The Influence of High-LET Particle Radiation on Cellular Communication via Tunneling Nanotubes

Der Einfluss von hoch-LET Teilchenstrahlung auf die Zellkommunikation über Tunneling Nanotubes

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Abstract

Cell-to-cell communication is crucial for the survival of cells in stressful situations such as during or after radiation exposure. In 2004, a new kind of intercellular communication was reported and termed as tunneling nanotubes (TNTs). TNTs are thin membrane channels with a diameter in the nanometer range that directly connect cells over long-distances. They facilitate the direct cell-to-cell transfer of several cargoes such as organelles, viruses and signals. This thesis deals with the role of TNTs in radio-biology. It has the aim to investigate the influence of radiation on communication networks built up by TNTs and to figure out to what extent cellular communication via TNTs can interfere the cellular survival upon radiation exposure.

Several membrane markers are tested in order to identify the most suitable TNT marker. Here, the CellMask® plasma membrane stain shows excellent properties. This non-toxic dye enables an uniform and intensive labeling of the cell membrane within 15 minutes. Due to its robustness against dye internalization by endocytosis, the staining offers the opportunity to study TNTs over long time periods.

Furthermore, TNTs in U87 glioblastoma cells are characterized using confocal and STED microscopy. Cell-to-cell connections can consist of one single TNT or of several dense packed TNTs. The appearance of TNTs is not always straight and stretched, instead they can have kinks, junctions and noodles. TNT formation by cell-dislodgement as well as the transport of a gondola are successfully imaged. The formation of the TNT occurs within one hour and the gondola moves along the TNT with an average speed of $(14.5 \pm 0.7) \text{ nm s}^{-1}$. In addition, the cytoskeleton content of TNTs is studied by additional labeling of F-actin and microtublin. Thick TNTs contain microtubules as well as F-actin. In thin TNTs, F-actin is only found as fragments at their origins close to the cell body. This finding indicates that F-actin is only needed at the TNT formation, but not for stability of TNTs. An accurate TNT diameter of $\sim 195 \text{ nm}$ is measured by STED nanoscopy. Although, TNTs are smaller than the resolution of a confocal microscope, in combination with bright and stable labeling they can be identified by confocal microscopy, when no accurate thickness measurement is needed.

Additionally, a first pilot experiment on the investigation of the impact of radiation on TNTs is performed. U87 glioblastoma cells are irradiated with $\alpha$-particles to a dose of 1.2 Gy at the $\alpha$-particle irradiation setup located in Neubiberg. After irradiation cells are labeled with CellMask® orange plasma membrane stain. The TNT network is then examined using live-cell confocal microscopy and compared to sham irradiated controls. In order to follow the evolution and expansion of the TNT network, samples are analyzed 1 h, 6 h, 24 h and 72 h after irradiation. The results of this experiment show that irradiated cells establish their
network faster within the first 6 h and have more cell-to-cell connections, which have a high TNT density, than sham irradiated controls after 24 h. These findings suggest that there is an additional trigger upon radiation damage, which results in fast and intensive network formation by TNTs, as an additional damage response mechanism.
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Chapter 1

Introduction

Every day we are exposed to natural and artificial radiation which has a harmful effect on our organism and damages our genetic material. This health burden accompanies every single person throughout his or her life. Natural radiation exposure in Germany amounts to an average of approx. 2.4 mSv per year and is composed of terrestrial and cosmic radiation exposure [1]. Radon is one of the main contributor of the terrestrial radiation exposure in Germany [2]. It arrives from the ground as gas and every human being incorporates it into his or her body by breathing and eating. The cosmic radiation increases with the height above sea level. The devastating consequences and damages that ionizing radiation can cause become particularly clear when one recalls events such as the nuclear accidents in Chernobyl in 1986 and in Fukushima in 2011 or the dropping of nuclear bombs on Hiroshima and Nagasaki in the second world war. The regions around Chernobyl and Fukushima are still uninhabitable today. Health effects of ionizing radiation range from long-term effects such as increased cancer risk and deformities of children to acute effects such as hair loss or organ failure. Furthermore, the survival of astronauts in space is highly endangered by acute radiation diseases. For this reason, the radiation exposure of astronauts, especially during planned long-term missions such as the trip to Mars, is an essential limiting factor. For the execution of such missions it is therefore indispensable to reduce the damaging effect of radiation by mission planning, shielding and possible drug treatment.

However, the harmful effect of radiation is not always unwelcome. In radiation therapy, the cellular damage effect of ionizing radiation is specifically exploited to kill selective tumor cells. Here, the tumor is irradiated in several sessions with a radiation dose that causes the cells to die. Usually, the irradiation takes place from outside, which means that healthy tissue also receives radiation. This leads to damages such as skin erosion and inflammation of the healthy tissue. Therefore, it is crucial to find an adequate treatment which protects the healthy tissue as much as possible, but kills the tumor cells at the same time.

To comprehend how radiation affects tissues and organisms it is essential to understand the principle mechanisms occurring in cells upon exposure. From a molecular biological point of view, ionizing radiation affects cellular life by depositing energy in biological matter, which causes breakages of chemical bonds. Therefore proteins, lipids, genetic material as well as other cellular components can be damaged by radiative exposure. A critical damage for the
survival of cells is the DNA double strand break (DSB), in which the DNA, the carrier of the genomic information, is completely severed [3]. An erroneous repair of this type of damage can lead to cell death or tumor formation.

However, in cellular networks such as tissues not only DNA damage, but also signal transduction plays a key role in the damage response. Here, direct cell-to-cell communication is of great importance. It has been observed that irradiated cells send signals to their neighboring cells and thus influence their cellular survival, too. This communication can lead to the so-called “Bystander effect”, in which non-irradiated cells are induced by signal-transferring from neighboring, irradiated cells to show biological radiative effects [4]. In contrast to that it was also reported that healthy cells can transport organelles, proteins or signals to damaged cells in order to support repair and cell survival [5]. This means in both cases, that cell-to-cell communication directly influences the biological effect caused by radiative stress. The underlying mechanism of this effect as well as to which extend cellular communication affects the cell survival and also genetic alterations after irradiation remain unclear. However, a better understanding of this direct cellular response to radiation might help to improve radiation therapies. New therapy approaches can be development in which the transfer of death signals amplifies the cell killing effect.

During evolution, cells developed several approaches to communicate. One special communication mechanism is the information exchange via tunneling nanotubes (TNTs). TNTs are thin membrane bridges that interconnect cells over long distances up to more than 100 µm. Several cellular components and signals can be directly transferred within these channels. This communication tool enables the cells to communicate with each other in a very quick and effective way. This property and the fact that TNTs are more frequently formed at stress situations [6–9] indicate that TNTs play an important role in the direct cellular response to radiation.

This thesis deals with the study of TNTs and their response to radiative stress. In this context, two essential questions are addressed - whether TNT communication networks are actually influenced by radiation and if cellular communication is enhanced due to radiation exposure. TNT research is mainly based on fluorescence microscopy. Here, fluorescent markers are used to specifically label cellular components of interest. In order to visualize TNTs and their interactions, the plasma membrane has to be fluorescent marked. In this work, different membrane markers were tested to ascertain the best suitable marker for the study of TNTs. Here, the CellMask© Plasma Membrane stain was selected, because its usage results in an uniform and intensive staining of the plasma membrane and thus the TNTs. This dye provides the foundation for the research of cellular communication via TNTs.

Another crucial criterion is the precise characterization of TNTs. For this purpose, the identity of TNTs in U87 glioblastoma cells was investigated using confocal microscopy. Here, several properties such as the morphology and cytoskeleton content of TNTs were addressed. Additionally, the accurate TNT diameter was determined by STED nanoscopy. The size measurement reveals that TNT have a diameter of ~195 nm. This is smaller than the resolution of a confocal microscope. Nevertheless, TNTs can be observed and studied with confocal microscopy when no accurate sizing is required.
For the investigation of the influence of radiation on TNTs, a first pilot experiment was performed. U87 cells were irradiated on cover glasses with high-LET α-particles to a dose of 1.2 Gy. The cells were labeled with CellMask© Orange and live-cell microscopy images were recorded. In this thesis, TNT networks were analyzed in a quantitative manner for the first time. Here, it was differentiation between isolated cells, which are not involved in the network, and connected cells, which contribute to the network. Furthermore, the cell-to-cell connections are subdivided with respect to the number of TNTs they consist of. This established TNT network analysis enables the characterization of the network regarding its strength and complexity. The irradiated samples were compared to sham irradiated controls to investigate the impact of the α-particle irradiation. The TNT network was evaluated for incubation times 1 h, 6 h, 24 h and 72 h after irradiation to gain insights of the temporal development of the cellular communication network. With this experiment it was figured out that the cellular communication is indeed influenced by radiation and there must be an additional mechanism which causes the irradiated cells to form TNTs faster and more frequently than normal.

The present work is structured as follows: Chapter 2 provides a review of the research on TNTs and introduces the today observed functions and roles of this cell-to-cell communication mechanism as well as the remaining open question about the TNTs. In chapter 3 the basics of fluorescence microscopy are outlined and the principles of confocal and STED microscopy are discussed. Chapter 4 deals with effect of ionizing radiation on biological matter. In chapter 5 the cell irradiation method is described. Chapter 6 covers the microscopy of TNTs in living U87 cells. In particular, the selection of CellMask© as the most suitable TNT marker is presented. The used methods for image processing and size measurement are described in chapter 7. Chapter 8 focuses on the characterization of TNTs in U87 cells and the first biological results obtained from the study of the influence of radiation on TNTs are presented in chapter 9. The work is rounded off with a final conclusion and an outlook provided in the chapter 10.
Chapter 2

Tunneling Nanotubes

Tunneling nanotubes (TNTs) are thin membrane tubes with a diameter in the nanometer scale that interconnect cells over long distances up to several micrometers. Through this tiny tubes small molecules such as vesicles, organelles and calcium signals can be exchanged directly from one cell to another. This mechanism enables cells to communicate with each other in a selective direct manner. In addition to TNTs, there are many others cell communication techniques. Some of them are briefly discussed here to provide an overview about the whole possibilities for cells to communicate. Afterwards the focus is placed on the TNTs and the most important topics in the research on this quite new kind of cell-to-cell communication are highlighted in this chapter.

2.1 Cellular Communication

For cells, it is crucial to have the possibility to communicate among each other in order to support themselves during cell survival and development. Without that essential tool cells are not able to coordinate and organize themselves in complex cellular systems such as tissue or organisms. For this purpose cells have developed various types of cellular communication mechanisms during evolution. Many are only poorly understood or even completely unknown. The best known communication mechanism of cells are gap junctions. It is the quickest and most direct way for cells to communicate with each other [10]. Gap junctions can be formed at cell-to-cell contact by the docking of two hemichannels also called connexons. These channels are located in the plasma membrane of the two adjacent cells and connect both in the extracellular space by hydrophobic interactions. A connexon consists of six junctional proteins named connexins forming a hexameric torus with an aqueous pore in the middle. This junctional channel with a pore size of 2 nm to 3 nm allows the direct exchange of small molecules and diffusion of small ions up to 1 kDa between the cytoplasm of cells [11]. Gap junctions play an essential role in development [12] and mutations of connexin genes can cause several diseases such as hearing loss [13].

Cellular communication is also possible even if the cells are separated and have no contact to another. One mechanism is the secretion of signal molecules which reach their receptor cells by diffusion through the extracellular space, called signal transduction. The signal molecule encounters a receptor protein which is located on the outer surface of the plasma membrane.
Chapter 2. Tunneling Nanotubes

The contact of signal molecule and receptor leads to intracellular signal in the receptor cell. The signal is then amplified by signaling cascades [14]. As a consequence of the diffusion process this kind of cellular communication requires a close proximity of cells. The secretion of molecules plays an important role in wound healing and cancer invasion [15].

Cells can also communicate if there is a obstacle located between them which prevents a chemical communication via secretion. Such a non-chemical and non-contact communication appears over physical ways as sound and electromagnetic radiation in the UV range [16]. However, this kind of communication does not allow the exchange or transport of cellular components.

In 2004, a new kind of intercellular communication was reported and termed as tunneling nanotubes (TNTs). Straight thin structures composed of membrane connecting cells directly over long-distances have been discovered by 3D live-cell microscopy in cultured rat pheochromocytoma PC12 cells. They contain F-actin, exhibit a diameter ranging from 50 to 200 nm and a variable length up to several cell diameters. They appear as stretched branches between cells connecting these at their nearest distance above the substrate and facilitates the transport of organelles [17]. After this discovery many similar findings in different cell lines were made and a deluge of biological processes were reported in which TNTs could be involved.

In the last two decades the research focused on TNTs reveals a large diversity about the morphology, composition and function of these membrane connections, which yields to new points of controversy and the role of TNTs in the cellular communication remains obscure.

2.2 Identity and Similar Structures

To date, a clear and totally agreed definition of TNTs does not exist. This is a consequence of numerous observations of similar structures which show on the one hand some comparable properties but on the other hand significant differences. Therefore it is difficult to distinguish these observations from each other and to establish an uniform classification. However, some key characteristics can be satisfied about TNTs.

TNTs are thin cytoplasmic membrane bridges with a diameter ranging from 50 nm to 1500 nm that interconnect cells over long distances up to several cell diameters length. This allows the direct cell-to-cell transfer of signals as well as cellular compounds [18, 19]. In vitro, they often appear as straight lines, but this is not a proper criterion since TNTs found in tissue or in a three dimensional extracellular matrix cultivated T-cells exhibit a curved morphology [20, 21]. Due to the flexible shape, TNTs are also able to connect cells even if the nearest distance between them is blocked by obstacles as other cells. In the most cases, TNTs are above the substrate in contrast to similar structures. Nevertheless, this is not a meaningful identification feature, since in tissue or three dimensional space a substrate is not always present. Between cell lines or even in the same cell line the morphology and cytoskeletal composition of TNTs varies, which suggests that different types of TNTs are responsible for particular functions.
Figure 2.1: (a) STED microscopy image and (b) the corresponding sfp rendering picture of a TNT connection between U87 glioblastoma cells labeled with the membrane marker PKH 26 (recorded in this thesis). The dimensions of the tube are approximately 320 nm in diameter and 33.6 µm in length. Additionally, the TNT is above (∼2.9 µm) the substrate and has a stretched appearance.
Whereas F-actin is found in most TNTs, usually only the thicker membranous connections contain also microtubules [19] or cytokeratin filaments [22]. The length of TNTs varies in a range of just a few to over 100 µm [21] and can be dynamically regulated if the interconnected cells migrate until the distance becomes too large and the tube disappears [19].

In figure 2.1 a STED microscopy image of a TNT connection between U87 glioblastoma cells is shown (recorded in this thesis). The x-y-view of one slice is displayed in (a) and the 3D-visualization in (b). The cells were labeled with the membrane marker PKH 26, which visualizes the plasma membrane as well as other lipids compositions such as vesicles inside the cells. The dark regions inside the cells caused by the missing of the fluorescent dye indicates the localizations of the cell nuclei. The tube is recognizable as a straight line between the cells with approximately 320 nm in diameter and 33.6 µm in length.

TNTs have to be distinguished from other similar membrane structures such as cytonemes and filopodia, which do not have cell-to-cell contact. Furthermore, there are membrane connections which also allow cell-to-cell communication, but are structurally distinct from TNTs, the plasmodesmata and EP bridges. In the following, these similar structures are described to work out an appropriate definition of TNTs. An overview and comparison of these structures is provided by the table 2.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Diameter</th>
<th>Length</th>
<th>Cell-to-Cell Contact</th>
<th>Occurrence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNTs</td>
<td>50 - 1500 nm</td>
<td>Few to over 100 µm</td>
<td>Yes</td>
<td>Animal cells</td>
<td>[19] [21]</td>
</tr>
<tr>
<td>Cytonemes</td>
<td>&lt; 200 nm</td>
<td>Up to 700 µm</td>
<td>No</td>
<td>Tissue</td>
<td>[23] [24]</td>
</tr>
<tr>
<td>Filopodia</td>
<td>100 - 300 nm</td>
<td>Several microns</td>
<td>No</td>
<td>Animal cells</td>
<td>[25]</td>
</tr>
<tr>
<td>Plasmodesmata</td>
<td>20 - 50 nm</td>
<td>Up to 90 nm</td>
<td>Yes</td>
<td>Plant cells</td>
<td>[11] [26]</td>
</tr>
<tr>
<td>EP bridges</td>
<td>1 - 20 µm</td>
<td>25 - 1000 µm</td>
<td>Yes</td>
<td>Animal cells</td>
<td>[27]</td>
</tr>
</tbody>
</table>

Table 2.1: Overview and comparison between intercellular connections and membranous protrusions similar to TNTs.

Cytonemes are straight, long actin-based cytoplasmic extensions found in various tissues. They were first described in *Drosophila* Imaginal Discs as very small, less than 200 nm in diameter, and remarkably straight protrusions which grow orientated to a chemoattractand. Since their growth was not stopped by the microtubule-destabilizing drug nocodazole, microtubules was excluded as cytoskeleton composition in cytonemes. In addition, they grow very fast, 25 µm per minute up to a length of ~ 700 µm [23]. The special polarization of their orientation distinguish them from their relatives, the filopodia. On cytonemes various signaling molecules and receptors of diverse signaling pathways are located. This reveals the specificity to the response of morphogen cargo leading to a regulated spatial orientation. Thus, cytonemes play an important role in patterning during development [24].

The filopodium, is also an actin-rich membranous protrusion with a diameter ranging from
100 nm to 300 nm [25]. As cytonemes, filopodia contain signaling molecules and receptor proteins. Thus, filopodia are also involved in signal transduction communication. Filopodia are often described as ‘antennae’ of the cell, since with these finger-like structures the cell explores its environment followed by migration. Inside the tips of filopodia diverse adhesion molecules such as cadherins and integrins can be found, which anchor the filopodia to the substrate and enables the cell to pull its body forward. Consequently, filopodia have a significant biological function in cell migration and cell spreading [28]. Macrophages use filopodia as tentacles to find pathogens. Once detected the filopodium binds to the pathogen which is then pulled to the cell body by retraction of the filopodium [29]. Furthermore, filopodia play an important role in cell-to-cell adhesion during wound healing and embryonic development or acting as guidance in neuronal growth cones [25].

TNTs have to be further distinguished from other cytoplasmic connections which mediate the exchange of cargoes, plasmodesmata and epithelial (EP) bridges. These cellular connections can be easily discriminated by structure and occurrence. Plasmodesmata only occur in plant cells, whereas TNTs and EP bridges are found in animals cells. Additionally, in contrast to TNTs plasmodesmata are thinner (20 nm to 50 nm in diameter) and their length can not be regulated since it is limited by the cell wall thicknesses [30]. On the other hand, EP bridges have a larger diameter ranging from 1 μm to 20 μm and an extended length of 25 μm to 1 mm in comparison to TNTs [27]. However, there is a small overlap recognizable between EP bridges and TNTs regarding the diameter. In human lung carcinoma (A549) cells TNTs were found, which exhibit diameters ranging from 400 nm to 1500 nm [31]. These connections which can be assigned to TNTs as well as EP bridges, symbolize the ridge hike between the individual designations.

To conclude, there are three main marks that characterize TNT best. First of all, they interconnect cells by cell-to-cell contact. Furthermore, their dimensions are 50 nm to 1500 nm in diameter and their length can dynamically be regulated from a few microns to over 100 μm. Finally, they facilitate the direct transport of various molecules and are consequently a tool for cell-to-cell communication. Although, these characteristics differentiate TNTs from similar cellular connections and membrane protrusions (see table 2.1), they do not supply a classification or assignment to the diversity regarding to morphology, composition and function observed at TNTs in different cell lines or even in the same cell line.

2.3 Functions of Tunneling Nanotubes in Cellular Behavior

TNTs are found to play crucial roles in many cellular processes. This section deals with the versatility of TNTs regarding their functions and roles and its consequences for disease development and cellular survival.

2.3.1 Exchange of Organelles and Particles

TNTs can be used as highways among cells to transfer and exchange cellular compounds from one cell to another. Organelles and particles, which are observed to be interchanged between
cells are mitochondria, vesicles, membrane as well as cytoplasmic components, nanoparticles and even more. The usage of video fluorescence microscopy enables the study of organelles exchange along membrane nanotubes.

Already at their discovery, Rustom et al. [17] showed that vesicles are transferred in one direction along TNTs with a speed of \((25.9 \pm 7.9)\) nm/s. The transfer of plasma-membrane components, lysosomes, endosomes as well as the motor protein myosin Va via TNTs was reported in cultured rat pheochromocytoma PC12 cells. However, the small cytoplasmic molecule, calcein, was not exchanged by the membrane tubes, which reveals some kind of selectivity in the transport mechanisms within or along TNTs. Furthermore, the research on TNTs pointed out that there are indeed different transport mechanisms which may be dependent on the cytoskeletal content, diameter as well as membrane continuity of the considered nanotube. For instance, in human macrophages two different types of TNTs are found, thinner ones which only contain F-actin as cytoskeleton component and thicker ones which consist of both F-actin and microtubules. Both types exhibit different kinds of functions. The transport of intracellular components including endosomes, lysosomes and mitochondria are only exchanged within thick tubes, whereas surfing of bacteria on the membrane surface is only detectable in thin TNTs [22]. Another fascinating effect was observed for the transfer of mitochondria via TNTs. Virus-infected or apoptotic cells that are connected to healthy cells via TNTs can be rescued if mitochondria are transferred from healthy towards unhealthy cells at an early apoptotic stage [5] [32]. It has also been reported, that mitochondria traffic through TNTs supports the invasiveness of bladder cancer cells or modulates chemoresistance [33]. These findings suggest that the transfer of mitochondria via TNTs may play a crucial role in cellular development and survival. TNTs are also suspected to transfer P-glycoprotein in cancer cells, a protein which can cause multidrug resistance [34, 35]. Besides organelles also nanoparticles such as quantum dots (QD) have been observed to be transferred by TNTs [31, 38]. Fluorescent streptavidin-coated CdSe/ZnS QDs were incorporated in rat cardiac myoblast H9c2 cells and actively transported via TNTs. The QDs move bidirectionally with a mean speed of 1.2 \(\mu\)m/s [38] along the TNTs. This knowledge can be used to further study the diverse transport mechanisms along TNTs and may open up new possibilities for the diffusion or selective transport of therapeutics inside the communication system of desired target cells [39].

2.3.2 Signal Transfer

Furthermore, it has been proven that TNTs have a prominent role in the propagation of signals. For instance, \(Ca^{2+}\) signals can be transferred via TNTs between remote cells. Usually, electrical signals are transmitted through neuronal synapses or gap junctions, latter need a close proximity for cell-to-cell communication. In contrast, these kinds of signals can be quickly transferred over long distances when cells are connected by TNTs. That phenomenon was initially reported in 2005. Myeloid-lineage dendritic cells and monocytes showed an amplified cellular response to chemical or mechanical stimulation by signal transfer within seconds across large cellular networks interconnected via TNTs [40]. Here, calcium signals have been transmitted unidirectional from a stimulated cell towards connected cells by TNTs, followed
by morphology changes of the nanotubes and the appearance of beads along these connec-
tions. Treatment with α-glycyrrhetinic acid, an inhibitor for the functionality of gap junctions, 
does not block the Ca\(^{2+}\) flux transfer, suggesting that gap junctions are not involved in this 
transmission. Simulations showed that passive diffusion of Ca\(^{2+}\) would be insufficient for an 
efficient transport through TNTs. Instead these signals are actively propagated by inositol 
triphosphate receptors (IP\(_3\)R) channels within the TNTs [41]. Recent studies using the 
sensitive membrane potential probe DiBAC\(_4\)(3) reveal that several cell types can be electrically 
coupled by TNTs, where gap junctions interposed at the membrane interface in one end of 
the connection allowing bi-directional passage of electrical currents in a very selective way [42] 
[43]. The strength of signal depends on the length and diameter of the nanotubes, the open 
probability of present gap junctions as well as the number of involved TNTs per connection. 
The fact, that there are both gap junction independent and gap junction dependent electrical 
transmissions by TNTs implies a significant diversity of TNTs with different properties and 
functions formed by various cell types. 
Beside electrical signals, death signals can also be transferred through TNTs. Apoptosis sig-
naling pathways are very important in the immune system. Death receptors such as Fas/CD95 
mediate rapid cell death after receptor ligation with cell-surface proteins like FasL leading to 
the elimination of diseased or needless cells. The involvement of TNTs in apoptosis signaling 
was found in T cells, in which death signals are propagated by TNTs and Fas stimulation leads 
to an enhanced TNT formation among cells. This formation is associated with diseases such 
as autoimmune lymphoproliferative syndrome (ALPS), whose cells are not able to establish 
such TNT networks after Fas stimulation [44]. Other important cells in the immune response 
system are neutral killer (NK) cells. They are able to recognize infectious or tumor cells and 
thus, pivotal in the defense system. In Chauveau et al. [45] reported, that activated NK cells 
form more frequently TNTs and connected target cells are more often lysed than unconnected 
target cells. This suggests that TNTs can help the immune system by the transfer of cytotoxic 
chemicals to distant target cells.

2.3.3 Pathogen Spreading

Of course, such a cellular communication system, which enables the direct exchange of 
molecules and particles among cells, does not only promote positive effects. Instead TNTs 
are equally involved in spreading pathogens and thus related to diseases. As already men-
tioned above, bacteria can surf on TNTs. Önfelt et al. [32] reported that Mycobacterium bovis 
BCG expressing GFP can surf on the surface of thin membrane nanotubes and internalize 
into the cell body after transport in human macrophages. The same effect was visible by 
the transport of streptavidin-coated fluorescent beads when cells were surface-biotinylated, 
suggesting a constitutive flow along the surface of the TNTs. Similar findings were obtained 
in synthetic nanotube-vesicle networks injected with bacteria. Here, the transfer of bacteria 
within as well as on the surface of the nanotube was observed. Both transport processes 
result from a continuous flow of the lipid bilayer system caused by membrane surface tension 
differences [10]. Also viruses such as the murine leukemia virus (MLV) can travel along the 
surface of nanotubes and thus spreading from infected cell to targets by the usage of cellular
Chapter 2. Tunneling Nanotubes

bridges as highways [6]. Cell-to-cell spreading of viruses can also appear if mitochondria are used as vehicle or Trojan horse by viral materials, as they can be transferred to non-infected cells through TNTs [33]. In addition, HIV-carriers were also found in TNTs and use them to infect other cells [7, 21]. Beside the spreading of bacteria and viruses, also prions and misfolded protein aggregates travel along TNTs which implies that TNTs play an crucial role for prion spreading during neuroinvasion and pathogenesis of neurodegenerative diseases such as Huntington, Parkinson and Alzheimer [18, 47, 48].

Figure 2.2: Schematic illustration of a TNT with possible cargo transfer inside as well as on the surface of the membrane tunnel. Several cargoes such as mitochondria, pathogens like prions, bacteria or viruses, signals, cytoplasmic or solved molecules and even more, which has been observed to be transfered via TNTs, are illustrated. The black springs, symbolizes the anchorage of the virus and the bacterium to the plasma membrane during surfing. The black box with the question mark represents the open question about the actual connection of the nanotube to the cell body.

Overall, these findings imply that direct cell-to-cell communication via TNTs can have potential roles in the immune respond system, cell development, repair and survival, cancer progression as well as in the spreading of pathogens such as bacteria, viruses or misfolded proteins. In the literature, there are much more functions and roles of TNTs reported as the ones pictured above and there are definitively much more which have not been discovered until now. However, the already known features of TNTs allow to speculate about hypothetical application of TNTs in therapy. Either the formation of TNTs can actively be blocked in order to interrupt the spreading of pathogens or TNTs are used as cellular highways for drug delivery [18]. As summarization of the reported functions of TNTs an illustration of a TNT showing the transfer of various cargoes is presented in figure 2.2. Furthermore, the consequent possible roles of TNTs by their usage as highway for several particles and molecules are depicted in the mind map shown in figure 2.3.
2.4 Main Questions about Tunneling Nanotubes

Although, many reports about TNTs in various cell types and situations were published there is little known about actual working proceeding of the transport mechanisms seen in TNTs. Also the triggering and regulation of their formation or stability and their connection to the cell body are widely unknown principles. In this section, the main questions about structure, regulation and transfer mechanisms of TNTs are outlined and several observed scientific facts around these questions are presented.

2.4.1 Formation and Regulation of Tunneling Nanotubes

How does the cell decide and regulate to form a nanotube, which signals might be involved or trigger the formation of TNTs? Furthermore, which mechanisms drive and regulate the process of TNT formation or the length regulation of the TNT? All these biological mechanisms are poorly understood to date.

Based on the observations of TNT formation in research two models of TNT establishment were proposed [49]. The first one is the de novo generation of nanotubes from filopodia-like...
Chapter 2. Tunneling Nanotubes

**Figure 2.4:** Illustrations of the two TNT formation models. On the left side, the formation of a nanotube by the actin-driven growth of membrane protrusions as filopodia is shown. a) The protrusion elongates until it reaches the target cell, where physical contact will be established by adhesion followed by a membrane fusion of tunnel and target cell. b) It might be also possible that two different membrane protrusions meet each other and establish a connection by adhesion and fusion. c) An open-ended nanotube connection will be generated. On the right side, the second formation model is illustrated, the TNT formation by cell dislodgement. d) Here, the cells migrate apart from each other after physical contact and e) during their migration the nanotunnel will be pulled out of the cells. f) At the end of the migration, the cells are still connected via the generated TNT. c) and f) represents the same end point, an established TNT connection between cells.

protrusions by an actin-driven process (depicted in fig. 2.4 on the left side). This kind of TNT formation was observed in neuronal cells such as PC12 and mouse catecholaminergic (CAD) cells, where TNTs were formed within several minutes [17, 48, 50]. In this model, a protrusion of the membrane probably initiated by Rho-family GTPases elongates by actin polymerization. If the tip of the protrusion reaches the target cell a physical contact will be establish by adhesion and possible membrane fusion (see fig. 2.4 a). However, it is uncertain whether the filopodia grows in precise direction driven by a chemoattractant as in the case of cytonemes [23] or not. Rustom et al. reports at the discovery of TNTs a “seemingly directed” growth of the filopodia [17]. Additionally, it is not explored whether a nanotube is also es-tablish when two tips of filopodia meet each other and make contact (depicted in fig. 2.4 b). Furthermore, this formation proceeding is ascertained to be actin-driven, since the treatment with the actin-depolymerize drug latrunculin-B results in elimination of TNTs [17]. Similar findings were made after treatment of PC12 cells with cytochalasin B which blocks the actin polymerization yielding to a selective inhibition of TNT formation [50]. Adhesion molecules are also required to establish physical contact. The role of fusion proteins is difficult to explore because spontaneous membrane fusion may also be possible depending on the curvature and lipid composition of the membrane [49].
The second model is the TNT formation at the detachment of cells after a short cell-to-cell contact. This mechanism has been observed in immune cells as T cells, NK cells or macrophages as well as in normal rat kidney (NRK) cells [21, 32, 42, 43, 45]. It is illustrated on the right side in figure 2.4. Here, cells form an immune synapse or fuse when they come into direct cell-to-cell contact. With the dislodgement of the cells a nanotube is pulled out (as shown in fig. 2.4 e). Whether one or both cells contribute to the establishment of the TNT is uncertain. Additionally, the involvement of adhesion, fusion or actin polymerization remains unknown in this formation mechanism [49]. However, studies in T cells reveal that formation of a TNT depends on the time of cell-to-cell contact, since TNTs are only hardly established if the cell-to-cell contact lasted under 4 minutes [21]. Even though, these two models might appear to be very distinct, they are not mutually exclusive since there are findings where both mechanisms are observed in the same cell type and there might exist a combination of both mechanisms. If connected cells migrate apart this can cause an elongation of filopodia extensions [49] [51]. Since the length of a TNT can be dynamically regulated there is the question whether actin polymerization does continue in one or both cells. Beside the two heterogeneous models, recent research reveals that several stress conditions lead to increased TNT formation and thus demonstrate that it is inducible. Such stress conditions are for instance infections, inflammation or hydrogen peroxide (H$_2$O$_2$) [6–9]. Additionally, there have been found several stimuli of TNT formation such as Fas-ligand receptor in the immune system or M-sec, a protein associated with the component Sec6 of the exocyst complex required for the docking of exocytic vesicles on the plasma membrane [44] [52]. The findings suggest that the TNT formation might be similar to the mechanisms of filopodia and lamelipodia regulation, but the actual machinery behind TNT formation and regulation remains unknown.

2.4.2 Membrane Continuity and Stability in Tunneling Nanotubes

Are TNTs open-ended at both ends and thus exhibit membrane continuity as feature? Or are they close-ended at one end and connected to the other cell by an interposed channel as a gap junction or an immune synapse as illustrated on the left side in figure 2.5. These questions lead to many controversies among scientists. For instance, electrical coupling of various cell types such as NRK cells was only detectable if the TNT connections contain gap junctions at one end. This reveals gap junction and therefore close-ended dependence of the TNT for electrical coupling [42] [43]. Similar observations were made in T cells, where studies with the fluorescent membrane markers DiO and DiD as well as transmission electron microscopy (TEM) demonstrate that the TNTs found in this cell types exhibit a distinct junction which can move dynamically within the tube and therefore these connections are not open-ended [21]. Additionally, TNTs in NK cells were also found to be close-ended and contain a submicron scale immune synapse [45]. This heterogeneity of membrane interface among TNTs in various cell types is one challenge of the current research about TNTs. It has to be resolved under which conditions the membrane at interface can fuse
Figure 2.5: Schematic representation of the two reported kinds of TNT connection to the cell body. On the right side an open-ended nanotube is drawn, there the membrane of the tunnel has been fused with the plasma membrane of the connected cell and thus a membrane continuity was generated. Via this kind of connection membrane components can be easily exchanged by lipid sliding. However, this connection is not fully open as illustrated here, since the exchange of small cytoplasmic dyes such as calcein via these connections was impeded [17]. These findings suggest that there must be some kind of selection barrier within the nanotube connection. On the left side a close-ended TNT is illustrated. This kind of connection was observed in several cell types such as T cells. Instead of membrane continuity there is a distinct junction between the connected cells recognizable. Such a junction is mostly found at one end of the nanotube as drawn here, but it has been also observed that these junction can dynamically shift along the membrane tunnel [21]. Possible junctions could be nanoscale immune synapses or an accumulation of gap junctions [42] [43] [45].

and therefore generating an open-ended channel. Furthermore, it has to be explored which gating mechanism operates in an open-ended TNT connection to generate the selectivity of the exchange of molecules. There must be an active mechanism, since the passive transfer of cytoplasmically expressed GFP and the small dye molecule calcein was impeded in open-ended TNT in PC12 cells [17]. It might be also beneficial to work out proper characterizations in order to differentiate TNTs in appropriate subclasses.

In addition, the exact role of the cytoskeletal content within a nanotube is unknown. Most TNTs contain F-actin which is thought to be the backbone of the tube. However, treatment of PC12 cells with the actin polymerization inhibitor cytochalasin B results only in a reduction of TNT formation, but has almost no influence on already formed TNTs. This suggests that actin polymerization is not necessary for the stability of existing TNTs [50]. Same results were made with the treatment of T24 urothelial cells with cytochalasin D. Here, the authors believe that a once formed TNT is mechanically stabilized by flexible membrane micro- and nanodomains which self-assemble in the most energetically favorable way [22]. Therefore F-actin may play an important role in the formation and growth of a TNT, but might not be required for the stability. Furthermore, F-actin seems to be non-decisive for the length or lifetime of TNTs, since these two features vary from cell to cell. The lifetime of a TNT is ranging from a few minutes in PC12 cells up to several hours in NRK cells. These features might be determined by the available membrane reservoirs for a given cell type [53]. Additionally, TNTs which contain microtubules may be more stable than those composed only of F-actin since microtubule-filaments exhibit a higher degree of stiffness [19].
2.4. Main Questions about Tunneling Nanotubes

2.4.3 Transport Mechanisms via Tunneling Nanotubes

As illustrated above, there are many situations where TNTs are responsible for the selective transfer of various kinds of cargoes. The research on TNTs uncovers a significant diversity of transport processes via nanotubes. To date, it remains unresolved how these individual transfer operations really act within or along TNTs and which mechanism is responsible for the selectivity in these transport processes as well as under which circumstances unidirectional or bidirectional carriage appears. However, there are probably various different kinds of transport mechanisms operating along TNTs.

One observed and suspected transport mechanism is the exchange of molecules by molecular motors. Indicators for a molecular-motor-mediated transfer is the presence of the actin-binding motor myosin Va in TNTs which also partly colocalizes with the exchanged organelles [17, 30]. Additionally, several transferred molecules and particles such as HIV-1 viral particles or lysosomal vesicles exhibit a velocity in a range similar to those of actin-driven molecular motors [49]. Furthermore, this myosin-driven transport mechanism is in agreement with the unidirectional one-way street feature, since this could be establish by assuming that the actin filaments are of the same polarity [51]. It could also be possible, that the cargoes are linked to actin themselves and transported by actin polymerization. Here, the actin could be seen as a rope on which the cargoes are anchored and dragged along the tube driven by actin polymerization at one end [30]. A further evidence for an actin-dependent transport mechanism inside TNTs is that after treatment with cytochalasin B which blocks the actin polymerization, a decrease of the organelle transfer was detectable in PC12 cells [17]. However, the transfer of vesicles also called gondolas were unaffected from the drug treatment which demonstrate that actin is not necessarily needed for all transport mechanisms [50].

Bidirectional transport of cargoes was only observed in TNTs which additionally contain microtubules as cytoskeletal content suggesting that a microtubule molecular motor could be responsible for this behavior [49]. It was also observed that bidirectional transfer of cargoes can change into an unidirectional transport proceeding after a certain stress situations such as injury [39]. It might also be possible, that there are transport mechanisms which are cytoskeleton independent such as the transfer by gondolas. Gondolas, are moving distentions in TNTs which can carry enclosed organelles which are bigger than the diameter of the respective nanotube itself. The formation and the generating force needed for the movement of such a bulk vessel are unexplored. It is possible that the movement is driven by differences in chemical potential regarding to the molecules inside the bulk solution and the interior of the target cell or to the compositions of the gondola membrane and the target cell membrane [22]. Additionally, the surfing of particles such as bacteria or viruses on the surface of the membrane nanotube is also unresolved. It may be independent of molecular motor since surfing was also observed in artificial vesicle networks without any cytoskeleton content driven by lipid sliding [46].

Further investigation has to be done in order to resolve how the carriage of cargoes occurs within tubes and which molecular motor or other proteins are involved in such a transfer system. Nevertheless, many reports demonstrate that the transfer of molecules and particles occurs in an active matter and not due to diffusion. For instance, studies with ATP depletion
reveal a blockage of organelle transfer via nanotubes pointing out that ATP is necessary for the respective transferring processes [48][32][53][39]. Same results were obtained by considering the transfer of electrical signals across TNTs, here simulations showed that a passive transfer is inefficient and experiments indicate that the signals are active generated and propagated within TNTs [41]. These reports show, that transport mechanisms via TNTs are active processes.

2.4.4 Relation to Stress

One further research topic of TNTs is their relation to stress. Cell-to-cell communication is crucial for the survival of cells in stressful situations. Several reports pointing out that the presence of TNTs in a cellular network correlates with several stress situations such as hydrogen peroxide (H$_2$O$_2$), infections or inflammation [6][9]. Furthermore, several studies reveal that healthy cells are able to rescue apoptotic cells by the exchange of functional mitochondria via TNTs [5][33]. One of these studies is based on ultraviolet (UV) radiation as stress factor. Additionally, a study of the membrane-bound heat shock protein mHsp70 located on TNTs reveals that the TNT connections are supported by cholesterol-rich microdomain clusters which contain these stress proteins [55]. These findings suggest, that TNTs have a special relation to stress.

In his recent paper, Amin Rustom used these findings to link them to introduce a new “mechanistic model of reactive oxygen species-dependent tunneling nanotube formation”. He describes the formation of TNTs according to the increase of reactive oxygen species (ROS) level in stressed cells [56]. These stressed cells transmit “call-for-help” signals to their surrounding. According to this model, TNTs will be formed by unstressed cells in order to establish an open communication channel to the stressed cell. Followed by the exchange of particles such as mitochondria to rescue the apoptotic cell or by the isolation and removal of the cells whose ROS level is too excessive. Based on this model, TNTs are a communication tool among cells used for the organization of their survival during stress.
Chapter 3

Fluorescence Microscopy

Fluorescence microscopy plays an essential role in TNT research. Due to the very fine structure of TNTs with a diameter of only a few hundred nanometers, fluorescence microscopy is the best way of observing and researching these small cell-to-cell connections. It is therefore not surprising that most research reports on TNTs are based on fluorescence microscopy. In this work, the TNTs of U87 cells were studied using confocal microscopy as well as STED nanoscopy. This chapter deals with the fundamentals of fluorescence microscopy followed by the introduction of confocal and STED microscopy.

3.1 Fundamentals of Fluorescence Microscopy

This section focuses on the basics of fluorescence microscopy. The phenomenon of fluorescence and its application in fluorescence microscopy are described in this section. Additionally, the resolution of conventional light microscopes is discussed.

3.1.1 What is Fluorescence?

Fluorescence is the property of certain molecules, the so-called fluorophores, to spontaneously emit light shortly after they have been irradiated with light of shorter wavelengths. This phenomenon is also known as luminescence. There are two different types of luminescence, fluorescence and phosphorescence. The two types are distinguished in the duration of light emission after the end of excitation. Fluorescence has a very short lifetime of $10^{-6} - 10^{-9}$ s, whereas phosphorescence has a very long lifetime of $10^{-3}$ s and more [57]. The term fluorescence was firstly introduced by George Gabriel Stokes in 1852 [58]. Followed by the introduction of the term luminescence by the physicist and science historian Eilhardt Wiedemann in 1888 [59]. In nature, fluorescence occurs in many living beings. For example, the most well-known fluorophore in research, the Green fluorescent protein (GFP), was discovered in the jellyfish Aequorea victoria by Shimomura et al [60] in 1962. Nowadays, natural and artificially produced fluorescent dyes are widely used in biology research to label proteins and other structures in cells and thus make them visible for studying.
Chapter 3. Fluorescence Microscopy

Figure 3.1: Simplified Jablonski energy diagram showing the term schema of excitation and emission. The molecules are transferred from the energetic ground state $S_0$ to the higher energetic state $S_1$ by absorbing photons (excitation, green arrows). For this transition the exciting photon must have at least an energy of $\Delta E = S_1 - S_0$. In the energetic higher state $S_1$, the molecules relax to the lowest vibrational level by internal conversion (dashed black arrow). At the subsequent dropping down to the vibrational and rotational levels within the ground state $S_0$, the molecules release their remaining energy by emitting light (fluorescence, orange arrows). This light exhibit shorter wavelengths than the absorbed photons due to the internal conversion and by relaxing down to energetic higher individual vibrational and rotational levels.

In order to cause a fluorescent molecule to emit light, it must be excited with a photon from a certain energy range, the so-called excitation spectrum. This process is illustrated as simplified Jablonski energy diagram depicted in figure 3.1. A photon of a certain wavelength is absorbed by the molecule, whereby one electron is transferred from its electronic ground state $S_0$ to the energetic higher electronic state $S_1$. In general, an energetic state is divided into many individual levels and is therefore not mono-energetic. These individuals are known as vibrational and rotational energy levels and are responsible for the spread of the excitation and emission spectra. For reasons of clarity, the rotational levels are not shown in most Jablonski term schemes [61]. Therefore, they are only indicated on the left in figure 3.1. The excitation of the molecules occurs within femtoseconds [62]. In this energetic higher state, the molecules relax within picoseconds radiation-free to the lowest vibrational level by internal conversion. Finally, after a molecular-dependent lifetime of typically a few nanoseconds, the molecules drops down again to the ground state resulting in the emission of light (fluorescence) by releasing their remaining energy. As a result of the previous relaxation of vibrational and rotational levels, this electromagnetic radiation exhibit longer wavelengths and thus is less energetic than the absorbed photons. This difference among the wavelengths is known as Stokes shift and its value depends on the fluorescent molecule [57]. In figure 3.2 the excitation and emission spectra of the fluorescence dye CellMask© Orange are depicted. The Stokes shift is the difference between the excitation and the emission maximum.
3.1. Fundamentals of Fluorescence Microscopy

Figure 3.2: Excitation and emission spectra of the fluorescent dye CellMask® Orange used in this work. The Stokes shift is the difference between the excitation and emission maximum and varies from one fluorophore to another. The shape of the spectra also depends on the fluorescent molecule according to their distribution of rotational and vibrational levels. The spectra data was obtained from [63].

3.1.2 Operating Principle of a Fluorescence Microscope

The principle of fluorescence microscopy is based on the essential stokes shift. Due to the fact, that the emitted light exhibit longer wavelengths than the excitation light, it is possible to separate them from each other. For this purpose, a dichroic mirror is used as beam splitter. It is only transparent for a certain range of wavelengths, light of a wavelength which does not fit to this range will be reflected. The light propagation in a filter cube of a fluorescence microscope is illustrated in figure 3.3.

Figure 3.3: Schematic illustration of the beam path in a filter cube of a fluorescence microscope. As beam splitter a dichroic mirror is used, which is selectively transparent for a specific range of wavelengths. Excitation and barrier filters ensures that only the desired light can enter and leave the cube.

The excitation light with shorter wavelengths are reflected towards the sample by the dichroic mirror. The emitted light of the sample can pass through the semitransparent mirror
Chapter 3. Fluorescence Microscopy

towards the detector. Additionally, the excitation and barrier filters ensures that only the desired light passes this beam path. The excitation filter is usually used in conventional fluorescence microscope, such as the epi-fluorescence microscope. Here, the excitation wavelength is filtered out from the spectrum of light source which is often a Halogen lamp [64]. Excitation light, which is back-reflected by the sample, can not reach the detector since it will be reflected away by the mirror. Therefore only the stained areas appear in the microscope image. In this way, individual organelles in cells as well as individual proteins and even metabolic processes can be made visible.

3.1.3 Point Spread Function and Optical Resolution

The optical resolution of a light microscope is in general limited by the diffraction of light on lens and apertures. Therefore the signal of an object will be transformed when imaged by an optical system. Hereby, each diffraction caused by an attribute of the optical system contributes to the overall transformation and the end result is the sum of individual transformations. The imaging of a point-like source by an optical system leads to characteristic diffraction pattern in the image plane, which is called Airy disk. The diffraction pattern of an imaged point-like source is always broader than the source itself. Additionally, the resulted image is not just a single point but consists of several circular rings of light with an intense central spot (see figure 3.4 (a)). In figure 3.4 (b) the intensity distribution of a cross-section through the center of the computer-generated Airy disk can be seen. The characteristic diffraction pattern of a point-like source exists not only in 2D but also in 3D and is known as point spread function (PSF). This function describes the response of point-like source when imaged by an optical system. The resulted three-dimensional diffraction pattern of a point-like source typically looks like an oval shaped central spot surrounded by several intensity rings. The oval shape of the central spot is the result of the fact that the resolution in the z-direction is lower than that in the x-y direction [65].

![Figure 3.4: Imaging of a point-like source by an optical system results in a characteristic diffraction pattern, also called Airy disk.](image)

(a) Computer-generated Airy disk of a point-like source in the image plane.   
(b) Intensity distribution of a cross-section through the center of the Airy disk.
The lateral resolution of an optical microscope is given by the smallest distance \( d \) of two point-like sources, where their separation is still possible in the image plane. The definition of this critical distance \( d \) is not unique and there are several approaches to it. The most known optical resolution limit for the microscopy was formulated by Ernst Abbe. He discovered that the maximum achievable resolution cannot be more than

\[
d_{\text{min,Abbe}} = \frac{\lambda}{2n\sin(\alpha)} = \frac{\lambda}{2\text{NA}}
\]

where \( \lambda \) is the wavelength of light and \( \text{NA} = n \sin(\alpha) \) is the numerical aperture of the objective, which is determined by the refractive index \( n \) of the medium located between objective and sample and the half aperture angle \( \alpha \) of the objective lens. This relation he ruled out by his experiments with light diffraction on the double slit. According to Ernst Abbe, an object can be resolved in the image plane if the \( \pm 1 \) diffraction orders can still be captured [66]. Very good objectives have a numerical aperture of \( \text{NA} = 1.4 \) and use oil as immersion medium with a refractive index of \( n = 1.518 \). Resulting in a theoretical achievable resolution of about \( \sim \frac{\lambda}{4} \). However, this resolution is never reached by conventional microscopes in praxis due to material deviations and uncertainties in the beam path. Consequently, the rule of thumb for most optical systems is \( d_{\text{min}} \approx \frac{\lambda}{2} \). This rule of thumb results in a theoretical maximum resolution of 300 nm for long-wavelength red light (\( \lambda \approx 600 \text{ nm} \)) and 225 nm for short-wavelength blue light (\( \lambda \approx 450 \text{ nm} \)) in x-y-direction.

As mentioned above the lateral resolution is better than the z-resolution resulting in an oval shaped appearance of three-dimensional PSF, which is surrounded by diffusing light rings. This distorted appearance is because light from other planes also contributes to the appearance when imaging a three-dimensional, fluorescent object in the focal plane. Whereby, the quality of the optical system as a crucial role. Systems of higher quality have a significantly smaller PSF than systems of lower quality. In this context, the better the optical system, the smaller the PSF. The distortion caused by diffusive light of other planes also contributes to a reduction in resolution. In order to enable the investigation of approximately 200 nm small objects and structures such as TNTs, it is therefore of great importance to achieve imaging close to or even beyond the diffraction limit.

### 3.2 Confocal Microscopy

With the confocal microscopy technique a resolution close to the Abbe-limit is achieved by step-by-step scanning of the sample and the usage of an additional pinhole in the beam path. The aperture pinhole enables a better depth of focus. Emission light which arises from other planes as the focus plane is not focused on the pinhole and therefore filtered out. The schematic beam path of a confocal microscope is illustrated in figure 3.5 (a). In order to achieve the scanning process, a laser is used as light source. The excitation light is reflected by a dichroic mirror towards the sample. In contrast to conventional fluorescence microscopes where the whole sample is illuminated, the sample is scanned point-by-point with a focused laser spot in confocal microscopy. The scanning is realized by a scanning box which contains several scanning mirrors [65]. The emitted light passages the scanning box as well
as the dichroic mirror and is focused on the detector. With the usage of a photomultiplier (PMT) the fluorescence intensities are recorded and the microscopy image is reconstructed on a PC. Where the intensity values are linked to their corresponding x-y-coordinates aimed by the scanning mirrors. Only the emission light which arises from the focal plane reaches the detector. The out-of-focus emission light is blocked by the aperture pinhole. In this way, confocal microscopy can achieve a better depth of focus than the conventional epifluorescence method.

(a) Schematic construction of a confocal microscope. The excitation light is reflected by the dichroic mirror. The sample is scanned point-by-point in x- and y-direction by a scanning mechanisms realized with a scanning box. The emitted light passages the scanning box as well as the dichroic mirror and reaches the detector. Out-of-focus emission light arising from other depths as the focal plane is filtered out by the pinhole.

(b) Schematic construction of a STED microscope. Based on a confocal microscope, an excitation laser is coupled in addition to the excitation laser. This laser has a doughnut-shape intensity profile (see fig. 3.6 (a)). The fluorophores inside this regime are excited to stimulate emission. The stimulated emission is filtered out by a STED filter. With this technique the fluorescent spot size is reduced and a resolution beyond the diffraction limit can be achieved.

Figure 3.5: Schematic constructions of a confocal and STED microscope.
3.3 High-Resolution STED Microscopy

The point-by-point illumination enhances the contrast and enables the acquisition of 3D images by moving the sample in a defined z-direction and recording an optical section at each depth \[67\]. Very good confocal microscopes achieve a resolution close to the diffraction limit in x- and y-direction.

The disadvantage of the confocal microscopy is that the scanning mechanism slows down the speed of image acquisition. Additionally, the high intensity of the focused laser spot is more harmful to the cells as compared to the intensities of LEDs used in epifluorescence \[68\]. Consequently, one has to be careful, when using confocal microscopy for live-cell imaging which is the case in this thesis. Therefore it is significant to keep the light exposure and the laser intensity as small as possible without losing information and high costs regarding resolution when imaging cells alive.

3.3 High-Resolution STED Microscopy

Stimulated Emission Depletion (STED) microscopy was developed by Stefan W. Hell in 1994, for which he was awarded the Nobel Prize in 2014. With this kind of microscopy a resolution better than the diffraction limit can be achieved \[69\]. The schematic construction of a STED microscope is shown in figure 3.5 (b). Based on the construction of the confocal microscope, an additional laser is coupled into the beam path. This laser is used to excite fluorophores in a certain area to stimulated emission. The wavelength of the stimulated emission is filtered out and therefore a depletion of this area is achieved. If the intensity profile of this laser is chosen in a proper way, it can be achieved that natural fluorescence only occurs in an area that is smaller than the diffraction limit. This requires a doughnut-shaped intensity profile of the STED laser, which means that the intensity is zero in the center and maximal in the outer edge, as illustrated in figure 3.6 (a). If the doughnut-shaped intensity profile of the laser fits perfectly to the confocal excitation spot, the overlay of the two lasers leads to a reduction of the florescence spot. Here, the alignment of the excitation and the STED laser plays a significant role. If a proper alignment is not achieved, the resulted reduced fluorescence spot size will be not as small as possible leading to a decrease of the resolution. For the reduction of the fluorescent spot an additionally filter is needed in order to filter out the STED laser wavelength.

Fluorescence remains inside the STED doughnut and stimulated emission occurs in the bright edge of the laser doughnut. The process of stimulated emission is shown in figure 3.6 (b). The fluorophore is excited by photons with a certain wavelength (green) to higher electronic state. Due to internal conversion the molecules relaxes towards the lowest vibrational level within the excitation state. Here, after short life time the molecules can regularly drop down again by emitting fluorescent light (orange) or they are forced to emit stimulated light (red). This is achieved by a higher STED wavelength before they have the chance to relax by their own. The molecule relaxes to the ground state and emits a photon with the same wavelength as the incident STED photon.

By exploiting the stimulated emission, the area illuminated by the STED laser is specifically
Chapter 3. Fluorescence Microscopy

(a) Schematic illustration of the intensity profiles in the STED microscope. The excitation and the STED laser are aligned, whereby the intensity profile of the STED laser is doughnut-shaped. The overlay of the lasers leads to stimulated emission in this specific region. The wavelength of the STED laser is filtered out. This results into a reduced fluorescence spot.

(b) Simplified Jablonski energy diagram showing the process of fluorescence (left) and stimulated emission (right).

Figure 3.6: Schematic illustration of the principle of STED microscopy. The usage a doughnut-shaped STED laser intensity profile leads to the reduction of the fluorescence spot by stimulated emission.

depleted and fluorescence only occurs through spontaneous emission in the central spot of the doughnut. Both the excitation and the STED beam are limited by diffraction, whereas the remaining fluorescence spot is not. This depletion of the fluorescence in the periphery of the excitation point leads to an increase of the lateral resolution. The achieved lateral resolution can be derived as follows:

\[ d_{\text{min,STED}} = \frac{\lambda}{2 \text{NA} \sqrt{1 + \frac{I_{\text{max,STED}}}{I_S}}} \]  

(3.2)

where \( \lambda \) is the STED wavelength, NA the numerical aperture and \( I_{\text{max,STED}} \) is the maximal STED intensity \[70\]. \( I_S \) is the so called saturation intensity, which is defined as the intensity at which the first 50 % or more of the fluorophores are already relaxed to the ground state \[71\]. If one compares the above formula 3.2 with the formula for an Abbe-limited resolution 3.1, one will find that it is the same except for the root term in the denominator. For infinitely high STED laser intensities, this factor goes to infinity and thus the lateral resolution \( d_{\text{max,STED}} \) goes to 0. However, in the practice the STED laser intensity is limited by the signal-to-noise ratio of the image. Here, the used fluorescent dye has a crucial role, since its properties highly influences the brightness and quality of the recorded image. If the intensity of the excitation laser is too high, too many molecules are excited resulting in a very dark image with a poor signal-to-noise ratio. For a sufficiently good image, about 15 to 20 photons per bright voxel
3.4 Resolution Enhancement by Deconvolution

The resolution of an image can be improved by trying to determining the PSF out of the captured images and thus getting the original signal instead of the distorted diffraction pattern. This approach is done by an iterative method, the deconvolution. As illustrated in section 3.1.3 the image of point source is rather a collection of several light rings spreading out from a centered intensity maximum then consisting of a single spot. The three-dimensional characteristic diffraction pattern is known as PSF. Mathematically the image formation process of an object can be treated as convolution

\[ g = f \ast h \] (3.3)

where \( g \) is the image, \( f \) the object and \( h \) the corresponding PSF. In other words, the microscope provides an image \( g \), which is a distorted version of the object. The distortion is due to the blur (convolution) caused by the PSF. This problem is also known as the inverse convolution problem. A general solution for this problem is given by the convolution theorem, which states that the Fourier transform of a convolution (\( G \)) of two functions is equal to the product of the Fourier transform of the two functions (\( F, H \)):

\[ G = F \cdot H \] (3.4)

Thus, in Fourier space the signal of the object can be determined by the quotient \( F = \frac{G}{H} \). However, there are significant problems with this relation. The Fourier transform of the PSF can have zero points, in which the quotient is not defined. In addition, the measured signal is not only composed of the signal of the object, rather it is the sum of the object signal and the background noise. Thus, the more exact formulation of the problem is

\[ g = f \ast h + n \] (3.5)
where the additionally term $n$ corresponds to the noise signal. The noise detectable in the image is mostly caused by photon noise. In Fourier space the relation is changed to

$$G = F \cdot H + N.$$  \hfill (3.6)

Thus, the final deconvolution problem looks as follows:

$$F = \frac{G}{H} - \frac{N}{H}.$$  \hfill (3.7)

One can notice, that the noise term dominates for high frequencies where $H$ gets close to zero. This additional term as well as possible zero points of the Fourier transform of the PSF cause that the native deconvolution fails completely and an analytical solution is impossible. In order to solve this deconvolution problem nevertheless, one uses iterative approaches. The quality of a restored image resulted by deconvolution depends on the efficiency of these approaches and of course on the quality of the measured image itself. In addition, z-stacks are essential for the deconvolution of images, since the complete signal of a point also includes signals arriving from other z-planes outside the focal plane. Thus, the deconvolution of one single slice often fails, because the deconvolution algorithm is then incapable to distinguish the signal of the focus plane from the diffusing signals of other planes.
Chapter 4

Effect of Ionizing Radiation on Biological Matter

Ionizing radiation is any particle or electromagnetic radiation that has sufficient energy to ionize atoms or molecules of the target material. In this process, one or more electrons are removed from the electrical potential of the target atom. The interaction of ionizing radiation with biological matter results in chemical bond breakages and thus in biological damage. In the tumor radiation therapy, this biological radiation effect is used to kill tumor cells. Irradiation acts as stress factor to cells and therefore they have to react specifically to it in order to survive. This chapter deals with the deposition of energy on biological matter by ions and the associated biological damage.

4.1 Energy Loss of Ions

Ions essentially lose their energy through collisions with the electrons of the target material. In the case of heavy ions, there can also be interactions with the nucleus. Thus, the energy loss of the ions in a target material is composed of electronic energy loss at the electrons of the atoms of the target and nuclear energy loss at the atomic nuclei. These two interactions are responsible for stopping process of ions when passing through matter. The stopping power of an ion is described by:

\[ S(E) = -\frac{1}{\rho} \frac{dE}{dx} \] (4.1)

where \( \rho \) is the target material density, \( E \) the energy of the ion and \( x \) corresponds to the path length during the passage through the matter.

The electronic interaction is dominated by Coulomb interactions between the electrons of the target material and the charged particles. In this interaction, the particles continuously lose energy through many individual collisions with the electrons of the target material \[73\]. The energy loss of the particles rises with increasing penetration depth. The rate of energy loss that a charged particle experiences when passing through matter is described by the Bethe-Bloch equation. This equation has the following form

\[ -\frac{dE}{dx} = \frac{4\pi n z^2}{m_e v^2} \left( \frac{e^2}{4\pi \varepsilon_0} \right)^2 \left[ \ln \left( \frac{2m_e c^2 \beta^2}{I \cdot (1 - \beta^2)} \right) - \beta^2 \right] \] (4.2)
where \( v, z, E \) characterize the moving charged particle by its velocity, charge and energy, respectively. The parameters \( n, I \) describe the target material by its electron density and mean excitation potential. The variable \( x \) describes the penetration depth, \( \varepsilon_0 \) is the electrical field constant, \( m_e \) is the electron mass, \( e \) is the elementary charge, \( c \) is the speed of light and as abbreviation \( \beta = \frac{v}{c} \) is used.

The nuclear energy loss is dominated by elastic scattering of the ions in the shielded Coulomb potential at the atomic nuclei of the target material and the strong interaction at the central collision with the atomic nucleus. This interaction requires an energy of the particles above the Coulomb barrier and can lead to fragmentation of both the charged particle and the target nucleus at high energies. Here, no excitation or ionization occurs. Consequently, the stopping process caused by nuclear interaction is only predominate for ions with small kinetic energy. In this work, \( \alpha \)-particles with a kinetic energy of 2.3 MeV were used to irradiate cells. In figure 4.1 the total, electronic and nuclear stopping power for \( \alpha \)-particles passing through water in dependence of their energy is shown. The vertical dashed black line marks the considered energy of 2.3 MeV. At this energy, the stopping process caused by electronic interaction is dominant and nuclear stopping can be neglected.

![Figure 4.1](image_url)

**Figure 4.1:** Total, electronic and nuclear stopping power in dependence of the kinetic energy for \( \alpha \)-particles passing through water. The nuclear stopping process contributes only at low kinetic energies to the total stopping power. The electronic stopping process is predominate for \( \alpha \)-particles having a kinetic energy of 2.3 MeV (dashed black line) as used in this work. The data for the plot was generated online by ASTAR [74].
4.2 Dose

During the stopping process, the charged particles deposit energy in the matter. The dose

\[ D = \frac{\Delta E}{\Delta m} \]  

(4.3)

indicates the amount of energy \( \Delta E \) deposited in a volume element of the mass \( \Delta m \). It is expressed in the unit Gray, 1 Gy = 1 J kg\(^{-1}\). The dose is not homogeneously deposited in matter, since the underlying processes are statistical in nature. Consequently, there are high fluctuations of the absorbed dose recognizable in the micro- and nanometer-range. These fluctuations are considered in microdosimetry \[75\]. The non homogeneous nature of dose deposition is beyond the scope of this thesis, because only the overall dose on the sample is used in the performed experiments.

4.3 Linear Energy Transfer (LET)

The mean energy deposition also varies along the irradiation direction. To describe the energy transfer of the charged particle along its traversal in biological matter, the linear energy transfer (LET) is introduced. This quantity results from the energy release \( \Delta E \) of the ion to matter per path length \( \Delta x \):

\[ \text{LET} = \frac{\Delta E}{\Delta x} \]  

(4.4)

and is specified in the dimensions keV \(\mu\text{m}\). The above expression looks very similar to the stopping power (see equation 4.1). Indeed, due to the conservation of energy both are closely related to each other, but there are important differences. One constraint is that the LET does not take Bremsstrahlung into account, whereas the stopping power does. However, the contribution of Bremsstrahlung during the passage of a charged particle with an energy in the MeV-range through matter, is very small \[72\]. Hence, the LET is a sufficient quantity to describe the stopping power of charged particles with high energies as used in radiation therapy and in this work. Additionally, the LET describes the local rate of energy transfer to biological matter, which is another essential difference \[76\]. The relation of the LET to the local pattern distribution of energy deposition utilizes it as a tool to understand the biological action of radiation. The LET depends on the type of particles as well as their energy and varies between 0.1 keV \(\mu\text{m}\) and more than 1000 keV \(\mu\text{m}\) \[73\]. The LET is also dependent on the target material. However, using water as biological matter is a very good approximation, since cells consist mainly of water. The LET for \(\alpha\)-particles with an energy of 2.3 MeV is 150 keV \(\mu\text{m}\) (calculated with SRIM \[77\]). In addition, the LET is a measure of the ionization density in the target material and can therefore be used in biological experiments as a characterizing property for different particles. There are two distinct LET ranges, the low and high LET range. The limit is defined at 10 keV \(\mu\text{m}\) in biology. Radiation with a LET < 10 keV \(\mu\text{m}\) causes isolated ionizations, whereas radiation with a LET > 10 keV \(\mu\text{m}\) such as heavy ions induces densely packed ionizations. The higher the LET, the higher the density of adjacent ionizations distributed along the track of the ionizing particle \[78\]. For this reason, one also speaks of sparsely or densely ionizing radiation.
If a sample is now irradiated with a specific LET, the absorbed dose can be calculated from the following correlation:

$$D = \frac{F \cdot \mathrm{LET}}{\rho}$$  \hspace{1cm} (4.5)$$

where $F$ is fluence (number of particles per area) of the ionizing radiation and $\rho$ the density of the target material.
Chapter 5

Cell Irradiation with $\alpha$-Particles

One aim of this thesis was to investigate the response of cell-to-cell communication via TNTs in U87 glioblastoma cells on radiation. For this purpose, homogeneous high-LET irradiation of U87 cells was performed with an $\alpha$-particle source at the Institut für Angewandte Physik und Messtechnik in Neubiberg. The $\alpha$-irradiation device was developed by H. Roos and A. M. Kellerer [79]. This device in cooperation with a high-resolution STED microscope enables the study of irradiation effects on cells including DNA double strand breaks and their repair mechanisms [72] [80] [81] or cellular communication behavior as in this master thesis. In this chapter, the $\alpha$-particle setup is illustrated as well as the cell cultivation and handling during the irradiation proceeding are described.

5.1 Setup

The schematic construction of the $\alpha$-irradiation device is shown in figure 5.1. The $\alpha$-particles are produced by an Americium-241 source which has an activity of 0.37 GBq. The source is disk-shaped with an active area of 85 mm in diameter. Additionally, the source is covered with a 2 $\mu$m thick gold-palladium alloy and has a half-life of 485 years. 85% of the generated $\alpha$-particles have an energy of 5.49 MeV. However, due to self-absorption and the gold-palladium alloy the energy of the particles is decreased to a value of approx. 4 MeV [79]. The source is mounted on a stainless steel disk with a diameter of 87 mm, a height of 5 mm and is turnable with several cycles per second. The rotation of the source reduces inhomogeneities in the radiation intensity over the surface of the source. Furthermore, there is a collimator system, which allows only the passage of those particles that exhibit an angle smaller than 12° to the normal. The channels inside the collimator system have a diameter of 3 mm and are arranged in a hexagonal pattern. This collimator is also moved by a wobbler to ensure that all cells are irradiated. If the collimator is not moved, some cell will be covered by the channel walls and would therefore not be irradiated. The transparency of the collimator is 80 %. Between the collimator and the exit window there is a computer-controlled shutter disk which regulates the exposure time. The whole source chamber containing the source, collimator and shutter disk is shielded by a vacuum-tight stainless steel container with a diameter of 400 mm and a height of 135 mm. The chamber is flushed with Helium at normal pressure. The Helium reduces the energy loss of the particles by scattering processes compared to air by 20 %.  

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Chapter 5. Cell Irradiation with α-Particles

(a) Schematic illustration of the α-irradiation device. (b) Schematic illustration of the sample chamber.

Figure 5.1: Schematic construction of the α-irradiation setup. The α-particles are generated by a turnable Americium-241 source with a diameter of 85 mm. Above the source there is a collimator system which limits the particle direction. This collimator system is moved by a wobbler. The source chamber is fumigated with helium at normal pressure to minimize scattering events of the α-particles. Between the 2.5 μm Mylar exit window and the collimator there is a computer-controlled shutter that regulates the exposure time. The samples are placed on a spacer above the tensioned 2.5 μm thick Mylar foil. Inside the sample chamber water filled cell culture dishes are placed in as water reservoirs to increase the humidity. The illustrations are adapted from [72].

Normal pressure is necessary, to avoid the compensation of pressure differences by the exit foil and therefore enables the usage of a very thin 2.5 μm Mylar foil as exit window to reduce the energy loss of the particles caused by the foil. After the exit through the Mylar foil, the particles still have an energy of $\sim 2.7$ MeV. For cell irradiation a 3 mm thick o-ring is placed on the Mylar foil. This spacer is used to avoid the direct contact of the cells with the exit foil. If the cells are placed on the exit foil without this o-ring, they will be scraped off by their removal after irradiation. Due to the energy loss by the passage of these 3 mm space in air, the particles have an energy of $\sim 2.3$ MeV when reaching the sample. This corresponds to a LET of $150 \text{ keV} \cdot \text{μm}^{-1}$ and a range of approximately 12 μm. It can be assumed, that the thin medium layer on the cells is approx. of 5 μm [72]. Thus, the α-particles have an energy of 1.4 MeV and a range of 7.4 μm when reaching the cell layer. This corresponds to a LET of $200 \text{ keV} \cdot \text{μm}^{-1}$ (calculated with SRIM [77]). The fluence rate of the radiation at the sample is approximately $4.7 \cdot 10^{-3} \frac{\text{ions}}{\text{μm}^2 \cdot \text{min}}$ [80]. This corresponds to a dose rate of roughly $0.12 \text{ Gy} / \text{min}$. To increase the humidity inside the chamber and thus avoiding the complete dehydration of the sample, 30 mm wide round petri dishes filled with water are used as water reservoirs in the sample chamber (see figure 5.1 (b)).

5.2 Cell Seeding and Irradiation

One day before irradiation, the U87 cells were seeded on round, high precision cover glasses with a diameter of 25 mm and a precise thickness of $(170 \pm 5) \text{ μm}$ (Marienfeld). The cells were incubated in 6-well plates filled with 4 ml medium (DMEM, high glucose containing 10%
FBS and 1% P/S) overnight at 37 °C, 100% humidity and 5 % CO₂. Per well 150,000 cells were seeded. This seeding density ensures that the cells are not too dense for the evaluation of an incubation time of 72 h after irradiation. For irradiation the cover glass was pot out of the well and the medium was removed by careful dabbing to a tissue without touching the cells. This step is necessary because otherwise too many α-particles are stopped by the medium layer and the cells are not irradiated with the desired dose. The cover glass was sucked in with a vacuum pin and placed up side down centrally on the 3 mm thick o-ring in the sample chamber. The cells were irradiated for 10 minutes, which corresponds to a final dose of 1.2 Gy. Previous studies at the α-particle irradiation device reveal that 1 % of the cells get a dose of more than 2 Gy and 1 % of cells are not hit during irradiation with 1.2 Gy [72]. After irradiation the cover glass was put back into a fresh medium filled 6-well plate and incubated at 37 °C, 100% humidity and 5 % CO₂ until evaluation. The old 6-well plate was conscious not reused in order to avoid unwanted influence of non-irradiated cells grown on the plate bottom.
Chapter 6

Microscopy of Tunneling Nanotubes in Living U87 Cells

This chapter deals with the microscopy methods used to study TNTs in living U87 cells. The live cell imaging system of our microscope and its improvements, which have been developed during this work are discussed. The imaging methods including the imaging settings as well as the mosaic-drive-mode provided by the microscope are outlined in this chapter. Furthermore, the used fluorescent dyes are presented and evaluated for their suitability in the research on TNTs.

6.1 Live-Cell Imaging Setup

This section deals with the live-cell imaging setup equipped on our STED microscope, where the general construction of the live-cell imaging setup as well as improvements developed during this thesis are described.

6.1.1 Setup Construction

The live-cell setup construction is depicted in figure 6.1. Our microscope is the commercial STED microscope Leica TCS SP8 3X. The temperature of the whole system is adjusted by the microscope temperature control system “The Cube & the Box” of the company Life Imaging Services (Basel, Switzerland) (see figure 6.1a). The system is based on an incubator box combined with a precision air heater and ensures the temperature control of both, the specimen and the microscope parts including stage, stand and objective. The temperature control by an incubation box is the best option for high resolution microscopy, because focus instabilities caused by even small temperature changes are only avoided when the whole setup is heated up equally [82]. The CO₂ supply is provided by a gas mixer “The Brick” (Life Imaging Services, Switzerland), with which one can adjust the CO₂ amount. The CO₂-air-mixture is passaged through a humidifier shown in figure 6.1b. This humidifier is filled up with distilled water and the gas is pressured through a diffuser, where the gas is split up into several bubbles. The bubbles take up the water as they ascend and therefore the humidity is increased in the atmosphere. The gas further travels through hoses to the sample and the gas
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(a) The temperature is controlled by an incubator box and an air heater. (b) Humidifier in the CO\textsubscript{2} supply system. The adjustment of the CO\textsubscript{2} supply is established by a gas-mixer. (c) The closure of the sample by a lid ensures a sufficient gas environment.

Figure 6.1: Photographs of the live-cell imaging setup of our STED microscope.

Figure 6.2: Special live-cell imaging container for live-cell imaging after irradiation at the $\alpha$-particle setup described in chapter 5. (a) The container is composed of three parts: Bottom part, middle part and lid (from left to right). (b) The cover glass is clamped by screwing the parts together.

For the live-cell imaging of irradiated and sham irradiated cells seeded on glass coverslips a special live-cell container was used. This container was developed by B. Schwarz and is composed of three parts (see figure 6.2a). The cover glass is clamped by screwing the bottom part to the middle part. The usage of O-rings ensures the tightness of the container and prevents the leakage of the medium during image acquisition. The container can be closed...
with a lid (see figure 6.2 b). This live-cell container enables the imaging of living cells after the irradiation at the α-particle setup described in chapter 5.

6.1.2 Improvements of the Live-Cell Imaging Setup

The live-cell imaging setup was improved during this thesis by the development of new dish holder to enable long-term imaging without an increased focus loss and the ascertainment of the correct setup settings that ensures cell survival for several days by the system.

6.1.2.1 Construction of a New Dish Holder for the STED Microscope

On of the most critical problems when imaging living cells over a long time duration, is the focus shift caused by the system, temperature changes and vibrations. If the focus plane is shifted during acquisition time, the images recorded after this shift are either not collecting the whole cell or are even completely black. This means, that information gets lost and the successful collection of the desired cell dynamics fails. Considering our system one will recognize that the temperature as well as vibrations are not the main causes for the focus shift [82]. The temperature control is established by an incubation box, where up-heated air is circulated by the cube. This means, that after a certain heating up time (9.75 h and 16.5 min for the microscope and refrigerated samples at 23 °C, respectively [72]) the system has been stabilized and an equally stable temperature is reached. Vibrations can be also excluded since the microscope stands on a vibration damped table. Consequently, if one gives the sample and the microscope enough time to reach temperature stabilization, the focus shift is caused by another reason. As main cause of the focus shift, the dish holder for 35 mm wide cell culture dishes was identified.

![Figure 6.3: The development of a new dish holder enables long-term imaging without an increased focus shift.](image)

As one can see in figure 6.3 a, the dish is placed into an adapter ring which is again placed into the holder of the microscope. The problem here is, that there is no fixation of the sample to the holder. Every part, the dish as well as the adapter ring, are loosely put together. This construction causes focus shifts when only one of the two parts is slightly moved. Conse-
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Consequently, the focus problem can be solved by the development of a new dish holder. For the construction of the new dish holder the program Autodesk Inventor Professional 2018 was used. The new dish holder is made of aluminum and weighs less than 100 g. The weight is decisive, since the galvo (high precision z-drive) of the microscope shall not be loaded heavier than a weight of 130 g, otherwise the spring system of the galvo will be damaged. Aluminum has the additional advantage that it has a high thermal conductivity. This means, that the holder heats up very fast and a temperature equilibrium in the system is reached more quickly. The design drawing can be found in the appendix C. The sample is fixed in position by two clamping springs (see figure 6.3 b). The new holder can be firmly screwed into the microscope just like the original holder. Different increments of the clamping springs enables the usage of dishes exhibiting different heights. Additionally, the holder was designed in such a way that each 35 mm dish fits to it. With this new dish holder it is was able to minimize the focus shift. For a time-lapse imaging of more than 12 hours a successfully image collection with a slight focus shift of 0.2 \( \mu m \) was achieved.

Furthermore, the new dish holder facilitates the gas environment provided by the CO\(_2\) supply (see figure 6.1 c). With the new dish holder a complete closure of the microscope stage and the lid is established, whereas the old dish holder has gaps where the gas can escape and get mixed with the heated, dry air of the temperature control. Thus, the new dish holder improves the live-cell imaging setup by solving two problems, the focus shift and the gas mixing with the heated dry air resulting in the loss of both, CO\(_2\) and humidity.

6.1.2.2 Ascertainment of the Correct Setup Settings

The live-cell imaging setup was further improved by the determination of the correct setup settings. The problem with the usual settings of 37 \(^\circ\)C and 5% CO\(_2\) was that the cells did not survive when cultured over the weekend in the live-cell imaging setup, even not when no imaging acquisition occurred during this time. Consequently, the settings are not reflecting the truth and either 37 \(^\circ\)C nor 5% CO\(_2\) are achieved. A temperature measurement with a thermometer reveals that the temperature reaches a value of \((39 \pm 1) ^\circ\)C at a setting of 37 \(^\circ\)C which is slightly too high. Additionally, the medium of the cell cultures was always pink after a short time duration. The pink color of the medium indicates that the pH is too low. Meaning that the percentage of CO\(_2\) is too low in the gas environment provided by the system.

For the ascertainment of the correct setup settings, 120 000 U87 cells were seeded in 35 mm glass bottom dishes (Ibidi) containing 2 ml medium. At least two dishes were prepared on Thursday. On Friday, the cells were imaged with the phase-contrast microscope Primover (Zeiss) and it was verified whether the cell densities are the same in each dish. One dish was put back in the incubator and the other one was placed in the running live-cell imaging setup on the switched off microscope over the weekend. For the cultivation, the dishes were tightly closed by locking feature of the lid. This special feature is a click system of the dish design and minimizes the evaporation. According to the manufacturer, this locking feature minimizes the moisture loss to approximately 10% per day in a dry 37 \(^\circ\)C environment. On Monday, the color of the medium was noted, the amount of medium loss was determined and again phase-contrast images of the cells were recorded for both, control and sample. This
6.1. Live-Cell Imaging Setup

(a) Control for (35 °C; 5% CO₂).
(b) Sample for (35 °C; 5% CO₂).
(c) Control for (33 °C; 15% CO₂).
(d) Sample for (33 °C; 15% CO₂).

Figure 6.4: U87 cells imaged on Friday before cultured in the incubator (control) or in the live-cell imaging setup (sample) over the weekend. The samples and the controls have comparable the same cell density. Scale bars: 200 µm.

The process was performed for the settings (35 °C; 5% CO₂) and (33 °C; 15% CO₂), the mass flow of the gas mixer was kept at 3 l/h for both.

In figure 6.4 the phase contrast images of the cells recorded on Friday before the incubation over the weekend are displayed. The cell density is comparable for the controls (cultivated in the incubator) and the samples (cultivated in the live-cell imaging setup). After the weekend the controls cultivated in the incubator at 37 °C, 5% CO₂ and 100% humidity were exponentially grown and reach a high cell density (see figure 6.5 (a) and (c)). The cells cultured in the live-cell imaging system with the setting of (35 °C; 5% CO₂) almost all died (see figure 6.5 (b)). The medium of this cell population was extremely pink i.e. the CO₂ level was definitely low. The medium color of the control was red-orange. The medium loss was estimated to be about 200 µl in the sample. Thus, 10% of the medium evaporates during 2 days culturing in the live-cell system. A medium loss of the controls cultivated in the incubator at 100% humidify was not detectable. The adjustment of the system settings to (33 °C; 15% CO₂) results in a survival of the cells (see figure 6.5 (d)). The cells cultivated in the live-cell system grew not as good as the controls, but their amount increases over the time of 2 days i.e. cell division has been appeared. This means, that the condition provided by the system
Chapter 6. Microscopy of Tunneling Nanotubes in Living U87 Cells

Figure 6.5: U87 cells imaged on Monday after the cultivation in the incubator (control) or in the live-cell imaging setup (sample) over the weekend. Almost all cells died upon the cultivation in the live-cell system at the settings (35 °C; 5% CO₂). The adjustment of the settings to (33 °C; 15% CO₂) results in an achieved cell survival. Scale bars: 200 µm.

are sufficient for cell growth. The color of the medium was pink-red, which means that the CO₂ level is still not high enough at these settings. However, a higher percentage of the CO₂ is not adjustable by the gas mixer, but one could instead try to increase the mass flow to achieve an even better environment for cell growth. The medium color of the control was again red-orange. The moisture loss of the sample was estimated to be approximately 100 µl.

6.2 Imaging Methods

The cells were imaged alive at 37 °C and a sufficient percentage of CO₂ fumigation for cell growth. Live-cell imaging of the TNT was preferred to avoid TNT breakage and distortion upon cell fixation. The images of this thesis are recorded with a commercial Leica TCS SP8 3X STED microscope, which exhibit three STED wavelengths, 592 nm, 660 nm, and 775 nm. The first two are CW (continuous wave) STED lasers and the last one is pulsed STED laser. The microscope uses a white light laser adjustable from 470 nm to 670 nm for excitation and an array of prisms for detection, which means that the wavelength range can be flexibly adjusted.
for the detection. The fluorescent light is detected by hybrid detectors (HyD) consisting of photomultiplier and avalanche photo-diode, which combine very good sensitivity with good noise suppression. A STED microscope resolution of 105 nm in lateral direction and approx. 200 nm in axial direction was measured\textsuperscript{72}. In this thesis, the resolution of the system was additionally investigated using GATTA Quant products (see appendix D). However, due to the low quantum yield of the fluorescent spots, an accurate resolution measurement was not possible. The images were acquired with a high resolution using the oil objective Leica HCX PLAPO 100× with a numerical aperture of 1.4 in combination with the halogen-free and fluorescence-free immersion oil Immersol\textsuperscript{TM} 518 F / 37 °C of the company Zeiss (Pulch + Lorenz GmbH; Catalog number: 444970-9010-000) which has a refractive index of 1.518 at 37 °C.

6.2.1 Live-cell Imaging Settings

During live-cell imaging the WLL laser intensity (Excitation laser intensity, 100% WLL intensity $\hat{=} \approx$ approx. 50 mW) was kept as low as possible to reduce the stress of the cells caused by the laser, but high enough to yield a sufficient image brightness. For the imaging of the U87 cells after the treatment with $\alpha$-particle radiation labeled by CellMask\textsuperscript{©} Orange a WLL intensity of 10% to 15% was chosen. For the collection of images where the cells were labeled with a weak staining such as CellLight\textsuperscript{®} or DiO, the WLL intensity was increased up to 100% to achieve a sufficient image brightness. For live-cell imaging of U87 cells fresh labeled with CellMask\textsuperscript{©} Orange or Green, a WLL laser intensity of 5% is sufficient, but has to be increased after a short time window of few minutes. For STED imaging of TNTs and filopodia diameters, the WLL intensity was not exceeding 45%. STED images are collected with 30 % ($\hat{=} \approx$ approx. 70 mW) STED laser intensity in x-y-direction and 70 % in z-direction.

The cells were scanned bidirectional and with a scanning speed of 600 Hz to ensure a fast image acquisition, which reduces movement artifacts and stressing of the cells caused by long light exposure time. The chosen excitation wavelength and the detector settings for the individual dyes can be found in the appendix B. The images were recorded with a set pixel size of 40 nm $\times$ 40 nm and z-plane spacing of 160 nm or 400 nm. The pixel sizes must be selected so that they meet the Nyquist criterion (pixel size $= \frac{1}{2.3}$ resolution\textsuperscript{72}) and their adequate choice is essential for a later image processing by deconvolution.

6.2.2 Mosaic-Drive-Mode

To record a large area at high resolution the mosaic-drive-mode of the microscope can be used. Imaging using the 100× oil objective and a set pixel size of 40 nm results in an image size of 116.25 µm $\times$ 116.25 µm. Hence the field of view is relatively small and only a few cells are contained in this area. Consequently, one has to enlarge this area to record more cells. This can be achieved by using an other objective with a smaller magnification but then the resolution of this enlarged image will be decreased. In order to still obtain high resolution in an enlarged recorded area one can use the mosaic function. It is available in the LAS X program of the microscope as special acquisition mode. In order to compensate possible z-stages disagreements due to unevennesses of the cover glasses a focus map tool is
provided. Where one can adjust the respective focus at certain positions distributed within the area of interest. This tool is very important to achieve a proper data acquisition since if the beginning of the z-stack is set too low, one would collect many dark pictures which contain no information. On the other hand, if the beginning is set too high, one will miss to record the interface of the cell to the substrate which is often a very interesting plane. Therefore, it is significant to ensure that the focus is set correctly in an appropriate range over the whole area of interest. For further information about the mosaic merging settings please visit the website of Leica Microsystems [84].

For the investigation of the response of TNT networks in U87 glioblastoma cells on radiation, mosaic images were collected with a field size of $10 \times 10$ partial images and an overlapping of 20%. The cells were imaged alive to avoid possible breakages of the connections, since it was reported that TNTs are sensible for fixation [17]. The z-stacks consist of 14 slices with a synchronized z-distance of 400 nm covering an overall height of 5.2 µm. The parameters were chosen in order to acquire the whole cell by leaving the overall acquisition time less than one hour. This limited imaging duration is to keep the changes of communication networks as small as possible. A rectangular distribution consisting of 9 points was chosen for the focus map.

### 6.3 Fluorescence Markers for the Plasma Membrane and Cytoskeleton Components

In this work, the plasma membrane as well as the cytoskeleton of the cells were labeled. In this section, the individual used markers are introduced. All dyes are non-toxic and suitable for live-cell imaging. The staining protocols are provided in the appendix A.

#### 6.3.1 Ascertainment of the Most Suitable Marker

Due to their small size, TNTs are studied by fluorescence microscopy. For their visualization the TNTs have to be fluorescent labeled. The staining of the TNTs is of great importance as it provides the basis for their research. In order to identify the most suitable dye for the visualization of TNTs by fluorescence microscopy, different staining methods of the plasma membrane to visualize TNTs were investigated in this work. The plasma membrane of the cell can be labeled by different techniques and dyes. The most common technique is the usage of lipid inserter as staining. A lipid insertor is composed of long aliphatic tails and a fluorescent head group. The plasma membrane is fluorescent labeled by the stable incorporation of these molecules into the lipid bilayer. Additionally, there are other ways to obtain a membrane staining like the selective labeling of a membrane component or transduction. During this study five critical points for TNT staining by membrane dyes were identified: cell-off background noise, quantum yield, homogeneity as well as reliability of the staining and the speed of internalization of the dye into the cell body by endocytosis. In the following, the individual membrane dyes are evaluated for their suitability as TNT markers according to these five critical points and their staining effort.
6.3. Fluorescence Markers for the Plasma Membrane and Cytoskeleton Components

6.3.1.1 WGA

The labeling of membrane protein such as the glycoprotein results in a plasma membrane staining. Wheat germ agglutinin (WGA) conjugates are widely common used fluorescent lectins which selectively bind to N-acetylglucosamine and N-acetylneuraminic acid residues. Glycoproteins that have these sugar moieties are probed by WGA conjugates. As labeling solution Hank’s balanced salt solution (HBSS) without phenol red is preferred since labeling with culture medium can lead to a higher cell-off background. An incubation time of 10 minutes is sufficient. In this thesis, the WGA, Alexa Fluor™ 633 conjugate (Thermo Fisher Scientific; Catalog number: W21404) was used. This dye has a max. excitation wavelength of 632 nm and a max. emission wavelength of 647 nm.

The staining with WGA results in a very noisy and speckled appearance of the cell membrane. In figure 6.6 two confocal microscope images of U87 stained with WGA are shown. One can see that the vesicles inside the cells are clearly visible and intensively stained whereas the plasma membrane is poorly stained and only weakly contrasts with the background. Additionally, there is a lot of cell-off background noise noticeable. This high background noise is not only the photon noise but also generated by fluorophores which are resolved in the medium. Since WGA selectively binds to sugar moieties which are probably also contained in the medium the cell-off background is increased. Due to this high cell-off background noise it is difficult to identify and to enhance the signal of the small membrane protrusions and TNTs. The TNTs are only visible as weak and dotted signals and some of them are almost vanished after the subtraction of the background noise. This high cell-off background noise in addition to the immediately overexposure of the intracellular vesicles after the labeling lead to the fact that the fine TNTs are not evaluable and thus excludes WGA as suitable TNT marker.

Figure 6.6: The staining of WGA results in a high cell-off background noise. TNTs are only weak and as patchy structures visible. Scale bars: 20 µm.
6.3.1.2 CellLight™

The fluorescent labeling of plasma membrane of live cells can also be achieved by CellLight® reagents. These reagents are ready-to-use constructs which enable the cellular labeling of the plasma membrane by transduction and the usage of the Myristoylation/palmitoylation sequence from Lck tyrosine kinase as target sequence. The cells are transfected in a simple, one-step process by the BacMam 2.0 transduction technology. Here, an insect cell virus (baculovirus) coupled with a mammalian promoter is used to transfer genes. Only the transfer-genes are expressed, whereas the baculoviral genes and their promoters are not recognized. Due to this transduction, fluorescent signal peptides or proteins fused to CFP, emerald GFP (emGFP), or TagRFP are expressed by the cells and a specific targeting of cellular structures is achieved. The usage of the CellLight® reagents is safe and provides a transient footprint-free labeling method of cells [86]. In this thesis CellLight™ Plasma Membrane-GFP, BacMam 2.0 (Thermo Fisher Scientific; Catalog number: C10607) was employed. This dye has a max. excitation wavelength of 488 nm and a max. emission wavelength of 509 nm.

Figure 6.7: With CellLight™ Plasma Membrane-GFP reagent successfully transfected cells exhibit various GFP expression rates with a very low quantum yield. Scale bars: 20 µm.

The big advantage using transfection and therefore manipulating the DNA to label the plasma membrane is that there is no dye rearrangement caused by endocytosis. Which means that the staining of the plasma membrane does not vanish over the time and the signal of the vesicles does not become more intensive than before. However, using CellLight™ GFP as labeling of TNTs turned out to be difficult since the signal yielded from the expressed GFP after the successful transfection of the cells, is very low. Therefore a high WLL intensity of 85 % is needed to achieve a sufficient brightness of the images. This high laser intensity again causes high background noise and is certainly not beneficial for live-cell imaging. In figure 6.7 two images are displayed which show cells with a very good GFP expression rate.
The cells look very bright, but the impression is misleading since the contrast of the images was extremely increased with the program ImageJ. The maximum gray values are 128 for (a) and 166 for (b). The plasma membrane as well as the fine membrane protrusions are clearly visible, but one can also see that not all cells expressing the same amount of GFP. Some cells are brighter than others (see figure 6.7 (b)) and in some cases only the vesicles of the cell are visible (see lower left corner of figure 6.7 (a)). This inhomogeneity of the staining also suggests that not all cells are successfully transfected. The increase of the PPC (particles per cell) during transfection has neither improved the quantum yield nor led to a more uniform GFP expression of the cells. Due to the low signal gained from the expressed GFP and the high number of very dark or even not visible cells, CellLight™ was excluded as TNT marker.

6.3.1.3 PKH

The fluorescent dye PKH was developed by Paul Karl Horan in 1989 \[87\] for long-term cell tracking. The marker is a lipid insertor that binds irreversibly to the cell membrane. There are several types of PKH with different fluorescence spectra and aliphatic tail structures. In this work PKH26 (Sigma-Aldrich, Catalog number: Midi26) was used. Its fluorescence lies in the yellow-orange region with a max. emission wavelength of 567 nm and a max. excitation wavelength of 551 nm.

PKH is one of the best known plasma membrane markers. However, for an efficient staining of the cells a dye-loading solution is needed. The solvent- and salt-free labeling solution Diluent C is iso-osmotic, which means that the cell membrane is not damaged by osmotic pressure caused by concentration differences. Additionally, the labeling of PKH is designed for the staining of cell suspensions. This means, that the cells have to be trypsized from the surface and resuspended in the labeling solution after a centrifugation step (see labeling protocol in appendix A). To remove the unbound remnants of the dye, the cells have to be washed several times by repeated centrifugation and resuspension in medium. For microscopy the cells have to be counted again and seeded in glass bottom cell culture dishes. The cells need about 4-5 h to adhere to the surface. The use of the dye-loading solution and the repeated centrifugation steps makes the staining with PKH very stressful for the cells.

To reduce the stress at the labeling of the cells, one can try to label them when they are still adherent to the surface. However, this leads to a more inhomogeneous staining of the cells by PKH. Which means, that some cells are more intensive stained than others (see figure 6.8). Furthermore, even if the dye-loading solution is iso-osmotic to the cells, it can happen that the cells are damaged by the labeling. Figure 6.8 (b) shows an image where this was the case and the cells have been damaged by the labeling solution.

Besides the stressful labeling process the inhomogeneity of the staining as well as the rearrangement of the dye by internalization processes are further problematic. The internalization process of PKH was studied. U87 cells were labeled in suspension and seeded in cell culture dishes. The cells were imaged 1 h, 5 h, 21 h, 2 days and 3 days after finishing the staining process. In figure 6.9 the corresponding images are displayed. Figure 6.9 (a) shows a cross section through the still round and suspended cells imaged 1 h after finishing the labeling process. The dye is mainly distributed at the cell surface. Additionally, one can see that the
cells show membrane bubbles which are indicators for the still fragile state of the cells after labeling. In figure 6.9 (b) a image recored after 5 h is depicted. The cells have adhered to the surface again and the internalization of the dye has continued. Now, in some cells the vesicles are brighter than the plasma membrane and one can see that the cells are not equally stained by PKH. This becomes extremely problematic if one looks at figure 6.9 (c). Here one can see that after 21 h some cells are completely non-visible. The TNTs depicted in the image end in emptiness and one can only assume that there are other cells which are hard to recognize. The overexposure of the vesicles increases over time and after 3 days the cell membrane is not visible anymore. The cells are not distinguishable of each other since there is no cell border noticeable (see figure 6.9 (e)).

Furthermore, the cells react sensible to the laser when labeled with PKH. The stress level of the cells is recognizable by membrane bubbling when illuminated by the laser. Cells which are in a more fragile or even apoptotic state exhibit more membrane bubbling than cells in a healthy state [88]. It has been reported, that the cell viability dramatically decreases if the cells are labeled with PKH and exposed to light [89]. These findings suggest that PKH has a high phototoxicity.

Due to the stressful labeling process, the high phototoxicity and the inhomogeneity of staining which becomes really problematic with progressing dye internalization, PKH was excluded as suitable TNT marker.

**Figure 6.8:** Adherent cells labeled with PKH using a solvent- and salt-free loading solution. Scale bars: 20 µm.
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Figure 6.9: The internalization of the PKH dye causes a dye redistribution within the cells. After three days the plasma membrane as cell border is no more detectable and only the intracellular vesicles are visualized by the staining. Scale bars: 20 µm.
6.3.1.4 DiO

DiO (DiOC$_{18}(3)$; 3,3’-Dioctadecyloxycarbocyanine Perchlorate; Thermo Fisher Scientific; Catalog number: D275) is a lipophilic dialkylcarbocyanine dye fluorescing in the green region with a max. excitation wavelength of 484 nm and a max. emission wavelength of 501 nm. It is a lipid insertor and as staining solution the medium is used.

The labeling with DiO requires an incubation time of several minutes. According to the manufacturer one should use 20 minutes as starting point and ascertain the actual needed incubation time by stepwise increasing or decreasing of the incubation time. If the incubation time is too short the labeling with DiO fails and the cells are only recognizable by some labeled vesicles. The cells can be labeled when adherent to the surface or in suspension similar to PKH (see protocol in appendix A). However, the appearance of the staining with DiO varies from cell type to cell type. Some cell lines are labeled very well with DiO and others are only punctuated labeled [90].

![Images of labeled cells](a) (b) (c)

**Figure 6.10:** The labeling with DiO results is very inhomogeneous and a broad variety of the brightness levels of the cells is noticeable. Scale bars: 20 µm.

In the case of the U87 cells the labeling by DiO appears very different. Some cells are intensive stained and appear very bright on the images, whereas other cells are hard to see (see figure 6.10). The problem with the unpredictable brightness levels of the cells was not solvable by increasing the incubation time or the dye concentration. An uniform cell membrane labeling was never achievable by DiO in U87 cells. In addition to this inhomogeneity, the DiO staining is not very reliable. During this work, the cells were initially cultivated in RPMI medium, where the labeling with DiO worked with an incubation time of 20 minutes, but due to the reached high passage number the cells were exchanged by U87 cells with a lower passage number which were cultivated in DMEM, high glucose medium. With the change of the medium, the labeling with DiO failed completely and an incubation time of more than 1 h results in a poor labeling of only a few cells. The dye internalization further contributes negative to a poor cell membrane signal. The internalization process of DiO was additionally studied. For this purpose, the cells were labeled in suspension as in the case of PKH and seeded in glass bottom cell culture dishes.
The cells were imaged 1 h, 24 h, 2 days and 3 days after finishing the labeling process. 1 h after the labeling the cells are still round and already exhibit different brightnesses (see figure 6.11(a)). The imaging of the cells on the next day reveals that some cells are not sufficiently stained by DiO (see figure 6.11(b)). They are only recognizable by the accumulations of their intracellular vesicles. The membrane as well as the fine membrane structures are only clearly visible at cells which are well stained. The vesicles of some cells are very overexposed. After more than 2 days the labeling of the plasma membrane is almost vanished and only the vesicles are clearly visible (see figure 6.11(c) and (d)).

Since the labeling of DiO results in a very inhomogeneous and not reliable cell membrane staining of U87 cells, this membrane marker is no longer used to visualize the TNTs.

Figure 6.11: Internalization of the dye DiO in U87 cells. Scale bars: 20 µm.
6.3.1.5 CellMask®

CellMask® plasma membrane stains are also lipid inserters. CellMask® is available in three colors green, orange and deep red. In this thesis, CellMask® green (Thermo Fisher Scientific; Catalog number: C37608) and CellMask® orange (Thermo Fisher Scientific; Catalog number: C10045) were employed. CellMask® green has a max. excitation wavelength of 522 nm and a max. emission wavelength of 551 nm. CellMask® orange has a max. excitation wavelength of 554 nm and a max. emission wavelength of 567 nm.

The labeling with the dye CellMask® results in an uniform and intensive cell membrane staining in U87 cells (see figure 6.12). Here, no dramatical variation of the brightness levels of the cells is recognizable. Each cell exhibits almost the same brightness with a high quantum yield. Therefore, a WLL intensity of 5% is sufficient resulting in a low background noise. The plasma membrane, filopodia as well as the TNTs are clearly visible. There is no difference recognizable when labeling with CellMask® Orange or Green. Both dyes results in a very good labeling of the plasma membrane. Only their properties regarding photostability, excitation and emission spectra are different. CellMask® Green is rapidly bleached when illuminated with the STED laser, whereas the signal of CellMask® Orange is more longer stable.

![Image](a) U87 cells labeled with CellMask® Green. (b) U87 cells labeled with CellMask® Orange.

**Figure 6.12:** CellMask® Green labels the plasma membrane as efficient as CellMask® Orange. For both dyes, the cells are uniformly stained. The cell membrane as well as membranous structures like TNTs are clearly visible due to an intensive labeling. Scale bars: 20 µm.

The labeling process is very easy and quick. The cells are labeled within 15 minutes and as staining solution the medium is used as described in the labeling protocol in the appendix. According to the manufacturer this dye is developed to be independent from the used cell type and to increase the narrowed window for cell membrane studies limited by the internalization process. The dye is negatively charged which slows down its internalization by endocytosis. In figure 6.13 images recorded a few minutes, 20 h, 26 h and 2 days after the labeling of the cells with CellMask® Green ((b) and (c)) or Orange ((a) and (d))
are displayed. In figure 6.13 (a) the cells are labeled with CellMask® Orange and exhibiting the same brightness. The plasma membranes of the cells are well stained and clearly visible. After 20 h and 26 h the distribution of the dye remains nearly the same (see figure 6.13 (b) and (c)). Only the signal decreases over time and a WLL intensity of 40% is needed to achieve a sufficient image brightness. After 2 days the internalization is recognizable (see figure 6.13 (d)). The intracellular vesicles are now overexposed, but the cell membrane is still visible. This demonstrates that CellMask® is more resistant to endocytosis than DiO or PKH.

Figure 6.13: The internalization of CellMask® occur slower than of DiO or PKH. Scale bars: 20 µm.

This membrane marker is characterized by its uniform, intensive and stable labeling of the cell membrane. Its internalization into the cell body occurs slowly and the labeling effort is low. The cells can easily and quickly be labeled within the medium. These features and the
fact, that the TNTs are good visible over a long time duration define CellMask© as the most suitable TNT marker.

6.3.2 Cytoskeleton Markers

In addition to the cell membrane, the cytoskeleton F-actin as well as microtubules were stained in this work. For the labeling of actin, CellLight™ Actin-GFP, BacMam 2.0 (Thermo Fisher Scientific; Catalog number: C10582), SiR-actin kit (Spirochrome; Catalog number: CY-SC001) and SiR700-actin kit (Spirochrome; Catalog number: SC013) was used. Microtubules staining was performed with SiR-tubulin kit (Spirochrome; Catalog number: SC002). SiR has a max. excitation wavelength of 652 nm and a max. emission wavelength of 674 nm. SiR700 has a max. excitation wavelength of 698 nm and a max. emission wavelength of 728 nm.

The CellLight© reagents method was previously discussed in the section 6.3.1.2 For the targeting of actin, GFP fused to human actin is expressed upon transfection with almost no cytotoxic effects [92].

The near-infrared fluorophore silicon-rhodamine (SiR) derivate was initially introduced by Lukinavičius et al in 2013 [93]. This dye is specially suitable for live-cell imaging and super-resolution STED microscopy. Due to its far red spectrum it is highly biocompatible, since phototoxicity and unwanted autofluorescence of the cells increase with decreasing wavelengths [94]. Furthermore, the dye is featured by a high photostability, brightness and permeability. The most excellent feature of SiR probes is that no washing steps are needed and a low dye concentration can be left in the medium during long-term live-cell imaging. This causes that the signal is kept constant by substantially binding of the dye to its target structure and thus photobleaching problems are avoided. The dynamics of the cytoskeleton is not affected when using a dye concentration below 100 nM [95].

SiR-actin consists of the fluorophore SiR and the actin binding natural product jasplakinolide and specifically labels f-actin [96], whereas the microtubules binding drug Docetaxel is the basis for SiR-tubulin [97]. In contrast to the most commonly used actin staining probe Phalloidin, SiR actin has a high cell permeability. This enables the staining of actin for live-cell imaging without transfection or invasion steps such as electroporation or glass-bead loading to introduced the dye in the cell body.
Chapter 7

Data Processing and Size Measurement

The microscopy images were analyzed using several methods. Deconvolution of microscopy data was applied for background noise reduction and resolution enhancement. It enables the subsequent quantitative evaluation of tube diameters and improves 3D Sfp rendering results. Sfp rendering was used for visualization of 3D microscopy images. The sizing of the tunneling nanotubes (TNTs) and filopodia diameters in U87 cells was realized by a correlation method using the Van Steensel Ansatz. The two-sample t-test was used for statistical significance verification. These analysis methods are introduced in the following chapter.

7.1 Deconvolution of Microscopy Raw Data

For background reduction and resolution enhancement the microscope raw data were deconvolved using the Huygens Professional Deconvolution program of the company Scientific Volume Imaging B.V. (Hilversum, Netherlands). The program uses a theoretically calculated point spread function (PSF) to accurately restore the microscope image. The calculation of that theoretical PSF is based on the used microscopy parameters such as voxel dimensions, numerical aperture, excitation- and emission-wavelengths etc. The deconvolution problem which was described in section 3.4 is solved by an iterative Classic Maximum Likelihood Estimation (CMLE) algorithm. This algorithm is based on a non-linear iterative method with the idea to iteratively optimize the likelihood of an estimate of the object issued by the measured image and a theoretical PSF. For this, an initial estimation of the object pictured in the image is generated and convolved with the PSF to a blurred estimate image, which is then compared to the measured image. With the difference (computed correction) of these two images a new estimation of the measured image is computed and the process is repeated. This iteration will be continued until the quality stop criterion is met. The CMLE algorithm is the most general restoration method and has the advantages that it restores the data with little information loss with a high quality. However, the disadvantage of this algorithm is its slowness. For very computer-intensive data such as long time-series faster algorithms as the Quick Maximum Likelihood Estimation-time (QMLE) algorithm are beneficial. For detailed information about
the Huygens Professional Deconvolution program please refer to the website of Scientific Volume Imaging B.V. [98].

For a qualitative good deconvolution result the signal-to-noise ratio (SNR) is of great importance. Since the quality criterion which computes the likelihood assumes that the photon noise in the image underlies Poisson statistics. If the criterion for the SNR is set too low, the resolution is not improved by the deconvolution process. On the other hand, if the criterion for SNR is set too high, statistical fluctuations are interpreted as signals and artifacts occur in the computed image. Therefore, it is significant to figure out which SNR still results in an artifact-free image by successively increasing the SNR. The SNR depends on the photon yield. STED microscopy images were recorded with a low photon yield therefore a low SNR should be used for deconvolution. However, the chosen SNR should not be too low, since otherwise there is no resolution enhancement by deconvolution. In her dissertation, Dr. Judith Reindl figured out that a SNR of 5 is a good choice to deconvolve STED microscopy images recorded with the same setup as used in this thesis [72]. For microscopy images with a higher photon yield such as confocal microscopy images a higher SNR can be used.

However, one has to draw attention to the photon yields for each dye and situation separately. For instance, whether the labeled cells are alive or fixed while imaging. The SNR is probably lower at live-cell imaging since the medium mostly contains phenol red which causes additional noise. Residues of the dye in the medium also leads to a lower SNR. The brightness of a dye also depends on the duration of time-lapse imaging. Due to photo-bleaching as well as rearrangements of the dye the local brightness can vary extremely. In figure 7.1 the deconvolution results of a confocal image using different SNRs are displayed where (a) shows the measured image. The cells were labeled with CellMask© Orange. Thus the plasma membrane as well as other lipid compositions like vesicles inside the cell are visible. The image shows a TNT which interconnects two cells with each other. Underneath the TNT an arm or offshoot of a third cell is visible. The deconvolution results with a SNR of 3 and 5 exhibit clear continuous membrane borders and the TNT is clearly separated from the offshoot (see 7.1 (b) and (c)). A SNR of 7 might also provide a good restoration of the image but the membrane borders get more and more a punchy appearance (see 7.1 (d)). However, with a SNR of 10 the result is not artifact-free anymore, recognizable by the dotted appearance of the continuous membrane (see 7.1 (e)). A deconvolution using the default SNR value of 20 for confocal microscopy images in the Huygens Professional program completely fails by image restoration (see 7.1 (f)). The membrane borders are not visible and the TNT can not be differentiated from the offshoot. These results demonstrate that an appropriate SNR has an essential role for image restoration by deconvolution.
7.1. Deconvolution of Microscopy Raw Data

Figure 7.1: Deconvolution results of a confocal microscopy image showing a TNT with different SNRs. Scale bar: 10 µm.
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7.2 Visualization of 3D Microscopy Images by Sfp Rendering

3D microscopy images are visualized as simulated 3D pictures by using the Huygens Simulated Fluorescence Process (SFP) Volume Renderer available by the Huygens Professional Deconvolution program (Scientific Volume Imaging B.V., Netherlands). This renderer computes the distribution of the fluorescent material from the intensities values of the 3D microscopy data. The results are displayed as a simulation of the appearance of the object when excited by the corresponding excitation wavelength. Herein, it models the physical light interaction with the fluorescent matter as it would have happened in reality when excited at the identical conditions as depicted in the microscopy data. At the end, a scene of the object is simulated, where the parts facing the viewer contribute more to the image than hidden parts. The direction of the excitation light as well as the viewpoint can be regulated by the user. Best results are achieved by using deconvolved images, since the noise will also be illustrated by the rendering. The Sfp rendering is a good tool for the visualization of 3D microscopy images and to get a better feeling how the labeled proteins and cellular components of interest are distributed within a cell.

7.3 Size Determination using the Van Steensel Ansatz

Microscopic structures as the tiny membrane protrusions and bridges can be analyzed by using correlation functions. Especially, the diameters of these linear membrane structures are of interest, since this is a main TNT characteristic. To establish a proper sizing of these diameters it is advantageous to use a method which does not only determine the diameter by looking at the intensity profile at one single position along the tube. Instead, the method should reflect a quantitative diameter profile of the entire length of the tube. The autocorrelation method using the Van Steensel Ansatz is such a method [99].

The cross-correlation analysis method of Van Steensel et al. [99] enables the quantitative evaluation of correlated and non-correlated patterns in complex dual-labeling images of the same dimensions. Herein, the two images to be compared are shifted to each other pixel-by-pixel in x- and y-direction from $-\Delta x$ to $\Delta x$ and from $-\Delta y$ to $\Delta y$. For each shift the Pearson correlation-coefficient $r_P$ is determined as follows:

$$ r_P(\Delta x, \Delta y) = \frac{\sum_i (A_i - \bar{a})(B_i - \bar{b})}{\sqrt{\sum_i (A_i - \bar{a})^2 \sum_i (B_i - \bar{b})^2}} $$  \hspace{1cm} (7.1)

where $A_i$ and $B_i$ are the gray values at the current pixel $i$ in first image A and second image B and $\bar{a}$ and $\bar{b}$ are the respective mean values. In an anti-correlation, the Pearson correlation-coefficient assumes the value -1 and in a full correlation, the value 1. Thus, the Pearson coefficient is within a range of [-1,1]. As the final result one obtains the 2D correlation function, which is a correlation matrix with the size of $(2\Delta x + 1) \times (2\Delta y + 1)$. By plotting the Pearson correlation-coefficients as a function of the shift $\Delta x$ for a fixed $\Delta y$ or vice versa, one can distinguish positively and negatively correlated patterns in two-labeled images. Important
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Here is the shape of the 1D cross-correlation function. If the pattern is positively correlated the size of overlapping distributions can be determined by measuring the peak width of the cross-correlation function at the half-maximum height.

This property can be further used for sizing microscopic structures [100]. Instead of taking a second image containing the intensity distribution of a second dye, one uses the copy of the original image, thus calculating the autocorrelation function. With the pixel-by-pixel overlapping technique one determines the size of the structure in a very precise and objective way. The autocorrelation function for no shift ($\Delta x = 0$, $\Delta y = 0$) equals 1, since here the two identical structures are completely overlapping. If the shift is increased in one direction, the Pearson correlation coefficient will decrease followed by changing its sign when the overlapping is more and more canceled by exclusion and a negatively correlation begins.

Since the autocorrelation function is a symmetric correlation problem, it can be simplified into a convolution problem [100] where the intensity distribution of the structure is convolved with itself. For simplification the intensity distribution of a TNT was assumed to be Gaussian-shaped. The convolution of two Gaussian functions results again in a Gaussian function with the width $\sigma = \sqrt{\sigma_1^2 + \sigma_2^2}$ [101], where $\sigma_1$ and $\sigma_2$ are the respective widths of two convolved Gaussian functions. Since the Gaussian-shaped structure with the width $\sigma_1$ is folded with itself, the relation can be simplified to $\sigma = \sigma_1\sqrt{2}$. Thus, the autocorrelation function is a Gaussian function with a full width at half maximum (fwhm) of $f_{\text{whm}} = 2\sqrt{\ln 2}\sigma$ and the diameter of an TNT can be calculated as follows:

$$d_{\text{measured}} = f_{\text{whm}1} = 2\sqrt{\ln 2}\sigma_1 = \frac{f_{\text{whm}}}{\sqrt{2}}$$  \hspace{1cm} (7.2)

To determine $\sigma$ the following Gaussian function was chosen as fit of the autocorrelation function:

$$f(x) = y_0 + \frac{A}{\sqrt{2\pi\sigma}}e^{-\frac{x^2}{2\sigma^2}}$$  \hspace{1cm} (7.3)

where $y_0$, $A$ are the mean Pearson correlation coefficient for large shift and the amplitude of the Gaussian function, respectively.

In figure 7.2 the sizing of TNT using the autocorrelation correlation method with the Van Steensel Ansatz is depicted. The STED microscopy image of the TNT visualized by the dye CellMask® Orange is displayed in figure 7.2 (a). The single pixels are visible. The image was recorded with a pixel size of 40 nm. The corresponding autocorrelation function is showed below (see 7.2 (b)). For fitting a range of $\Delta x \in [-6; 6]$ was chosen, since in this range the autocorrelation function is still positive and the structures still are overlapping each other.

The actual TNT diameter is then calculated by quadratic subtraction of the STED resolution:

$$d_{\text{actual}} = \sqrt{d_{\text{measured}}^2 - d_{\text{resolution}}^2}.$$  \hspace{1cm} (7.4)

Here, a STED resolution of 105 nm was assumed, because this value was measured in previous experiments at similar imaging conditions [72].
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(a) STED image of a TNT. (b) Autocorrelation function of the TNT. The Gaussian function (red line) fits for a x shift from -6 to 6, where the autocorrelation function remains positive. The pixelsize is 40 nm.

Figure 7.2: Sizing of a TNT using the correlation method. The resulted positive autocorrelation is fitted with a Gaussian function to determine the TNT diameter by measuring the fwhm. Scale bar: 500 nm

7.4 Statistical Evaluation with the Two-Sample T-Test

In order to resolve significant differences regarding to cellular communication via TNTs the two-sample t-test was used. With the independent two-sample t-test one can verify whether the mean values of two independent samples are identical or not (mean value comparison). Prerequisite for the two-sample t-test are that the data are normally distributed and the variance of both samples is statistically the same. At the t-test the null hypothesis $H_0$ assumes that the difference between the two groups is very small and hence both belong to the same population. Which means that the two mean values are the same ($H_0: \mu_1 = \mu_2$). In contrast, the alternative hypothesis $H_1$ represents the opposite, where the two mean values are very different and therefore the two groups most likely originate from two distinct populations ($H_1: \mu_1 \neq \mu_2$). With the t-test it will be evaluated which hypothesis should be discarded. For this purpose, the $t$ value is calculated as follows:

$$
t = \sqrt{\frac{N_1 N_2}{N_1 + N_2}} \frac{\mu_1 - \mu_2}{s_p} \tag{7.5}
$$

where $N_1, N_2$ is the number of samples of group 1 and 2, respectively and $s_p$ is the pooled standard deviation. The pooled standard deviation can be arrived from the individual standard
derivations \( s_1 \) and \( s_2 \) of two mean values:

\[
s_p = \sqrt{\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2}}
\]  

(7.6)

To figure out whether the null hypothesis is refused or not, one needs the critical \( t \)-value. The critical \( t \)-value is determined by the degrees of freedom \( (N_1 + N_2 - 2) \), for the two-sample \( t \)-test) and the level of significance \( \alpha \). The null hypothesis will be refused if the \( t \)-value is more away from 0 than the critical \( t \)-value. Critical \( t \)-values can be looked up in tables. Often the significance is marked by a small \( p \). This \( p \) stands for the p-value and is the area under the symmetric, zero centered \( t \)-distribution left to the negative \( t \)-value and right to the positive \( t \)-value. To verify the significance one can also compare the level of significance \( \alpha \) with the \( p \)-value instead of comparing the \( t \)-value with the critical \( t \)-value. If the \( p \)-value is smaller than the level significance, the null hypothesis will be refused. At this point one can say that the two compared mean values are significantly different from each other. Typically the level of significance is taken at 5% for statistical highly significant results [102]. Thus, with the specification of \( (p < 0.05) \) one indicates significance.
Chapter 8

Characterization of TNTs in U87 Cells

This chapter deals with the morphology of the U87 glioblastoma cells, and with the knowledge about their TNT connections regarding their morphology and properties gained in this thesis. In addition, STED nanoscopic measurements were performed on TNTs and filopodia diameters to resolve their structures and identify whether filopodia and TNTs in U87 cells have the same diameter.

8.1 Morphology of U87 Glioblastoma Cells

Gliomas are the most common and most aggressive brain tumors. They are characterized by their high invasiveness and recurrence, which means that despite multimodal treatment, patients have a median survival of no more than 15 months [103]. The cell line used in this thesis was deposited from a male human by J. Ponten in 1966 [104] and is called U87 MG (ATCC, HTB-14). The cells have an epithelial morphology and grow adherent. For microscopy adherent cells are beneficial, because they are very flat attached to the surface and axial movement is low. The cells were cultivated in DMEM, high glucose medium (Sigma Aldrich) with the additives 10 % FCS (Sigma Aldrich) and 1 % PenStrep (100 mg/ml Penicillin Streptomycin, Sigma Aldrich) at 37 °C, 100 % humidity and 5 % CO2 fumigation. They were passaged twice a week, and single-cell suspensions were derived by 5 min treatment with Trypsin/EDTA (Sigma-Aldrich). A subcultivation of 1:5 was recommended. U87 cells have a doubling time of approximately 34 h [105], which means that they grow slowly compared to other cell lines such as HeLa. The doubling time is quite important, since it is an indicator for the required seeding cell density when preparing an experiment. In the process of this thesis, it was figured out that seeded cells need more than one day culturing until they start to exponentially grow again. Additionally, the cells need even longer to grow when seeded to a low cell density.

The U87 cell line was selectively chosen for this thesis, because in previous studies it was observed that these cells exhibit a high number of TNT networks [55]. The high cell connectivity can also be presumed by their morphology. In figure 8.1 (a) a phase-contrast image of a grow-
Chapter 8. Characterization of TNTs in U87 Cells

(a) Human U87 glioblastoma cells  (b) Mouse GL261 glioblastoma cells

Figure 8.1: Phase-contrast images of the cell lines, U87 and GL261. Scale bar: 100 µm.

ing U87 cell population is depicted. One can see that these cells do not grow homogeneously like e.g. mouse GL261 glioblastoma cells (see figure 8.1 (b)), which were also cultivated by the author of this work. The GL261 cells grow in monolayer by spreading over the entire surface and even distances between the cells are formed. In contrast to this, U87 cells are densely packed at some locations with wide spacings. Instead of growing next to each other, U87 cells tend to form clusters and grow in multilayer on top of each other. The tendency of U87 cells to form such spheroids is increasingly shown at high cell densities and under stress situations such as hypoxia and ATP treatment [106] [107] [108]. During growth they stretch out strongly and form long thin lines, which connect the individual nodes with each other. Due to this behaviour, the cells appear to grow like a strongly cross-linked spider’s web. This networking is also reflected in the microscopic range. Here the cells are interconnected by a large number of TNTs (see figure 8.2). Due to this high cross-linking and the high frequency of TNTs, this cell line was selected for further investigation in this master thesis.
8.2 Morphology and Properties of TNTs in U87 Cells

In the next section selected microscopy images are shown, to explain the features of TNTs regarding their appearance, formation, transport and cytoskeletal content. In this section all images were derived by labeling the plasma membrane with an lipid insertor, unless it’s explicitly stated otherwise.

8.2.1 Morphology and Appearance

TNTs mostly appear as straight thin lines. Their length ranges between few micrometers up to more than 108 µm (field size of the 100× objective). They occur at different cell heights and often lie obliquely in space, which means that z-stacks are required to fully map them. In figure 8.3 (a) a single TNT is shown. Single tubes are often found far above the sub-
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Figure 8.3: TNTs can occur alone or as accumulations of several dense packed TNTs. Scale bars: 10 µm.

The strate. In this image the small sizes of the cell nuclei, especially of the cell at the bottom of the image is remarkable. Since the plasma membrane is labeled by a lipid insertor, the unlabeled cell nucleus appears black on the image. Cells have a fried egg-shape appearance when adhered to the substrate. The cell nucleus has an oval spherical shape and is located inside the cell slightly above the substrate. The cell nucleus of the cell located at the bottom of fig. 8.3 (a) has a small size, indicating that this image is recorded at a height, close to the upper end of the cell. TNT do not only occur alone, but also as whole collections. These accumulations of many tubes, as shown in the figure 8.3 (b), are packed so densely that the individual tubes are no longer distinguishable from each other. This phenomenon becomes problematic if one wants to measure their diameters or consider individual tubes on their own.

Figure 8.4: STED microscopy images of branched TNTs. Scale bars: (a) 10 µm; (b) 5 µm.
TNTs are not always continuous straight connections, in fact they can also form kinks and branches (see fig. 8.4). It also occurs that a tube does not connect two but three cells. These tubes have three tube ends which are connected by a junction. However, these branches and junctions of TNTs are rarely found in U87 cells.

In most cases the TNTs get thicker towards their ends and some of them have nodules as the TNT shown in figure 8.5. These nodules can contain some cargoes, but their exact content or role is unclear.

![Figure 8.5: STED microscopy image of a TNT which gets thicker to its end and contains nodules. Scale bar: 10 µm.](image)

In figure 8.6 (a) a Sfp rendering of a TNT connection with a special shape is depicted. This tube has several kinks and its middle section lies on the substrate. The most reports define TNTs as straight line located above the substrate [17, 19]. Since this is not the case here, the question arises whether this can be identified as TNT or not. In my opinion it is a TNT because it establishes cell-to-cell contact over several micrometers and its diameter of ~ 480nm is in the range of usual TNT diameters (see chapter 2, table 2.1). The smaller and branched structures are filopodia or retraction fibers. These two structures are not distinguishable in one single microscope image, since both have the same diameter and appearance.

![Figure 8.6: Sfp renderings of observed special TNT connections.](image)
Chapter 8. Characterization of TNTs in U87 Cells

8.2.2 Formation

TNT formation can be established by several mechanisms as described in chapter 2. By considering the Sfp renderings shown in figure 8.6 one can imagine that the TNT illustrated in (a) was formed by filopodia interplay, whereas the TNTs in (b) probably occur after cell-to-cell division. TNT formation by filopodia interplay is presumable in (a) because the filopodia of the two cells are pointing in the direction of the tube towards each other. Filopodia growth as TNT formation could also explain why this TNT has kinks and substrate contact. If one imagines that this channel was built up after two filopodia tips had met each other and membrane fusion had appeared, the newly formed TNT will need some time to detach from the substrate. After its detachment it will be stretched by the cells due to energy benefits and the kinks will vanish.

When considering figure 8.6 (b) this scene looks like a TNT formation after cell division. The cells are very round and have a lot of retraction fibers or filopodia. It is probable that they’ve just divided and are about to stretch out again using the filopodia. TNTs are often found in associated cells [49, 109]. Thus, it might be that TNTs are also established by cell division and serve as connections between daughter cells.

As one can see by this two simple examples, the imaging of such a TNT formation is quite difficult, because one has to assess the correct time ranges. Additionally, live-cell imaging has to be established in a way that the cells are not influenced by the imaging. The labeling of the membrane is a further aggravation because it is the largest area of the cell that can be labeled. Therefore phototoxic effects are accordingly decisive. Consequently, the cells react very sensible on the laser when stimulating the whole membrane to fluorescence. Nevertheless, the whole process of TNT formation was successfully recorded in this thesis and can be seen in figure 8.7. The TNT was formed within one hour after two adjacent cell moved apart. Recorded was this TNT formation by live-cell confocal video microscopy, where image acquisition happen every thirty minutes over several hours.

TNT formation by filopodia growth or cell division is not excluded by this result because it could be that several TNT mechanisms are active within one cell line. Therefore live-cell imaging of cell division and filopodia interplay are still pursued.
8.2. Morphology and Properties of TNTs in U87 Cells

(a) Two adjacent cells joining cell-to-cell contact.
(b) When the cells move apart from each other membrane fusion occurs. Well recognizable by the accumulation of the fluorescent signal in the lipid multilayer.
(c) A nearly formed TNT appear.

Figure 8.7: Video microscopy images of a TNT formation in U87 cells. The TNT was formed within one hour by cell dislodgement. Scale bars: 10 µm.

8.2.3 Gondola Transport

Additionally, the transport of gondolas were observed and recorded. In figure 8.8 (a) time frames selected from a video microscopy image of a transport are shown. The arrows with different colors indicate the previous positions of the gondola. Every 10 seconds one image was recorded for several minutes. The gondola moved irregularly with an average speed of $(14.5 \pm 0.7) \text{ nm/s}$ forward. Within 5 minutes it covered a distance of $(4.37 \pm 0.20) \mu m$. The gondola has a total length of $(2.96 \pm 0.18) \mu m$ and a width of $(1.94 \pm 0.04) \mu m$. It contains several lysosomes or exosomes, recognizable by the dye accumulations inside the gondola. Furthermore, the gondola is probably filled with cytoplasm which appears dark in the image because it is not labeled by the dye.
Chapter 8. Characterization of TNTs in U87 Cells

(a) Confocal video microscopy images of a gondola transport via a TNT. The arrows with different colors indicate the previous positions of the gondola.

(b) Gondola transport via a TNT in U87 cells. Scale bar: 5 µm. (b) Magnification of the gondola. The gondola contains several lysomes or exosomes and probably cytoplasma which appears black because it is not labeled by the plasma membrane dye. Scale bar: 1 µm.

Figure 8.8: (a) Gondola transport via a TNT in U87 cells. Scale bar: 5 µm. (b) Magnification of the gondola. The gondola contains several lysomes or exosomes and probably cytoplasma which appears black because it is not labeled by the plasma membrane dye. Scale bar: 1 µm.

Gondola transports were rarely observed in U87 cells. The microscopy image depicted in figure 8.9 probably shows the establishment of a gondola or the endocytosis into the target cell at one end of the TNT. A broad cytoplasmic cell-to-cell connection can additionally be seen in this image. This connections is probably an EP bridge as described in chapter 2. Within the EP bridge several vesicles and cytoplasmic materials can be exchanged as visible in this image. It was also observed, that TNTs can be established by the shrinkage of these connections. Furthermore, often TNTs are connected to these broad bridges.

Figure 8.9: Beginning of a gondola transport or endocytosis of a gondola in the target cell. Close to the TNT an EP bridge containing several vesicles and cytoplasmic material, is visible. Scale bar: 10 µm.
8.2.4 Cytoskeletal Content

In this section, the cytoskeletal content of TNTs in U87 cells is investigated. For this purpose, the cells were labeled with SiR-actin and SiR-Tubulin additionally to cell membrane marker DiO. Two- or three-colored confocal microscopy images were recorded. The SiR-actin labels F-actin filaments and SiR-tubilin labels microtubules as described in chapter 6.

F-actin was found only occasionally as fragments or very close to the cell body, i.e. in the tails of the connections. In figure 8.10 microscopy images of U87 labeled with SiR-actin are depicted. One nicely sees the stress fibers of the cytoskeleton inside the cells, but thin protrusions of the cells as filopodia and TNTs were never completely visible with the labeling of the F-actin. Usually the beginning and ends of the tubes contain F-actin, whereas the total length of the TNT was only identifiable by some F-actin fragments distributed randomly inside the TNT. In figure 8.11 the double labeling with DiO reveals that the most tubes are not labeled by SiR-actin. Only in some tails F-actin was found.

![Figure 8.10](image)

**Figure 8.10:** U87 cells labeled with SiR-actin. The TNTs are only partly visible and the dye concentration is too insufficient in the TNTs for TNT visualization. F-actin seems to be only present as fragments inside the TNTs and its location probability gets higher towards the cell body. Scale bars: 10 μm.

The three-color labeling with SiR-tubulin, SiR700-actin and DiO reveals that the thin TNTs and filopodia mostly contain no microtubules. However, thick TNTs contain both, F-actin and microtubules (see figure 8.12 (a), (b), (c) and (d)) and microtubules were additionally found in some origins of filopodia (see figure 8.12 (c) and (e)).

Now the question arises if there is indeed no F-actin present in the membrane protrusions or the dye does not reach the F-actin filaments inside the tiny tubes and filopodia and thus not all actin filaments are visible on the microscopy image. Especially, filopodia which should definitely contain F-actin are not stained by SiR-actin. Therefore the incubation times were increased and Verapamil, an efflux pump inhibitor, was used, but both treatments do not
change the labeling. TNTs and filopodia are still only partially stained by SiR-actin. Hence, TNTs can not sufficiently be labeled by SiR-actin and the dye was excluded for live-cell time-lapse imaging studies of TNTs.

![Image of cell membrane and F-actin](image1)

(a) Cell membrane.  
(b) F-actin.

![Merged image](image2)

(c) Merged image.

**Figure 8.11:** F-actin is partly detectable in the beginnings of the TNTs near the cell body, but the most TNTs exhibit no F-actin content. Scale bars: 20 µm.
8.2. Morphology and Properties of TNTs in U87 Cells

(a) Plasma membrane labeled with DiO.
(b) F-actin labeled with SiR700-actin.
(c) Microtubules labeled with SiR-tubulin.
(d) Merged image.
(e) Magnification of the filopodia in the merged image.

Figure 8.12: Cytoskeletal content of TNTs in U87 cells. Thick tubes contain both, F-actin and microtubules. Thin tubes and filopodia contain mostly no or only fragments of F-actin. Microtubules can be found in some origins of filopodia. Scale bars: (a), (b), (c) and (d): 10 µm; (e) 5 µm.
To verify whether F-actin is indeed not present in the thin TNTs of U87 cells, it was tried to label the actin per transduction using the CellLight™ Actin-GFP, BacMam 2.0 technology. In figure 8.13, the resulted images after the labeling with CellLight™ Actin-GFP at a PPC number of 100 are displayed.

The imaging of cells, which has been incubated over night as described in the appendix A was very difficult because the photon yield was very low. Thus, the cells were imaged with a line accumulation of 6 and with 100 % WLL intensity. Not all cells were stained by...
the BacMam technology and the cells, which were stained show different expression yields of GFP (see figure 8.13 (a)). Additionally, it does not look like actin staining. The cells express GFP but not located in the F-actin filaments. The stress fibers, which were nicely visible by the SiR-actin staining, are not identifiable, punctuated accumulations of expressed GFP are rather detectable inside the cells. Furthermore, the staining of cells with high GFP expression look more like a cytoplasmic staining than a cytoskeletal staining.

It could be possible, that GFP is overexpressed by the cells and therefore the expressed GFP is distributed in the whole cell. To figure out whether the wrong expression of GFP is canceled after a longer cultivation of the transfected cells, the cells were imaged three days and one week after transfection. The figures 8.13 (b), (c) and (d) show that the wrong GFP expression was not solved by longer cultivation of the cells. The only advantage of a longer cultivation is that the signal was increased over this time and no line accumulation and 70 % WLL intensity was sufficient to capture an appropriate image. Resulting that the recording of z-stacks was possible. The figures 8.13 (b), (c) and (d), show maximum projections of z-stacks, whereas figure 8.13 (a) shows only one single slice. In figure 8.13 (d) a magnification of (c) can be seen, showing a thick TNT and several filopodia. The TNT contains the expressed GFP and is fully visible. On the other hand, the thin filopodia are only partially visible, but the distribution of the GFP is more regular as compared to the staining with SiR-actin. However, since GFP is not necessarily located at actin filaments one can not conclude that the visible GFP inside the filopodia and the TNT marks F-actin. Additionally, some cells either hardly or not at all express GFP and are therefore not visible in the microscope. These facts exclude CellLight™ Actin-GFP as a reliable dye and for further experiments.

8.3 Structural Analysis using STED Microscopy

The structure of the TNT in U87 cells was analyzed using STED nanoscopy for the determination of its accurate diameter. These measurements were performed with different dyes with different excitation wavelengths and emission spectra. Here, the diameters of TNTs as well as filopodia were evaluated to figure out whether these similar structures can be distinguished by their diameters in U87 cells. Additionally, the sizing of the TNT diameter was performed by using STED as well as confocal microscopy to reveal the differences between the two techniques.

8.3.1 Comparison of Tubes and Filopodia Diameters

TNTs and filopodia of U87 cells were imaged by confocal and STED microscopy. The cells were imaged alive to avoid possible structure deformations by fixation. The plasma membrane of the cells was labeled by the green fluorescent dye DiO. During the imaging, confocal and STED microscope images were alternately acquired from the same location, thus the identical TNTs and filopodia were analyzed for the sizing using deconvolved, confocal and STED microscope images. The images were deconvolved, the regions of interest were cut out and the size of the diameters were analyzed using the correlation method with the Van Steensel Ansatz as described in chapter [7]. Aim of this measurement was to figure out whether filopodia and
Chapter 8. Characterization of TNTs in U87 Cells

Figure 8.14: Diameters of TNTs and filopodia when imaged with a confocal microscope. The usage of the zoom option of the microscope does not affect the results. TNTs and filopodia have comparable the same diameters. The resulted sizes of diameters are at the resolution limit of the confocal system.

TNTs exhibit different diameters or not. Since TNTs can be formed by filopodia interplay and thus filopodia are the precursors of the TNTs it is assumed that they are of the same dimensions.

In figure 8.14 the results of the filopodia and TNT diameters when imaged by confocal microscopy are depicted. Here, it was additionally distinguished whether the image was collected using the zoom option of the microscope or not. The results show that zooming does not affect the measurement, which was expected since the pixelsize does not change using the zoom option. TNTs and filopodia have the same average diameters with a TNT diameter of \((237 \pm 27)\) nm and a filopodia diameter of \((213 \pm 18)\) nm when using the zoom option. The TNT diameters tend to be slightly higher than those of the filopodia. Nevertheless, if one remembers the diffraction limit that was described in section 3.1.3 in chapter 3, the rule of thumb for the resolution of the most optical system was \(d_{\text{min}} \approx \frac{\lambda}{3}\).

The green fluorescent dye DiO has a maximum emission wavelength of 514 nm. Hence, the resolution of the confocal microscope is \(\sim 257\) nm when using the DiO staining. This means, that we are actually at the limit of the confocal microscopy system and the accurate size of the diameters can be not resolved by the confocal microscope. Therefore it is necessary to use STED nanoscopy for the accurate determination of TNT and filopodia diameters.

In figure 8.15 the resulted diameters of TNTs and filopodia imaged via STED microscopy are depicted. Here, a STED resolution of 105 nm was assumed and the actual diameters were calculated by quadratic subtraction of the resolution (see eq. 7.4 in chapter 7). The diameters of TNTs and filopodia have the same values with \((178 \pm 21)\) nm for TNTs and \((179 \pm 16)\) nm for filopodia. Thus, the both structures can be not distinguished by their dimensions in U87 cells. They can be only differentiated from each other by the fact, that TNTs promotes cell-to-cell contact which filopodia do not.
8.3. Structural Analysis using STED Microscopy

Figure 8.15: Actual diameters of TNTs and filopodia determined by using STED nanoscopy. TNTs and filopodia have the same average diameter. A STED resolution of 105 nm was quadratic subtracted from the measured values.

If one now compares the resulted TNT diameters of the STED measurement with those of the confocal measurements depicted in figure 8.16, one sees that the STED measurements reveals a significant smaller mean TNT diameter value of \(178 \pm 21\) nm as compared to the mean TNT diameter value of \(237 \pm 27\) nm resulted by the confocal measurement \((p < 0.0001)\). Regarding the distribution of the individual data points, one can say that they are normally distributed in all measurements. Thus, the resulted mean values and standard derivations are reliable results.

Figure 8.16: Comparison of the resulted TNT diameters determined by STED and confocal microscopy. For the STED measurement, a STED resolution of 105 nm was quadratic subtracted from the measured values.

8.3.2 Dye Comparison in Tube Diameter Determination

The efficiency of the STED strongly depends on the properties of the used dye as described in chapter 8. To investigate whether the measurement of the TNT diameter is influenced by the chosen staining, the sizing of the TNT was additionally evaluated using the dyes
CellMask® Green and Orange. DiO and CellMask® Green are green fluorescent dyes with excitation wavelengths of 484 nm and 522 nm and maximum emission wavelengths of 514 nm and 545 nm, respectively. CellMask® Orange is a yellow-orange fluorescent dye with an excitation wavelength of 554 nm and a maximum emission wavelength of 572 nm. For the green fluorescent dyes, the STED wavelength 592 nm was chosen and for CellMask® Orange the STED wavelength 660 nm. The respective spectra and detector regions for each dye are depicted in the appendix B. All three dyes are lipid insertors and label the plasma membrane as well as other lipid compositions of the cell. However, the labeling of DiO was not as good as the labeling of the CellMask® dyes as described in chapter 6.

Figure 8.17: Actual TNT diameters measured by STED microscopy. For the labeling three different dyes, CellMask® Orange, CellMask® Green and DiO, were used. A STED resolution of 105 nm was quadratic subtracted from the measured values.

Figure 8.17 shows the resulting TNT diameters of the three different STED measurements using the dyes CellMask® Orange, CellMask® Green and DiO. The resulted mean TNT diameter values are comparable the same with (194 ± 30) nm, (197 ± 31) nm and (178 ± 21) nm for CellMask® Orange, CellMask® Green and DiO, respectively. Nevertheless, it seems that the resulted TNT diameter using DiO as dye tend to be smaller than the resulted TNT diameter using the other dyes. The reason for this tendency is, that during the evaluation different SNR values were chosen for the deconvolution. For the CellMask® measurements a SNR value of 5 was usually chosen and in some cases when a higher WLL intensity was taken or the image was very noisy a SNR value of 3 was selected. For the CellMask® Green measurement, a SNR value of 3 was often chosen, since this dye bleached very quickly and to obtain an appropriate signal strength the WLL intensity was stepwise increased during imaging. For the deconvolution of the STED measurement with DiO a higher SNR value of 7 was chosen. In order to investigate the impact of the chosen SNR value, the images obtained with DiO were additionally evaluated with a selected SNR value of 3 at the deconvolution. The respective results of the two distinct analysis are depicted in figure 8.18 for comparison. A SNR value of 3 results in a mean TNT diameter of (194 ± 19) nm, whereas a SNR of 7 results in
8.4 Conclusion

U87 cells are well suited for the study of TNTs as they have a high number of TNTs. They do not grow homogeneously but tend to form spheroids and grow in multilayers on top of each other. TNTs are not always just straight thin lines between distant cells, instead they can also be branched or show kinks. Due to junctions it is possible, that even three cells can be linked by one single TNT. Cargoes can be transported by gondolas via TNTs. The transport of gondolas which contain vesicles as well as cytoplasm has been observed in U87 cells. Regarding the TNT formation in U87 cells, a TNT formation by cell dislodgement was successfully recorded where the TNT was formed within one hour. TNT formation caused by filopodia growth or cell division was not excluded in U87 cells but not imaged.

The staining of F-actin and microtubules with the dyes SiR-actin and SiR-tubulin reveals that thick TNTs contain both as cytoskeletal content and in the most common thin TNTs only fragments of F-actin was detectable. In addition, F-actin as well as microtubules was found in the origins of filopodia close to the cell body. The fact that F-actin was only present in the TNTs in a very incomplete form could confirm the theory that actin is only required for the formation of TNTs and not for their stability. Instead, the stability of the TNTs could also be ensured by microdomains in the lipid layers as described in [22]. However, then the question arises whether the transport by actin-driven molecular motors is still present inside the TNTs or not. For the transfer of cargoes by molecular motors a continuous actin-rod is needed. The actin labeling with CellLight™ Actin-GFP failed in U87 cells. The staining resulted more in a cytoplasmic and not in a cytoskeletal staining of the cells. Longer incubation times did not solve this problem. Therefore, the question remains open whether the fragmentary occurrence

(178 ± 21) nm resulting in a diameter difference of (16 ± 28) nm when using a SNR value of 3 instead of a SNR value of 7.

Figure 8.18: Investigation of the impact of the SNR value on the sizing analysis. Resulted TNT diameters measured by STED microscopy with DiO when using different SNR values for the deconvolution. A STED resolution of 105 nm was quadratic subtracted from the measured values.

8.4 Conclusion

U87 cells are well suited for the study of TNTs as they have a high number of TNTs. They do not grow homogeneously but tend to form spheroids and grow in multilayers on top of each other. TNTs are not always just straight thin lines between distant cells, instead they can also be branched or show kinks. Due to junctions it is possible, that even three cells can be linked by one single TNT. Cargoes can be transported by gondolas via TNTs. The transport of gondolas which contain vesicles as well as cytoplasm has been observed in U87 cells. Regarding the TNT formation in U87 cells, a TNT formation by cell dislodgement was successfully recorded where the TNT was formed within one hour. TNT formation caused by filopodia growth or cell division was not excluded in U87 cells but not imaged.

The staining of F-actin and microtubules with the dyes SiR-actin and SiR-tubulin reveals that thick TNTs contain both as cytoskeletal content and in the most common thin TNTs only fragments of F-actin was detectable. In addition, F-actin as well as microtubules was found in the origins of filopodia close to the cell body. The fact that F-actin was only present in the TNTs in a very incomplete form could confirm the theory that actin is only required for the formation of TNTs and not for their stability. Instead, the stability of the TNTs could also be ensured by microdomains in the lipid layers as described in [22]. However, then the question arises whether the transport by actin-driven molecular motors is still present inside the TNTs or not. For the transfer of cargoes by molecular motors a continuous actin-rod is needed. The actin labeling with CellLight™ Actin-GFP failed in U87 cells. The staining resulted more in a cytoplasmic and not in a cytoskeletal staining of the cells. Longer incubation times did not solve this problem. Therefore, the question remains open whether the fragmentary occurrence

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of F-actin inside the TNTs is caused by insufficient labeling of F-actin by SiR or not. For the verification of F-actin lack inside the TNTs of U87 cells another actin-marker is needed in order to exclude insufficient labelling as cause.

Filopodia and TNT have the same diameters in U87 cells and are therefore not proper distinguishable by their structures but whether they promote cell-to-cell contact or not. Furthermore, confocal microscopy is not sufficient for the accurate determination of the TNT diameters, since the resulted size is close to the resolution limit caused by diffraction. Therefore STED microscopy is needed for the diameter sizing. The resulted TNT diameters of the STED measurements performed with three different dyes are in the range of $\sim 195$ nm which fits to the initially reported TNT diameter of (50 - 200) nm by Rustom et al [17]. The resulted diameter is smaller than the resolution of a confocal microscope. Nevertheless, confocal microscopy is sufficient to identify TNTs.

The chosen SNR value at the deconvolution of the STED microscopy images plays an essential role for the size analysis. For a SNR value change from 3 to 7, a size difference of $(16 \pm 28)$ nm was detectable. This leads to a relative uncertainty of about 8%, when looking at a 194 nm wide structure. However, the determination of the correct SNR value remains critical during evaluation since the artefacts in deconvolved images are hardly noticeable. Nevertheless, a SNR value of 5 seems to be a good choice for the convolution of STED images showing TNTs.
Chapter 9

Influence of Radiation on TNTs in U87 Cells

In this work, the response of cellular communication via TNTs in U87 glioblastoma cells to homogeneous radiation with $\alpha$-particles as stress factor was investigated. The aim of this study was to figure out whether the connections via TNTs are influenced by radiation and if cellular communication was enhanced upon irradiation. For this purpose, cells were homogeneously irradiated with $\alpha$-particles up to a dose of 1.2 Gy. The irradiation setup and method is described in chapter 5. After incubation times of 1 h, 6 h, 24 h and 72 h, the cells were labeled with the membrane marker CellMask Orange according to the protocol in the appendix A. The nanotube communication network was analyzed by studying tube density per connection and cell-to-cell connectivity using confocal microscopy of living cells. The TNT network was evaluated at several times in order to comprehend possible communication stages during the recovery phase after irradiation. For each time slot at least two samples were evaluated. Finally, the irradiated samples were compared to sham irradiated controls as reference to establish the impact of the radiation on TNTs and neither the influence of dehydration during the irradiation procedure or the labeling with the fluorescent dye and imaging. The impact of the dehydration during irradiation was explored separately.

Altogether, this established TNT network analysis enables the evaluation of the direct cellular response to radiation and how the cell-to-cell communication influences the survival of cells. In the next sections, the evaluation of the TNT communication network as well as the results of this study are exemplified and discussed.

9.1 Determination of Tube Density per Connection and Cell-to-Cell Connectivity by Confocal Fluorescent Microscopy

The cells were imaged alive at 37 °C in a special live cell imaging container on the live-cell system of the microscope (see section 6.1 in chapter 6). Imaging of the cells was performed when the cells are still alive in order to avoid possible breakages of the sensible nanoscaled TNT-connections caused by fixation as reported in [17]. Additionally, the complete image acquisition duration was less than one hour to ensure that as few network changes as possible
Influence of Radiation on TNTs in U87 Cells

occur during image capture related to the result in section 8.2.2, where a nanotube in U87 cells was formed within one hour. In order to record a large area with the best resolution, imaging was performed using the mosaic function of the microscope as described in section 6.2.2. The high resolution was needed to resolve the single nanotubes, therefore the usage of an objective with a larger field of view was excluded. The mosaic-pictures with an area of about $(670 \times 670) \mu m^2$ contain in average $94 \pm 48$ cells per sample. Overall, 2524 cells were analyzed in the whole experiment.

After image acquisition and merging of the 100 single images to a large overall picture with 14 stacks, the tubes were counted by hand while scrolling through the image and looking at each cell separately. At the evaluation it was distinguish whether the cells were connected by 1, 2, 3, 4 or more than 4 tubes. This separation serves as indicator for the tube density within a connection and thus reveals the strength of the individual connection. In addition, the determined connections were drawn in a transparent copy of the respective sample, where each cell is marked by a black dot. A final result of an analyzed image is shown in figure 9.1 (a). Here, the original picture is faded in the background and the cells are visible as bright structures. The connections are drawn as lines and the density of the tubes within a connection is indicated by the respective used color. Maximum projections of a very dense tube connection and a single tube connection are shown in figure 9.1 (b) and (c), respectively. These are the corresponding enlargements of the selections marked by red frames in the above picture.

With this method the connection frequency per cell subdivided by the corresponding tube density within the connection can be determined. This is done by counting the respective colored lines. Additionally, one can ascertain how dense the cells are connected among each other. This cell-to-cell connectivity can be studied by counting the connected lines at each dot. In other words, counting the number of cells to which the currently viewed cell is connected. With these measurement variables it is possible, to make qualitative and quantitative statements of the cellular communication systems composed of TNTs.
9.1. Determination of Tube Density per Connection and Cell-to-Cell Connectivity by Confocal Fluorescent Microscopy

(a) Drawn picture of one sample. The original image is transparent in the background where the cells are visible as white structures. Each cell is marked as a black dot. The colors of the lines represent the different tube density per connection.

(b) Dense tube connection.
(c) Single tube connection.

Figure 9.1: Resulted drawn image after evaluation (a). The enlargements correspond to the respective selection indicated with red frames, showing a dense tube connection (b) and a single tube connection (c). These images are maximum projection of the corresponding confocal z-stacks. Scale bars: (a) 50 µm, (b) and (c) 5 µm.
9.2 Investigation of the Influence of Dehydration during Irradiation

At the begin of the irradiation procedure the cells are only covered by a thin medium layer (< 5 µm) as described in chapter 5. Consequently, the cells are not only stressed by irradiation, but also by moisture loss during the irradiation procedure. To figure out whether the dehydration affects the TNT network in addition to the irradiation, the sham irradiated controls are compared to further controls, which were left in the incubator during irradiation and were always be covered by 2 ml medium.

![Figure 9.2: Comparison cell-to-cell connectivity in the sham irradiated controls, which has been dehydrated during irradiation process, to undried controls. These controls were always be covered by medium.](image)

In figure 9.2, the results regarding the cell-to-cell connectivity are shown as a histogram. By looking at the proportion of isolated cells (number of connected cells = 0) one can immediately see, that the dried samples exhibit much more cells without any connection to any other cell. Furthermore, the amount of cells which are connected to one, two, three or at least four other cells are always greater in the undried control than in the dried sham irradiated controls. Especially, cells connected to more than three cells are completely missing in the dried samples. All differences are significant (p < 0.05) according to the two-sample t-test. These findings suggest that the cellular communication network is much more pronounced in the undried cells as compared to the dehydrated cells. This fact is also revealed when looking at the connection frequencies (see figure 9.3). Here, two histograms are depicted. Figure 9.3 (a) shows the overall connection frequency, i.e. how many connections can be found in total in the samples. This frequency is normalized by the total amount of cells within one sample. Whereas the frequency of connections subdivided by the tube density within a connection as depicted in fig 9.3 (b), is normalized by the amount of total number of connections. This histogram shows the distribution of all connections with respect to the tube density within a connection i.e. reveals the counted number of the individually colored lines as explained.
above. The distribution of the connections regarding the tube density is the same for the undried and dried samples. However, if one considers the total number of connections, one can immediately see that the undried control have significantly more connections than the dehydrated samples (p < 0.05). Thus, the total tube frequency within the dehydrated sample is extremely decreased.

![Graphs showing comparison of dried and undried controls regarding the frequency of connections and their distribution with respect to the tube density within one connection.](image)

(a) Total number of connections found in the respective samples. Normalized by the total number of cells within a sample.

(b) Distribution of the connections in respect to the tube density within a connection. Normalized by the total number of found connections.

Figure 9.3: Comparison of dried and undried controls regarding the frequency of connections and their distribution with respect to the tube density within one connection.

These findings suggest, that the dehydration during irradiation indeed has an additional affect on the TNT network. The cellular communication network in the control samples is more pronounced than that in the dehydrated samples. Probably the already existing nanotubes are breaking by the dehydration and therefore their number is significantly decreased.

9.3 Impact of the Cell Density on the TNT Network

Throughout the entire experiment, strong fluctuations in cell density were observed between the individual samples. As discussed in chapter 2, the formation of a TNT can occur by two mechanisms, cell dislodgement and filopodia growth. Since the probability of cell-to-cell contact is higher in denser cell populations, TNT formation might also be increased. Hence the TNT frequency probably also correlates with the distance of the corresponding cells. With incubation times up to 72 h the cell density will change during the experiment, due to cell division. Additionally, the cell density also underlies strong, local changes within a single sample, because the cell line used here tends to grow in clusters and is not homogeneously distributed (see section 8.1 in chapter 8). As result strong differences regarding the averaged cell densities are recognizable. These fluctuations may influence the results of this experiment. Therefore, it is indispensable to consider these variations in more detail in order to assess the impact of the cell density and to figure out whether the samples are comparable to each other or not.
In figure 9.4, the averaged cell densities of irradiated and sham-irradiated samples at different incubation times are depicted. For 1 h, 6 h and 72 h the averaged cell density of irradiated and sham-irradiated samples are comparable. However, at the 24 h time slot the irradiated samples contain much more cells than the control ($p < 0.05$). The huge error bars of the irradiated samples at 1 h and 24 h reveal that at these time slots there are strong fluctuations in the cell density even between the individual samples. Additionally, not an exponential growth is seen, as generally expected when considering growth curves. Possibly, the general form of the cell growth curve can not be figured out due to the low number of samples. Furthermore, the recorded area within one sample was selectively chosen, cell-rich areas for samples with low cell density and cell-poor areas for samples with high cell density. Thus, the samples are not randomly distributed with respect to the cell density, but chosen in a way to keep the cell density as constant as possible among the individual samples. Additionally, samples which contain a huge total number of cells were not evaluated, since then a proper assignment of the TNTs to the individual cells is not possible anymore. The fact that the cell density remains constant up to 24 h in the sham controls is probably caused by the low seeded cell density since this cell line needs a proper cell density for exponential growth. If the cells are not seeded confluent, they will need more than 24 h to start growing again as described in section 8.1. This probably has happened, therefore it is not an exponential growth. Furthermore due to the high error bars of the irradiated cells at e.g. 1 h also the question appears whether these samples can still be compared to each other or not. Therefore, it is necessary to investigate the extent to which cell density affects the TNT communication network. For this purpose, the networks of the individual samples were additionally examined at the critical times of 1 h and 24 h for their dependence on cell density. In figure 9.5, the results are shown. Here, the fraction of the connected cells is depicted in dependence on the cell density. The fraction of the interconnected cells indicates how many cells in the entire sample are involved in the network and thus also contribute to the establishment and expansion of the network. Thus cells that actually are networking.
9.4 Cell-to-Cell Connectivity

During the experiment, there have been four irradiated samples recorded for the time slot 1 h. Two of these samples exhibit a low total number of cells, thus a low cell density, and the other two samples contain a high number of cells, thus a high cell density. The sham irradiated controls consisting of three individual samples exhibit a low cell density. In the following, the averages of the fraction of the connected cells have been determined with respect to the cell density and irradiated or not. These are depicted as the three individual dots in figure 9.5 (a). It can be seen that the results agree within the scope of their measurement accuracy. If one now also considers the proportion of the connected cells of the 24 h samples, one can see that these also agree despite the high difference in cell density. These findings suggest that the fraction of cells, which indeed correspond and contribute to the cellular communication network are scarcely affected by the cell density. Hence, one can conclude that the cell density has probably not a crucial role to the TNT network. Of course, one can find more connections in samples with a high total cell number compared to those with a low cell density, but if one normalizes this to the total amount of cells and considers the fraction, it remains the same. This conclusion can be reached, since there is no significant relevance of the cell density recognizable when considering the fractions of cells, which indeed do networking. Consequently, treated and non-treated samples are comparable to each other even if the cell densities are not.

9.4 Cell-to-Cell Connectivity

In this work, the TNT network is investigated with regard to cell-to-cell connectivity, i.e. how strongly the cells are interconnected. Critical parameters are the type and strength of the individual connections. This section deals with the connectivity among the cells. First, the irradiated cells are compared separately with control samples for the individual incubation periods. Subsequently, the temporal development of cell-to-cell connectivity is discussed.
9.4.1 Comparison at Certain Times

In the followed figures [9.6] [9.7] [9.8] and [9.9] two histograms are displayed for each timepoint 1 h, 6 h, 24 h and 72 h, respectively. The left one shows the fraction of interconnected and isolated cells, thus those cells which contribute to the network and vice versa. The histogram on the right shows an insight into the connected cells themselves by showing the partitioning of these cells according to the number of cells they are connected to. In other words, if one will sum up the columns in the right histogram one will get the value of the fraction of connected cells showed in the left one. Therefore, the right histogram is a refinement of the columns in the left histogram corresponding to the connected cells.

(a) Distribution of the cells to those which are interconnected or isolated. (b) Partitioning of the interconnected cells according to the number of cells they are connected to.

Figure 9.6: Cell-to-cell connectivity in irradiated samples and controls for an incubation time of 1 h.

In figure [9.6] the results of irradiated and sham irradiated samples for an incubation time of 1 h are shown. The proportions of connected and isolated cells are the same after 1 h. For both groups, irradiated and sham irradiated, the fractions of isolated and interconnected cells are roughly half. The fraction of connected cells are \((44 \pm 11)\%\) and \((42 \pm 7)\%\) for irradiated samples and shams, respectively. Also the partitioning of the connected cells is similar in both, irradiated and non-irradiated samples. Most of the cells are connected to one other cell (Irradiated: \((27 \pm 4)\%\), sham: \((30.5 \pm 2)\%\)) and the partition decreases with the number of cells to which one cell is interconnected. Cells connected to three or four other cells are hardly represented (Irradiated: \((5 \pm 4)\%\) and \((2 \pm 4)\%\), sham: \((1 \pm 0.9)\%\) and 0% for 3 and \((\geq 4)\), respectively). However, for both groups this trend remains the same. Thus, no significant difference between irradiated cell and control populations is recognizable after 1 h incubation time.
9.4. Cell-to-Cell Connectivity

(a) Distribution of the cells to those which are interconnected or isolated.

(b) Partitioning of the interconnected cells according to the number of cells they are connected to.

Figure 9.7: Cell-to-cell connectivity in irradiated samples and controls for an incubation time of 6 h.

The results for an incubation time of 6 h are depicted in figure 9.7. Here, the distribution of the cells has been changed. There are much more interconnected cells (Irradiated: $(79 \pm 9)$ %, sham: $(61 \pm 15)$ %) than isolated cells (Irradiated: $(21 \pm 9)$ %, sham: $(39 \pm 10)$ %). This shift tends to be more pronounced in irradiated cells than in non-irradiated cells. By looking at the partitioning of the connected cells, one sees that the trend remains the same for both groups. The most cells are connected to one (Irradiated: $(38 \pm 7)$ %, sham: $(28 \pm 10)$ %) and the fraction of cells linearly decreases with the number of cells to which one cell is connected. However, also here the fractions of irradiated cells tend to be higher than those of the shams.

(a) Distribution of the cells to those which are interconnected or isolated.

(b) Partitioning of the interconnected cells according to the number of cells they are connected to.

Figure 9.8: Cell-to-cell connectivity in irradiated samples and controls for an incubation time of 24 h.
In figure 9.8 the results of the cell-to-cell connectivity of irradiated and non-irradiated cell populations after an incubation time of 24 h are shown. One sees, that the proportions of the isolated cells are further decreased, especially in the shams. Here, the fraction of isolated cells changed from \((39 \pm 10)\%\) at 6 h to a fraction of \((28 \pm 11)\%\) at 24 h. However, the fractions of connected (Irradiated: \((80 \pm 12)\%\), sham: \((72 \pm 9)\%\)) and isolated cells (Irradiated: \((20 \pm 12)\%\), sham: \((28 \pm 11)\%\)) are the same for irradiated and non-irradiated samples. Nevertheless, there are differences between irradiated and sham irradiated cell populations. If one considers the partitioning of the linked cells, one can see that sham irradiated cells are increasingly connected to only one single cell (Irradiated: \((31 \pm 7)\%\), sham: \((45 \pm 5)\%\)). Cells that are connected to more than one other cells are more common in irradiated samples than in non-irradiated ones. This means that the irradiated cells exhibit a more complex interconnections than sham irradiated controls.

(a) Distribution of the cells to those which are interconnected or isolated.  
(b) Partitioning of the interconnected cells according to the number of cells they are connected to.

**Figure 9.9:** Cell-to-cell connectivity in irradiated samples and controls for an incubation time of 72 h.

After 72 h the distributions according to the differentiation of interconnected and isolated cells as well as the partitioning of the interconnected cells is the same for both groups (see fig. 9.9). Here, cells have reached an equivalent state with a quite high fraction of cells which are interconnected ((88 ± 5) % in shams and (84 ± 10) % in irradiated samples). Most of the cells are connected to one single cells (Irradiated: (29 ± 7) %, sham: (30.8 ± 0.6) %), but there are also considerable more cells which are interconnected to more than one single cell. There is again a linear decrease recognizable.

### 9.4.2 Temporal Development

After the separative consideration of the communication network in irradiated and non-irradiated cells for the each single incubation time, this subsection deals with the time development of the cell connectivity. This is done by looking at the changes according to the fraction of those cells, which are interconnected and therefore contribute to the network.
9.4. Cell-to-Cell Connectivity

![Figure 9.10: Time development of the cell-to-cell connectivity. The fraction of connected cells is depicted in dependence of the cell density. The incubation times are labeled by different colors. Sham and irradiated samples are distinguished by blank and filled symbols, respectively.](image)

The time development of the fraction of connected cells can be tracked in figure 9.10. In this plot, the portion of cells, which are involved in the network, is displayed against the cell density. The cell density also changes with time. For the identification of the four distinct incubation times different colors were used. Sham irradiated samples are marked by blank symbols, whereas irradiated samples are indicated by filled symbols. There is a baseline for an incubation time of 1 h noticeable, since the fraction of the interconnected cells is the same independent from the cell density and treatment. With growing time, the cross-linked cell fraction increases in both groups, irradiated and non-irradiated. However, here are differences according to the speed and the trend of the temporal development recognizable. The sham irradiated samples initially show a growth of their networked cell fraction, although their cell density remains almost constant. Recognizable by the fact that the values of the incubation times 1 h, 6 h and 24 h of the control samples are directly above each other and thus form a vertical line upwards. In contrast to this, the irradiated samples form a horizontal line. There is a noticeable jump accordingly to the connected cell fraction between an incubation time of 1 h and 6 h at the irradiated samples visible. During this jump, the cross-linked cell fraction is almost doubled by increasing from a value of (44 ± 11) % to a value of (79 ± 9) %. This difference is significant (p < 0.05), whereas the cell density does not significantly change in this time. After this jump, the cross-linked cell fraction in the irradiated samples remains constant up to a maximum incubation time of 72 hours. The cell density, on the other hand, varies during this time, so that a horizontal course is recognizable. After 72 h incubation time irradiated and non-irradiated samples are the same accordingly to the cell density as well as to the fraction of cells which are interconnected.
9.5 Tube Density per Connection

In addition to cell-to-cell connectivity, the frequency and strictness of the connections are investigated. In this context, the connections were subdivided according to the number of tubes they consist of. This subdivision was made by using different colors to identify the individual connections between the cells, as explained in section 9.1. The underlying idea of the differentiation of connections according to the tube density is, that the exchange of signals and cargoes is enhanced when more tubes are available for the transport. In this section, the results were presented in the same way as for the cell-to-cell connectivity. First both groups, irradiated and non-irradiated, are separately compared to each other for each incubation time and then the focus is on the temporal development of connection frequency and tube density per connection.

9.5.1 Comparison at Certain Times

For quantitative evaluation a simplification is made as connections containing exactly 2, 3 or 4 tubes are rarely found. Not a partition of the connections into five but instead in two subgroups is used. Connections containing 1 or 2 tubes are referred as simple connections, whereas those consisting of 3 or more tubes are referred as complex connections.

In the figures 9.11, 9.12, 9.13 and 9.14 again two histograms are displayed for the timepoints 1 h, 6 h, 24 h and 72 h, respectively. The left ones show the normalized frequencies of connections totally found within the samples at the respective incubation times. The partitions of those connections are depicted in the right histograms for the respective incubation periods.

The overall frequency of the connections is determined by summing up all lines which have been drawn in the picture during evaluation. The frequencies of the subgroups, simple and complex, are determined by the summing up all blue and pink lines for the simple connections.

**Figure 9.11:** Frequency of the connections totally found and their subdivision into the subgroups, simple and complex, for an incubation time of 1 h.

(a) Frequency of connections found in total. Normalized by the total number of cells.

(b) Partition of the connections into the two subgroups, simple and complex connections. Normalized by the total number of connections.

1 h, 6 h, 24 h and 72 h, respectively. The left ones show the normalized frequencies of connections totally found within the samples at the respective incubation times. The partitions of those connections are depicted in the right histograms for the respective incubation periods. The overall frequency of the connections is determined by summing up all lines which have been drawn in the picture during evaluation. The frequencies of the subgroups, simple and complex, are determined by the summing up all blue and pink lines for the simple connections.
or all green, violet and yellow lines for the complex connections.

In figure 9.11 the results for an incubation time of 1 h are shown. The frequency of connections found in total are the same for irradiated and non-irradiated cell populations. The values are $0.26 \pm 0.09$ for the controls and $0.37 \pm 0.24$ for the irradiated samples, i.e. about every third cell has a connection. Also the partitioning of the connections are the same for irradiated and sham irradiated cells. There are more simple (Irradiated: $0.79 \pm 0.07$, sham: $0.59 \pm 0.28$) than complex connections (Irradiated: $0.24 \pm 0.07$, sham: $0.41 \pm 0.28$).

![Graphs](image)

(a) Frequency of connections found in total. Normalized by the total number of cells.

(b) Partition of the connections into the two subgroups, simple and complex connections. Normalized by the total number of connections.

**Figure 9.12:** Frequency of the connections totally found and their subdivision into the subgroups, simple and complex, for an incubation time of 6 h.

After 6 h the frequency of connections is still the same for both groups, but is increased compared to the values at 1 h (see fig. 9.12). Now, for every second cell there is a connection. The distribution into the two subgroups is again identically for irradiated and non-irradiated cells. There are still more simple connections present than complex connections. About 2:3 of the connections are simple and 1:3 are complex.

For an incubation period of 24 h after irradiation there are differences between irradiated and sham irradiated cell populations recognizable (see fig. 9.13). The irradiated cells tend to establish more connections than the sham irradiated cells because the frequency of connections is generally higher in irradiated samples. In irradiated cells connections can be found with a frequency of $0.87 \pm 0.23$, whereas in the controls approx. one connection is found to only every second cell ($0.54 \pm 0.18$). Additionally, the partition into the two subgroups has changed in the irradiated cells. There are still more simple connections present (Irradiated: $0.57 \pm 0.03$, sham: $0.71 \pm 0.06$), but the distribution is shifted towards complex connections in irradiated samples (Irradiated: $0.43 \pm 0.03$, sham: $0.29 \pm 0.06$). They exhibit significantly more complex connection than the sham irradiated controls ($p < 0.05$). On the other hand, there are significantly more simple connections in sham irradiated cells as compared to irradiated cell populations ($p < 0.05$).
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(a) Frequency of connections found in total. Normalized by the total number of cells.

(b) Partition of the connections into the two subgroups, simple and complex connections. Normalized by the total number of connections.

Figure 9.13: Frequency of the connections totally found and their subdivision into the subgroups, simple and complex, for an incubation time of 24 h.

The results for an incubation time of 72 h after irradiation are depicted in figure 9.14. At this time the frequency of connections are the same for both groups. The frequency of connections is grown to a value of 0.967 ± 0.012 in sham irradiated and 0.93 ± 0.13 for irradiated cell populations. Also the partitioning of the connections into the subgroups, simple and complex, is identical for irradiated and non-irradiated samples after 72 h. In both groups, there are more simple connections (Irradiated: (0.6 ± 0.04) %, sham: (0.561 ± 0.017) %) than complex connections (Irradiated: (0.4 ± 0.04) %, sham: (0.439 ± 0.017) %). However, the distributions are almost balanced between two different kinds of connections.

(a) Frequency of connections found in total. Normalized by the total number of cells.

(b) Partition of the connections into the two subgroups, simple and complex connections. Normalized by the total number of connections.

Figure 9.14: Frequency of the connections totally found and their subdivision into the subgroups, simple and complex, for an incubation time of 72 h.
9.5.2 Temporal Development

This subsection deals with the temporal development of the frequency of connections as well as for the partitioning of the connections regarding the tube density per connection. In figure 9.15 the frequency of connection totally found in the samples is displayed against the time after irradiation. It can be seen that within the three days of observation the connection frequency has more than doubled in both cases. Cell-to-cell connections tend to be found more frequently in irradiated samples than in non-irradiated ones. However, the values of the corresponding hourly values always overlap so that no significant difference can be observed. The strongest increase of the connection frequency can be observed between the 1 h samples and the 6 h samples. Subsequently, the growth is attenuated in the irradiated cells and completely vanished in the shams. Here, the connection frequency remains constant between 6 h and 24 h after sham irradiation. Suggesting that the irradiated cells are still focused after 6 h to further condense their TNT network, whereas the shams are calmer in expanding of their network. However, after 72 h both groups exhibit the same frequency of connections.

In figure 9.16 the temporal development regarding the redistribution between the two kinds of connection, simple and complex, for irradiated and sham irradiated samples can be seen. The frequencies are normalized by the overall number of connections, which are found in total in the respective samples. This normalization gives the plot a symmetric appearance. There are always more simple connections than complex connections regardless of time and treatment. However, the exact distribution between the different connection types is neither independent of time nor treatment. At the beginning the partitioning of the connections is the same for both groups, since the error bars are overlapping. The distribution does not change after 6 h. Thereby an averaged frequency was observed of $0.66 \pm 0.01$ for simple connections and $0.34 \pm 0.01$ for complex connections. At the incubation period of 24 h there are significant
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Figure 9.16: Temporal Development of the subdivision of the connections into simple and complex connections in irradiated and sham irradiated cell populations. Those frequencies are normalized by the total number of found connections.

9.6 Discussion of the Results and the Network Analysis

During the irradiation the cells are not covered by medium as described in chapter 5. The investigation of the influence of the dehydration during irradiation reveals that the frequency of the TNTs is significantly decreased in dehydrated samples when compared to non-dehydrated controls. Probably the sensible TNTs are breaking when the cells are not covered with a proper amount of medium. The study of the impact of the cell density on the TNT formation indicates that the cell density play only a subordinate role at TNT formation. Thus, samples with different cell confluences can be compared if the parameters are normalized by the total number of the cells contained in the relative samples. The comparison of irradiated cells to shams reveals that the trend of cell-to-cell connectivity as well as connection frequency remains the same. Both, irradiated and non-irradiated cell expand and upgrade their communication network during growth. However, the irradiated cells establish their TNT communication network faster than the shams. Noticeable in the temporal development of the connectivity where the fraction of cells, which are already connected and thus involved in the network, jumps to a higher level in irradiated samples within 6 h after irradiation. Whereas in sham irradiated cell populations this value is only reached after about 24 hours.
These findings suggest that there are different triggers which induce the TNT formation in irradiated and non-irradiated cells. It seems that in irradiated cells the TNT formation and the establishment of the cellular communication network is accelerated by an additional mechanism, which is not active in the controls. With this faster establishment of their TNT communication network, irradiated cells are more able to survive stress situations.

TNT formation can be realized by cell dislodgement after cell-to-cell contact or by filopodia growth as illustrated in section 2.4. In this work, it was observed and recorded that TNT formation in U87 cells occurs via cell dislodgement (see section 8.2), but this does not exclude that TNT formation can additionally be realized by filopodia growth in U87 cells. Consequently, both mechanisms can occur in U87 cells. It might be possible that the selective TNT formation is realized by filopodia growth and the usual communication network is established by cell dislodgement after cell division or encountering of cells. Therefore, it could be possible that the communication network in cells is enhanced upon irradiation and an additional mechanism causes an increased triggering of TNT formation by filopodia growth. This would explain the immediately rise of the interconnected cell fraction within 6 h in irradiated cell populations. It could be that the release of stress signals into the medium, which originates from the irradiated, stressed cells, targets the filopodia growth and leads to an orientated TNT formation.

After this immediate jump, the networking cell fraction does not change in irradiated cell populations during the complete observation period up to 72 h. Suggesting that after 6 hours the additional triggering of TNT formation is attenuated or a saturation regarding the establishment of the TNT network has been reached. In a cellular communication network there are cells, which are interconnected with several cells, i.e. more than one other cell. This portion of cells can be seen as indicator of the complexity of a communication network. Here, more highly connected cells are present in irradiated samples than in controls after 24 h. Additionally, after 24 h there are significant more complex connections found in irradiated cells as compared to sham irradiated controls. Demonstrating that after the expansion of TNT network by involving as much as possible cells within 6 hours, the irradiated cells are now focused on condensing and strengthening their network. Whereas the control samples build their network more leisurely and more non-complex, mostly by the formation of simple tube connections between one other cell.

After three days, irradiated and sham irradiated samples have aligned themselves and exhibit the same values regarding connected cell fraction, distribution of the cells according to the number of cells they are connected to, frequency of connections as well as partition of the connections into simple and complex connections. Thus, they equalized completely after this observation period. This suggest that the additional triggering of TNT formation in irradiated cells has been stopped and the network is not further expanded and strengthened by the irradiated cells at a certain point. Since the partition of complex connections decreases again between 24 and 72 h. It could be possible that there might be a saturation regarding to the establishment of the TNT network or irradiated cells are at a certain point not longer able to further expand their communication network. Or it could be that the most cellular repairs are finished and the cells stop the orientated TNT formation and serve the remaining TNT
connections to isolated apoptotic cells, which cannot be rescued (as similar to the model of Rustom in [56]). Consequently, it would be interesting to evaluate longer incubation times than three days, to figure out if the controls are further able to expand and strengthen their network and thus will pass the irradiated cells. If this is true, it would then demonstrate that the irradiated cells are in their communication via TNTs hampered by the irradiation. Or it could be also possible that the damaged cells die during this period and the normal state is reached where the establishment of the network occurs in the same way as for the controls. Overall, these findings demonstrate that the communication network via TNTs is influenced by irradiation and its establishment is accelerated. Irradiated cells built up and condense their communication network faster than non-irradiated cells within the first 24 hours. However, after this period the controls caught up and equalized with them within 72 hours after irradiation. The results gained in this experiment are not statistically significant. To obtain more significant results more samples have to be evaluated. Since this experiment was only a pilot experiment, only very few samples were evaluated for time reasons. The aim of this pilot experiment was to determine whether or not there are any differences in cell communication by TNTs between irradiated and non-irradiated cells. This line was achieved with a small number of samples.

In this thesis, TNT networks were evaluated in a quantitative manner for the first time. The network analysis enables the characterization of these cellular communication networks regarding their size and complexity. Thereby, the TNT network is described by the investigation of cell-to-cell connectivity and connection frequency. The cell-to-cell connectivity is determined by the differentiation of isolated and connected cells. Here, the fraction of connected cells plays a crucial role, since these cells are actually involved in the communication network and thus contribute to it. A further refinement of these cell proportion is realized by its distributions according to the number of cells they are connected to. This partitioning of the cells serves as indicator for the complexity of the communication network. The connection frequency displays the establishment and the condensing of the network. By studying the tube density per connection two subgroups of connections are distinguish. Simple connections contain only one single or two TNTs, whereas complex connections consists of more than two TNTs. The tube density per connection can be seen as indicator for the strength of a bond, since studies with Ca$^{+2}$ signals revealed that the higher the tube number the higher the amplification of the signals [42] [43]. These parameter characterizes a communication network established by TNTs and thus allow to work out qualitative and quantitative statements about the network.

The experiment can be repeated since the method is reproducible. Prerequisites for the performing of such an experiment are a confocal microscope with live-cell imaging setup and an opportunity to homogeneously irradiated cells with α-particles to a dose of 1.2 Gy. CellMask© Orange and U87 cells are commercial available. A further condition is that the cells have to be dried for 10 minutes, since the pre-experiment shows that the dehydration affects the TNT network. The dehydration of the cells probably leads to the breakage of the sensible TNTs, since their frequency is extremely decreased by this treatment. This effect probably also ex-
plains the high increase of the connection frequency within the first 6 h, which is identically to both, irradiated and non-irradiated cells. As a result of the inhomogeneous growth of the U87 cells (see section 8.1) and the irregularities caused by the seeding of the cells, there are very strong fluctuations of the cell density noticeable. However, the results show that the increase of the interconnected cell fraction in irradiated as well as non-irradiated samples is independent of the cell density. Suggesting that the cell density plays an subordinate role in TNT formation.

At the evaluation it was sometimes difficult to identify where one TNT begins or ends and to which cell it belongs to. This was especially the case, if the cell density was very high and the cells lay on top of each other. To estimate the uncertainties regarding the cell assignment and counting of the TNTs, one sample was evaluated twice. This resulted in a maximum deviation of 3 % for the distribution of cross-linked cells in relation to the number of cells with which they are connected. The fraction of interconnected cells exhibit an uncertainty of 1.5 %, whereas the determined connection frequency remains the same. The partitioning of the connections into the two subgroups, simple and complex, results with an uncertainty of 0.005. Consequently, these uncertainties are negligibly small compared to the uncertainties caused by variations between the individual samples themselves.
Chapter 10

Conclusion and Outlook

The goal of this thesis was to establish a backbone for the research field which focuses on the role of membranous communication channels, called tunneling nanotubes (TNTs). The aim of the new research field is to investigate the influence of radiation on a TNT communication network and to figure out how the cellular communication via TNTs affects cell survival and cellular behavior after radiative stress. This thesis should serve as a template and therefore provides the basics for studying TNTs and their response to radiation.

One aim of this thesis was to figure out how TNTs can be identified and studied. For this purpose, several fluorescent membrane marker were tested to identify the best suitable TNT marker. Here, CellMask© was found to be the best marker. This dye is a lipid inserter and has excellent properties which include an uniform, intensive and stable labeling of the plasma membrane with low background-noise. The labeling of the cells is easy and quickly performed within 15 minutes. The stain is non-toxic to the cells and very robust against its internalization into the cell body by endocytosis. Furthermore, the TNTs in U87 glioblastoma cancer cells were characterized by STED and confocal microscopy. The U87 cell line was selected because of their high ability to form TNTs. It was found out that TNTs are not always continuous straight lines, instead they have kinks and junctions. Additionally, some TNTs have noodles and become thicker towards the cell body. The investigation of their cytoskeleton content reveals that F-actin and microtubules can be found in thick TNTs, whereas thin TNTs contain only fragments of F-actin. This might indicate that actin is only needed for the formation, but not for the stability of TNTs. However, it also raises the question whether the transport via molecular motors is still possible. Additionally, the TNT formation by cell-dislodgement was successfully imaged in this thesis, which serves as indicator for the duration of a TNT formation. The accurate diameter of TNTs and filopodia were determined using STED nanoscopy. It was found out that TNT and filopodia have comparable diameters. This result is an evidence for the relation of these two structures and therefore makes it more plausible that TNTs can also be formed by orientated filopodia growth. The TNTs in U87 cells have a diameter of ~ 195 nm, thus smaller than the resolution of a confocal microscope. Nevertheless, confocal microscopy is sufficient for the TNT identification. STED nanoscopy is only necessary if one wants to determine the size of a cargo transfered inside the tube, but for the observation of TNTs confocal microscopy is recommended to avoid additional stress.
of the cells caused by high laser intensities. Live-cell microscopy is preferred to study TNTs since these connections are very sensible and might break during fixation. In this thesis, the live-cell imaging setup at the STED microscope was improved by the development of a new dish holder and by the ascertainment of the correct setup settings. Now, it is possible to keep the cell alive during a time period of 2 days when cultured in the live-cell system. Further improvements are in progress. For instance, the live-cell system does not provide 100% humidity. This is very problematic since it leads to an increase of moisture loss by evaporation and this in turn causes concentration changes in the culture medium which become toxic for the cells. Here, the development of a special cell culture dish which is closed by a FEP foil might solve this problem. FEP foil is permeable for CO$_2$ and O$_2$, but impermeable for water vapor [110]. Thus, by exploiting these features, long-term studies in a non-humidified environment are possible.

The final goal of this thesis was a first pilot experiment on the investigation of the influence of radiation as stress factor on a TNT communication network. For this purpose, U87 cells were seeded on cover glasses and homogeneously irradiated with high-LET $\alpha$-particles with a final dose of 1.2 Gy. After the irradiation, the cells were incubated in the culture incubator until evaluation. The cells were labeled with CellMask$^\text{©}$ Orange and a large area of the cover glass was imaged by confocal live-cell microscopy, exploiting the mosaic-drive mode of the confocal microscope. Irradiated cells were compared to sham irradiated controls and the intercellular communication network was evaluated 1h, 6h, 24h and 72h after irradiation in order to comprehend possible communication stages during the recovery phase.

The TNT network was analyzed by addressing new parameters regarding the cell-to-cell connectivity and the TNT density within one connection. Here, it was differentiated between isolated and connected cells which contribute to the communication network. The connections were subdivided into simple and complex connections, which contain more or less TNTs to dissolve the strictness of the individual connections. This established TNT analysis enables the evaluation of the direct cellular communication response on radiation and gives insights about the influence of cellular communication on the survival of cells and their behavior upon radiation.

The results of this pilot experiment reveal that irradiated and sham irradiated cell populations exhibit the same trend regarding the temporal development of their network. Both cell populations expand and enhance their network during time, but irradiated cells establish their TNT network faster than the shams within the first 6 hours after irradiation. In irradiated cell populations, the fraction of cells, which are already connected and thus involved in the network, jumps to a higher level within 6 h after irradiation. In contrast, this value is reached after 24 h in sham irradiated cell populations. Thus, the communication network is indeed influenced by the radiation and the establishment of the TNT network is accelerated. It might be that the irradiated cells release signal molecules into the medium which causes an increased TNT formation by filopodia growth. Furthermore, it was found out that irradiated cell populations have more complex connections consisting of several TNTs as compared to non-irradiated cell populations after 24 hours. Thus, the irradiated cells strengthen their TNT network more intensive than non-treated cells. This indicates an increased communication
via TNTs. Since the more TNTs, the more cargoes can be transferred at the same time. After 72 h incubation all features of the TNT network are the same for both, irradiated and non-irradiated samples. These findings suggest that there is an additional trigger upon radiation damage which results in fast and intensive network formation by TNTs as an additional damage response mechanism. It also indicates that there are different TNT formation mechanisms within one cell type. During the pilot experiment, high fluctuations of the cell density were recognizable. Evidence was found that indicates a subordinate role of cell density in the TNT formation.

Based on the results of the pilot experiment one can establish a TNT formation model. Here, the hypothesis is that in non-treated cells, the TNT formation initially occurs in an oriented manner and after the cells start to grow, the TNT formation is dominated by cell division and simple connections, consisting of one TNT, are mostly formed. After the establishment of these simple connections the communication network is strengthened by further TNT formation and the simple connections become complex connections with a high TNT density. This higher TNT density probably leads to an amplification of the transferred signals. It has been reported that a higher number of TNTs leads to an amplification of electrical signals [42]. In contrast to the non-treated cells, the TNT formation in irradiated and therefore stressed cells is dominated by orientated filopodia growth and not by cell division. In addition to the orientated TNT formation, the newly formed connection are immediately solidified which leads to an increase of the complex connection frequency in stressed cells.

To proof this hypothesis, it is necessary to perform live-cell imaging videos of TNT formations in irradiated and non-irradiated cells. Here, cell-tracking of single cells would be beneficial. It is also important to find out whether cell division leads to the formation of one single TNT or to several dense packed TNTs.

The performance of this pilot experiment, the characterization of TNTs in U87 cells, the ascertainment of a suitable marker and imaging method as well as the literature research about TNTs provide the foundations for further studies of TNTs and their response upon radiative stress. With this research it is possible to further understand the direct cellular response to irradiation and how the cell-to-cell communication influences the survival of cellular networks such as tissues. On the basis of the derived knowledge about TNTs and the first pilot experiment carried out in this work, further follow-up projects are conceivable.

In order to avoid high cell density fluctuations in further experiments, one could imagine to grow the cells by colony formation instead of cell seeding. The seeding of the cells leads to unpredictable, inhomogeneous gaps between the individual cells and this results into very high local differences in the cell density. In contrast to this, cell populations grown by colony formation exhibit very similar cell-to-cell distances and the cell density is more globally stable. This is caused by the fact, that all cells arise from one single cell by colony growth. However, U87 cells are not a proper cell line for colony formation, because they grow very slowly and not homogeneously. Their tendency to grow in clusters excludes them from establishing a homogeneous cell density distribution. Consequently, it might be advantageous to use a different cell line in further experiments.
Alternatively, one can imagine to grow the cells on a certain grid, like in a Block-Cell-Printing device [111]. However, here it is unclear whether the printing of the cells on a grid would influence the TNT formation or not. The anchorage of the cells at certain positions means that they will not touch each other and therefore TNT formation by cell dislodgement is nearly impossible. Thus, the growing of the cells by colony growth seems to be the best option to ensure homogeneous cell growth and thus enables to keep the cell density constant during the observation time.

Additionally, the role of cell division in the establishment of the TNT network can be further studied by deliberative prevention of cell division. This could be achieved by decreasing the serum level to 1% in the cell culture medium. The cells are still viable and the cellular metabolism is not affected by this treatment but the cell division is stopped by this. With the performance of an experiment under this condition, one can figure out whether TNTs are predominately formed between associated cells upon cell division or not. Depending on the gained results of this experiment, it could serve as evidence or as refusal of the hypothesis of a predominated TNT formation by cell division.

One could also imagine to use holotomography instead of confocal microscopy for live-cell imaging studies. This quite new technology measures the physical properties of a cell, i.e. the refraction index. The big advantage of this method is that the cell membrane can be studied without the need of a fluorescent marker and therefore issues such as phototoxicity, photobleaching or the interference of the dye with the cellular dynamics are avoided. Additionally, the lateral resolution of the holotomography method is below 100 nm and thus provides high resolution nanoscopy of living cells [112]. The image acquisition is more cell-friendly because it is faster and only a low laser intensity is needed. Using holotomography as microscopy method would also solve the problem of the limited imaging window caused by the internalization of the membrane stain.

Furthermore, using the microscopy and analysis methods together with targeted irradiation, available at the ion microprobe SNAKE of our institute located at the 14 MV tandem accelerator in Garching, opens the opportunity to further study bystander effects. The usage of this microbeam allows the targeted irradiation of single cells or cell groups with precise doses of radiation. This powerful tool facilitates the study of bystander effects because this phenomenon is predominately at low doses (< 0.2 Gy) and was observed at exposures that are as low as a single proton or helium ion can deliver to a single cell [4]. Here, it would be interesting to investigate the behavior of neighboring cells, which are located in the surroundings of an irradiated area. Especially, the cellular communication at the interface between irradiated and non-irradiated areas is essential to further understand the underlying mechanism of the bystander effect. A better knowledge about this phenomenon would help to improve cancer radiotherapy by the development of cell-to-cell communication mechanisms that trigger the transfer of damage sensor signals and thus enables the amplification of cell-killing effects [113].

In addition, it would also be interesting to figure out whether complex connections consisting of several TNTs predominately exist after the exposure of high-LET radiation or if their frequency also increases after the exposure of low-LET radiation. If the frequency of these dense connections is rather increased upon high-LET radiation, this would imply a relation between
these cellular connections and the bystander effect because this response is more frequent found after densely ionizing radiation \([114]\). A further interesting follow-up project are co-cultivation studies, where irradiated and non-irradiated cells are cultured together. With the co-culturing method, one can study the observed “rescue effect” and may proof the model of ROS-dependent TNT formation introduced by Rustom \([56]\). This effect is also a bystander effect, since the non-exposed cells respond to the signals released by the irradiated cells. It would be very interesting to figure out which kind of cells form the open communication channel, the non-irradiated cells to the irradiated cells or vice versa. For these studies, a good fluorescent cell-tracker for the differentiation of the cells is indispensable and has to be ascertained.

Finally, the identification of the transferred cargoes is essential because this would provide a better understanding about the interfering mechanisms of cell-to-cell communication and would be the evidence that there is indeed an exchange of information via TNTs. The further projects and ideas presented here are extremely extensive and go beyond the scope of this work. However, they also show the potential of using this thesis as backbone for the further research on TNTs to answer radiobiological questions concerning cell-to-cell communication. Such experiments would provide a better understanding of the direct cellular response to radiative stress and how the cell-to-cell communication influences the cell survival. A better understanding of cellular communication would help to develop new therapy approaches in which the transfer of drugs or death signals is induced to selectively kill or help target cells.
Appendix A

Staining Protocols

CellLight™

The labeling of CellLight™ reagents is optimized for adherent cells. CellLight™ reagents are provided as solutions with a particle concentration of $1 \times 10^8$ particles/ml. The cells are seeded to a desired confluence (no more than 70% to achieve best results) and grown overnight to give them sufficient time to adhere. Before labeling the appropriate volume has to be calculated according to the followed equation:

$$\text{Volume of CellLight™ reagent (ml)} = \frac{\text{number of cells} \times \text{desired PPC}}{1 \times 10^6 \text{ particles/ml}} \quad (A.1)$$

where PPC is the number of particle per cell and the number of cells is the estimated total number of cells at the time of labeling. For the labeling of U87 cells with CellLight™ Plasma Membrane-GFP and CellLight™ Actin-GFP a PPC of 30-50 and 100 was chosen, respectively.

- Mix the CellLight™ reagent solution gently, do not vortex
- Mix the calculated volume of CellLight™ reagent together with a proper volume of culture medium
- Remove the old medium of the cells and add the fresh medium containing the desired amount of particles
- Return the cells to the culture incubator overnight ($\geq 16$ hours)
- On the next day, the cells are ready for imaging

CellMask©

CellMask© Plasma Membrane Stains are provided as 1000× concentrated stock solutions. The labeling protocol is optimized for adherent cells. The cells are seeded to a desired confluence on cover glasses or cell culture dishes one day before labeling.

- Prepare a fresh 0.5×-1.5× staining solution in culture medium (for 1 ml 1.5× staining solution: add 1.5 µl of the provided 1000× stock solution to 1 ml cell culture medium)
- Remove the old medium of the cells and add the fresh prepared staining solution
Appendix

- Incubate for 10 minutes at 37 °C, 100% humidity and 5% CO₂
- Remove the staining solution and wash the cells 3× with culture medium
- Add fresh culture medium to the cells
- The cells are ready for imaging

DiO

DiO is provided as 1 mM concentrated stock staining solution.

Adherent Cells

The cells are seeded to a desired confluence in cell culture dishes and grown for a sufficient time to let them adhere (usually one day before labeling). U87 cells need approx. 4-5 hours to adhere.

- Prepare a fresh 5 µM concentrated staining solution by adding 5 µL of the provided stock staining solution to 1 ml culture medium
- Remove the old medium of the cells and add the fresh prepared staining solution
- Incubate for at least 20 minutes at 37 °C, 100% humidity and 5% CO₂
- Remove the staining solution and wash the cells 3× with culture medium. For each wash cycle incubate the cells in the incubator for 10 minutes.
- Add fresh culture medium to the cells
- The cells are ready for imaging

Cells in Suspension

- Suspend cells at a density of $1 \times 10^6$ cells/ml in serum-free culture medium in a conical bottom polypropylene tube
- Add 5 µl stock staining solution to 1 ml Suspension (≈ 5 µM concentrated staining solution)
- Incubate at least 20 minutes in the incubator
- Centrifuge the labeled suspension at 1500 rpm for 5 minutes
- Remove the supernatant and resuspend the cells in fresh culture medium
- Repeat the last two steps two more times
- Seed the labeled cells to a desired confluence in cell culture dishes
- Give the cells a few minutes to settle down before imaging
The PKH dye is provided as a 1 mM stock staining solution dissolved in ethanol. Additionally, PKH-Linker kits contain the iso-osmotic dye-loading solution Diluent C. The staining protocol of PKH is optimized for the labeling of cells in suspension, but the labeling of adherent cells is also possible.

**Adherent Cells**

The cells are seeded to a desired confluence in cell culture dishes and grown for a sufficient time to let them adhere (usually one day before labeling).

- Remove the old medium of the cells and wash the cells 2× with PBS to remove all medium residues
- Prepare a fresh 2 μM concentrated staining solution by adding 2 μL of the provided stock staining solution to 1 ml Diluent C
- Rapidly add 500 μl staining solution to the cells
- Incubate for 5 minutes at 37 °C, 100% humidity and 5% CO₂
- Stop the labeling process by adding 500 μl culture medium and incubate for < 1 minute
- Remove the staining solution and wash the cells 3× with culture medium
- Add fresh culture medium to the cells
- The cells are ready for imaging

**Cells in Suspension**

- Prepare a cell suspension containing $2 \times 10^7$ cells in serum-free culture medium in a conical bottom polypropylene tube
- Centrifuge the cells at 1000 rpm for 5 minutes
- Resuspend the cells in PBS and repeat last step
- Resuspend the cells in 1 ml Diluent C
- Prepare a fresh 2× staining solution by adding 4 μL of the provided stock staining solution to 1 ml Diluent C
- Rapidly add the staining solution and incubate for 5 minutes while periodic mixing
- Stop the staining process by adding 10 ml culture medium
- Centrifuge the labeled suspension at 1000 rpm for 10 minutes
- Remove the supernatant and resuspend the cells with 10 ml fresh culture medium
- Transfer the suspension in a fresh conical bottom polypropylene tube
• Centrifuge the labeled suspension at 1000 rpm for 10 minutes
• Remove the supernatant and resuspend the cell pellet with 2 ml culture medium
• Repeat the last two steps two more times
• Seed the labeled cells to a desired confluence in cell culture dishes
• Give the cells a few minutes to settle down before imaging

SiR

The stains SiR-actin, SiR700-actin and SiR-tubulin are provided as solids with a content of \(1 \times 50\, \text{nmol}\). To prepare a 1 mM stock solution, the content has to be dissolved with 50 µl anhydrous dimethyl sulfoxide (DMSO). The staining protocol is optimized for adherent cells. The cells are seeded to a desired confluence in cell culture dishes and grown for a sufficient time to let them adhere (usually one day before labeling). The concentration of the working staining solution depends on the desired incubation time:

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Table A.1

• Prepare a fresh staining solution in culture medium with a concentration that fits to the desired incubation time
• Remove the old medium of the cells and add the fresh prepared staining solution
• Incubate for the desired incubation time at 37 °C, 100% humidity and 5% CO\(_2\)
• (Optionally) remove the staining solution and add fresh culture medium
• The cells are ready for imaging

A washing step of the cells is not needed, but is recommended to keep the dye concentration \(\geq 100\, \text{nM}\) during imaging to get a constant signal and to avoid influences on cytoskeleton dynamics caused by the dye.

WGA

The WGA conjugate stain is lyophilized provided with an amount of 5 mg. To prepare a 1 mg/ml stock solution, the 5 mg of lyophilized WGA conjugate is dissolved in 5 ml PBS or water. It is recommend to aliquot this stock solution, because repeated freeze/shaw cycles
The labeling protocol is optimized for adherent cells. The cells are seeded to a desired confluence in cell culture dishes and grown for a sufficient time to let them adhere (usually one day before labeling).

- Prepare a fresh 5 \( \mu \text{g/ml} \) concentrated staining solution by adding 5 \( \mu \text{L} \) of the provided stock staining solution to 1 ml Hank’s balanced salt solution (HBSS)
- Remove the old medium of the cells and add the fresh prepared staining solution
- Incubate 10 minutes at 37 °C, 100% humidity and 5% CO\(_2\)
- Remove the staining solution and wash the cells 3\( \times \) with HBSS
- Add fresh culture medium to the cells
- The cells are ready for imaging
Appendix B

Excitation and Emission Spectra of the Used Dyes

On the next pages, the emission and excitation spectra of the used dyes are depicted. In each spectra a blue line and a transparent blue box are drawn in. The blue line indicates the excitation wavelength of the WLL and the transparent blue box marks the detector settings for the emission wavelength region of interest during imaging. A red line indicates the chosen STED wavelength. The transparent green box in figure B.1(b) indicates a further detected region with which the actin signal of three-colored images was recorded. The third detector region at the three-labeling experiment was the same as drawn at the spectra of DiO depicted in figure B.2(d).

Figure B.1: The emission and excitation spectra data was adopted from [115] and [116].
Appendix

(a) GATTAquant nanoruler with Alexa Fluor 488 as fluorophore.

(b) CellMask© Green.

(c) CellMask© Orange.

(d) DiO.

Figure B.2: The emission and excitation spectra data of (a), (c) and (d) were adopted from [63]. The image (b) was modified from [91].
(a) CellLight™ Plasma Membrane- and Actin-GFP (emerald GFP).

(b) PKH26.

(c) Alexa Fluor 633 WGA conjugates.

Figure B.3: The emission and excitation spectra data of (a) and (c) were adopted from [63]. The image (b) was modified from [117].
Appendix C

Design Drawing of the New Dish Holder

On the next page, the design drawing of the new dish holder is shown. It shows schematically the construction of the holder with all relevant technical dimensions in mm. The representation is 1:1 on DIN A2 paper. The dish holder contains several milling grooves to keep the weight as small as possible. A dish holder weighing more than 130 g would damage the spring system of the galvo flow (high precision z-drive of the microscope) over time.
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Appendix D

Investigation of the System Resolution using GattaQuant Products

The resolution of our STED and confocal microscope was studied using STED and confocal nanoruler (GATTAquant GmbH, Germany). The nanoruler are DNA origami structures on which fluorescent marks are located in a specific distance [118]. The used confocal and STED nanorulers have a mark-to-mark distance of 350 nm and 120 nm, respectively.

![Figure D.1: STED nanoruler with a mark-to-mark distance of 120 nm imaged by STED microscopy. The two fluorescent marks are not distinguishable. (a-c) Three individual nanoruler imaged with a pixelsize of 40 nm. (d-e) Three individual nanoruler imaged with a pixelsize of 20 nm. Scale bars: 200 nm.](image)

STED and confocal microscopy was performed at 23 °C with the 100× objective using the immersion oil with a refraction index of 1.518 at 23 °C. The used nanorules have Alexa Fluor 488 molecules as fluorescent marks. This molecule has a maximum emission wavelength of 520 nm and is a green fluorescent dye. The spectra and the chosen settings for the STED wavelength and the HyD detector can be found in the appendix [B]. Since this dye was extremely fast bleached by the STED Laser, only 2D images and no z-stacks were recorded. The scanning direction was unidirectional. The images were not deconvolved to avoid artifacts due to the missing information in z-direction. Instead the images were Gaussian blurred with a pixel-radius of 2, the background was subtracted and the contrast was enhanced using the program ImageJ.

Nanorulers with the distance 120 nm and Alexa Fluor 488 as fluorescent marks are not resolvable by our STED system (see figure D.1). The above images (a-c) are imaged with a pixelsize
of 40 nm and the below images (d-e) are imaged with a pixelsize of 20 nm. The variation of either the pixelsize nor the scanning speed and STED laser intensity resulted in a resolved structure. The images (a-c) were recorded with a WLL laser and STED intensity of 100 %. For the images (d-f) a STED laser intensity of 30 % (\approx 70 \text{ mW}) was set. Since the signal of the nanoruler was very weak, a line-accumulation of 10 and a frame-accumulation of 4 were chosen while imaging. The high accumulation rates causes a longer acquisition time which in turn can cause movement artifacts and a stronger blurring of the signal.

On the other hand, the 350 nm separated fluorescent marks of the confocal nanoruler are re-

![Images](image1.png) ![Images](image2.png)

**Figure D.2:** Individual confocal nanorulers with a mark-to-mark distance of 350 nm imaged by confocal (a-c) and STED microscopy (d-f). The two fluorescent marks are distinguishable by both systems. Scale bars: 200 nm.

solvable by both systems, confocal and STED microscope. Confocal microscopy images of the 350 nm nanoruler are displayed in figure [D.2](image1.png) (a-c) and in figure [D.2](image2.png) (d-f) STED microscopy images of the 350 nm-nanoruler are depicted. The images were recorded with a pixel size of 40 nm, a scanning speed of 700 Hz and with a WLL intensity of 100 % (\approx 50 mW) for both confocal and STED microscopy. Additionally, a line-accumulation of 10 and a frame-accumulation of 2 was applied to increase the photon yield. A STED laser intensity of 30 % (\approx 70 mW) was selected for the STED imaging. Thus, for confocal and STED imaging the same settings were chosen and the only difference between the imaging is the 30 % STED laser intensity.

On the first glance of these images in figure [D.2](image1.png) there is no particular difference recognizable. The images of the confocal nanoruler are further analyzed by determining the diameters of the fluorescent marks. For this, the nanorulers were cut out, aligned horizontally and the sum of the intensities for each x-pixel was determined. For an automatic evaluation of the last step, a macro was written in ImageJ, which is listed in the appendix [E](image3.png). The intensity profile was then analyzed with the program origin, where two Gaussians are fitted to the two peaks by using the replica option. The Gaussian fit function has the following expression:

$$f(x) = y_0 + \frac{A}{\sqrt{2\pi}\sigma}e^{-\frac{(x-x_c)^2}{2\sigma^2}}$$  \hspace{1cm} (D.1)

where \(y_0\) is the background offset, \(A\) the amplitude of the Gaussian function, \(x_c\) the x-position.
of the peak maximum and $\sigma$ is the standard deviation of the Gaussian. The diameter of the fluorescent spot is then given by the full width at half maximum (fwhm) of the Gaussian fit. In figure D.3 (a) an intensity profile of an imaged 350 nm nanoruler typically gained when using confocal microscopy is depicted. The two Gaussian functions (red and green lines) nicely fits to the intensities peaks of the two separated fluorescent spots. The blue line corresponds to the cumulative fit, which is the sum of the both Gaussian functions. Here, one can see that the measured intensity values are matched by the cumulative fit. Beside the intensity profile gained with confocal microscopy, an intensity profile of a 350 nm nanoruler typically gained with STED microscopy is depicted in figure D.3 (b). The first difference which catches the eye is the that the intensity profile of the STED measurement exhibit lower gray values as compared to intensity profile gained with confocal microscopy. In the confocal measurement a maximum gray value sum of 770 was gained, whereas at the STED measurement only a maximum gray value sum of 450 was reached. Thus, the photon yield is significant decreased by the STED laser. In addition to the low gray values, the drop between the two intensity maxima is more distinct in the STED measurement than in the confocal measurement. Consequently, the two marks are better distinguishable by the STED microscopy.

In figure D.4 the resulted spot diameters for the confocal and the STED measurement are illustrated as box-diagrams. In both measurements, the data points are normally distributed. This is nicely recognizable by the diamond shape formed by the data points. For STED measurements, a smaller spot diameter tends to be targeted compared to the spot diameters achieved with confocal microscopy. A mean spot diameter of $(d_{\text{measured}} = 231 \pm 36)$ nm was achieved with STED microscopy, whereas with confocal microscopy a slightly bigger mean spot diameter with a value of $(d_{\text{measured}} = 275 \pm 31)$ nm was obtained. This small difference is significant ($p < 0.0001$) due to the high number of data points (confocal: 222; STED: 226). The fluorescent marks are 25 nm long (in the direction of the connection axis) and 7 nm wide.

Figure D.3: Two selected intensity profiles of 350 nm nanorulers imaged with a pixelsize of 40 nm by confocal and STED microscopy. The intensity profile of the STED imaging exhibit significant lower gray values and the drop between the two peaks is more distinct.
Thus, they have a very small size and the resolution of the microscopes can be calculated as follows:

\[ d_{\text{resolution}} = \sqrt{d_{\text{measured}}^2 - (25 \text{nm})^2}. \]  

(D.2)

This results in a resolution of \( d_{\text{resolution}} = (230 \pm 36) \text{ nm} \) and \( d_{\text{resolution}} = (274 \pm 31) \text{ nm} \) for the STED and confocal microscope, respectively. These measured resolutions are poor resolutions for both systems, confocal and STED.

![Figure D.4](image)

**Figure D.4:** Spot diameters of the 350 nm nanoruler achieved by confocal and STED microscopy.

**Conclusion**

The investigation of the resolutions achievable by our confocal and STED system using nanorulers of the company GATTAquant GmbH reveals poor resolutions for both, confocal and STED microscope. The STED nanoruler with a mark-to-mark distance of 120 nm was not resolvable by our STED system. On the other hand, the confocal nanoruler with a mark-to-mark distance of 350 nm was resolvable by both systems, confocal and STED system. The measurement of spot diameters reveals a poor resolution of \( d_{\text{measured}} = (275 \pm 31) \text{ nm} \) for the confocal microscope and a poor resolution of \( d_{\text{measured}} = (231 \pm 36) \text{ nm} \) for the STED microscope. Probably the low photon yield and the lack of deconvolution cause that poor resolution results.

The high amount of line- and frame-accumulation and the fact, that the WLL laser intensity was set to 100 %, lead to a high background noise which in turn causes a huge blurring of the spots. However, these settings were necessary to even locate the small fluorescent spots during microscopy. Additionally, it was not able to record z-stacks of the nanorulers because the green fluorescent dye Alexa Fluor 488 is extremely fast bleached by the STED laser. Due to the low signal and the circumstances caused by this, such as the lack of the deconvolution process and the high number of accumulations, the method is not suitable for determining the actual resolution of the microscopes. Nevertheless, it would be interesting whether a STED nanoruler with a mark-to-mark distance of 170 nm can be resolved by our system or not. This distance correspond to the smallest TNT diameter measured in chapter 8. However, for a proper resolution measurement the conditions have to be identical or at least very similar to
the conditions under which the investigated structure is imaged. This was not the case here, because the fluorescence signal of the nanorulers was definitely much lower than the signal gained from the TNTs. Therefore, the measured resolution by GATTAquant-analysis is not comparable to the accurate resolution obtainable at the TNT imaging. As a result of this, the in this section measured resolution was not used for the accurate sizing of the TNTs in chapter 8. Instead, a resolution of 105 nm was assumed. This resolution was determined under conditions similar to microscopy of TNTs [72]. However, for future analysis using GATTAQuant products a red fluorescent dye instead of the green fluorescent dye is preferred because red dyes are more photoresistant [93]. The green dye Alexa Fluor 488 was only selected because of the context to the used green fluorescent dye DiO.
Appendix E

ImageJ Macro for GattaQuant Analysis

The following ImageJ macro was used to get the intensity profiles of the imaged GattaQuant nanoruler spots as described in the subsection of chapter 6. It enables the automatic evaluation of all images contained in one single folder. Thereby, the program asks the user to select the actual directory of the folder. The images are read in as a list and the program calculates the intensity profile of each image separately. The image is opened and the intensity profile is calculated by summing up all gray values of each pixel which have the same x-coordinate. The intensity profile is then saved as txt.-file in the selected order. The program then closes the “results” window and the current evaluated image. After the closure, the program opens the next image in the folder and the process is repeated until each image has been evaluated by the program.

Path = getDirectory('Choose Source Directory');
list = getFileList(Path);
for (i = 0; i < list.length; i++){
    open (Path + list[i]);
    Height = getHeigt();
    Width = getWidth();
    Title = getTitle();
    intensity = newArray(0);
    for (x = 0; Width > x; x++) {
        Sum = 0;
        for (y = 0; Height > y; y++) {
            Sum += getPixel(x, y);
        }
        intensity = Array.concat(intensity, Sum);
    }
    Plot.create("Intensity-Profile", "X-Coordinate", "Gray Value", intensity);
    Plot.show();
Appendix

Plot.getValues(xpoints, ypoints);
Plot.showValues();
saveAs("results", Path + Title + ".txt");
close();
selectWindow(list[i]);
close();
}
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[105] ATCC. Thawing, propagation and cryopreservation protocol of nci-pbcf-htb14 (u-87 mg).


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