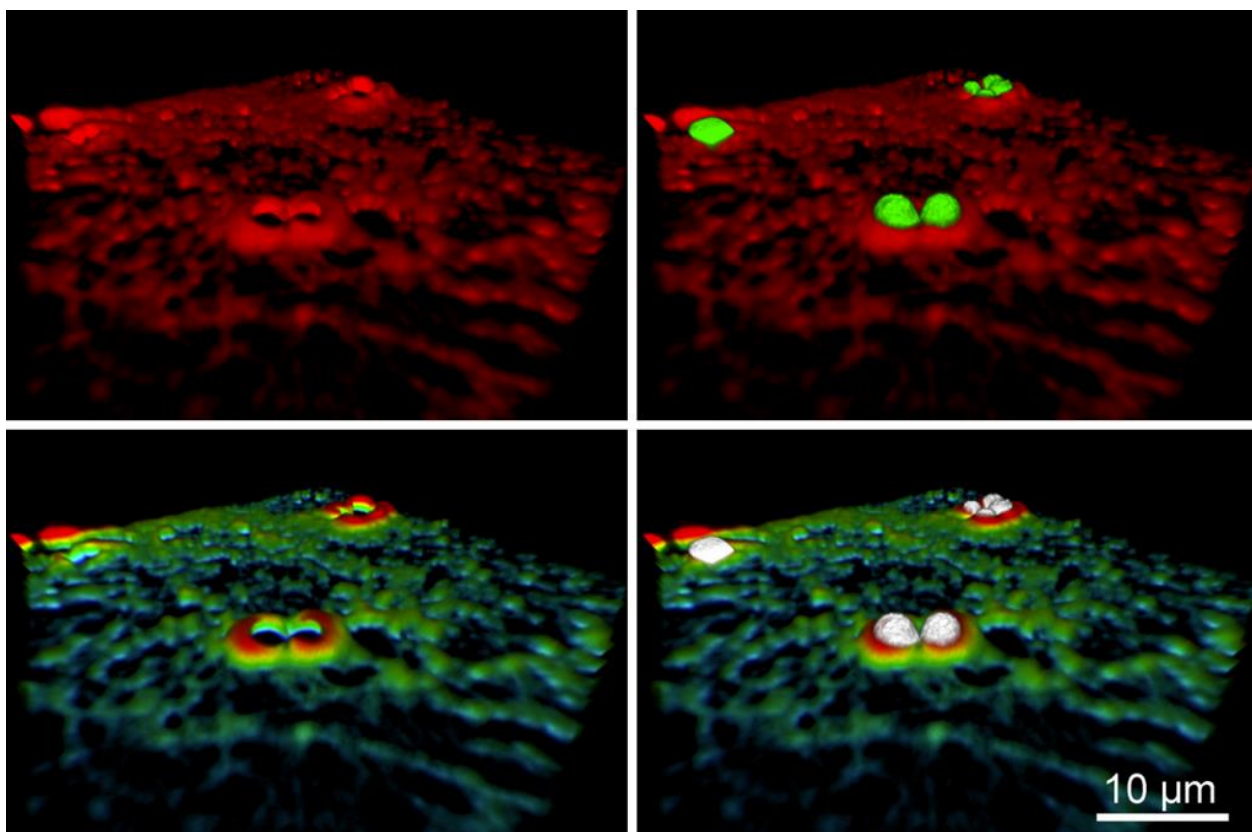


Figure 39. A 3D rendering of the microtubule cup-like structure. Upper panels: Beads (green) are surrounded by a microtubule surface (red). Lower panels: Latex beads are white and the color-height labelling for microtubule filaments (blue → green → yellow → red: low → high). Image posted with permission from *International Journal of Molecular Sciences MDPI*.

The observations showed the development of structures similar to the actin phagocytic cup, formed by microtubules, which, as it has been postulated thus far, do not take an active role in the process of endocytosis. This could indicate that the microtubule cytoskeleton actively participates in the phagocytosis process.

An exemplary visual representation of a cup is presented in **Figures 38 and 39**. The place where microtubules form the cup structure is depicted and zoomed in **Figure 38**. Additionally, the sideview image shows that the spheroid is fully enveloped with filaments. **Figure 39** depicts the identical cell fragment as in Figure 38, which is a three-dimensional projection that exposes the cup and cavity structure. The image was generated in *Fluorender* using color-height labelling to represent the microtubule filaments signal.

4.3 MICROTUBULE ASSOCIATED PROTEINS AND REMODELLING OF MICROTUBULE CYTOSKELETON

The second part of study aims to point out which MAP family or related proteins may be involved in the formation of microtubule cup-like structures. Three proteins were tested: CLIP1 (CAP-Gly domain-containing linker protein 1), IQGAP1 (Ras GTPase-activating-like protein) and MARK2 (microtubule affinity regulating kinase 2).

There were no noticeable differences in the behavior of MAP proteins when comparing substrates with an elasticity of 8 kPa and 40 kPa.

4.3.1 CAP-GLY DOMAIN CONTAINING LINKER PROTEIN 1 (CLIP1)

The first stage of this research was to visualise the cellular localization of investigated protein during the bead - cell interaction process. 3D image reconstruction technique, using the Z-stack of confocal images, was employed to pinpoint the position of the protein throughout the cell body. By applying maximum intensity projection to determine the intracellular location of the protein concentration, it has been possible to identify which external factors, such as the elasticity of the substrate or the presence of beads, exert the most significant influence on the protein localization [**Figure 40**].

MEF 3T3 cells were grown as described before, one sample was cultured overnight on substrate 40 kPa and the other one on the glass. Next day cells were fixed, stained against tubulin and CLIP1 protein, imaged, and analyzed.

Although it is typically challenging to determine the protein content in the cytoplasm based on fluorescence intensity, its distribution inside the cell can be assessed with high precision. The distribution of the CLIP1 protein is different in cells grown on a substrate with an elasticity of 40 kPa when compared to cells grown on glass. In the case of glass, CLIP1 is evenly distributed throughout the cell cytoplasm. However, in the case of an elastic substrate, higher concentrations could be observed in the nuclear areas, as shown in the example image [**Figure 40, A**]. Additionally, CLIP1 was also present in areas where the density of microtubules was lower [**Figure 40, B**].

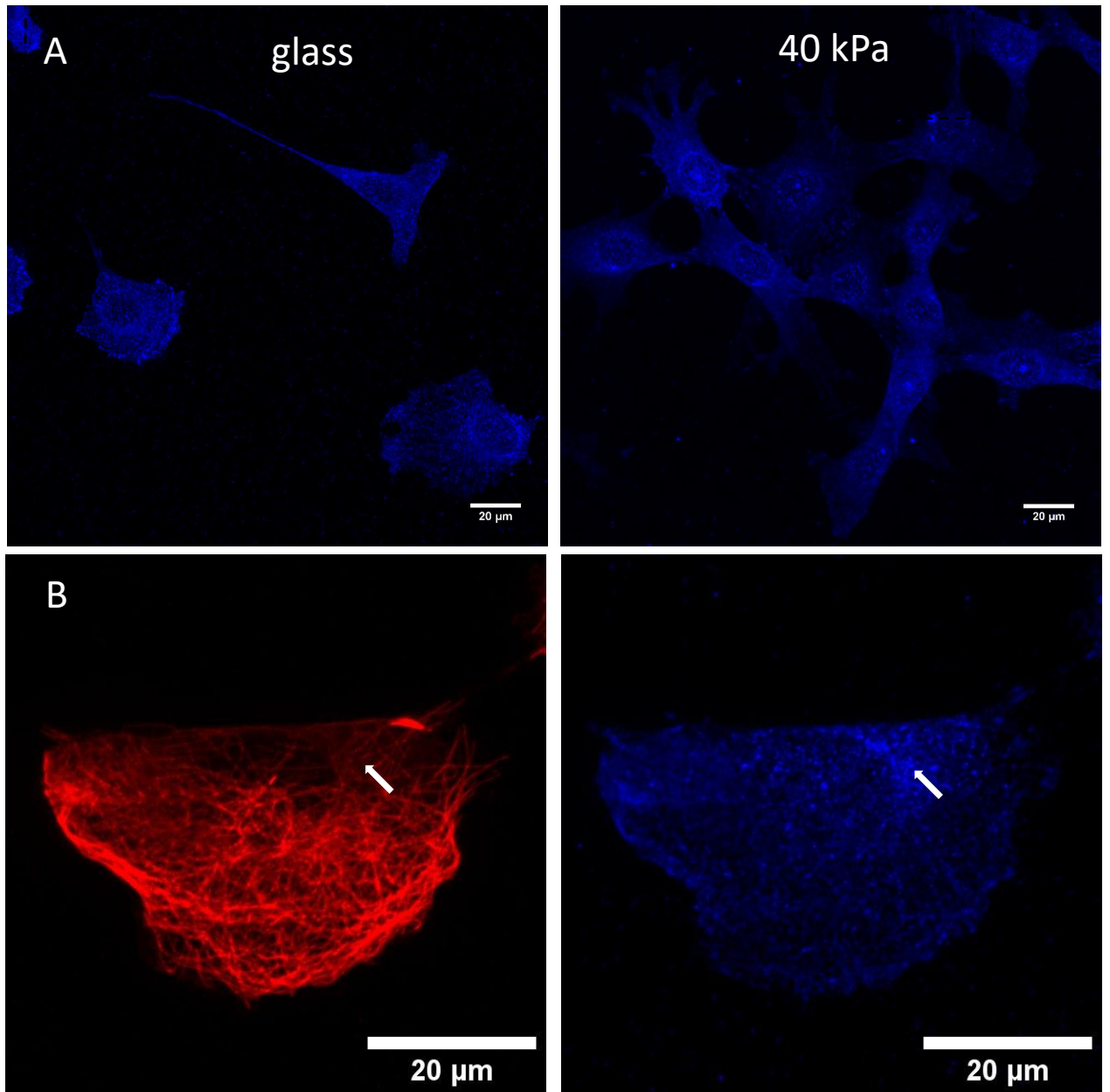


Figure 40. (A) Comparison of CLIP1 localization in a cell grown on glass and on an elastic substrate (40 kPa). (B) Images of microtubules (red) and CLIP1 (blue), arrow points a spot in cell where we observe the lower fluorescent signal from microtubules and despite this we observe a higher signal from CLIP1 in the same time; cells were growing on the glass and beads were no added.

Next, the distribution of CLIP1 in the presence of latex beads was examined (for uncoated and fibronectin - coated beads).

In these experiments, MEF 3T3 cells were plated and cultured overnight on 40 kPa substrates. Cells were then incubated for 1 hour with uncoated or fibronectin - coated carboxylated YG fluorescent latex beads, then fixed, stained against tubulin and CLIP1, imaged, and analyzed as described above.

The exemplary results are shown on **Figure 41 and 43**.

CLIP1 accumulation around the beads can be observed for both uncoated and fibronectin-coated beads [**Figures 41, 43**]. Some accumulation of microtubules can be seen around beads in the images, but it is not as pronounced. Important information is that the cells were fixed after 1 hour of bead- cell interaction, so it is possible that the tubulin ring has not yet had time to form and would have formed over time. This is intriguing because it reveals which protein, apart from tubulin, may be involved in the formation of the ring surrounding the bead.

For each of the image color channels, plots that show the dependence of pixel brightness intensity were generated, as shown on exemplary **Figures 42 and 44**. These plots are constructed using relative values (grey values) for specific image pixels along the white lines posted on the images. Because of this, it is possible to assess in both cases the approximate distribution of CLIP1 and microtubules around the bead.

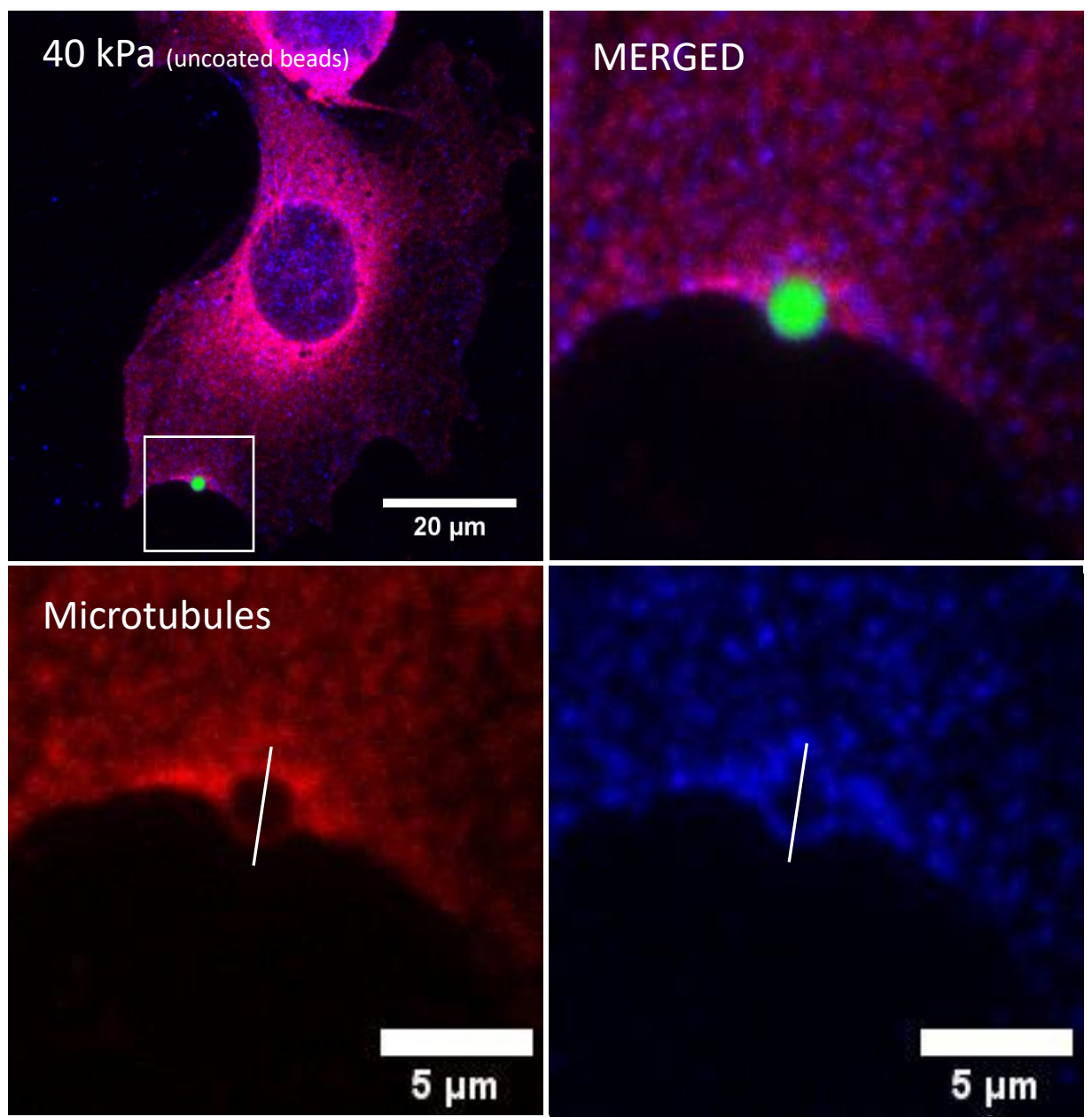


Figure 41. Images of microtubules structures (red), CLIP1 (blue) and fluorescent uncoated latex beads (green). Cells were grown on 40 kPa substrate. Fragment of picture marked with white square has been zoomed in separately for each channel.

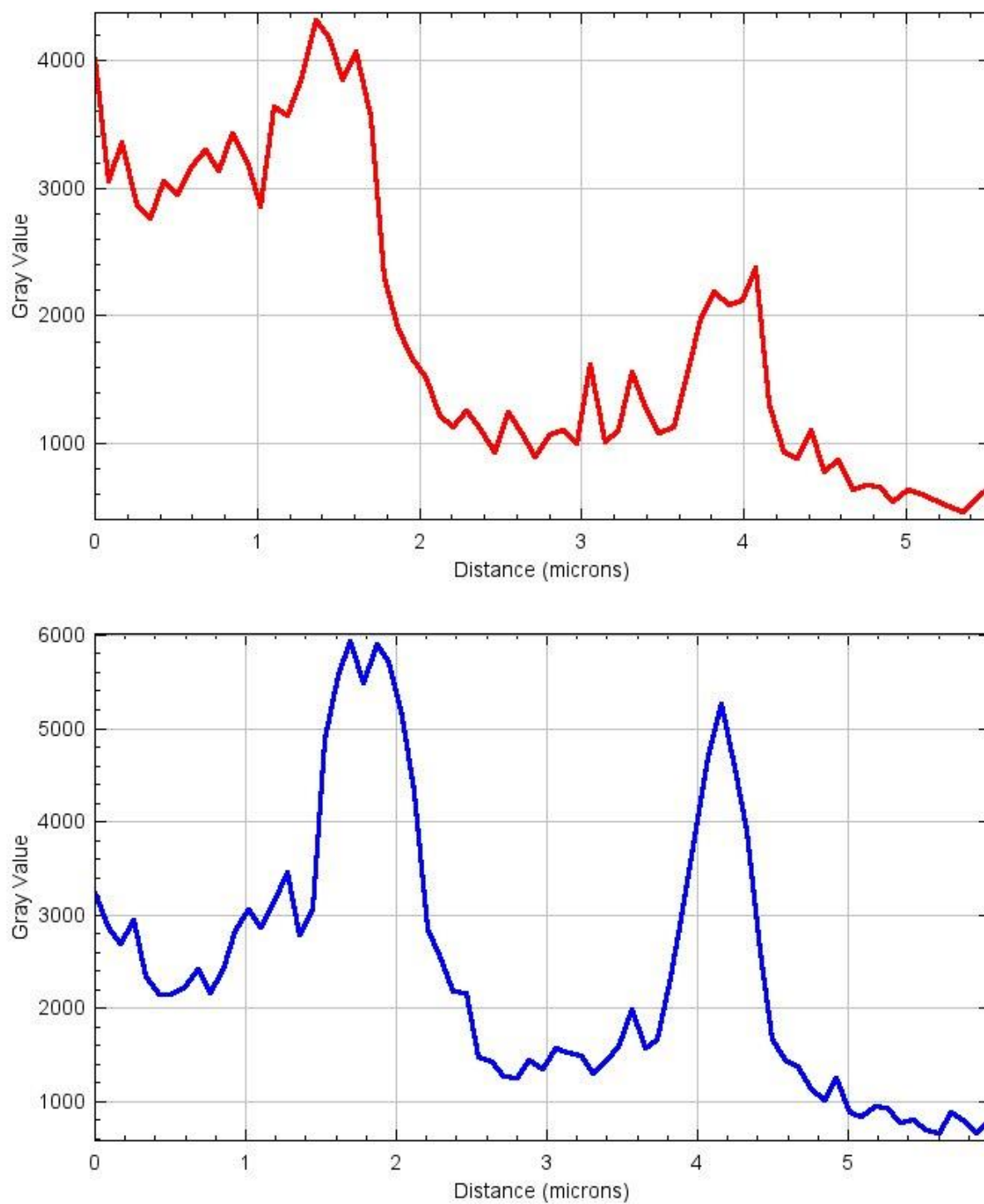


Figure 42. Color plot profile of grey value parameter (pixel brightness) for microtubules (red) and CLIP1 (blue) for Figure 41 (fluorescence intensity profile measured along a white line).

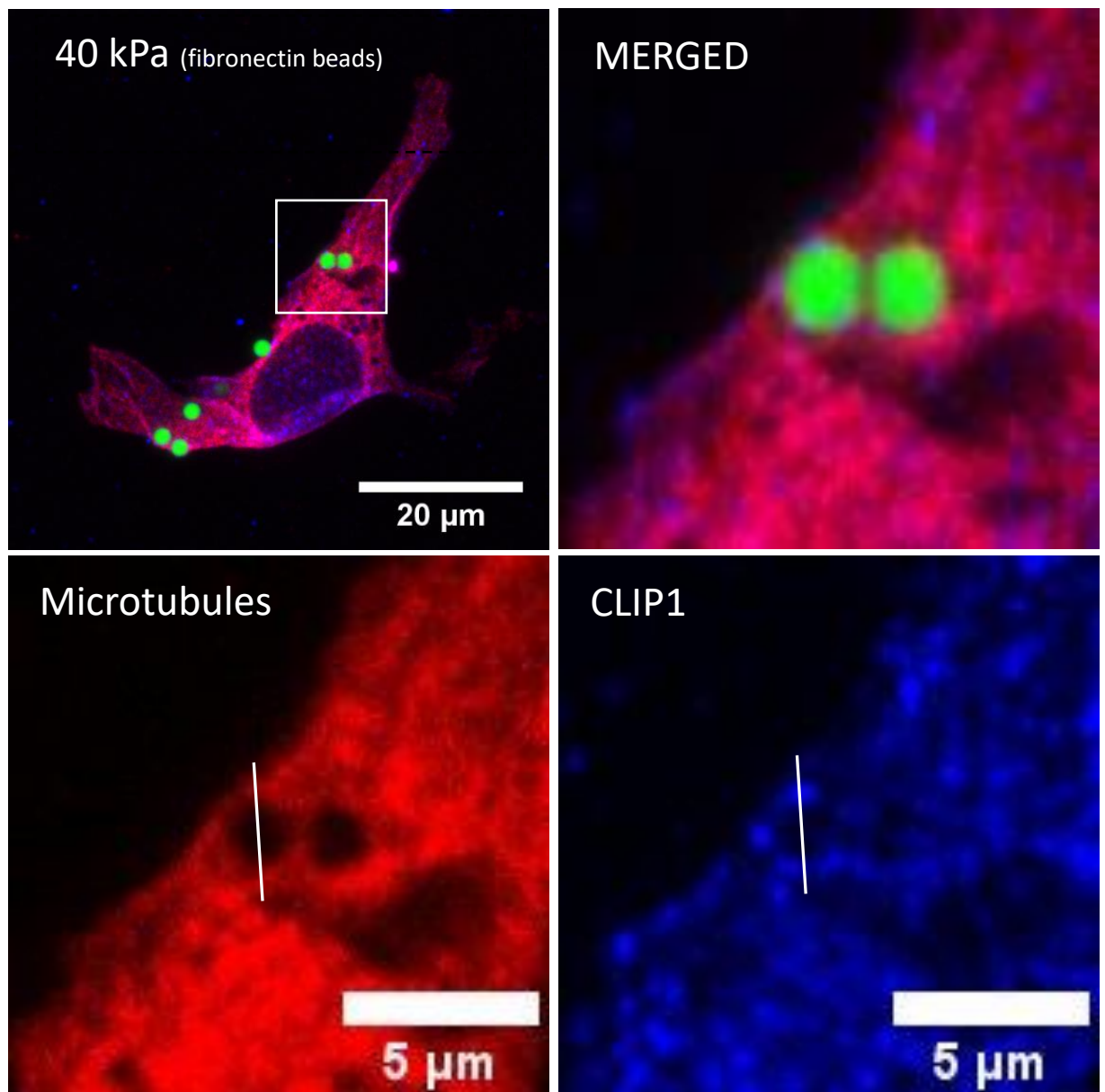


Figure 43. Images of microtubules structures (red), CLIP1 (blue) and fluorescent fibronectin coated latex beads (green). Cells were grown on 40 kPa substrate. Fragment of picture marked with white square has been zoomed in separately for each channel.

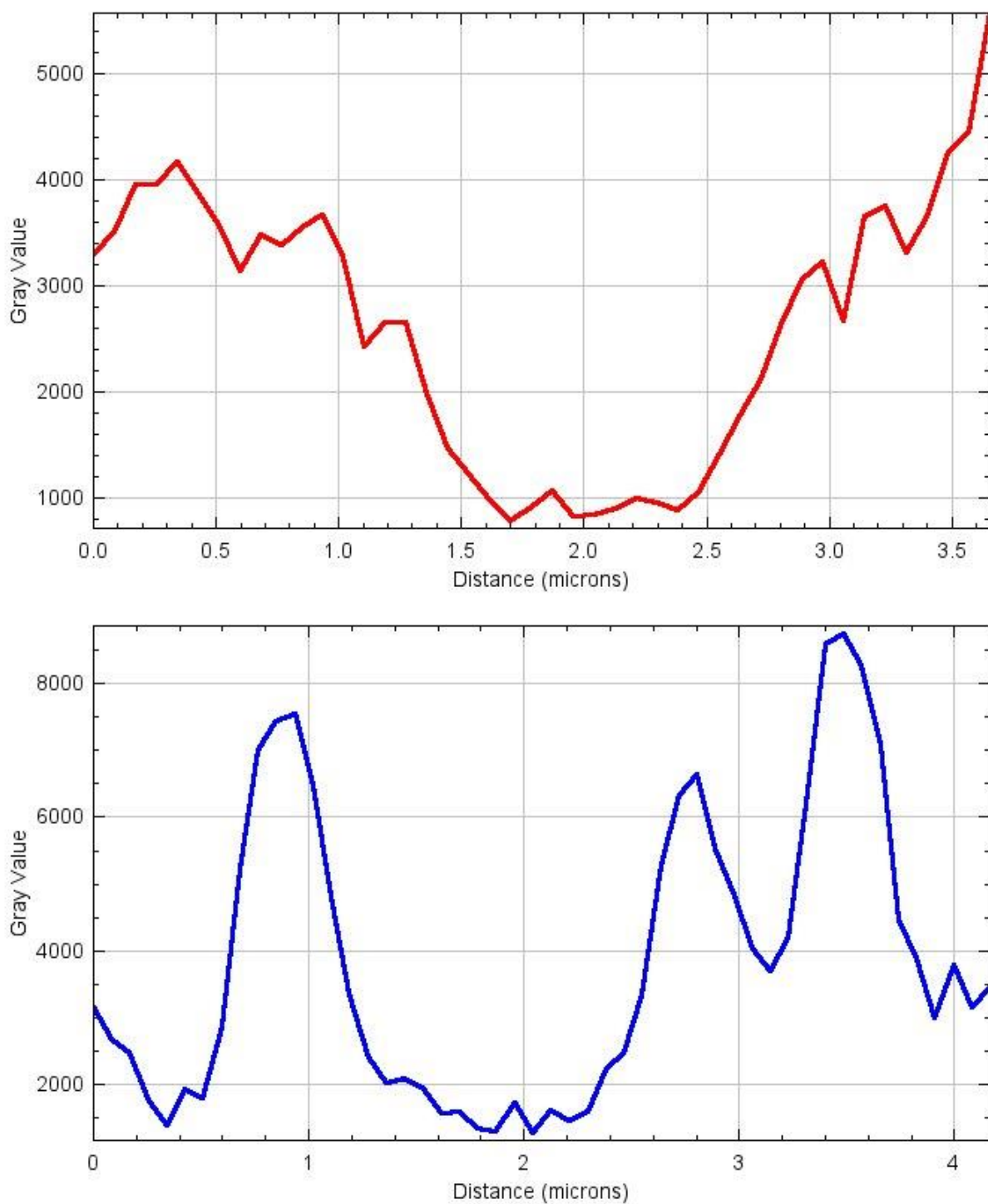


Figure 44. Color plot profile of grey value parameter (pixel brightness) for microtubules (red) and CLIP1 (blue) for Figure 43 (fluorescence intensity profile measured along a white line).

4.2.2 RAS GTPase – ACTIVATING – LIKE PROTEIN (IQGAP1)

IQGAP1 was the next protein that was investigated. This protein mainly interacts with the actin cytoskeleton [145], but it has also been proven to be related to microtubules [150], [152]. Moreover, it interacts with the CLIP1.

First the spatial distribution of the protein in the cell was assessed. In the case of cells grown on glass, we observe that IQGAP1 localizes within the nucleus but when the cells are plated on an elastic substrate, IQGAP1 localizes in the cytoplasm. This effect is very pronounced, and the results are shown in **Figure 45**. This is not seen with CLIP1 protein.

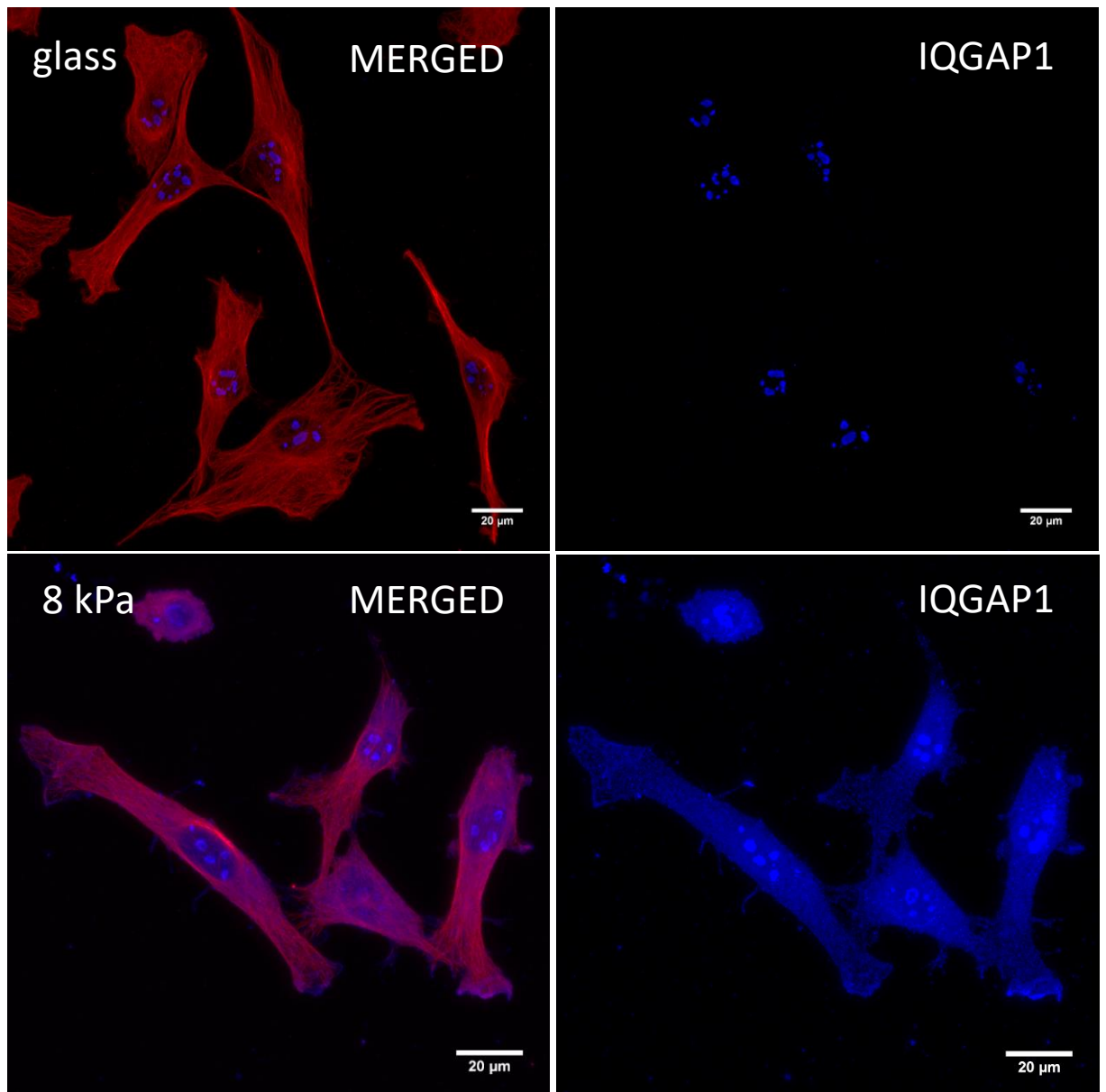


Figure 45. Comparison of the spatial distribution of the IQGAP1 protein (blue) and tubulin (red) in the cell plated on a glass (upper panel) and on substrate with an elasticity 8 kPa (lower panel).

The next step was to check how the IQGAP1 protein behaves in the presence of latex beads that are non-functionalized or coated with fibronectin. As shown in **Figures 46 and 47**, no organisation or formation of cup - like structures around the beads can be observed.

In the first image [for uncoated beads- **Figure 46**], there is a large accumulation of microtubules around the bead. However, IQGAP1 does not form rings or any visible structures in the place of cell contact with the bead, even when microtubules do.

The result is a bit surprising despite the fact that IQGAP1 interacts with CLIP1 and that an accumulation of the CLIP1 protein around the beads has been described in the previous section. IQGAP1 is also closely related to the actin that forms the phagocytic cups, which could also suggest that this protein should exhibit similar organisation when cells interact with beads.

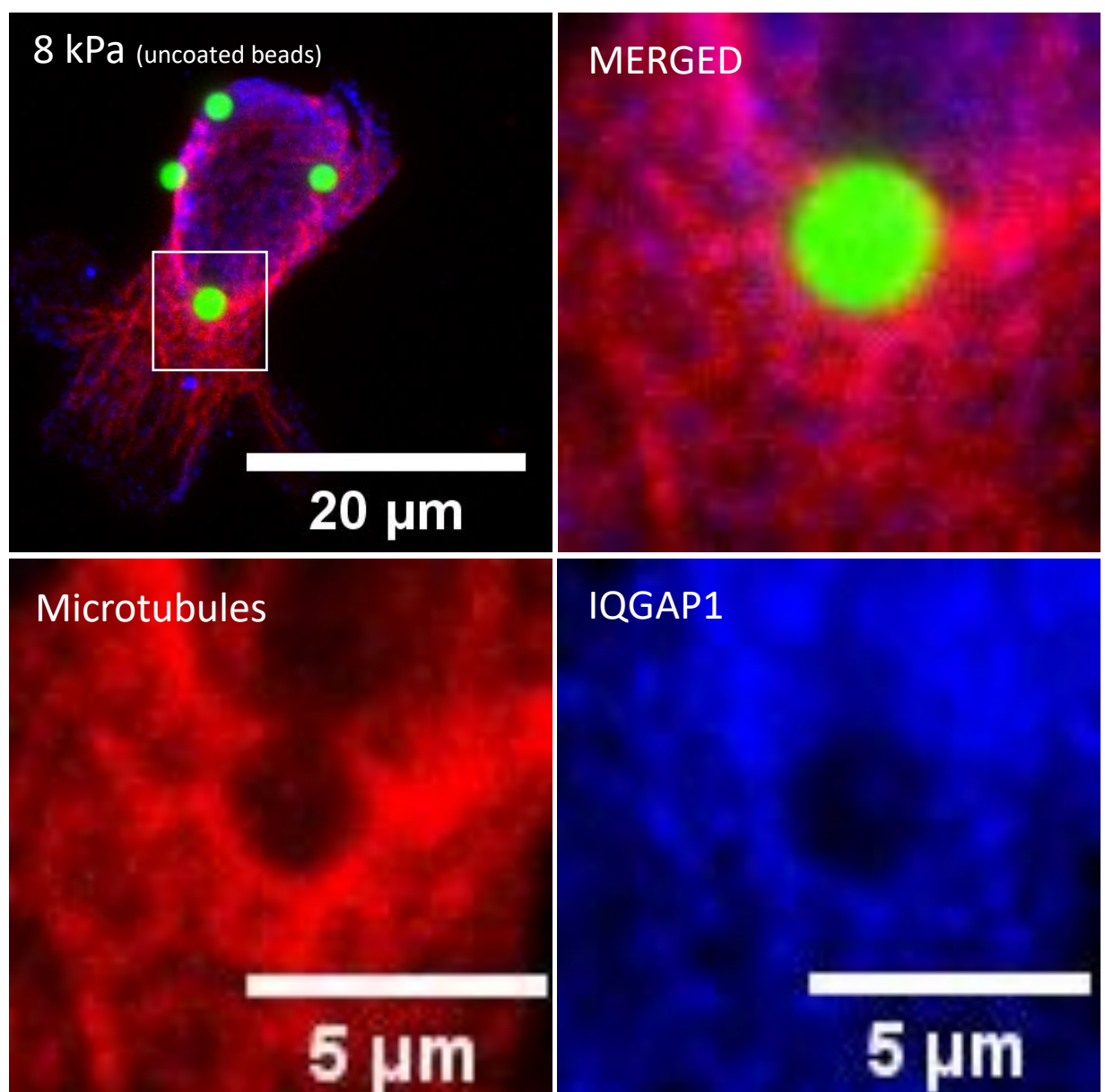


Figure 46. Images of microtubules structures (red), IQGAP1 (blue) and fluorescent uncoated latex beads (green). Cells were grown on 8 kPa substrate. Fragment of picture marked with white square has been zoomed in separately for each channel.

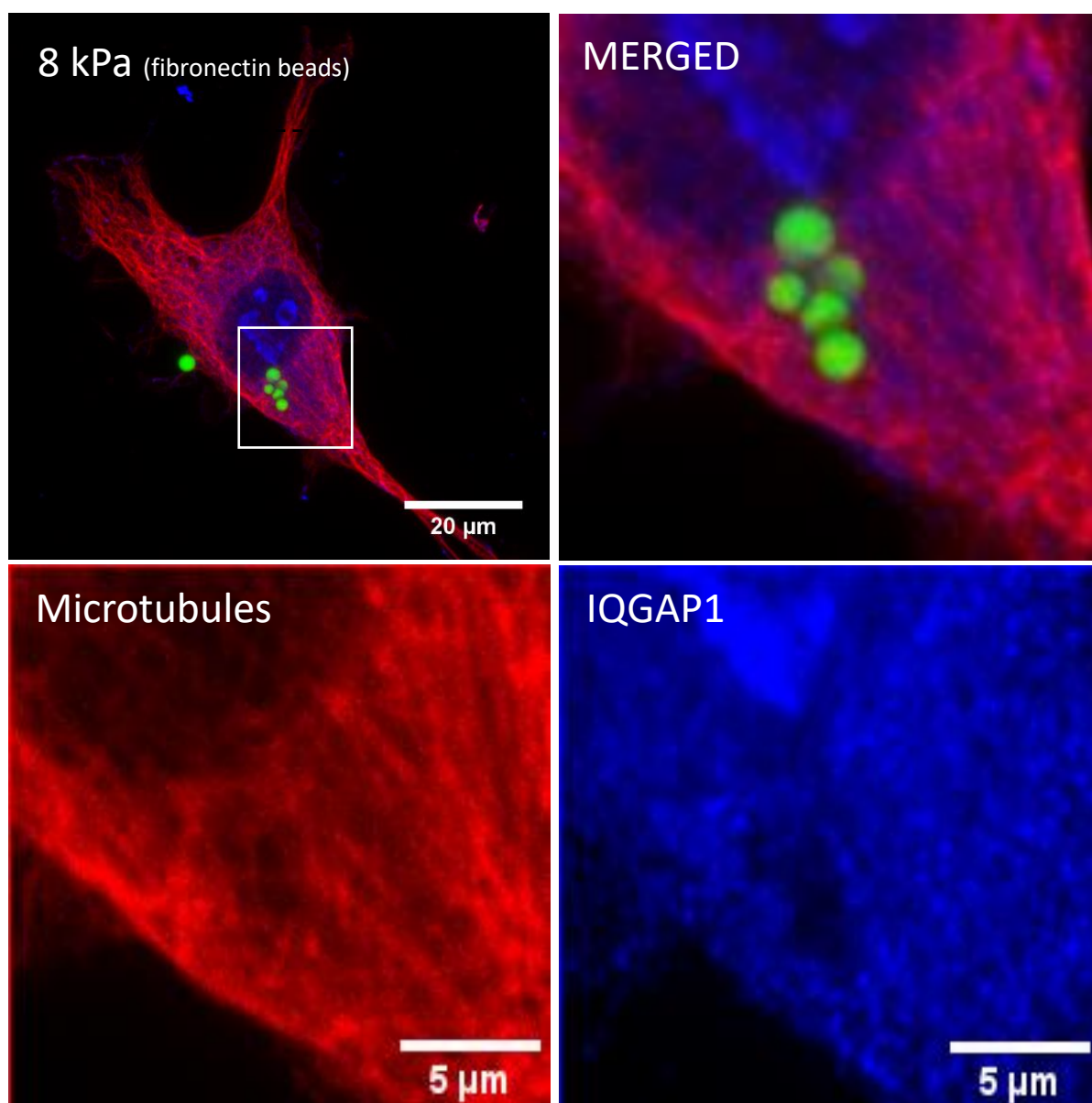


Figure 47. Images of microtubules structures (red), IQGAP1 (blue) and fluorescent fibronectin-coated latex beads (green). Cells were grown on 8 kPa substrate. Fragment of picture marked with white square has been zoomed in separately for each channel.

4.2.3 MICROTUBULE AFFINITY – REGULATING KINASE 2 (MARK2)

The third protein that was tested was MARK2, a protein that phosphorylates microtubule-stabilising proteins from the MAP family.

As one can see, in **Figure 48** MARK2 shows a strong accumulation in the nuclear area with weaker cytoplasmic component, in both glass and elastic substrates.

As previously pointed out (Chapter 4.1.1), the microspheres change the architecture of each cytoskeletal filaments architecture component. The goal of this research was to determine how MARK2 responds to cell-bead contact. In most cases, beads induce microtubule rearrangement and MARK2 voids in the place where beads are located [**Figure 48, A**]. The rest of cases show no changes in either the organisation of microtubules or MARK2 presence [**Figure 48, B**].

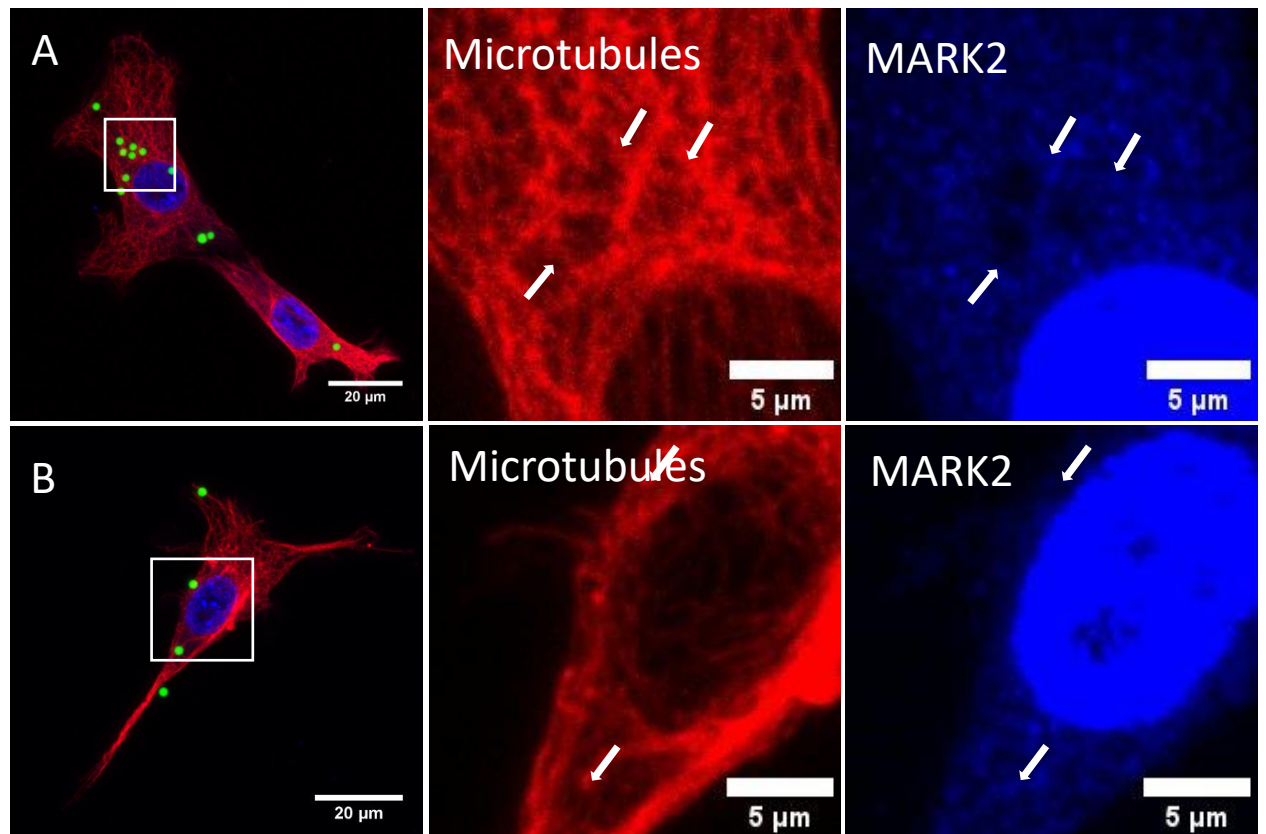


Figure 48. Images of microtubules structures (red), MARK2 (blue) and fluorescent fibronectin-coated latex beads (green). (A) Images of microtubules structures and localization of MARK2, arrows point a spots where beads **cause rearrangement** of microtubules and lack of MARK2 in the same time (B) The same stained proteins, arrows point spots where beads **do not cause rearrangement** of microtubules and do not cause the lack of MARK2.

5. FINAL CONCLUSIONS

- The beads force the cytoskeleton of the fibroblast to rearrange in just as little as 15 minutes of interaction, and many of the beads are completely enveloped by the cytoskeleton.
- After 3 hours of incubation, the respective cytoskeleton engulfs more fibronectin-coated beads, though the percentage of engulfed beads varies depending on the cytoskeleton component, varying from 28% for microtubules to 45% for actin.
- For uncoated beads, the results are different for every type of filament; vimentin envelops the majority of absorbed beads (50%), followed by actin (30%), and microtubules (10%).
- In the case of microtubules, uncoated beads are more effectively engulfed after 15 minutes than after 3 hours of incubation. This result might indicate that actin, vimentin and microtubules have separate strategies to engulf beads.
- After application of the cytochalasin D, the fraction of fully enveloped beads by actin filaments decreases 10 times in comparison with untreated cells.
- The elastic substrates affect the bead envelopment by microtubules.
- In some cases microtubules surround the beads forming „cup-like“ structures.
- Accumulation of CLIP1 around the latex beads is observed. In the some cases there is no similar accumulation of microtubules.
- IQGAP1 and MARK2 do not exhibit any specific organisation around the microspheres.

6. DISCUSSION

6.1 CYTOSKELETAL REARRANGEMENT AS A RESULT OF CELL – BEAD INTERACTION

The cytoskeleton architecture was investigated before and after interactions of cells with latex beads with a diameter of 2 μm , either functionalized with fibronectin or nonfunctionalized. These kinds of beads are frequently used in endocytosis studies and optical tweezers-based measurements. Our study shows that interaction of cells with beads deposited on their surface significantly changes the cytoskeleton structure in as little as 15 minutes. Images of actin, microtubules, and vimentin in cells grown on a glass substrate clearly demonstrate what appears to be a void area of the respective filaments where the beads were present **[Figures 29 - 32]**. The beads push apart the cytoskeletal filaments, which creates an empty space. The differences in the filaments architecture surrounding beads may be observed 15 minutes and 3 hours after introduction of beads to the cellular culture.

Actin periodically (~20% of all cases) created what seemed to be like a cup-like structure around the bead **[Figure 36]**.

As expected, this effect is weaker with uncoated beads **[Figures 29 and 31]**.

An in-depth analysis of the orthogonal cross- sections of 3D pictures of cells with stained components of the cytoskeleton reveals that beads are frequently engulfed by the respective filaments **[Figure 38, cyan panel]**. After 15 minutes, the actin and vimentin cytoskeleton exhibit signs of an effort to engulf the bead, as evidenced by raised edges of the respective filaments surrounding the bead **[Figure 29 and 30]**. Due to the very weak fluorescence coming from the top layer, these places appear as voids in the filaments. After 15 minutes and 3 hours of bead - cell interaction, fractions were compared to determine the effectiveness of latex beads being enveloped by each filament type.

Results show that 20% of the fibronectin-coated (FC) beads, that were set down on the substrate, are engulfed after 15 minutes, compared to small fraction of the uncoated

(UC) beads, in the case of actin. After 3 hours, it is noticeable that over 30% of the UC beads and around 45% of the FC beads have been completely enveloped. Vimentin, however, exhibits a different dynamic in the process of bead envelopment: at first, vimentin envelops 5% of UC and 15% of FC beads, but after 3 hours, more UC beads (50%) than FC beads (45%) are enveloped by vimentin. The situation changes in the case of microtubules: after 15 minutes, nearly 30% of UC beads and only 8% of FC beads are enveloped by microtubules. The percentage of UC beads that are microtubule enveloped then decreases to 8% after 3 hours of incubation, while the percentage of FC beads increases to 27%. This demonstrates that the interactions between the cytoskeleton components and the UC and FC beads have different dynamics. These results also demonstrate that the cytoskeleton plays a more complex role in endocytosis than previously believed, particularly when taking into consideration the time frame of such a process.

6.2 CYTOCHALASIN D INFLUENCES THE BEAD ENVELOPMENT PROCESS

In this part, it was observed how cytoskeletal drugs affect observable changes in the architecture of filaments after latex bead settlement. The impact of cytochalasin D, which inhibits actin polymerization was studied [228]. In this case, the changes in the structure of actin filaments after cytochalasin application were observed - visible stress fibers were difficult to observe, and a small percentage of the beads were enveloped (5% fibronectin-coated beads, 3% uncoated, compared to previously recorded 30–40%). This suggests that cytoskeleton homeostasis regulation dysfunction reduces the ability of cells to respond to mechanical stimuli. It also shows how the architecture of the normal cytoskeleton is significantly impacted by the deformations forced by the beads used in optical tweezers-based methods.

6.3 INFLUENCE OF ELASTIC POLYACRYLAMIDE SUBSTRATES ON MICROTUBULE ARRANGMENT

Cells behave differently when grown on substrates with different stiffness, as shown in a number of previous studies [202], [203]. The changes of the architecture of the microtubule cytoskeleton caused by the cell – bead interaction of fibroblast plated on polyacrylamide elastic substrates with different elasticities — 40 kPa, 22 kPa, and 2 kPa — were analysed against those plated on glass.

Both uncoated and fibronectin-coated beads were used as in previous experiments (Chapters 7.1 and 7.2). After analysing 3D images quantitatively, an interesting relationship was discovered. First, the percentage difference between the envelopment of unfunctionalized and fibronectin-coated beads was higher in the case of glass and a 40 kPa substrates than in the case of softer substrates—17 kPa and 2 kPa **[Figure 37]**. This suggests that on softer substrates, both coated and uncoated beads are almost identically efficiently enveloped.

The formation of unique, cup-like microtubule structures, which were not observed before, was initially seen in cells grown on elastic substrates of 22 kPa and 40 kPa **[Figures 35, 38 and 39]**. This result was unexpected because, to our knowledge, the microtubule cytoskeleton has not been known to directly take part in phagocytosis.

Importantly, according to current knowledge in the literature, the ability of microtubules to develop cup-like structures during phagocytosis has not yet been verified [231].

In conclusion, these results demonstrate that the architecture of actin, microtubules, and the intermediate cytoskeleton of fibroblast cells is modified by latex beads interacting with cells. When beads were functionalized with fibronectin, the effect was even stronger. The cytoskeleton's remodelling was practically invisible when cytoskeletal drug, Cytochalasin D, was used to interfere with its function.

These results may be important for research on the function and behavior of microtubules. Currently, it is believed that microtubules do not contribute to the endocytosis process, but our results suggest that this may not be the case. Microtubules ability to form cup-like structures on elastic substrates suggests these cytoskeletal components might participate in the endocytosis process in physiological

conditions. This means that the results of these studies need to be considered in future investigations where polystyrene or latex beads are used, such as in phagocytosis studies or Optical tweezers method. Sometimes it is crucial to preserve intact structures in living cells, hence it is important to take into consideration every factor that could affect those structures.

To further explore these phenomena, it was important to examine how proteins that are directly or indirectly involved in the dynamics of microtubules behave. The behavior of the microtubule-related proteins MARK2, CLIP1, and IQGAP1 was examined. It was observed what happens to them when the cellular substrate has different elasticity and when microspheres are added. It is intriguing how differently each of these proteins reacted.

6.4 ASSESSMENT OF THE MAPs BEHAVIOR DURING THE CELL - BEAD INTERACTION ON ELASTIC SUBSTRATES

6.4.1 CLIP1

The protein that showed the accumulation around the beads turned out to be CLIP1. In the context of ring structure formation, this is the most intriguing discovery because in some cases it forms a ring structure around beads even when microtubules do not do that. In our experiments, the time of cell-microsphere interaction was 1 hour; after that, the cells were fixed and stained for CLIP1. One hypothesis may be that the CLIP1-containing ring formation could precede the microtubule ring structure formation. However, at this stage it is still a hypothesis. The structures formed by CLIP1 are occurring for both uncoated and fibronectin-coated beads **[Figures 41 and 43]**. The signal coming from the CLIP1 rings has a higher grey value on pictures than that from the microtubules at that time **[Figures 42 and 44]**.

The role of CLIP1 protein in cells is still poorly understood, and not much is known about how it interacts with other proteins. Every discovery that advances our understanding of it is important, especially considering how closely related malfunction

or overexpression of CLIP1 is to pathologies like Hodgkin's lymphoma [232] or autosomal recessive intellectual impairment [233]. The most recent research demonstrates that it is also responsible for breast cancer metastasis. According to the authors, increased CLIP1 (CLIP170) tension facilitated invasion and metastasis by cortactin-associated filopodia and lamellipodia formation [234]. It is crucial to get a greater understanding of CLIP1 characteristics because both of these tumours are malignant and lethal. If overexpression of CLIP1 is closely related to the process of metastasis, it is worth knowing what external factors may affect the overexpression and functions of this protein. The question is whether the elasticity of the environment of the cells (i.e., one that occurs naturally in the tissue) affects overexpression. As shown in this work, we see subtle changes in protein distribution in cells on elastic substrates when compared to those grown on glass. However, we did not investigate whether they are connected with overexpression of the protein.

Concerning the initial interaction between endocytic carrier vesicles, potentially different structures and microtubules, CLIP1 is hypothesised to act as a general linker. The authors [144] claim that the capture of endocytic carrier vesicles by microtubules via CLIP-170 may be followed by phosphorylation of CLIP1 to weaken the microtubule interaction and allow their movement along microtubules with motor proteins [144] [235]. It could mean that CLIP1 is involved in macropinocytosis. This could be useful to explain the fact how quickly it organised around the 2 μm diameter bead.

CLIP1 may also be the key element that connects the formation of microtubule rings (similar to endocytic rings) with the process of broadly understood endocytosis. It was also observed that the shape and behavior of a cell are affected by the type of substrate it is growing on [236], [237]. Until now, glass substrate has been the primary material for *in vitro* cell research. This differs from their natural microenvironment, and that is why elastic substrates with tissue-like elasticity are being used more frequently in recent studies [238]. On an elastic substrate, the CLIP1 protein is distributed differently than on the glass; however, the most common observation is the accumulation of CLIP1 around the bead on elastic substrates.

6.4.2 IQGAP1

IQGAP1 was the next examined protein. This protein is connected with the activity of the actin cytoskeleton, where it is essential in control of the dynamics of microfilament assembly [212]. It has also been shown that IQGAP1 regulates cell migration by connecting the dynamic plus ends of microtubules with the cell cortex [155]. Despite the fact that this specific protein mostly interacts with actin, we chose it, because it has also been proven to be associated with microtubules. In addition, IQGAP1 binds CLIP1 and microtubules [213], which could give a broader view of the results obtained with CLIP1 protein (Chapter 7.4). Importantly, expression of the protein IQGAP is upregulated by gene amplification in two gastric cancer cell lines. It could indicate that IQGAP1 is responsible for the features that make this tumor malignant [214].

In this work, it was shown that IQGAP1 is present in the cytoplasm for cells grown on elastic substrates, but not on glass **[Figure 45]**. In the context of protein overexpression and its consequences, this result is important. Overexpression of IQGAP1 is associated with enhanced tumor proliferation, invasion, and angiogenesis which means that the protein plays a role in the mobilization of cancer cells, which is undesirable [215].

When observing the behavior of the IQGAP protein around the bead during cell-bead interactions, it could be clearly seen that there was a lack of accumulation and creation of ring- like structure **[Figures 46 and 47]**. Even when we see enhanced concentration of microtubules, mobilization of IQGAP1 is not visible and it is not contributing to the ring creation. As a result, we can postulate the lack of its involvement in microtubules formation of cup- like structures. This supports our hypothesis that not all microtubule-associated proteins contribute to the ring formation.

6.4.3 MARK2

Among the proteins we chose, MARK2 is probably the most well - known and well described. It has a broad spectrum of functions in cells [239], [240]. It may be directly or indirectly, but in a very significant way, responsible for the health problems that plague humanity today. It phosphorylates microtubule-associated proteins, resulting in their disassembly [241]. It is strongly related to the control of microtubule dynamics. One of the proteins that is phosphorylated by MARK2 is the Tau protein, which aggregates with microtubules, causing Alzheimer's disease [242].

As shown in **Figure 48**, the bead is capable of causing a cytoplasmic rearrangement of the MARK2 protein. This phenomenon is commonly correlated with the parallel reorganization of microtubules. The absence of microtubule rearrangement results in the absence of rearrangement of the MARK2 protein.

In summary, each of these proteins responded to external factors in a different manner. Some conclusions are still difficult to fully grasp, such as how the MARK2 protein interacts with microtubules during the cytoskeletal reorganisation. It is possible that this protein has an entirely novel function that has not been identified yet.

All of this raises the fundamental question of whether microtubules play any role in the endocytosis process? This seems to be supported by the literature data of the CLIP1 protein research, because this is a protein whose participation in macropinocytosis has already been confirmed. Moreover it has a strong correlation with microtubule dynamics. Furthermore, it was important to us that each of these proteins is related to an extreme pathogenic state in the human organism like Alzheimer's disease or cancers. Although it is a disease of civilization, Alzheimer's disease is still incurable. There are various types of malignant and deadly cancers for which effective treatment is still impossible for humanity. Our study brings us closer to understanding the nature and behavioral models of these proteins. Therefore, these results could be helpful in the study of cancer cells.

7. FINAL SUMMARY AND FUTURE RESEARCH

This work, which deals with the cytoskeleton and the influence of external factors on its architecture, may have a significant impact on future research.

First, it may influence the reliability of planned experiments by taking into consideration the influence of microspheres on the architecture of each of the three types of cytoskeleton. This includes, for example, cell elasticity studies using microspheres. The intact cytoskeletal framework is important in these investigations.

The second issue is to expand knowledge about the properties and activities of three proteins (MARK2, CLIP1, and IQGAP1) from the large MAPs family. Each of these three proteins is, to some extent, related to a serious pathological condition in the body. The results of this research may help with research on methods of preventing or treating these diseases, for example, certain types of malignant tumours.

The most significant aspect of this work is observation of the development of novel microtubule cup-like cytoskeletal structures during endocytosis.

Endocytosis is generally a very complex process, so at this point, it is difficult to clearly answer the question of whether microtubules can participate in it. Previous studies have mainly pointed to the involvement of the actin cytoskeleton, but our research shows that a hypothesis can be put forward that microtubules are involved in the formation of endocytic vesicles. This would be an important finding in the context of endocytosis research.

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SCIENTIFIC ACHIEVEMENTS OF AUTHOR

PUBLICATIONS

- Adamczyk O, Baster Z, Szczypior M, Rajfur Z. Substrate Stiffness Mediates Formation of Novel Cytoskeletal Structures in Fibroblasts during Cell–Microspheres Interaction. *International Journal of Molecular Sciences*. 2021; 22(2):960. <https://doi.org/10.3390/ijms22020960>
- Kizovský M, Pilát Z, Mylenko M, Hrouzek P, Kuta J, Skoupý R, Krzyžánek V, Hrubanová K, Adamczyk O, Ježek J, Bernatová S, Klementová T, Gjevik A, Šiler M, Samek O, Zemánek P. Raman Microspectroscopic Analysis of Selenium Bioaccumulation by Green Alga *Chlorella vulgaris*. *Biosensors*. 2021; 11(4):115. <https://doi.org/10.3390/bios11040115>
- Czaja M, Skirlińska-Nosek K, Adamczyk O, Sofińska K, Wilkosz N, Rajfur Z, Szymoński M, Lipiec E. Detection of bleomycin-induced DNA strand breaks and repair processes in living cells by Raman microscopy. *Int. J. Mol. Sci.* 2022, 23(7), 3524; <https://doi.org/10.3390/ijms23073524>
- Czaja M, Giergiel M, Skirlińska-Nosek K, Sofińska K, Adamczyk O, Lupa D, Seweryn S, Rajfur Z, Birarda G, Piccirilli F, Vaccari L, Szymoński M, Lipiec E. Alterations in DNA conformation and histone phosphorylation resulting from double-strand breaks induced in HeLa cells – a spectroscopic and immunostaining approach. Under revision in *Scientific Reports*; <https://doi.org/10.21203/rs.3.rs-3427641/v1>

SCIENTIFIC INTERNSHIPS

- | | |
|------------|---|
| 05-08.2022 | Institute of Biotechnology, Czech Academy of Science, Prague, Czech Republic
Visiting Scholar in Lansky and Braun group |
| 06-09.2019 | Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary
Visiting Scholar in prof. Miklós Kellermayer group |
| 04-06.2018 | Institute of Scientific Instruments, The Czech Academy of Science, Brno, Czech Republic
Visiting Scholar in prof. Pavel Zemanek group |

CONFERENCES, SEMINARS AND WORKSHOPS ATTENDANCE

- 1st C-Trap User Meeting, Amsterdam, Netherlands, 5-6 October 2022;
- 66th Biophysical Society Annual Meeting, San Francisco, USA, 19-23 February 2022;
- 11th International Conference Analytical Cytometry, Ostrava, Czech Republic, 2-5 October 2021 (oral presentation);
- 6th INTERM- International Congress on Microscopy & Spectroscopy, Oludeniz, Turkey, 12- 18 May 2019;
- The 21st Czech-Polish-Slovak Optical Conference on Wave and Quantum Aspects of Contemporary Optics, Lednice, Czech Republic, 3-7 September 2018;
- VI International Conference of Biophysics Students, Cracow, 19-21 May 2017;
- 9th Workshop on Applications of Scanning Probe Microscopy – STM/AFM, Zakopane, 30 November- 4 December 2016;
- V Conference of Molecular Biology, Łódź, 7-9 April 2016
- XLIII Winter School Faculty of Biochemistry, Biophysics and Biotechnology Jagiellonian University, Zakopane, 16- 20 February 2016;
- 3rd General Meeting of Polish Physicists, Kielce, 6- 11 September 2015 (oral presentation)

PROJECTS AND GRANTS

06.2021-02.2022	MNS 2021 [Study of novel cytoskeletal structures' formation and the role of proteins related to the regulation of the cytoskeleton dynamics and structure in cell mechanotransduction]
03.2021- 03.2022	DigiWorld minigrant competition for the young [Creation of microscopy 3D images database for algorithm classifying specific cytoskeleton components interrelated to culture substates elasticity]
06.2020-01.2021	MNS 2020 [Study of novel cytoskeletal structures obtained with optical techniques in cell biology research]
07.2019-04.2020	DSC 2019 [Study of cell mechanotransduction using advanced optical techniques]