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Light-sheet fluorescent microscopy in tardigrade anoxybiosis

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ABBREVIATIONS

ALSFM	Airy beam light sheet fluorescent microscopy
AO	Acridine Orange
AQP	Aquaporin protein
ATP	Adenosine 5'-triphosphate
CAHS	Cytosolic abundant heat soluble (protein)
Dsup	Damage suppressor protein
FOV	Field of view
GFP	Green fluorescent protein
HSP	Heat shock protein
IDP	Intrinsically disordered protein
LEA	Late embryogenesis abundant protein
LSFM	Light sheet fluorescence microscopy
MAHS	Mitochondrial abundant heat soluble (protein)
MRD	Metabolic rate depression
MSD	Mean squared displacement (µm2)
NA	Numerical aperture
NR	Nile Red
PSF	Point spread function
RFP	Red fluorescent protein
RI	Refractive index
ROI	Region of interest
ROS	Reactive oxygen species
SAHS	Secreted abundant heat soluble (protein)
SC	Storage cells
SOD	Superoxide dismutase
TDP	Tardigrade disordered protein (often referred to as IDPs)

ABSTRACT

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Tardigrades are invertebrates that are known for their tolerance to extreme conditions. This research focuses on anoxia, the total lack of oxygen, and the adaptation and survival of anoxia-induced experiments. Cryptobiosis is a form of dormancy that enables the survival of the animals, however, many forms of cryptobiosis are still poorly understood and the mechanisms and physiological responses are not entirely explained.

This study aimed to create a protocol that utilizes fluorescent dyes to visualize the transition into anoxybiosis and morphometric changes at the cellular level involved in the phenomenon. Light-sheet fluorescent microscopy enabled fast 3D volumetric scanning of the transition and revealed what happened to the animal when anoxia was chemically applied.

Results were aligned with the expectations: during anoxybiosis, tardigrades became immobilized and swollen leading to the relocation and reorganization of cells. Importantly it was observed that variation throughout the experiments was quite significant and in further studies, this should be outlined. In this research, two specimens of *M. ripperi* were used and obtained data were compared including cell number, volume, and displacement over time. The most fundamental issue is how to gain stable and reproducible results. Tardigrade cuticle and overall variation of body state of the animals create sources of error, particularly dyeing by soaking. By soaking, there are very few possibilities to control, how the dye is distributed and attached to the cellular compartments, therefore in this study we did not pay so close attention to the statistical significance of the results. Animals chosen for the experiments were random and therefore variables such as sex, age, and fasting were not included.

Fluorescent microscopy is a widely used method in biological studies and this study showed that it can be used in live imaging of tardigrades. However, as a pilot experiment, this led to many open questions and features to improve, especially in the image analysis part. Large datasets need a lighter pipeline to gain a higher throughput method. In the future, the (light sheet) fluorescent imaging can be a beneficial tool for similar cellular studies; however, the individual sources of variation need to be minimized. Tardigrades can be a promising model organism for studies including cell survival, cancer research, and storage and storing solutions for various drug components when understanding the mechanisms that enable the animal stress tolerance and survival.

Keywords: tardigrades, stress tolerance, anoxia, cryptobiosis, LSFM, arivis 4D Vision

1. Literature overview

Living organisms encounter harsh environmental conditions and over time they needed to develop strategies to overcome seasonal changes or more sudden phenomenon like desiccation. The most extreme adaptation to threat caused by environment is cryptobiosis. Among tardigrades cryptobiosis is a common response to tolerate extreme conditions. Within phylum Tardigrada, closely related to Onychophora and Arthropoda, there are over 1300 species[1,2], and their unique ability to tolerate extreme stress varies, however presumably every one of the species is resistant. Crowe categorized four types of cryptobiosis already half century ago: (i) anhydrobiosis: lack of water, (ii) cryobiosis: response to low temperatures/freezing, (iii) anoxybiosis: lack of oxygen, and (iv) osmobiosis: increased solute (salt) concentration[3]. Besides general cryptobiotic forms, tardigrades tolerate high pressures and radiation[2,4]. Among cryptobiosis, anhydrobiosis is considered the most common and well-studied form[5]. As a result of desiccation, the animals become dormant, contract their body, and form a tun. Anoxybiosis, which is highlighted in this thesis project, is a less studied dormancy state and vastly different from anhydrobiosis, as it lacks the tun state. This literature review aims to investigate survival strategies and mechanism of tardigrades. In addition, light sheet fluorescence imaging modality as a research tool to visualize these transitions into dormancy at the cellular level is introduced. Importantly, fluorescent microscopy is not commonly used as a part of tardigrade research, therefore a protocol for fluorescent imaging has been developed.

1.1. What is a tardigrade?

Tardigrades are microscopic animals, varying from 50 µm to 1200 µm in lenght[6], and are famous for their survival skills. Their body is surrounded by a layer of water to keep them in active state and enables gas exchange (i.e., oxygen uptake) by diffusion through the cuticle[7]. Thus, tardigrades are considered as aquatic animals. However, they are found everywhere around the globe – from glaciers of Antarctica to forests and even urban environment[3,8]. They can be considered one of the first multicellular organisms on Earth and lived through most mass extinction events. Based on tardigrades' position in the tree of life, it is fair to say that tardigrades evolved over 500 million years ago[9]. Based on both morphological features and molecular data, tardigrades are ecdysozoans like nematodes and arthopods[10]. Ecdysozoans share the though, flexible sometimes multilayer cuticle,

which is a common feature within the phyla. This exoskeleton can be shed in a process called ecdysis as a result of growing or sometimes in stress response[11]. The cuticle protects tardigrades from exogenous stress, predators, and other external threats like parasites[12]. The phylum Tardigrada is often considered as a sister taxon to the arthropods (i.e., spiders, crabs, insects) and onychophorans (i.e., velvet worms)[9]. The exact place of this phylum has been debated heavily among biologists, and still there is no consensus. However, the main indicator of relationships among genera is morphological features and by today it has been proposed that tardigrades, onychophorans, and arthropods form a monophyletic clade called Panarthropoda[11].

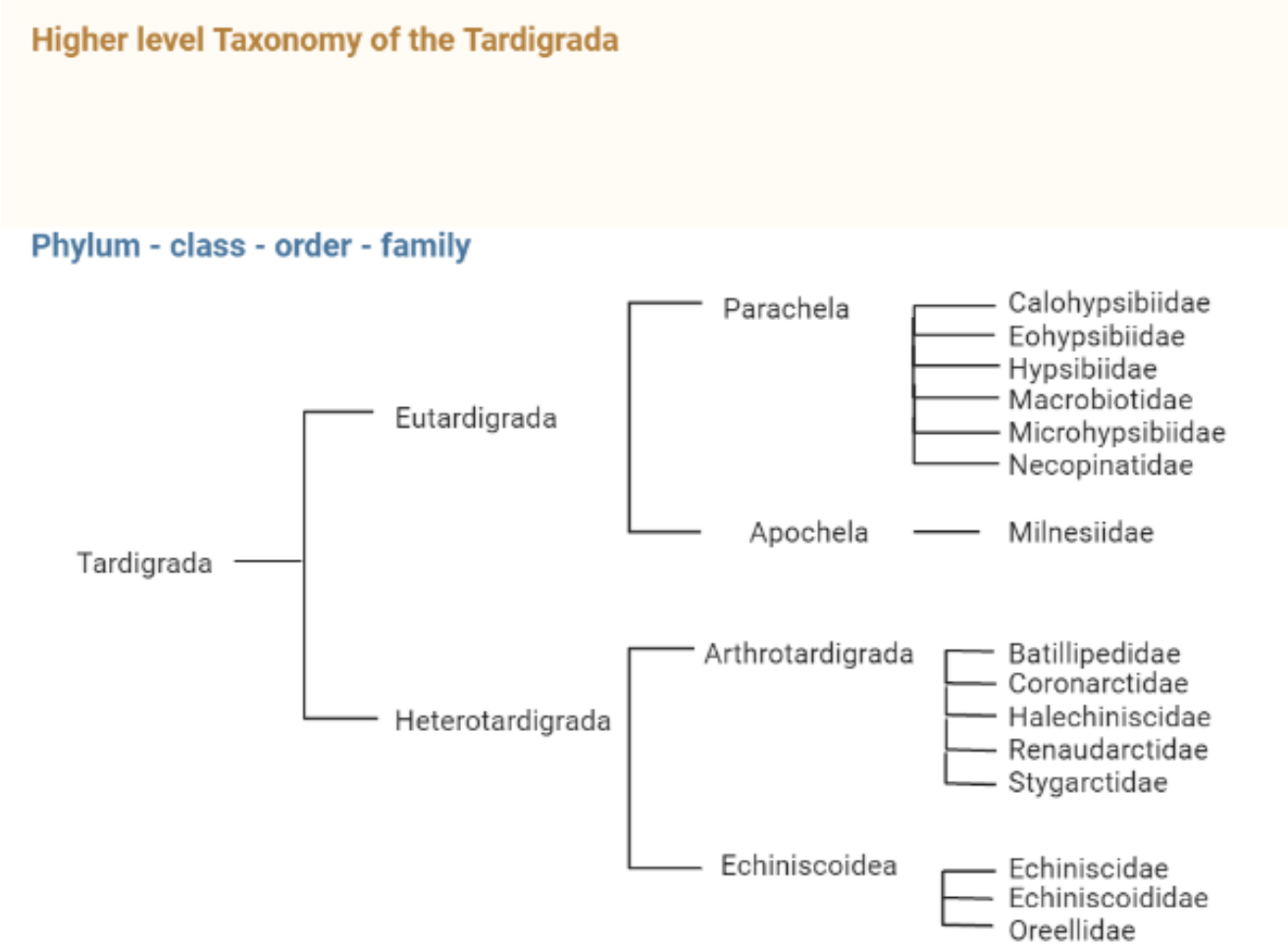

Figure 1. Phylum Tardigrada is diverse and includes almost 1400 species. The two classes, eutardigrades and heterotardigrades, include four orders and within orders many families. Tardigrades are divided into their classes based on habitat, morphology, and molecular studies. Figure adapted from D. Nelson, R. Guidetti (2015)[6].

Although in recent years tardigrades have gained a more reputation as potential model organisms, they are still less known than e.g., *C. elegans* (roundworm) or *D. melanogaster* (fruit fly). The first studies of tardigrades date back to the late 1700s[13]. Zoologist Johann Götze found these animals in 1773 and named them small water bears ("kleine Wasserbär") and a few years later Italian biologist Lazzaro Spallanzani studied these animals and renamed them Tardigrada ("slow steppers"). Afterward tardigrades have been studied by many scientists and today, 1380 species are discovered, and several research articles are published in a year[1,9].

Duration of active life among tardigrades ranges from one month to 2–3 years, and cryptobiotic periods can expand the lifespan by several years[3]. The lifespan of active tardigrades has been estimated to be from 3–30 months[14]. The lifespan may vary based on the habitat, and it was shown in study of Ramazzotti and Maucci (1983) that freshwater species of *Macrobiotus* and *Hypsibius* lived a few years and terrestrial counterparts of the same genera average 4–12 years[14]. To understand and gain knowledge of tardigrade lifespan, culturing these animals is essential. So far mainly eutardigrades have been cultured in laboratory conditions and the reason heterotardigrades do not adapt well in artificial environment is obscure[15,16]. However, in recent years a few research groups have managed to conduct their study on heterotardigrades in laboratory environments[16,17].

Reproduction of tardigrades is species specific and usually dependent on habitats[18,19]. It has been concluded, based on tardigrade behavioral analysis, that in favorable conditions the reproduction is most active, occurring rapidly and in high numbers[19]. Reproduction can be sexual or asexual[18]. The asexual process is called parthenogenesis, and so far, it is the only asexual mode confirmed among tardigrades[20]. Both hermaphroditic and dioecious tardigrades are known, however it is not shown that among hermaphrodites cross-fertilization is possible[20]. Parthenogenesis and hermaphroditism are not possible among the same species; thus self-fertilization will only evolve where parthenogenesis has never occurred[19]. Females lay up to 30-40 of eggs that are can be laid in groups in leaf pockets or in shed/molted cuticle[18].

1.1.1. Tardigrade anatomy

Tardigrades are divided into two main evolutionary lineages: eutardigrades and heterotardigrades (see figure 1.). The division is based on molecular and morphological traits of the animals, but habitat can also serve as a basis for division[6]. Within heterotardigrades, arthrotardigrades are

marine species with one exception: echiniscoids are primarily terrestrial species with an armored, thick cuticle[14]. The class Eutardigrada includes the unarmored orders Apochela, which is terrestrial, and Parachela family includes terrestrial and freshwater species[14]. Habitat also affects tardigrade anatomy; however, the basic components are the same in each species (see fig.2). There are neither respiratory nor circulatory organs in tardigrades and gas exchange happens through cuticle by diffusion[14].

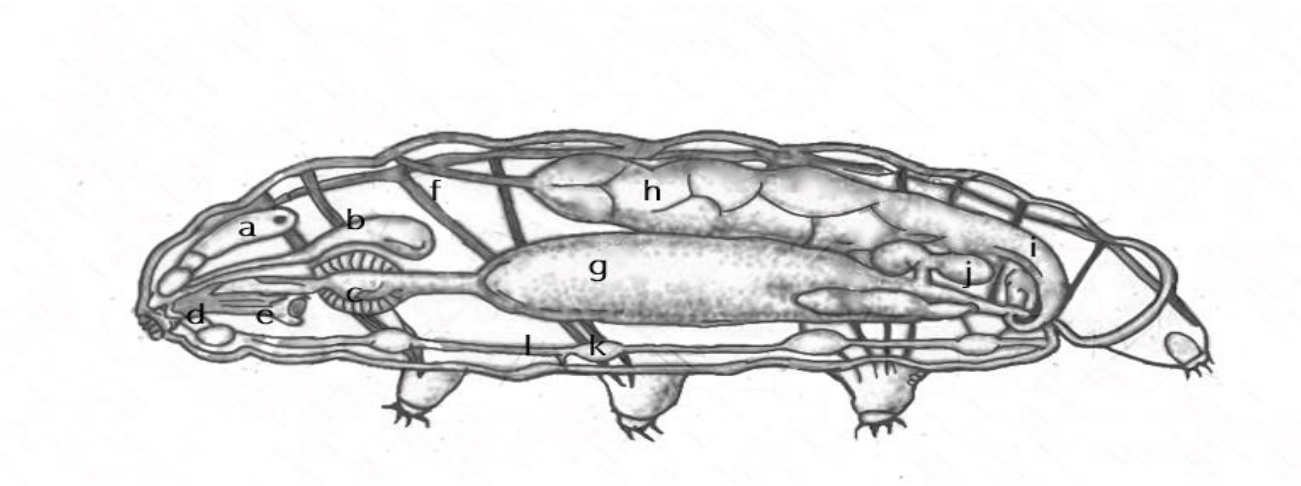

Figure 2. Illustration of basic tardigrade anatomy including brain and simple eye with opsins attach to it (a), salivary gland (b), pharynx, which creates the suction for feeding along with buccal tube (c), mouth (d), stylets ("tardigrade teeth") (e), muscular structures (f), midgut (g), ovary (h), oviduct (i). Malpighian glands, which are considered as excretory and osmoregulatory organs (j), and ventral ganglion, which is central part of the tardigrade nervous system (k) and ventral nerve cord (l). Storage cells are important for this study and fill the cavities of tardigrade body. Image adapted and modified from student project by Haldrup *et al.* (2015)[21].

Morphological differences among the species are based on buccal-pharyngeal apparatus, claws, cuticle morphology and eggs[6]. The buccal–pharyngeal apparatus is used to identify species in Eutardigrade class, including the shape of stylets, structure of buccal tube and cavity armature[6]. Eutardigrades, as often limno-terrestrial and freshwater ones, have smoother, more simply sculptured cuticle covered by pores and granulation, and cuticle of heterotardigrades, may have variation in number of spines, filaments or sensory appendages[6].

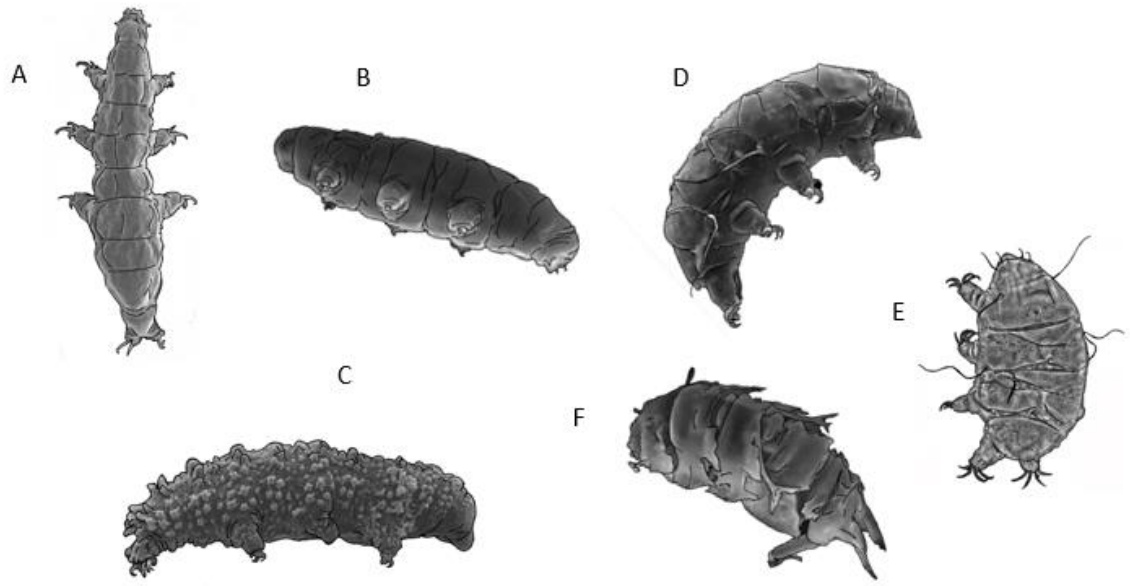

Figure 3. Morphology of tardigrades differs from species to species. It can be said that eutardigrades have smoother cuticles and heterotardigrades have various kinds of attachment like filaments or spines as sensory elements. *Milsenium pentapapillatum* sp. (a)[22], *Paramacrobiotus* sp. (b)[23], *Calohypsibius schusteri* sp. nov. (c)[24] are eutardigrade species with simpler cuticle structure, and *Mopsechiniscus franciscae* (d)[25], *Testechiniscus spitsbergensis* (e)[26] and *Stygarctus keralensis* sp. (f)[27] belong to the heterotardigrade class and the cuticle is more armored and with multiple spines. Sketches based on images from several studies.

Tardigrades are structurally quite simple; they have four pairs of legs with claws which they use to move on different surfaces[6]. Three pairs of legs are located on the side and one pair at the end of the body[6]. Legs in the back are unique for tardigrades and animals use them more as hands; to attach or climb[14]. Each leg has 4-8 claws depending on the species[14].

Tardigrades feed on plants, algae, bacteria, and other small invertebrates (i.e., rotifers, nematodes) and sometimes even other smaller species of tardigrades[4]. Digestive system of tardigrade consists of three-part system, starting with an anterior subterminal mouth, stylets and a buccal tube leading through a muscular sucking pharynx into a voluminous midgut (stomach), and eventually posterior ventral hindgut which empties the undigested parts through anus[6].

The nervous system includes the brain, one pair of nerve strands and four ventral ganglia. The dorsal lobed brain which is attached to a subsequent chain of four ventral trunk ganglia, one in each body segment, which are responsible for producing nerve fibers[6,28]. Each ganglia is attached and connected to each other, except the first trunk ganglia which is attached to the brain with inner and outer connectives[28,29]. Each ganglion is formed by two smaller hemi ganglia which are then attached

to each other by the fiber mass[28]. Even though there has been intensive research on tardigrade brain and nervous system, the results have been controversial and created huge debate of the functions and interaction of these two: whether the brain of tardigrade is composed of a single segmental ganglion or several segmental parts (smaller hemi ganglia)[30,31]? The nervous system and brain of tardigrades is a good example of how little is known, yet the nervous system was first described in 1840[29,30].

1.1.2. Cuticle

The role of the cuticle is highlighted in this project because of the fluorescent imaging and dye absorbing qualities. There has been fluorescent imaging done for tardigrades (e.g., Russell (2001), McGreevy (2018), deCarvalho (2019)), however the used dyes have been microinjected to the animal or used for embryos[32–34]. Our approach is to let dye penetrate through the cuticle layer and attached to cellular components by simply soaking the animals in dye solution.

The cuticle is more complex with several layers that protect the soft tissue of inner cellular organisms of the animals and works as mechanical/physiological protectant in stress tolerance[12]. Cuticle needs to be flexible for tun formation, the dormancy state in anhydro- and cryobiosis. A cuticle consists of three layers with different components, all having their purpose of protecting the animal from environmental stress factors[12]. The somatic muscles are attached to tardigrade cuticle and these muscles enable movement and morphological changes (i.e., tun formation). As ecdysozoans, tardigrade shed their cuticle for growth and can do it several times[14]. Sometimes molting can be a result of stress[12]. Molting does not concern only the cuticle, since tardigrades also shed the claws and the lining of fore and hindgut leading for days-long state, and because of this they cannot feed[12,14].

There is great variation in cuticular layers among tardigrade species. It has been observed that the thickness of the cuticle in freshwater species is greater than in marine ones, and in terrestrial species than in aquatic ones[12]. In many cases, the more tolerant the species is, the thicker the cuticle[12]. The outermost layer of the cuticle, epicuticle, varies the most among the species[12]. Flocculent coat in epicuticle is species-specific and some species do not have it. Besides the species specificity, its presence can vary from active state to cryptobiotic state. The flocculent coat is the thickest on legs[12]. The flocculent coat consists of acid mucopolysaccharides, and surrounding layers (inner/outer

epicuticle) are neutral polysaccharides and lipoproteins[12]. After molting both the old and new cuticles lack the flocculent coat; the animal is unable to mold new intracuticle until it has performed sclerotization[12]. Intracuticle (middle part of the cuticle structure) consists of phospholipids and neutral fats and works as diffusion barrier. Besides lipids there are chitin, untanned glycoproteins, and lipoproteins. During the molting lipid rich epidermal cells travel through procuticle to the upper layers (through pro- and intracuticle as far as epicuticle)[12]. However, granular layer is often missing in recently molded cuticles[12]. During the secreting part of the new cuticle, the old one is still attached to somatic muscles via cuticular filaments[12]. The cuticular filaments connect and enable the transition between old and new cuticle[12]. When the new cuticle encounters the surrounding environment, molecular phenomena start to occur enzymatic activity outermost epicuticle can be a trigger of releasing polysaccharides and forming a new flocculent coat[12]. The innermost layer of cuticle structure is the procuticle which is located just over epidermis. This layer of cuticle consists of irregularly arranged microfibers and is always chitinous[12]. Procuticle may receive components from epidermis, such lipids, or pigments, or even waste material[12]. Procuticle is also the most tension resistant layer of tardigrade cuticle because of its chemical composition: quinone-tanning and chitin[12].

Understanding the structure and composition of the tardigrade cuticle can improve fluorescent imaging of tardigrades and conducting research on living animals. Choosing the correct dyes for specific cellular compartments can be demanding since the dye needs to penetrate through multiple layers until it reaches the internal structure of the animal. Besides topics related to this project, understanding cuticular structures can lead to better understanding of protective molecular mechanisms related to cryptobiotic states discussed in further sections.

1.2. How do tardigrades respond to stress?

Tardigrades are one of the most stress tolerance organisms known to man. Even though there are several other extremophiles, tardigrades hold many records. They can survive extended periods without water or oxygen, tolerate extreme cold and hot environments, they can bear ionizing radiation and high pressures and besides tolerating one harsh factor, they are considered as polyextremotolerants, which indicates tolerating multiple stress factors simultaneously[35].

Stress does not have to be extreme for animals to response. Responding to seasonal changes is an adaptation for unfavorable conditions and triggers coping mechanism such as encystment. These adaptations include resting stages, defined as dormant[36]. During dormancy organisms or animals temporally suspend active life leading to reduced or suspended metabolism[36]. Reduced metabolic activity is a key factor in a state called diapause. In tardigrades, diapause is referred as encystment, which is response to seasonal changes and considered more as process than a state[36]. Encystment is regulated by hormones or other signaling factors usually depending on the developmental state[36]. Encystment is like molting, which was discussed in previous section, leading to decreased body size, and removing parts of the buccopharyngeal apparatus[12].

Cryptobiosis, compared to encystment, is a more immediate response to external stimuli. As Guidetti (2011) rephrased Jönsson's very well said words: "Cryptobiosis represents an ''*escape in time*'' from hostile habitat conditions, as opposed to an ''*escape in space*'' performed by organisms with an ability to migrate away from unfavorable conditions"[7,36]. Cryptobiosis is reversible state of suspended animation, and a general term for anhydro- and cryobiosis[13]. Anoxybiosis and osmobiosis are included in cryptobiotic form, even though this morphological response lacks tun formation[5]. For a rapid transition into dormancy, these animals use their specific proteins (bioprotectants) to form a state that enables prolonged periods of suppressed metabolism and down regulated cellular mechanisms[37]. Besides these active molecules participating stress response, structural and physiological features are playing crucial role for tolerating hostile environments[36]. Lack of water is commonly studied stress factor, and in anhydrobiosis unique proteins protect cellular proteins of dehydrated animals. The proteins are classified as molecular chaperones, which are involved in conformation (folding/unfolding) of proteins[37]. During anhydrobiosis there are several factors including elevated temperature, high humidity and high oxygen partial pressures that can alter and reduce the survival rate of the animals[36]. Besides anhydrobiosis, these factors can be involved also in other cryptobiotic forms such as anoxybiosis. Recovery time is associated in abiotic conditions and metabolic activity needed to repair molecular and cellular damages[36].

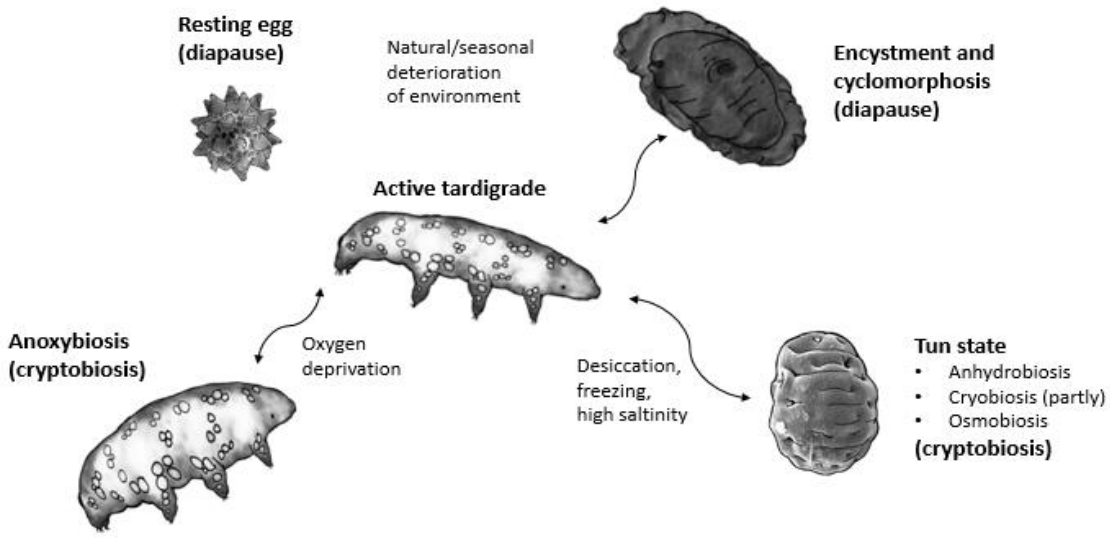

Figure 4. Response to exogenous stimuli and stress in tardigrades. Diapause is a prolonged event that is usually an adaptation for seasonal changes whereas more sudden changes in environmental conditions lead to cryptobiotic phenomenon. Illustration adapted from Mjøberd and Neves article [5].

Withers (2009) summarized the phenomena of cryptobiosis as a state where metabolic rate is extremely low, even unmeasurable[38]. Decreased metabolism is more likely to result from a lack of cell water, rather than changes (molecular/physiological) in metabolic enzyme cascades of normally hydrated tissues[38]. In the case of anoxybiosis, the intracellular water content is kept the same, but the metabolic depression is as low as in other cryptobiotic forms[38]. This leads to the question of what happens during anoxybiosis. In general, insects (in which tardigrades are included) have high metabolic rates[39]. In contrast to the mammalian water-requiring metabolism, insect metabolism is associated with aerobic ATP production[39].

Technology is now more advanced, and protein expression can be studied fast and with deeper insight. Research conducted to define stress tolerance in tardigrades has revealed a group of molecules that are tardigrade specific – intrinsically disordered protein (IDP) family. Besides IDPs there are Heat shock proteins (HSP) which are responsible for stress tolerance in many invertebrates[37,40,41]. The role of molecular components during cryptobiosis is still under research, and the whole picture of stress responsive mechanisms is yet to be revealed.

1.2.1. Cuticle as a shield for stress

The role of cuticle flexibility and outstanding ability to withstand stress is a principal factor in cryptobiotic survival. Cryo- and anhydrobiosis are states that lead to tun formation. Tardigrade cuticle is designed for aquatic environment and therefore water permeability is high, however varying among species and among cuticle area[12]. It has been discussed that during anhydrobiosis, the area of body parts that have higher water permeability are reduced by tun formation[12]. Dehydration or other stress led to the reduction of cell volume as it causes the contraction of the whole body and cell structures are in danger of collapsing[42]. Thus, cells are exposed to mechanical stress[42]. However, the tun formation and role of the cuticle is strongly related to the chemical composition of the cuticle and multiple layers that are designed to protect the animal. In the study of radiation tolerance, Jönsson (2019) concluded that not just avoiding DNA damage but also repairing the DNA, are involved in tardigrade survival[7]. There are several protective proteins that work as molecular switches for ensuring genome integrity[7].

Before the discovery of these specific molecular components, it was widely thought that trehalose and LEA (late embryogenesis abundant) proteins were the main components that protected cellular homeostasis during desiccation[37,42] and especially that both the sugar and LEA are present at cuticular layers. First trehalose was considered the key component in tardigrade survival, but shortly it was observed the accumulation of trehalose was surprisingly insignificant compared to other organisms that use trehalose as vitrifying their cellular contents[43]. To clarify the function of trehalose, there are two main hypotheses: either to avoid protein aggregation and denaturation and membrane collapsing, trehalose is used to create supporting matrix within the cells; or trehalose can prevent the damages by working as water replacement by replacing the hydrogen bonds[43]. However, the low levels of trehalose, which are found under the epicuticle of tardigrades forming a protective wall along with glycerol, does not entirely explain the survival but suggests that other molecules can be involved in vitrification[12,43]. The role of trehalose is also declined by proving that some tardigrades species lack canonical genes that are required to produce trehalose and this finding supports that there must be other components that enable the tolerance[37]. Boothby (2017) summarizes that intrinsically disordered proteins (IDPs) vitrify when dried, indicating that tardigrades use these proteins in equivalent manner as other anhydrobiotic animals use trehalose[43]. Similar observations have occurred with LEA proteins[42]. Recently, several studies have indicated

that the role of both trehalose and LEA proteins are not major components of tardigrade stress tolerance but the role of IDPs is highlighted [35,37].

Somatic muscles, that are attached to the cuticle, are also responsible for energy regulation and energy homeostasis[44]. As stated previously, the insect metabolism is related to energy production and comsumpition[39]. Storage cells are the main energy stores in tardigrades[7]. They are round, free-floating cells in the body cavity of tardigrades[45]. Storage cells store and transport lipids and glycogen[45]. The number of storage cells varies from a few hundred up to 1000 cells according to the species[46]. Diameter of storage cells vary from 10 to 15 µm[46]. The role of storage cells in cryptobiotic states (including anoxybiosis) is not well studied, however their role should be important in stress tolerance. As storage cells are responsible for energy storing and nutrient transporting, their role in a state where energy related metabolic activity is extremely low, must be connected.

1.2.2. Molecular components as a shield for hostile environment

Mechanical and physiological response to stress is related to tardigrade cuticle and muscular structure. Even though desiccation does not seem to influence tardigrade ageing and longevity, damage is accumulated in proportion to the time spent in dormancy[45,47]. Whether this statement is true in a case of anoxybiosis, is still unknown. Not just tardigrades, but other anhydrobiotic metazoans like rotifers or nematodes can reduce their size (surface reduction) as an adaptive process in unfavorable conditions[47]. However, the cuticle is not alone the key for survival, and it is brave to claim that the main mechanism that enables survival in such extreme conditions can be explained by cellular interaction and specific proteins such as intrinsically disordered proteins (IDPs) or heat shock proteins (HSPs). Besides these most famous components of tardigrade survival kit, there are also osmolytes that maintain integrity similarly to trehalose, antioxidants that are involved in oxidative stress, aquaporins (AQs) which are part of water transport and late embryogenesis abundant proteins (LEA) which are involved with IDPs[45].

Several research have indicated that extreme tolerance is related to the genomic structure of tardigrades and cellular interactions[37,42]. Current dogma has put aside the common trehalose-LEA hypotheses in the case of tardigrades. However, there must be a key factor that enables these animals to survive and recover from such stress. Genomic data have revealed different protein related coping mechanisms such as Heat shock proteins (HSPs) and intrinsically disordered proteins

(IDPs), that are now the main suspect for tardigrade highly developed survival skills[40,42,43]. Anhydrobiosis, which has been the most studied cryptobiotic state, has shown correlation in increased oxidative stress response in genes, loss of stress signaling pathways and the active role of IDPs[40]. HSPs work as molecular chaperones by interacting with other proteins and ensuring proper functionality[40]. Study with D. melanogaster showed that in anoxic conditions expression of HSPs were upregulated and ubiquitin 4 (UB4) and superoxide dismutase (SOD) downregulated[48]. SODs are enzymes that detoxify superoxide radicals and reduction of reactive oxygen species (ROS) can lead to bearing oxidative stress[49]. Hashimoto (2016) claimed in his research that significantly expanded gene families of superoxide SOD can be also one of the factors causing increased stress tolerance in tardigrades[49]. However, the study involved only one species of tardigrades, but the level of SODs expressed was significantly higher than in other organisms[49]. Oxidative stress is related to anhydrobiotic phenomenon and desiccation, but it can be involved with anoxybiosis[49]. Hashimoto *et al.,* also found that the peroxisomal pathway of the metabolism had been eliminated, thus avoiding the production hydrogen peroxide from β-oxidation during fatty acid catabolism and at the same time oxygen deprivation is known to lead to protein degradation and lipid peroxidation[49]. In this sense, the role of storage cells is highlighted.

Intrinsically disordered proteins (IDPs), which are specific protein groups in tardigrades, include secretory (SAHS), cytoplasmic (CAHS), and mitochondrial abundant heat soluble (MAHS) proteins and RvLEAM (group A LEA mitochondrial protein)[43]. IDPs lack stable 3D structure which therefore allows them to freely organize within protein conformations and create protective shield[43]. These components are unique and exist only within Phylum Tardigrada and this was confirmed in the study of Hashimoto (2016)[49]. These proteins are present both in adult tardigrades as well as embryotic state[49]. Boothby (2017) has done extensive research on TDPs/IDPs and highlighted the significance of these proteins as a part of stress tolerance[43]. As IDPs are highly disordered and resist change in their structure, factors such as high-temperatures or extreme pHs do not lead to aggregation or precipitation of these molecules[37]. Studies related to IDPs are mostly related to anhydrobiotic form of tardigrades and many are still hypothetical, even though extremely valuable information has already been gained.

The MAHS family is to protect mitochondria. Normal cellular metabolism can be maintained due to mitochondrial activity (and help from peroxisomes) and reactive oxygen species (ROS) controlled[37]. When exposed to desiccation, Richaud et al. (2020), did notice that mitochondria are smaller and

less membranous with a clear loss of inner membrane cristae in anhydrobiotic tardigrade[2]. This simplifies that animals that are dehydrated are also metabolically less active, thus generate less ROS[2]. The CAHS family, like SAHS family work as much in the same manner as LEA when supporting desiccation tolerance. A simplified case is that cytoplasm and nucleus can be protected by CAHS protein, when the extracellular components are secreted by SAHS protein[50]. Roughly said, IDPs are working as cell membrane protectors, in comparable manner to LEA in other metazoans and HSPs as supporting factors (as chaperones).

Dsup (damage suppressor) is unstructured nuclear protein which is involved in DNA repair and unique to tardigrades[49]. Dsup is a nucleosome-binding protein and its main function is to prevent chromatin from cleavage, leading to protection of DNA[51]. Chromatin cleavage is caused by hydroxyl radicals[51]. Thus, the tardigrade genome is protected from hydroxyl radicals by Dsup[51]. Reactive oxygen species (ROS), hydroxyl radicals being one of the most powerful ones, are involved in metabolic activity of the tardigrades, which is related to the cryptobiotic events, therefore also the role of Dsup is emphasized[51].

All these molecular mechanisms are quite novel and need research to deeper insight and understanding the complexity. However, it is observed that tardigrades express their genes ever during cryptobiotic survival, which increases the interest towards these unique coping mechanisms.

1.3. Anoxia tolerance

About two billion years ago, primitive life forms and protozoan adapted the ability to use oxygen for energy production. Evolution took tremendous steps when utilizing oxygen-linked catabolism, however it created dependency on oxygen with many species. Anoxia refers to total absence of oxygen and hypoxia decreased levels of oxygen tension, typically in the range of 1–5% O_2 and among tardigrades the response to anoxic conditions is called anoxybiosis[52]. Whereas normoxia is a state where oxygen tension lies between 10–21%[52]. There are several animals that are tolerant to oxygen deprivation, mostly marine vertebrates, and smaller invertebrates such rotifers, nematodes, and tardigrades[52]. The tolerance for anoxic environment is a result from evolved ability to maintain tissue and cellular homeostasis[36]. Oxygen limitation appears in many pathologies, often related to high tissue metabolism or deprivation of O_2[48]. It is still quite unclear how anoxia mechanisms negatively impact organisms, although consequences can be fatal. There is a chance of being

exposed to anoxic conditions in nature, therefore animals had to develop ways and manners to survive these harsh conditions and restore the oxygen levels[53]. Some vertebrates, like fish and reptiles, and many invertebrates may tolerate moderately long time in anoxia[48]. In contrast, within mammals, the anoxia tolerance is poor. On average, only a few-minute exposure of anoxia can cause severe brain damage in humans[48]. The naked mole rat is considered so far, the most tolerant mammal by surviving 18 minutes of anoxic exposure[48].

Hochacka (2000) described in his studies of hypoxia and anoxia tolerance that the survival from this very severe phenomenon can be divided into two phases[54]. Both phases include more specific functions of how animals respond to oxygen deprivation[54,55]. The first phase is *defense phase*, in which animal (or tissue, cells) respond to the lack of oxygen by sensing the change in oxygen levels, followed by regulation of several cellular mechanism and especially ATP homeostasis and finally suppressing ATP turnover rates[55]. After the oxygen levels starts to improve again, the *rescue phase* begins by sensing the environmental conditions followed by many repair mechanisms such as expressions of different transcription factors that later control the genes that are important in survival in low ATP turnover[55]. Some of the mechanisms were unexplained and researchers started to focus on how animals sense these conditions. After Hochacka claimed in his research that animals can sense hypoxia/anoxia and how this can trigger signaling pathways involved and regulated when exposed to anoxic conditions[55]. Studies on the cellular mechanisms that were responsible for ensuring and stabilizing membranes and molecules that were linked to oxygen deprivation were particularly popular topics[55].

Two decades later, within tardigrades, research has revealed underlying mechanisms that can explain stress tolerance (see 1.2.2.). The difference between anoxybiotic animals versus other cryptobiotic animals is the state of body water which can cause alterations in ion/osmotic balance. Metabolic depression enables aerobic animals to withstand with low oxygen environment, and thus anerobic metabolic rate can be decreased under 0,005 in resting animal – indicating that is unmeasurable[38]. This leads to the fact that tardigrade metabolic rate in anoxybiosis is still unknown and the mechanism behind this cryptobiotic form is unclear. Tardigrade anoxybiosis is very species specific, some species can tolerate hours and others even days of anoxic conditions, however the marine species are more tolerant for anoxia[6]. As discussed in previous sections, some indicators like temperature can negatively affect tolerance. If exposed to anoxia too fast, animals are not able to store oxygen or otherwise prepare for lack of oxygen and it leads to death[6,43].

The predominant theory relies on the fact that anoxia tolerant species can regulate and control both ATP supply and demand when oxygen levels are low[48]. This has been observed in some vertebrates and highly-anoxia tolerant marine invertebrates[48]. This applies also to other cryptobiotic forms, and the ability to maintain ATP levels beneficial for the animal during stress[35]. Downregulating energy turnover or upregulating anaerobic ATP production is the key method to tolerate oxygen deprivation[35,55]. This mechanism among anoxia tolerant species has led them to cope with reduced ATP production. Reduced ATP production has severe consequences like disrupted ion homeostasis, that can lead to cell injury and even death[55]. When species can control the demand and supply of ATP production, thus maintain the ATP levels even in anoxia, it can prevent decreased pH, altered calcium homeostasis, increased intracellular osmotic pressure, and/or mitochondrial damage and tolerate lack of oxygen[48]. Campbell *et al*. (2019), suggested that in anoxia tolerance, there are some factors more involved than others, downregulating metabolically demanding activities (e.g., protein synthesis) being one of them[48]. Boothby (2019) claimed that hypoxia tolerance cannot be explained just by one or two mechanisms or molecular interaction, but it involves both catabolic and anabolic pathway capacity[35]. Based on his studies, IDPs can have a surprising effect on anoxia tolerance[35].

It is easier to understand that anhydrobiosis and cryobiosis lead to a reversible metabolic standstill because of the presence of liquid water which is required to permit metabolism. Anoxybiosis differs from other forms of cryptobiosis, because of the water. Clegg (2000) questioned the old dogma related to anoxia and metabolism: when water and thermal energy is present, biological processes are to happen[56]. Animals that are well-adapted to anoxic environment can reduce the overall metabolic rate, as low as 1% of aerobic levels[57]. Metabolic rate depression (MRD) is commonly studied among intertidal invertebrates that often expose to anoxia[56]. Adjusting the intermediary metabolism can have a significant role in adaptions in anoxia[56]. In the beginning of 2000s, this led to many open questions of metabolic standstill in anoxybiosis – in general the role of free energy in cell viability and cellular integrity. Clegg stated in this article that tardigrades are sensitive if there are sudden changes in levels of oxygen and oxygen tension; the partial pressure of oxygen (PO_2), and prolonged reduction of oxygen can eventually lead to osmoregulatory failure[58]. This leads to the inability to control osmosis, which then leads to excess water entering the animal[4,56]. During the anoxybiosis, as a result from osmoregulatory failure, animals become immobilized and swollen[4]. The body turns stick-like, fully extended with limbs stretched on side, turning into bilaterally symmetrical creature[4].

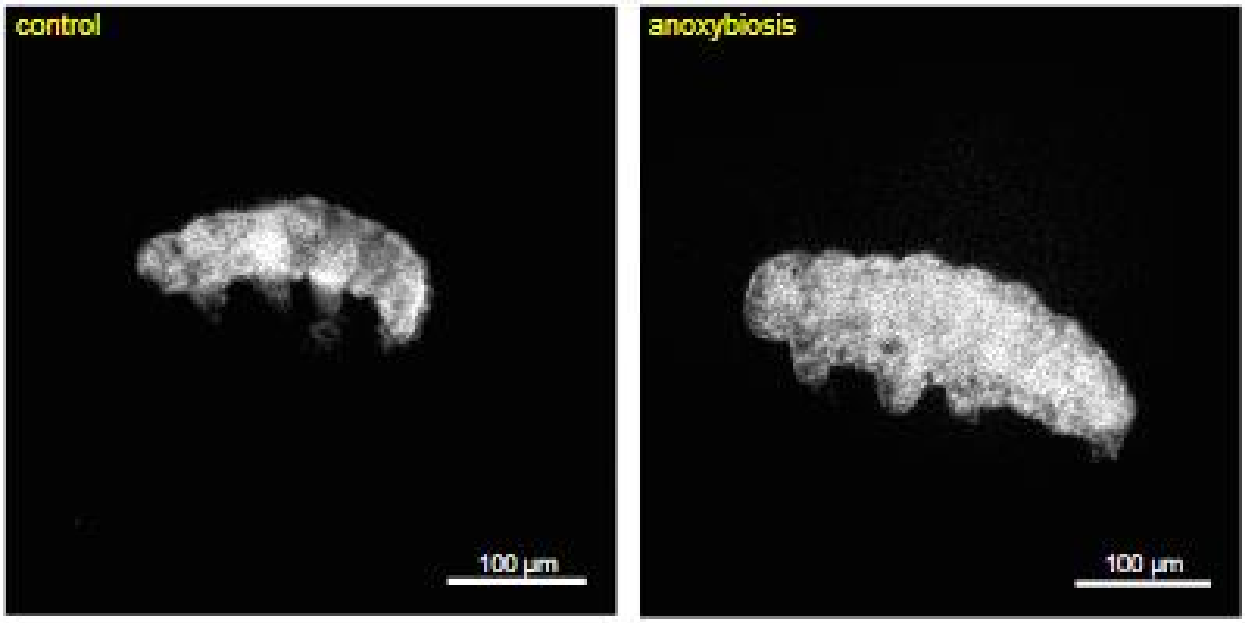

Fig 5. *Macrobiotus ripperi* in normal conditions and in anoxybiosis (Zeiss AxioZoom microscope, magnification 112x). The turgid body is highly emphasized in the right image. Anoxybiosis is a poorly studied form of tardigrade cryptobiosis. The impact of oxygen deprivation suggests that there are two phases: defense and rescue phase[35,54]. This means that the defense phase is where organisms are balancing the supply and demand of oxygen and downregulating ATP consumptive processes. This leads to reducing protein synthesis and membrane ion-pumping, which are the main cellular functions consuming oxygen and can be the reason for morphological changes in the body.

Keilin (1959) and Withers & Cooper (2009) used different terminology in their research, distinguishing between ametabolic state of cryptobiosis including anhydro-, cryo-, anoxy- and osmobiosis and dormancy state where metabolic activity is highly reduced but not entirely lacking[13,38]. Whether the anoxybiosis in tardigrades reflects a total lack of metabolic activity is unclear. The presence of water can indicate that animals are more in “dormancy” than other cryptobiotic forms as they do not enter the tun. Other cryptobiotic states are induced by altering the cell water content, but in anoxybiosis cell water remains in normal conditions[38]. Several studies of anoxybiosis across species have shown that it is exceedingly rare condition for oxygen dependent life as and can be tolerated only brief time without developing severe consequences and eventually death. Besides several metabolic adaptations, both ATP-consuming and producing pathways are linked to anoxia tolerance[54]. As discussed in this section, in tardigrades anoxybiosis can be combination of metabolic shutdown, protein interaction and ATP regulation.

1.4. Imaging tardigrades

Imaging is performed for better understanding of the phenomenon under study. Visualization of biological processes is extremely important to create an overall view of responses caused either stress, drugs or any other factor that impacts cellular homeostasis leading to change in morphology, regulation of biological or physiological process and for example disease progression.

Tardigrades are microscopic animals and, for example, require at least stereomicroscopy with 10x magnification to be observed. Largest species can be seen by naked eye, but informative observation cannot be obtained. Several imaging modalities have been used for tardigrade research: Perry *et al*. (2015) used epifluorescence microscopy to gain informative images and this method is based on the use of autofluorescence[59]. Autofluorescence can be an effective tool to visualize tardigrade structures, however in conventional fluorescence imaging is not desired[59]. Møbjerg (2011), Gross (2018) and Russell *et al*. (2001) have applied confocal microscopy on tardigrades gaining moderately reliable results, however animals were fixed and live imaging with species is not done[32,60,61]. These studies created some guidelines for live fluorescent imaging which is the aim in this project. Biologist de Carvalho used microinjections and fluorophores to dye internal organs of tardigrades and became “Global Image of the Year” award winner in 2019[34]. Gross (2019) used X-ray imaging for studying internal anatomy of tardigrades[62]. Scanning electron microscopy (SEM) is used to study morphology of the animals in several different studies; however, electron microscopy is not a suitable technique with living samples and in live imaging. Mayer (2013) used immunostaining for selective neuronal staining of tardigrades and his studies revealed that immunostaining can be also an alternative to investigate small organisms[29]. To conclude, the advantages of fluorescent imaging have not been applied for tardigrade research and even less imaging is done on living animals. Therefore, the aim in this project is to create protocol for regular staining of tardigrades by using commercial fluorescent dyes and perform time-lapse imaging of transition into anoxybiosis by light sheet fluorescent microscope.

1.4.1. Fluorescent microscopy

Fluorescence microscopy is an essential tool in biology and the biomedical sciences. The goal of fluorescence microscopy is to separate emitted light from excitation light and the principle behind the fluorescence is the luminescent emission which is caused by absorbed photons[63]. The use of fluorescent dyes allowed the identification of cells and submicroscopic cellular components and with high-resolution microscopes specificity at molecular level can be reached[63]. Although the spatial resolution of conventional fluorescent microscope cannot provide accurate features of the specific characteristics of a sample, it is still particularly useful device for regular imaging because the advantages of fluorophores, fluorescent proteins, and dyes[64]. Once the target of the fluorescent dye is known, it is easy to distinguish, for example, the nucleus or the lipophilic structures. The sample is illuminated through the lens with a larger energy source (such as lamp or laser) and illuminated light is absorbed into the fluorophores (within the sample) which causes them to emit longer, lower energy wavelengths of light[65]. This energy shift from excitation to emission is more commonly known as Stokes' shift[63].

When the values of Stokes' shift increase, the use of fluorescent filters facilitates the separation of excitation from emission light and therefore the separation of the spectral of excitation and emission, which then indicates more controlled imaging outcome[65]. Fluorescence microscope utilizes filter(s) that transmits only radiation of a wavelength corresponding to the fluorescent material and blocks other light[65]. Objectives with higher numerical aperture (NA) value are more efficient, while they gather more radiation[64]. In general, the use of multiple lenses in light microscopy can improve the image quality, however when using fluorescent microscopes, it causes issues[63]. In fluorescent microscopy one lens optic is more suitable, because the multi optics are preventing fluorescence transmission into UV range[63]. This consensus between image correction and high transmission are terms to balance about[65].

Other usual challenges with fluorescent imaging are related to phototoxicity and autofluorescence[63]. The general term for a reduction of fluorescence emission intensity is fading, including quenching and photobleaching[65]. Live fluorescence microscopy has some common issues like phototoxicity that are more fatal compared imaging with fixed samples leading to quite severe consequences[63,66]. It can cause damage to cellular macromolecules, thus harming the sample physiology and lead to sample death[66]. Careful consideration should be given to data acquisition

parameters (i.e., exposure time, laser efficiency) to avoid degradation of the imaging and damage to the sample.

Fluorescence microscopy is used widely in different studies, it is suitable for biological or biomedical imaging, but the reason for developing improved techniques based on fluorescent imaging is simple: to gain higher resolution and reach for 3/4D imaging.

1.4.2. Light-sheet fluorescent microscopy

Aiming for better image quality (high resolution, less artifacts, less phototoxicity to name a few) requires compromising in one or two factors. Light sheet fluorescent microscopy (LSFM) is a method that provides high optical resolution, and it is an amazingly fast method in 3/4D fluorescent imaging compared to commonly used confocal microscopy[67]. Therefore, LSFM is especially used for motility and migration studies and when imaging dynamic processes features like fast temporal resolution and subcellular spatial resolution are desired in 3/4D imaging[67]. Transparent biological samples are suitable for volumetric scanning of LSFM[68]. Disadvantages of LSFM are related to imaging geometry which creates issues with mounting the sample and some limits how to do the imaging[69].

Main principle of light sheet is to utilize plane sheets of light to create stack of induvial images through a sample, and these geometric difficulties create challenges to align two sheets of light perfectly in 3D space[69]. As a result, the quality of image set appears off focus therefore indicating that diffraction is limiting the field-of-view (FOV)[70]. This reduces axial resolution throughout the sample, except in the center of FOV, and weakens the contrast[70]. These issues are possible to fix and there has been developed “subunits” of LSFM that are tackling these geometric issues: Airy beam light sheet microscope, which is used in this study, utilizes Airy beam illumination profile instead of the common Gaussian beam. Compared to Gaussian light sheet, Airy beam provides ten times larger FOV leading to stronger contrast and higher resolution and advantage on Bessel light sheet, Airy beam has step change enabling[71]. Airy beam illumination profile utilizes curved trajectory leading to asymmetrical and extended transverse structure[71]. These features can seem unfavorable for light sheet imaging; however, the misalignments can be easily fixed by simple mathematical formula: deconvolution[71]. Fluorophore distribution in the sample can be used as base for the light sheet image scan and converted mathematically from convolution as high resolution image[71].

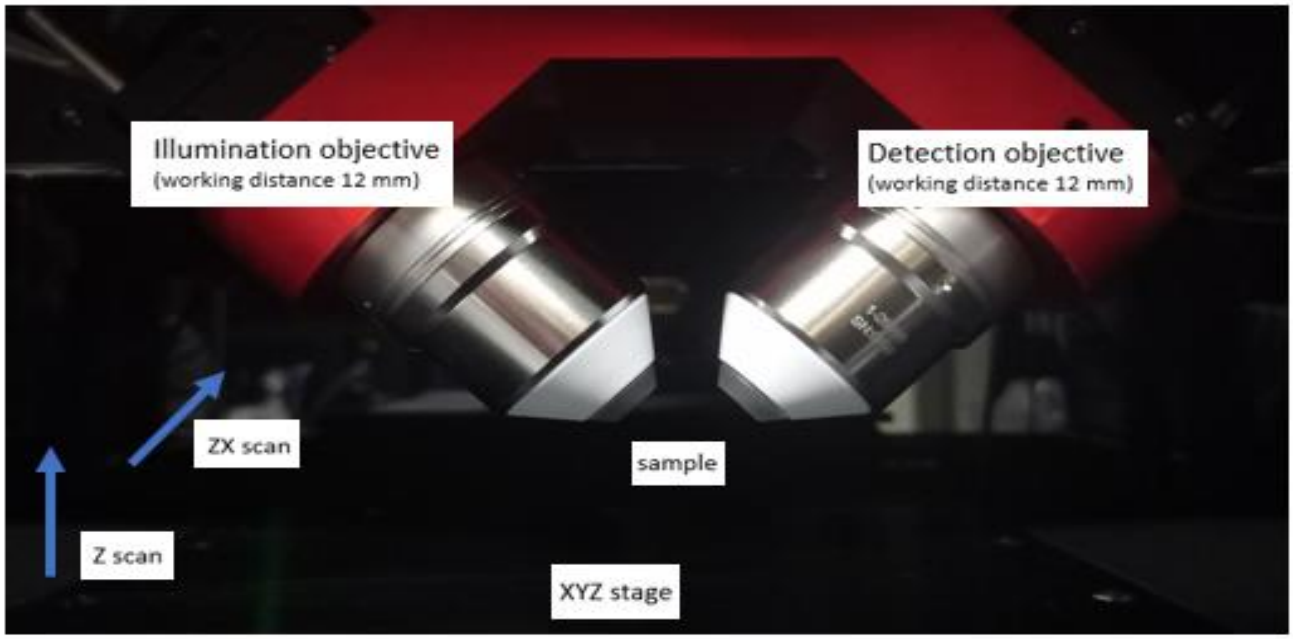

Figure 6. Illustration of airy beam working principle; both the illumination and detection objects are located at 45-degree angle. This working principle provides greater field of view, reducing diffraction, leading to higher resolution and stronger contrast.

1.4.1. Deconvolution and image analysis

The raw data gained from light sheet imaging is not informative and it requires image processing. Airy beam illumination has multiple advantages compared to traditional Gaussian beam illumination, however data obtained needs to be processed – deconvolved. In microscopy, it is good to remember how light behaves: planar wave transforms into a cylindrical form as it spreads when passing the splitter which resembles point source[72]. The larger the slit, the more point sources, therefore more wave fronts begin to interfere with imaging causing diffraction patterns[72]. The same event can be observed when the lens of the microscope is acting as a circular aperture for transmitted light[72]. By the time light reaches the camera, diffraction causes light from the original point to pass, describing this one single point source as a geometric pattern called the point spread function (PSF)[72]. PSF is shown as a bright circle when it is projected onto a plane sheet[72]. This phenomenon can be also called Airy function or Airy pattern[72]. The center area of this pattern is called the "Airy disk"[72]. Resolution can be defined from two partially overlapping airy patterns, but the patters can still be considered as separate entities which indicates that the emission light creates small point in size and therefore the original point is not exactly replicated[72]. The microscope optics are causing the blurriness. This blurriness, however, can be aligned and corrected by PSF. Applying

PSF to all images is called convolution, which can be explained as a mathematical mask/filter where the original light of the sample is convolved with microscope's PSF. Deconvolution is "reserved" action of convolution and either uses the measured or simulated PSF to correct the blurriness cause by optics. Deconvolution also increases the resolution of the image and especially with Airy beam illumination the effect of deconvolution improves the axial resolution[70].

Deconvolution is a crucial step in light sheet fluorescent microscopy; however, image analysis always requires high quality images to enable further processing such as tracking cells. Segmentation of the region of interest (ROI) is extremely time consuming and laborious, but even more so if the quality of the image set is blurry, low in contrast, exposed to autofluorescence or artifacts. Raw data must be dealt with in a comparable manner throughout the experiments and the image acquisition must be kept in same values throughout the study.

Quantitative measurements, in this case cell migration (µm) and size or volume (μm^2/μm^3), must be dealt with segmentation to rule out the background and objects that are not part of the experiment. ROIs can be extremely laborious to draw manually, especially with large data sets in 3D format. Fortunately, multiple software is recently developed to tackle these issues, however the enormous size of the data is still one limiting factor. When talking about segmentation, the aim is to separate ROIs from background, and it can be based on size, shape, sphericity, or intensity. When the segmentation is enough reliable and it is possible to tell the object from background, the tracking is based on the identification of the same object in subsequent frames[73]. Tracking provides several variables to work with the gained data, i.e., track length, displacements, speed, acceleration. To optimize the experimental setup, there are some factors that impacts straight to the image quality: avoid overlapping dyes, make sure the cell number is enough high to generate reliable data, but not too much while it causes issues with tracking[74]. Saturation should be avoided, but intensity of the cells must be enough strong to stand out from the background[74]. Time lapse imaging also includes artefacts that need to be considered latest in the analysis part: two in proximity cells can be split manually if there is possible to interfere to detection and double tracking which can be corrected by detecting cells that are in proximity and travelling same directions[74].

In this project, the aim is to track cellular structures/cells during anoxybiosis. A key factor for support the hypotheses, is to choose variables that reveal the best overall concept of the phenomenon of morphometric changes.

2. Hypotheses

"Transition into anoxybiosis state is associated with morphogenetic cell movements."

As discussed in the previous section, the tardigrades' ability to tolerate extreme conditions has been known for centuries. However, there is extraordinarily little fluorescent imaging done on living tardigrades, and of their cryptobiotic transitions. Imaging this phenomenon, transition into anoxybiosis, could provide the first insights into what is happening biologically to animals that suffer from oxygen deprivation. High resolution devices can visualize cell migration or accumulation, overall cell movement and morphogenetic changes and adaptations. Therefore, these tools can improve understanding and create more specific questions of how tardigrades tolerate hostile exogenous stimuli.

The aims of the thesis are: (i) develop an imaging protocol for living tardigrades by fluorescent microscopy and (ii) visualize transition of anoxybiosis at cellular level. Anoxybiosis is still poorly studied, and the underlying mechanisms are still a challenge for researchers. In this study, it is not possible to comment on molecular events, but morphometric changes and/or an accumulation of (storage) cells can be seen; these observations can inspire novel studies.

3. Materials and methods

A three-step protocol was developed (Fig. 7) including following step: i) fluorescent staining / imaging ii) anoxia and iii) light sheet imaging and image analysis.

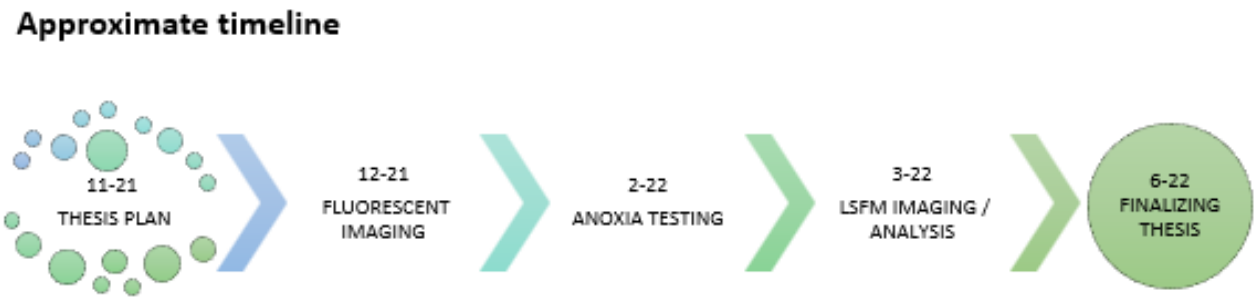

Figure 7. Approximate timeline was divided into 3-sections which together covered 6-month period that was estimated time for thesis project. Fluorescent imaging included the dye testing and optimization, and in addition, learning the general tardigrade laboratory work. Anoxia testing was short period, because the first method worked as expected. Finally, the most time-consuming part was to analyze the data, the light sheet imaging was not that time-consuming.

In the beginning of the project, the timeline was evaluated based on expected workload. Most laborious part was to optimize the fluorescent dyes and protocol for basic imaging. Anoxia experiments and LSFM imaging were quite simple and fast, however there were issues with PSF calibration which caused delay. Finally, the image analysis required patience with the large datasets. Image quality of LSFM data was not optimal and there were issues with overlapping dyes causing blurriness leading to challenges in image analysis, cell detection and tracking. Image analysis was the most time-consuming part during the projected as was expected.

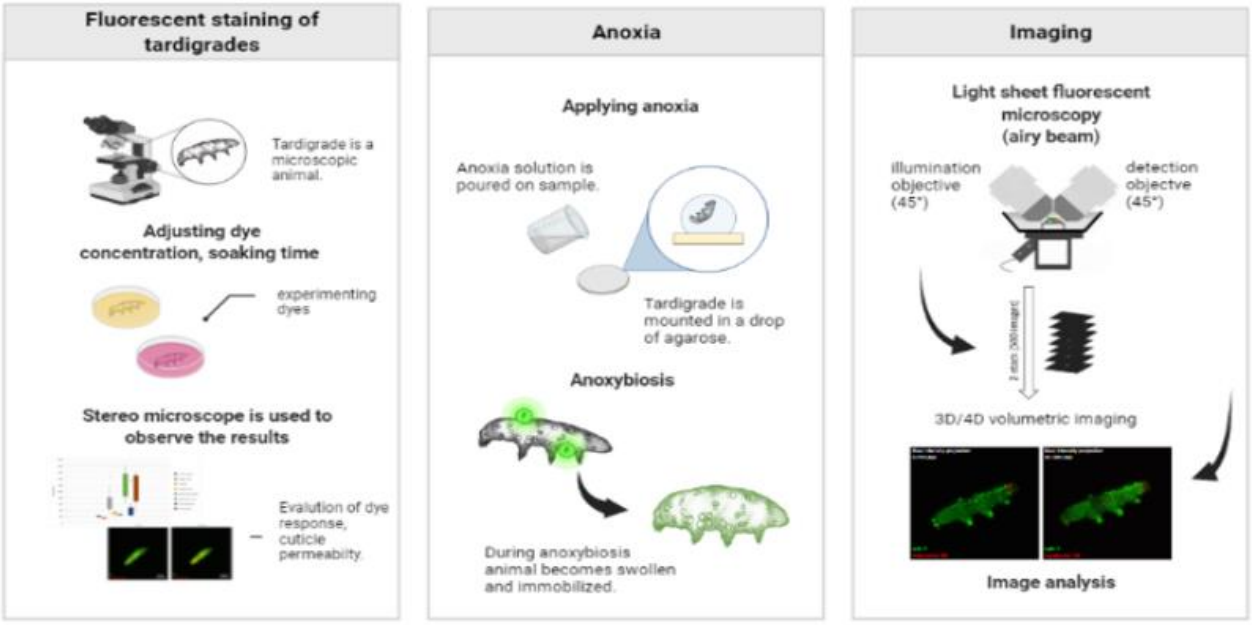

Figure 8. It was beneficial to divide the project in parts, in sense that all the parts can be seen as independent complexes. This made it easier to plan experiments and stay in the schedule.

3.1. Tardigrade culture

Two Eutardigrada species collected from moss in Jyväskylä, Finland in 2014: *Macrobiotus ripperi* and *Paramacrobiotus fairbanksi* (Parachela, Macrobiotidae). Animals were kept on scratched Petri dishes (ø50mm, height 23mm, Thermo Fischer, Germany) filled with spring water (Kotimaista lähdevesi, Multila spring, Finland) at room temperature. No light/dark cycle was applied, and animals were kept in styrofoam box in laboratory. Keeping the dishes in the dark prevented mold, algae overgrowth, and bacteria growth. However, the dark might affect the reproduction of the animals, which was extremely slow.

Populations were fed once a week with algae mix (50:50 Chlorococcum sp. and Chlorella vulgaris), pipetting “a few droplets” (5-10 µl) on dish. Water was changed once a week, by carefully pipetting half of the dish water away and filling the dish with new spring water. Water change was performed under stereomicroscope (Stemi DV4, Carl Zeiss Microimaging GmbH, Göttingen, Germany) to avoid losing any animals or eggs.

3.2. Anesthesia

Animals were anesthetized before imaging. Even though tardigrades are slow steppers, they move actively and can migrate in low melting point agarose (1,5w% of in spring water). For anesthesia 4 mg/ml stock solution of Tricaine (Ethyl 3-aminobenzoate methane sulfonate, 98%, Sigma Aldrich) was used. Tricaine was mixed to 1,5 w% agarose, 50:50, resulting 2 mg/ml Tricaine solution. Anesthesia was achieved rapidly, within 5-10 minutes. Recovery time from anesthesia was 15-20 minutes. Survival rate from anesthesia 100 %.

3.3. Fluorescent dyes and dyeing

There was quite little fluorescent imaging done on living tardigrades and all dyes, dye concentrations and soaking times were tested several times until optimal methodology was found. Tardigrades were dyed on a small petri dish (Cellstar®, ø35 mm, height 10 mm, Geiger Bio-One). In this small dish the total volume of dye solution was kept 2 ml and calculations were based on this. To create the same conditions for each animal, the animals were collected individually by using a loop and placed in 1 ml of spring water on a dyeing dish. After the animals were collected, the rest of the water and chosen fluorescent dyes were added. The dish was mixed gently on the table so that dye can be distributed evenly. The dye solution with animals was set in the dark waiting for the imaging – no need for shaking or incubation at specific temperature. Soaking time was first tested within minutes (5-60 minutes) and the results indicated that dye was only attached to the cuticle, or little was penetrated through it and/or digested. After multiple tests, it was decided to take a long soaking time (overnight,18-24 hours) and results were better. Main experiments were done with long soaking time. Fluorescent dyes tested on tardigrades are introduced in table 1.

Table 1. Fluorescent dyes used/tested in tardigrade anoxybiosis experiment.

Fluorescent dye	Manufacturer	Ex/em	Colour	target
Acridine Orange	Sigma-Aldrich	490/520	green	500⁄526 (DNA), 460⁄650 (RNA)
Nile Red	Sigma-Aldrich	450/500(<528)	yellow/orange/ red	hydrophobic molecules; lipids, lysosomes and cell membrane
SYTO™ 59 Red Fluorescent Nucleic Acid Stain	Invitrogen™, Thermo Fischer Scientific	622/645	red	Nucleic Acids
SYTO™ 9 Green Fluorescent Nucleic Acid Stain	Invitrogen™, Thermo Fischer Scientific	486/501, 485/498	green	Nucleic Acids
CellTracker™ Red CMTPX Dye	Invitrogen™, Thermo Fischer Scientific	577/602	red	Pass freely into cell, transformes into cell-impermeant reaction products (cell detection)
CellTracker™ Green CMFDA Dye	Invitrogen™, Thermo Fischer Scientific	492/517	green	Pass freely into cell, transformes into cell-impermeant fluorescent products (cell detection)
CellTracker™ CM-DiI Dye	Invitrogen™, Thermo Fischer Scientific	553/570	yellow	cell membrane, lipids
LysoTracker™ Green DND-26	Invitrogen™, Thermo Fischer Scientific	504/511	green	lysosomes
Calcein, AM, cell-permeant dye	Invitrogen™, Thermo Fischer Scientific	490/515	green	determine cell viability
5-(and-6)-Carboxy SNARF™-1	Invitrogen™, Thermo Fischer Scientific	580/640	yellow/orange - deep red	pH indicator

When choosing a dye for imaging and the aim is to follow up cellular migration, it is important to find a dye that attaches to the cellular components and do not dye other “stable” structures, such as different layers of cuticle. Our set up on LSFM had two lasers as energy sources, therefore dyes were chosen to correspond these specific excitation / emission wavelengths (i.e., green, and red channel) and to avoid overlapping. The fluorescence SpectraViewer application, provided by Thermo Fischer, was used to determine theoretically suitable dyes (see fig. 9).

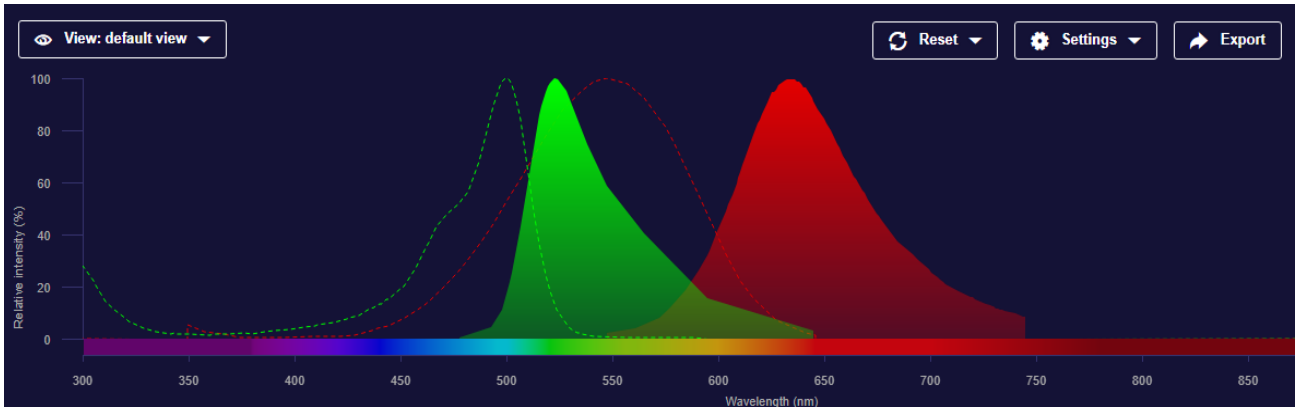

Figure 9. SpectraViewer default view of Acridine orange and NileRed. Overlapping can happen, but as it is shown the peaks are clearly separated from each other. Colocalization analysis can be performed when

analyzing the results. In the end of the experiments, we observed overlapping of AO and NR, however with strong response and cheap cost, this pair of dyes were chosen.

The main experiment with LSFM was performed with Acridine orange 1 µM solution in spring water for green channel and NileRed 15 µM solution in DMSO/spring water for red channel. Acridine orange was expected to dye nucleic acids (detecting therefore RNA/DNA) and NileRed is more likely to attach liposomal structures/lipids and often dyes cell membranes (figure 10). AO was attached to nuclei (green dots). AO unfortunately did not only dye specific compartments but also attached to membranes and cuticular structures leaving coat-like lighter shade of green all over the animal. Same phenomenon was observed with Nile Red and many other tested dyes. To avoid uncontrolled dyeing, the only reasonable way to achieve exact the desired results, is to use microinjection. However, the outcome of soaked animals provided enough informative and clear response in LSFM, and cellular migration and movement were observed.

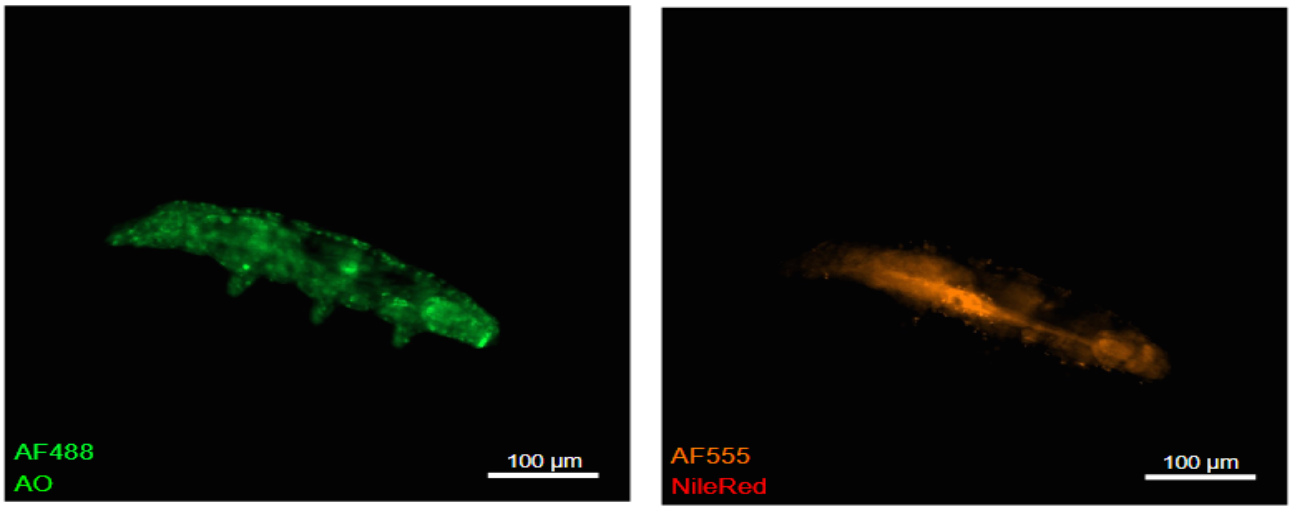

Figure 10. *M. ripperi* after 24-hour soaking. Dye penetration through cuticle was achieved which can be seen especially in green channel. As mentioned in table 1. Nile Red is slightly inconsistent and causes variation in response and in this specific example, NR has dyed multiple structures and concentrated to gonad area and digestive part. The outcome of NR was improved when it was diluted in DMSO instead of methanol – also creating slightly better option for tardigrades to bear with. Ratio of DMSO/total volume was kept approximately 1:100 and there no effect on the animals well-being.

3.4. Anoxia

Anoxia was applied chemically by sodium metabisulfite (baker analyzed, 97%, J.T. Baker) which is commonly used as oxygen scavenger. 1 w% solution was prepared and diluted into the spring water. To remove gases, the water was boiled in microwave for 5 minutes, after cooling down the sodium

metabisulfite was added. Sodium metabisulfite decreases the pH therefore adjusting the pH to 6,5 (as spring water) with sodium hydroxide was needed. Oxygen levels can be evaluated with drop tests (Aquatest, ProJBL). The solution can be used 4-5 days after preparation.

3.5. Mounting the animals

Imaging with AXIO Zoom, a glass bottom dish (#D35-14-1.5GO, Cellvis) was used. Agarose must be heated so that it can be pipetted. For each dish 100 µl agarose was enough. When collecting the experimental animal, agarose had time to cool down, thus did not cause any harm to the animal. Before agarose solidified, animals can be adjusted in an advantageous position, which however can be very tricky. 100 µl of Tricaine was pipetted on top. Tricaine will absorb agarose and anesthetize the animal within a few minutes.

Imaging with MSquare Aurora Light sheet needed specialized plastic molds with magnetic spots to hold them onto the media chamber (see figure 11.). The drop of agarose was placed onto the mold and animal settled in nice position into the droplet. This step requires concentration and speed because tiny drop of agarose solidifies extremely fast. Tricaine was applied on the agarose drop. Mold was settled in the chamber and filled it with water/anoxia solution. Anoxia solution was absorbed into the droplet while setting the imaging-acquisition.

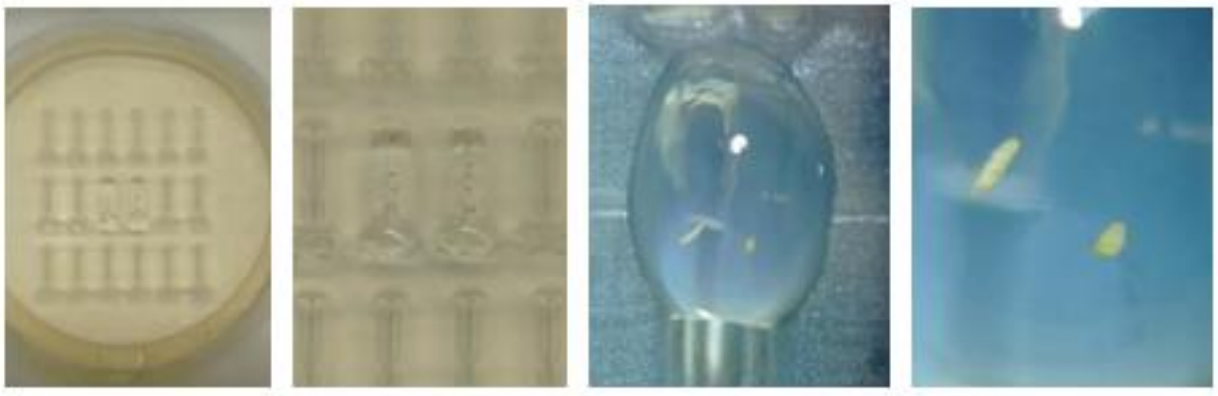

Figure 11. The plastic mold used in imaging was developed for zebrafish embryos, however it was suitable also for tardigrades. The mold was enough small to hold droplets of agarose. Dyed tardigrades are also more visible by naked eye as it can be observed in first two images from left. In the third image from left two M. ripperi were mounted in a drop of agarose. As it is shown the position of tardigrades can be challenging (animal on the right). The amount of agarose was little, so it solidified quickly and therefore adjusting the position of the animals was not possible afterwards. It was observed that if the position is not beneficial from the beginning, the mounting should be repeated.

3.6. Imaging stereomicroscope

Fluorescence AxioZoom.V16 stereomicroscope (Carl Zeiss, Oberkochen, Germany) was used to perform the regular/daily basis imaging, i.e., dye optimization and anoxia tolerance experiments. Hamamatsu sCMOS Orca Flash4.0 LT+ (2048 x 2048 pixels) camera can be used to gain moderately high-resolution images and visualize the response to dyes and anoxic behavior. The dye penetration can be observed high enough quality. Image acquisition was kept the same in all fluorescent imaging by creating procedure for these experiments in the very beginning of the project. AxioZoom had all together four filters for gathering the light, and our experiments utilized two of them: green channel Alexa 488 - filter set 38 HE excitation: BP 470/40nm and emission: BP 525/50nm and red channels Alexa 568 - filter set 45 excitation: BP 560/40nm and emission: BP 630/75. Bright field (BF) was used for anoxia testing. Before imaging, animals were mounted as described in previous section and dyes were chosen based on Fluorescence SpectraViewer application (Thermo Fischer). Although exposure time varied slightly in some imaging sessions (taken into consideration when analyzing the data), in common test series it was kept moderately low (100 ms).

Tardigrades were imaged at maximum 112x magnification which made it possible to some extent to observe the tardigrade anatomical structure. Before imaging was started, mercury lamp needed be turned on to heat up for 30 minutes, camera needed to be checked (fluorescent or bright field) and stage needed to be changed to black plate in fluorescence imaging. The transparent glass stage can affect negatively to the results but can be useful in bright field imaging.

3.7. Imaging light sheet fluorescent microscope

Aurora™ MSquared airy beam light-sheet microscope (Science Park, Glasgow, United Kingdom) is an upright microscope indicating the light is coming from above (in this case in 45° angle). Light-sheet technique was utilized to image 3D dataset of tardigrades in anoxic solution (water-agarose). Excitation and detection objectives (Special Optics, New Jersey, USA) for dipping media where refractive index (RI) range from 1.33 to 1.56 (NA 0.37-0.43). Magnification range of 15.3x-17.9 across the RI ranges and field of view <870 µm with a 12mm diameter imaging sphere. Microscope provided two lasers: 488 nm and 568 nm (Coherent OBIS Laser Box) and three filters including longer length green (GFP LP (500nm)), shorter green (GFP BP (520/40nm)) and for red channel (RFP LP (570nm)). In green channel imaging the short GFP was used. Hamamatsu sCMOS Orca Flash4.0

camera (Hamamatsu, Japan) (2048 x 2048 pixels) was used to capture the emitted light. The used step size was kept 0.4 µm by XZ translation of the sample through the focal plane, as the sample size varied around 500 images (200 µm). Eventually the output stacks were deconvoluted to provide an improved axial resolution. Green channel including dyes such as AO, Lysotracker green, and Cell Tracker green, utilized laser excitation wavelength of 488 nm. Excitation wavelength of 568 nm was used for red channel dyes such as Nile red, Sytotracker 59 and CellTracker Red. Exposure adjusted for optimal signal to noise ratio for muti-channel images: 20-30 ms when optimizing.

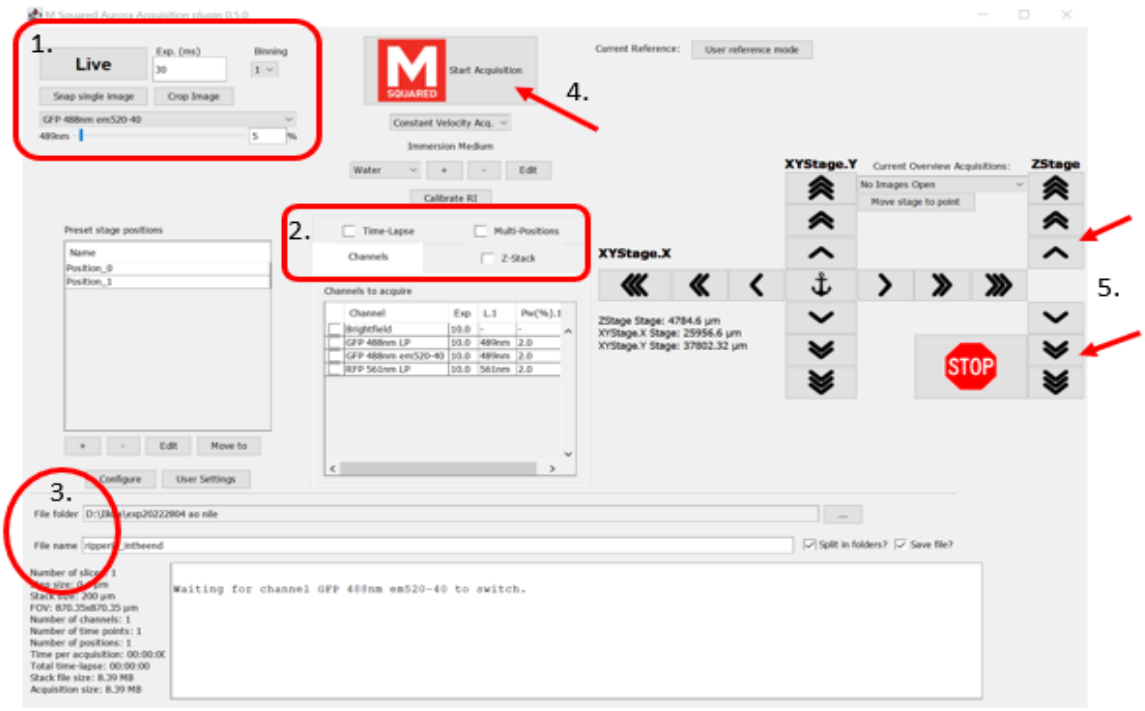

Figure 12. Performing the imaging, the platform to operate with MSquared microscope was robust and clear. To adjust the general acquisition including exposure time, binning and efficacy can be easily optimized in box 1. Box 2. is for imaging settings, with spreadsheets time lapse, multi-position, channels, and Z-stack. 3. File names and folder for saved data. 4. Start the acquisition button which starts the imaging. Arrows (5.) are for moving the plate in xyz-position (i.e., setting the z-stack planes).

Acquisition required setting the exposure time (30 ms) and laser efficiency (5%), and it is preferable to increase the efficiency rather than higher the exposure time if response is weak. Binning was 1.

After imaging, to process the raw data, M2 Deconvolution software was used. Point spread function was calibrated for water/agarose media with 488/568 wavelength. Image depth varied 200-300 µm (position, tardigrade size) leading to approximately 500 planes in each stack. Time-lapse imaging was divided into 7-10 timepoints, 4 minutes interval considering the time needed to achieve anoxia

response. Imaging one stack took less than a minute, so the method is extremely fast leading to over 80 GB datasets of one timelapse experiment. Working with data this size requires computers enough capacity to run it through.

Fiji, open-source platform for image analysis, was used to measure the intensities of chosen dyes and estimating the soaking times based on the values. Main analysis was done with arivis Vision4D (arivis AG, headquarters, Munich, Germany), which provides easy and user-friendly platform for object detection and tracking cellular movement.

Fiji workflow included manual segmentation of the region of interests (ROIs) (tardigrades) and measuring the intensities (see Fig. 13). This could have been performed by simple macro, but the workload was manageable just by drawing ROIs manually. Segmentation and tracking cells, on the other hand, would have needed automatization of the segmentation process and therefore arivis was used.

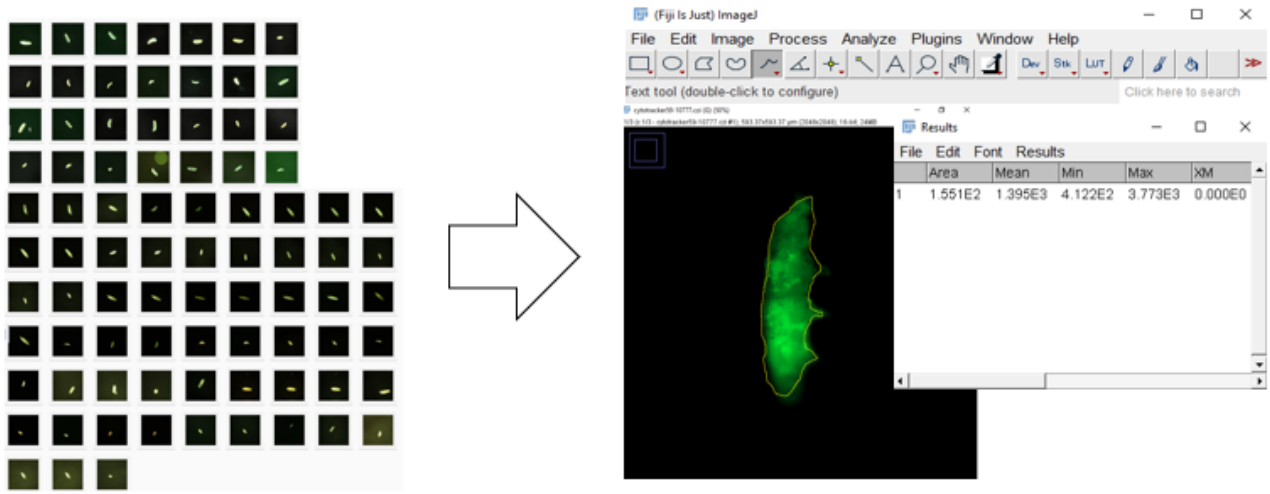

Figure 13. Even though there were approximately 100 images, manual segmentation was easily done (evaluated the best dye response for measuring intensity value). ROI was drawn free handed, and mean value corresponded the intensity of the ROI (1395). This was enough accurate methods to evaluate the fluorescent response – considering the acquisition is same with each test series.

Arivis workflow included image enhancements such as subtracting background, denoising, shape detection to improve the quality and reduce blurriness. Data needed to be converted to .sis files as usually every software uses its own format. The work itself was performed by pipeline for cell and particle tracking or basic cell/particle detection (workflow introduced in Fig. 14).

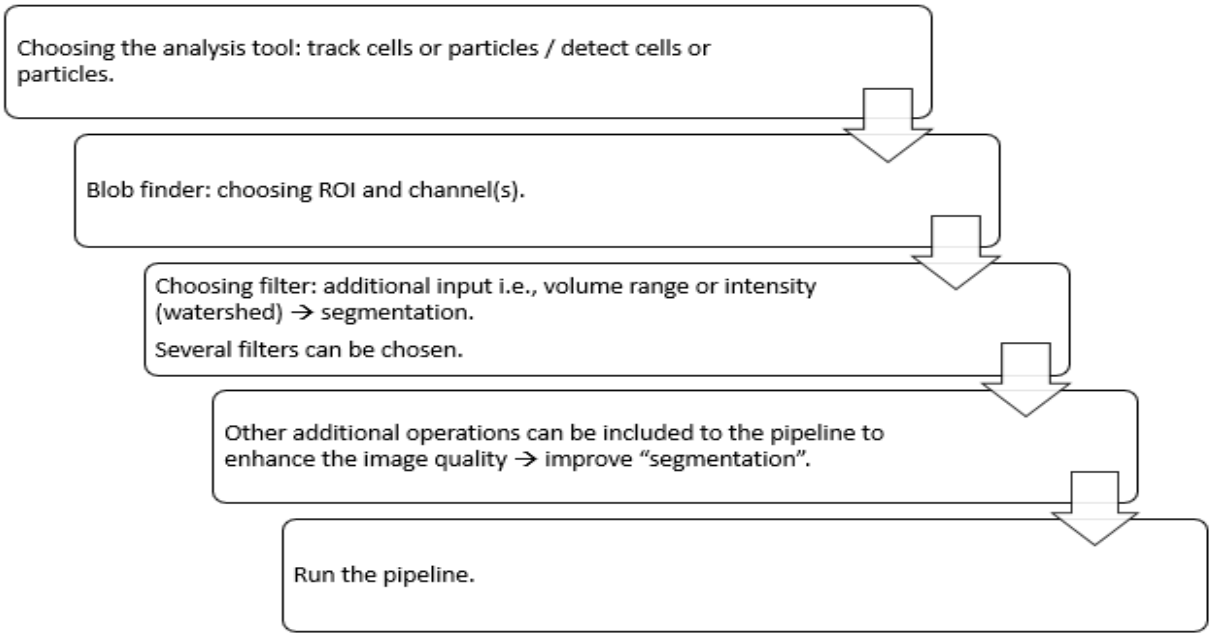

Figure 14. Pipeline included several steps that enabled the "manual" interference of the execution. Arivis had a wide selection of image processing tools for tracking. The segmentation is still extremely difficult and time consuming with such large data sets and even though arivis is built as user friendly platform, running 4D tracking complex takes several hours (i.e., subtracting background, shape detection, denoising, blob finder).

Segmentation parameters were based on values that have been provided by other researchers, such as the size of nuclei and size of storage cells. Shape detection operation has options such as object shape and size setting which allows to focus on specific size range: as in this case acridine orange, which dyes nuclei range varying from 2 µm – 4 µm and with Nile red object ranging from 5 µm – 15 µm as storage cells are approximately 10 µm by diameter. It is also possible to enhance the detection in blob finder and add additional filters (i.e., volume, surface area, sphericity) to segment specific particles.

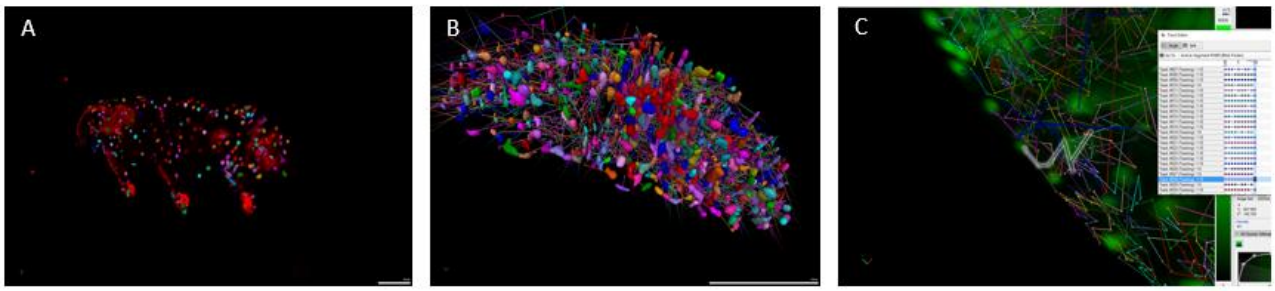

Figure 15. The executed pipeline gave information of both the objects (A) and tracks (B). User can choose the variables that are needed for the study from object volume, surface, sphericity to track speed, displacements, and length of the track. Tracking provided excellent visualization and individual tracks were easy to follow through timeframes (C).

4. Results

Maintaining the tardigrades in culture was successful. Animals were not kept exactly in the same conditions as in University of Jyväskylä (JYU): Tardigrades stayed in constant darkness during the research, as in contrast in JYU 2L:22D cycle is applied and temperature is kept at 16 °C. This explains the observed slower reproductive rate (especially *P. fairbanksi*).

After multiple tests of dye optimization results indicated that both species dealt well with the application of fluorescent dyes and anesthesia applied for imaging. The recovery rate from basic fluorescent microscopy was 100%. With anoxia related tests, there was significant difference between the species, and *M. ripperi* was more appropriate study species (with respect to anoxia recovery, see section 4.2.).

The results obtained at the end of the project showed that the assumed hypothesis was true, and the movement of cells was clearly observed during anoxybiosis. Even though there were some challenges during the experiments and image quality was not entirely ideal, the overall results and outcome were successful.

4.1. Fluorescent dyes

As highlighted in the literature overview, tardigrade cuticle is a tough, multilayer, and complex structure. Thus, dyeing by soaking was considered challenging from the beginning. However, the imaging revealed that the dye penetrates through the cuticle after a sufficiently long (i.e., hours) soaking time. It was also observed once the cuticle was shed, the dyes were removed as well, indicating that dyes do attach the cuticular structures and cause challenges in imaging. The dyes that were absorbed and attached to the cuticle can be responsible for the blurry image quality observed after main experiments and deconvolution.

Several dyes were tested (table 2.) and evaluated based on visual observation (whether the insides of the tardigrade are dyed) and intensity measurements (Fig. 16). A total n=500 individuals were used in the development of the dyeing protocol. The intensity measurements were done for the most promising dye candidates based on visual observation (Nile Red, Acridine Orange, Sytotracker 59 and Cell Tracker Red).

Table 2. Results of tested dyes, optimized working solution and soaking and visual evaluation of the fluorescent response.

Fluorescent dye	Manufacturer	Ex/Es	Colour	Stock (mM)	Working solution (µM)	Solute	Soaking time	Response
SYTO™ 59 Red Fluorescent Nucleic Acid Stain	Invitrogen™, Thermo Fischer Scientific	622/645	red	5	5	spring water/DMSO	over night	good
SYTO™ 9 Green Fluorescent Nucleic Acid Stain	Invitrogen™, Thermo Fischer Scientific	486/501, 485/498	green	5	5	spring water/DMSO	over night	fairly good, but animals die
CellTracker™ Red CMTPX Dye	Invitrogen™, Thermo Fischer Scientific	577/602	red	5	5	spring water/DMSO	over night	good
CellTracker™ Green CMFDA Dye	Invitrogen™, Thermo Fischer Scientific	492/517	green	10	5	spring water/DMSO	over night	poor
CellTracker™ CM-DiI Dye	Invitrogen™, Thermo Fischer Scientific	553/570	yellow	4	2,5	spring water/DMSO	over night	poor
LysoTracker™ Green DND-26	Invitrogen™, Thermo Fischer Scientific	504/511	green	1	5	spring water/DMSO	over night	no response
Calcein, AM, cell-permeant dye	Invitrogen™, Thermo Fischer Scientific	490/515	green (dead cells)	1	5	spring water/DMSO	over night	no response
5-(and-6)-Carboxy SNARF™-1	Invitrogen™, Thermo Fischer Scientific	580/640	yellow/orange - deep red	1,7	5	spring water/DMSO	over night	no response
Nile RED	Sigma-Aldrich	450/500(<528)	yellow/orange/ red	3,1	15	DMSO/ethanol/ methanol	over night	good, although inconsistent
Acridine Orange	Sigma-Aldrich	490/520	green	10	1	spring water	over night	good

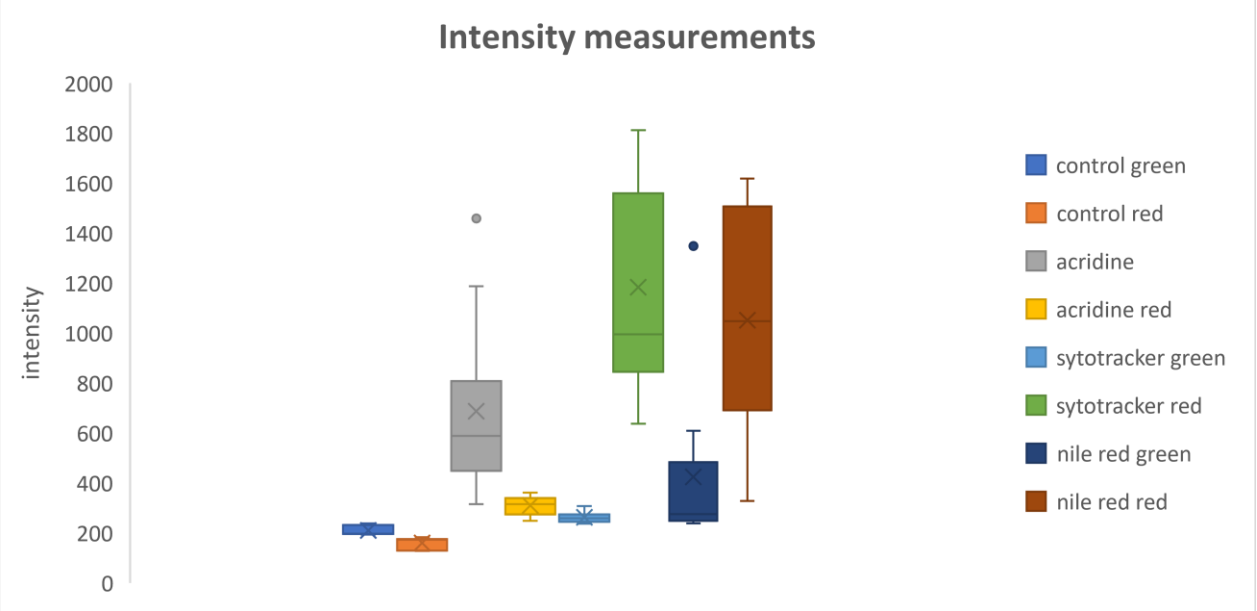

Figure 16. Fluorescent dye tests were done under AxioZoom microscope, exposure time 100 ms and used filters 488 nm (GFP) and 561 nm (Texas red). As the graph illustrates the strongest response in green channel is Acridine Orange and red channel Sytotracker 59 and Nile red. However, variation is quite large, indicating that dyeing quality can vary from animal to animal. Control animals revealed autofluorescence which was taken into consideration during experiments.

The most promising dyes were Nile Red (NR) and Acridine Orange (AO). The problematic aspect was the inconsistency in results, as is shown in the figure above. The variation in intensities could be due to state of the cell as well as differences between the animals (female/male, age, molting or digestion stage). In this experiment, we did not ensure the uniformity of the samples, but it was observed that these variables in subsequent studies should be considered more closely (see 3.11.). The use of one dye can be a more beneficial approach; especially if there are some statistical aspects to consider. With fluorescent imaging overlapping or other factors interfering with the outcome are common sources of error and to minimize these, controlling one dye is easier.

4.2. Anoxia tolerance

We found that the two species had quite different anoxybiosis tolerance, with *M. ripperi* outperforming *P. fairbanski*. All together 25 *M. ripperi* and 23 *P. fairbanksi* were used at timepoints: 15 minutes, 30 minutes, 1 hour and 4 hours. Figure 17. illustrates the anoxia tolerance and variation between two species.

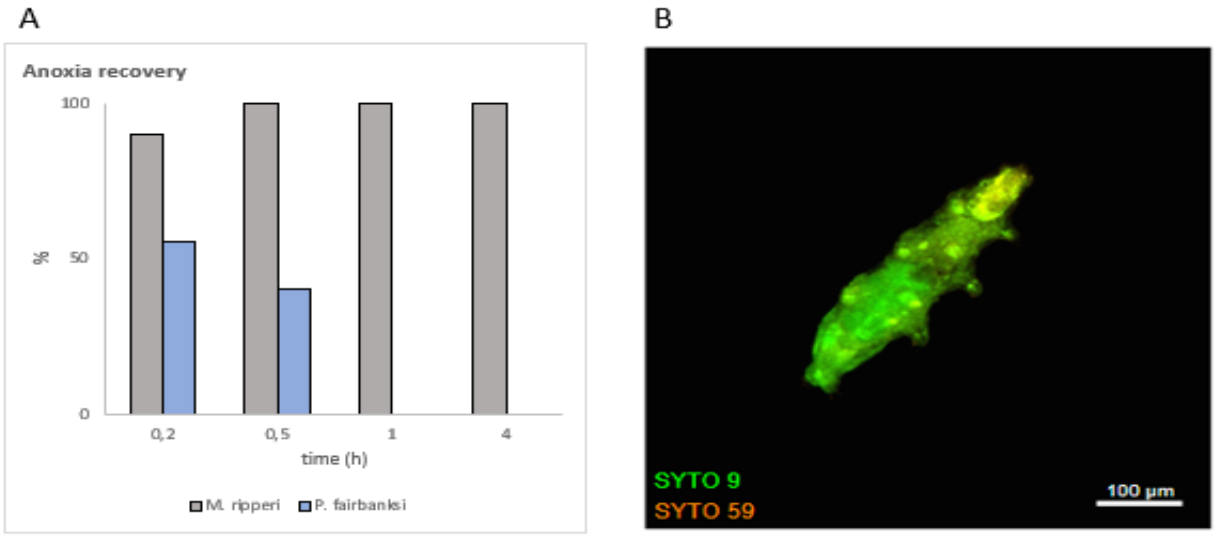

Figure 17. Anoxia tolerance is species specific. Based on this knowledge gained from other research, results were not surprising. *M. ripperi* did manage to survive even hours long anoxia whereas *P. fairbanksi* can recover only from brief period of anoxic conditions (A). The swollen and turgid body of dyed *M. ripperi* in 40 min anoxia (B).

Anoxia was achieved rapidly by chemically reducing oxygen in the water-based medium. The morphological changes (anoxybiosis) did also happen quite rapidly, thus the 15-minute anoxia was the shortest period of testing. As discussed in introduction, anoxybiosis is not a well-studied cryptobiotic mechanism. It was known, based on literature, that anoxia tolerance is species specific,

and these experiments strongly indicated that *M. ripperi* tolerates anoxic conditions better than *P. fairbanksi*. Therefore, in the subsequent steps *M. ripperi* was used.

4.3. Cellular tracking and image analysis

This thesis hypothesizes that the transition into anoxybiosis is associated with cell movement. Therefore, only the cells that were dyed successfully could be investigated. Based on observation and visualization by fluorescent microscopy methods, Acridine orange dyes smaller cellular compartments (as expected) and Nile red, as lipophilic, seems to attach to membranes of the storage cells, which are particularly lipid rich.

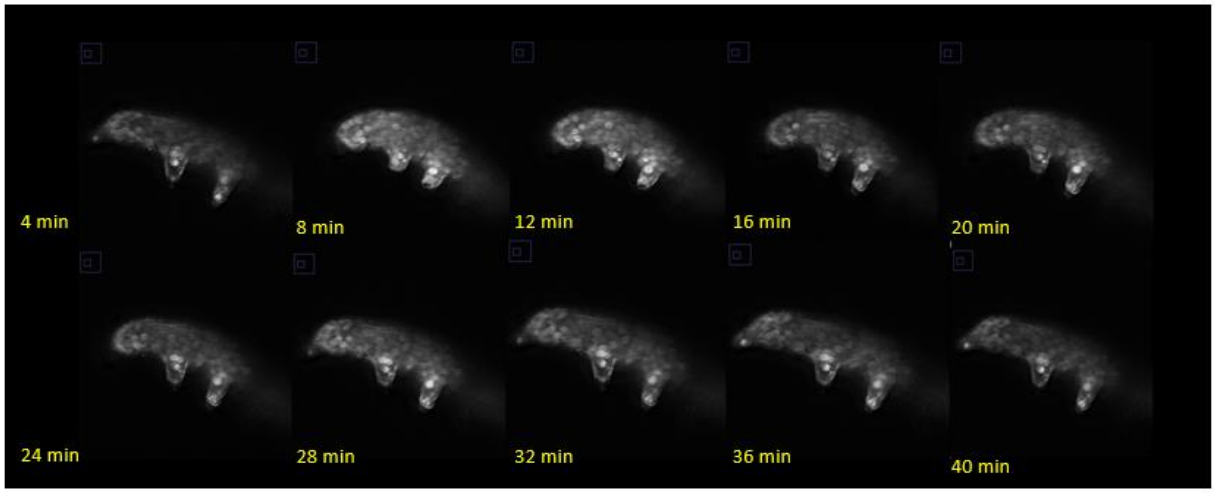

Figure 18. Maximum intensity projection performed by Fiji on each time point during the transition. As it can be observed, there is intensity variation and the whole body of the tardigrade is changing as well as closer look reveals storage cells relocation during anoxybiosis.

The tracking was extremely difficult because the whole body moved during the transition, first contracted, and then muscles were “released” leading to a floating entity. This was observed during every experiment. Altogether 7 anoxia light sheet imaging trials were obtained. However, due to issues either with saturation or position of the animal, we chose two fine quality image sets and compared results from those. Two different specimens of *M. ripperis* were dyed with AO and NR, soaked overnight, and prepped in the same day. Both channels were studied, and results were crosschecked. Colocalization analysis was performed to see how well the dyes stayed separated, or in other words, how strongly they are co-existing. As expected, there was overlapping of the channels with AO and NR (known excitation and emission wavelengths), and this was shown with basic colocalization tool. Overlapping interferes with the results.

Image analysis was performed by 4D Vision arivis, including object segmentation and cell tracking. The parameters for segmentation were extremely difficult to define, while the deconvoluted data was blurry. This can be a result from overlapping of dyes and/or unsuccessful dyeing (dye has attached to cuticle layers, leading to whole body coat phenomenon). Despite the blurriness, the cellular structures were visible (see Fig. 19).

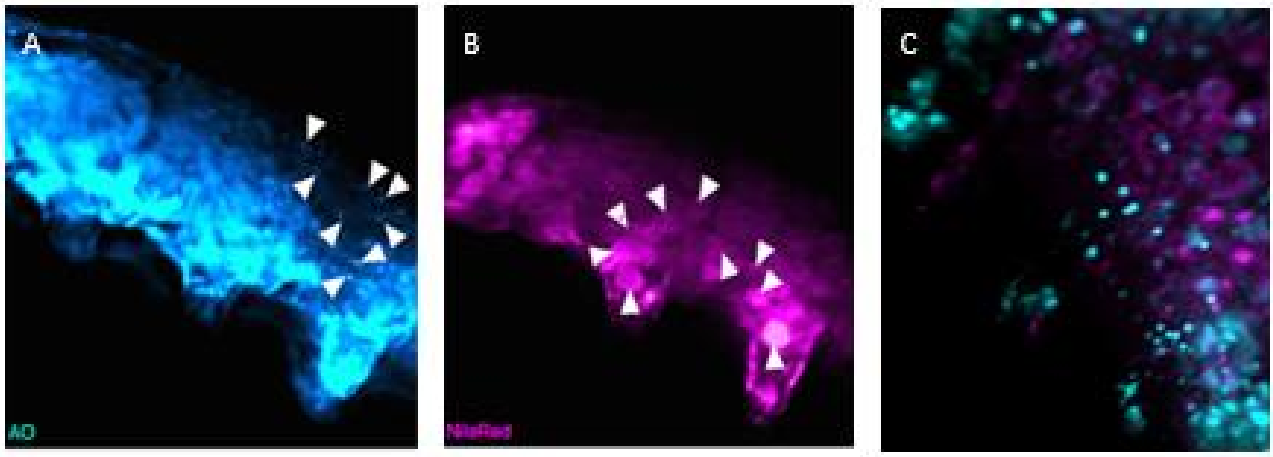

Figure 19. 2D planes to illustrate the dye distribution, and to visualize the targeted staining. Acridine orange dyed most likely nuclei, manually measured diameters varied from 1µm – 3 µm (A). Nile red dye worked well, clearly attached to the cell membranes, thus storage cells are visible (B). Storage cell diameters vary from 9 µm to 12 µm. The merged image (C) illustrates the dyes attached to specific compartments.

The first part of image analysis included object detection and it was performed on both channels (both dyes). As followed the same step of detection results revealed much better object detection in red channel (NR). To compare the channels the object volume was compared with both animals.

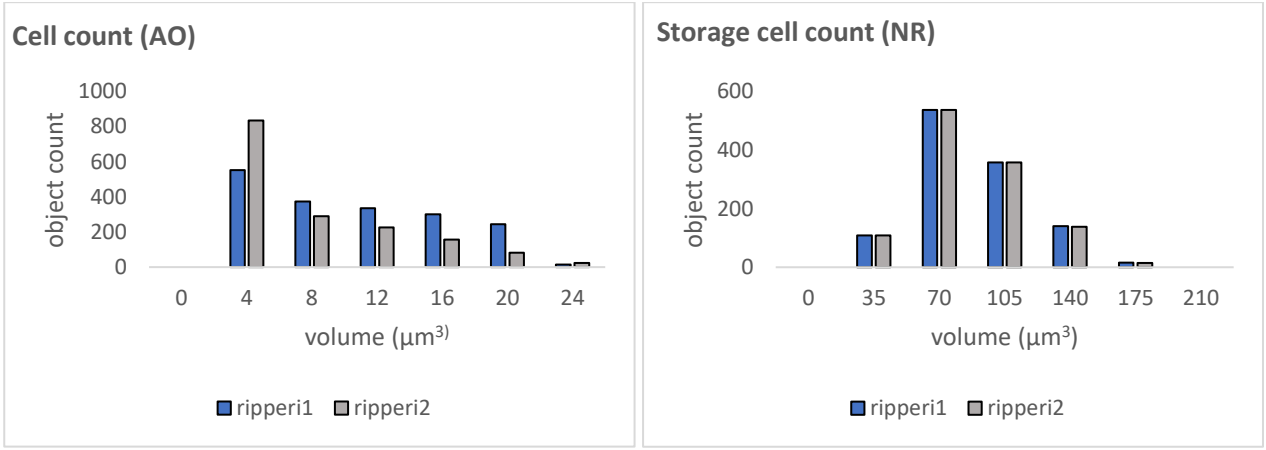

Figure 20. Object count of two ripperis varied, in both cases *ripperi2* had, according to results, more cells. AO was expected to dye nucleic acids and thus dyed objects are nuclei and results included all cells. NR was thought to dye membranes, as lipophilic dye. According to visual observation and knowledge that storage

cells (SC) contain lipids, it was possible to conclude that dyed objects were in fact SCs. Identical numbers were not expected, because the number and size of storage cells are dependent on the nutritional state of the animal[46]. ($n_{green,\ rip1}$=3788, $n_{green,\ rip2}$=3745, $n_{red,\ rip1}$=1160, $n_{red,\ rip2}$=1156)

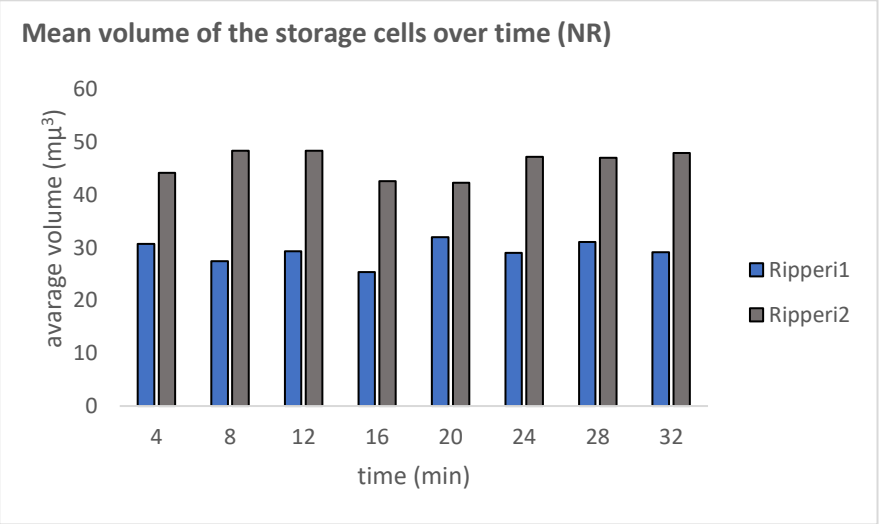

Figure 21. The mean volume (size) of the storage cells were much smaller in *ripperi1*. However, in both specimens the mean size stayed quite the same during the experiment. There was no clear reduction or increase in the size as the anoxia was applied, even though the size and morphology of the animal changes over time.

Object detection results indicated better segmentation on NR dyed objects, therefore cellular migration analysis was done with red channel detection. Moreover, we were interested in storage cells because of their expected crucial role in cryptobiosis and moderately large size. Storage cell numbers were also near literature review numbers, indicating the total amount of 1000 SCs. The amount of all cells in tardigrades varies from 4000 to 40000, therefore it is hard to evaluate if the segmentation was successful based on green channel values (project internal variation was high, and the total amount of *M. ripperi* cell count is unknown). To clarify the objects, ruling out missegemented objects, the diameter of cell was calculated manually (the feature was not included in software parameters). It was known that SCs diameter is approximately 10 µm. To calculate the diameter, we decided to consider the cells round objects and calculate diameter from surface area. Object sphericity quantifies the degree of cell roundness, and this is linked to the volume and surface area of the cell. Tardigrade storage cells are expected to be roundish and to make this analysis slightly less laborious we are considering the object spheres and when calculating the diameter of the object we use.

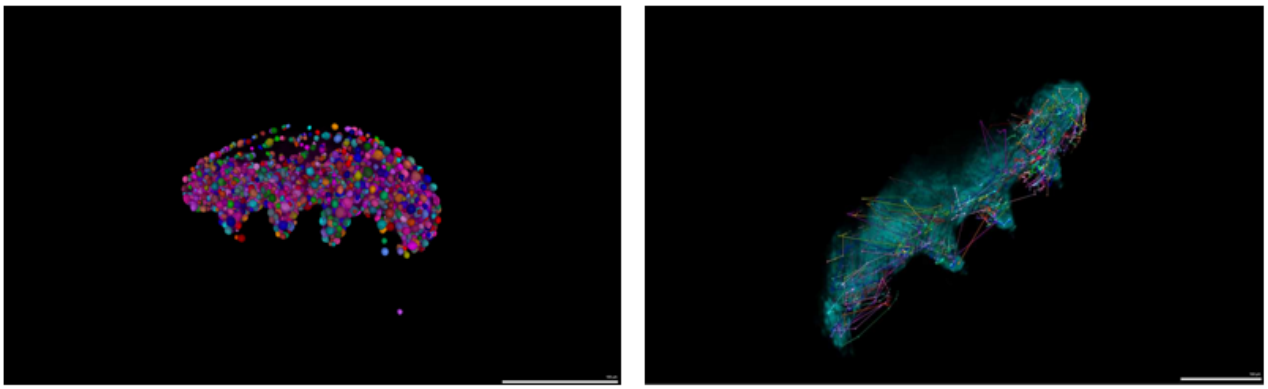

Figure 22. Main tools used in this project were segmentation tools in addition to image enhancement tools such as subtracting background or denoising. Simple pipeline for detecting objects was blob finder and its results are shown in image on left. Other main function was tracking which is shown in image on right. As a side note, this picture pair is a fine example of morphological change during anoxybiosis. Image on left is animal that is just exposed to anoxia (within the first minutes) and still in "normal" condition and image on right, the animal is in full anoxybiosis.

To illustrate the motility and cell movement, the parameters were chosen based on the features that are often involved in cell migration studies. These features are track length, speed, and displacement. The shortest distance between two time points (relocated position of cell or object) is called the displacement[74]. This deviation of displacement is compared to the total length of a track[74]. In other words, how much is the average transition between two timepoints? Typically, motility can be illustrated by mean displacement / square root of time = confinement ratio[74]. Mean squared displacement (MSD) is the most used measure of describing ransom cellular movement[75].

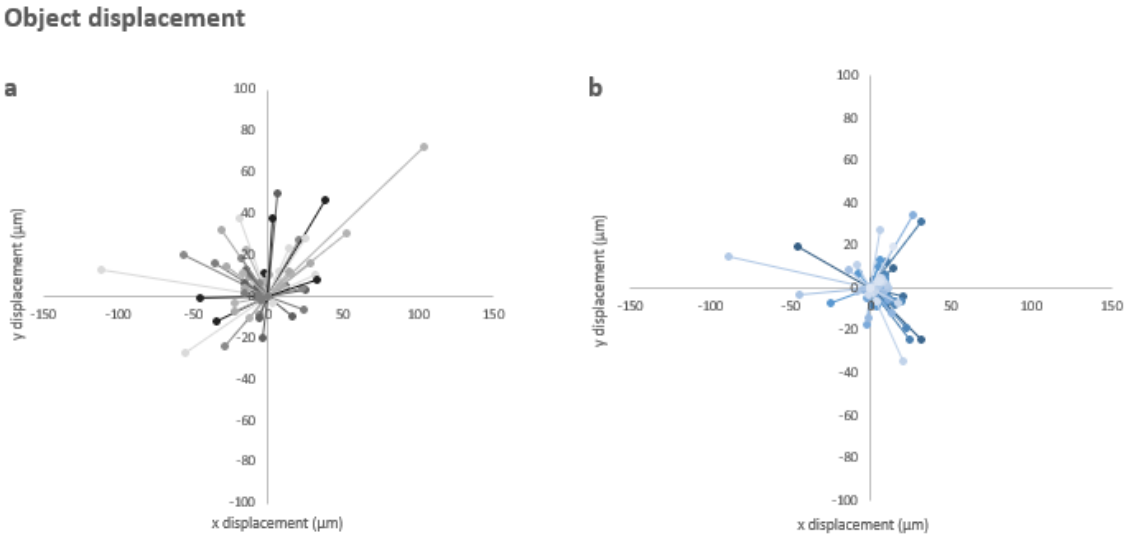

Figure 23. Object displacement at timepoint 8 (32 minutes after exposing to anoxia). As can be observed the behavior is slightly different: a) *M. ripperi* 1 has stronger variation on delocalization (n = 63) and b) *M. ripperi* 2 delocalization is more moderate (n = 73).

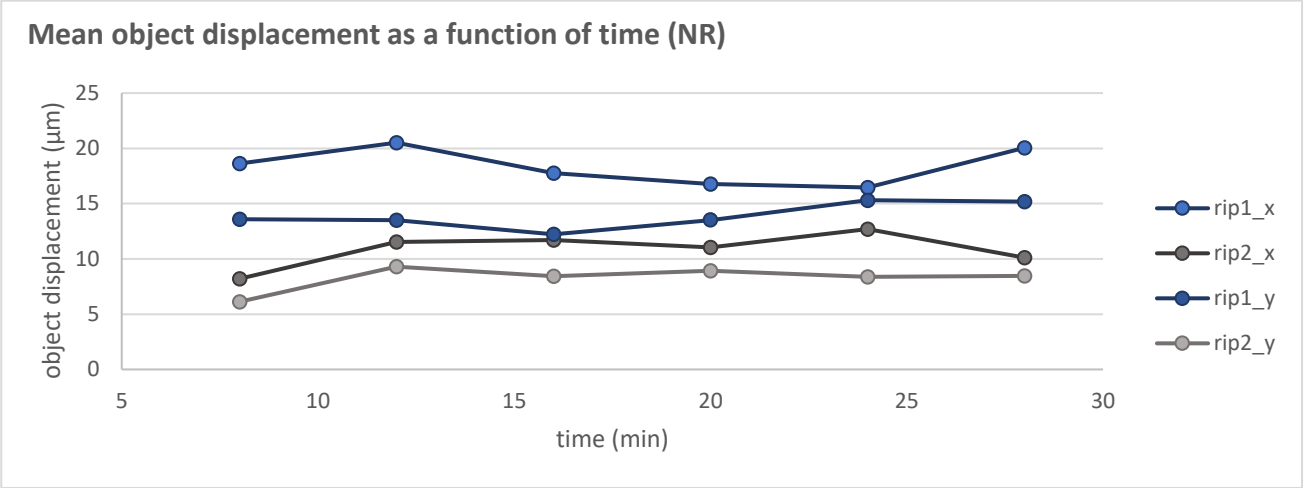

Figure 24. Mean displacement as a function of time (timepoints 2-7), did not reveal any specific pattern such as shorten the dislocation or slower velocity of the object over time. As seen in the figure, displacement is more controlled in ripperi2 and distances stay in both xy-coordinates moderately low, maximum 13 µm (mean). The average length of displacement was calculated by taking square root from MSD value.

The average speed of the cell can be estimated by dividing the distance travelled by a single cell (µm) by the time between the two sequential time frames (s)[74]. Cells do not migrate straight forward, therefore the estimation is underrated, and the actual speed is higher[74]. The error is greater when the interval is longer[74]. In this study as explained in the beginning of this section, the statistical significance is secondary and not so heavily weighed.

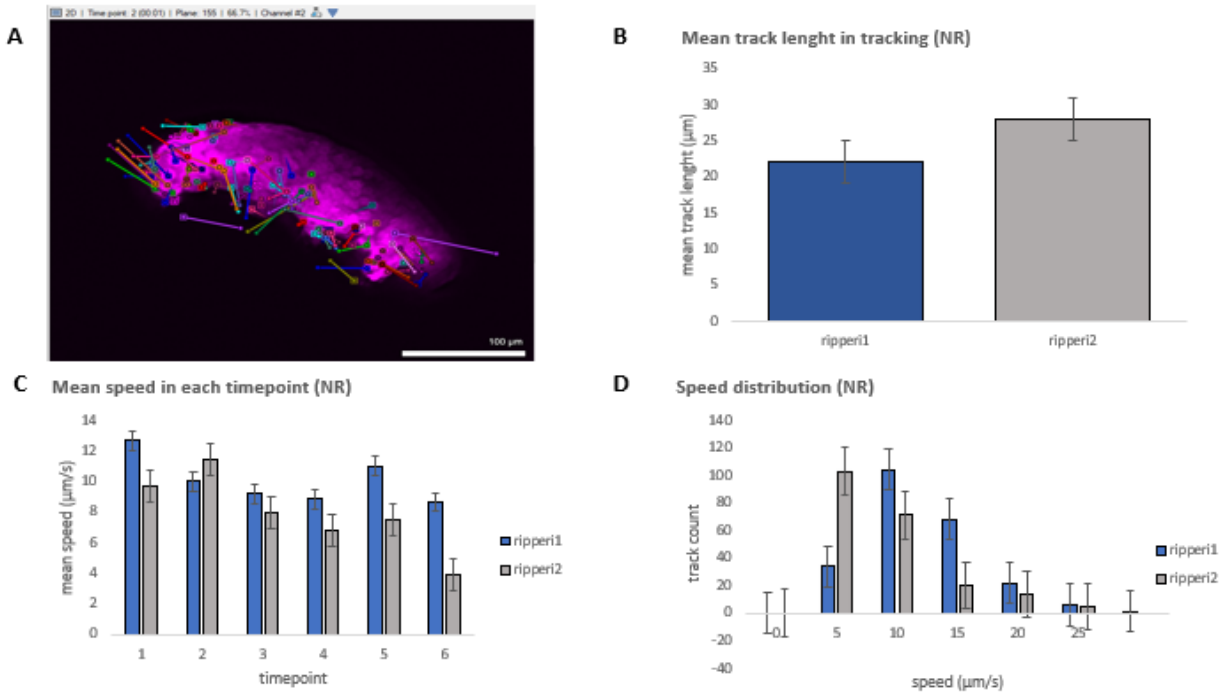

Figure 25. Results of NR tracking. Example of the view, objects, and tracks after running the pipeline (A). It was expected, based on segmentation that the length count would the same, however there were 16 tracks more in ripperi1 (B): $\bar{x}_{rip1}$= 22,2 µm, $\bar{x}_{rip2}$ =28,1 µm, p > 0,003, Student's t-test. unpaired (B). Mean speed in different timepoints had variation yet indicating that there is decrease in velocity the longer time spent in anoxia (especially ripperi2). Mean speed varied from 4 µm/s to almost 14 µm/s (C). Speed distribution indicated that velocities stayed often under 10 µm/s (D).

As two different dyes were used and overlapping of these dyes were expected based on Spectraviewer spectrum (see Fig. 8), colocalization analysis was good to perform to determine the intensity of the overlapping. Based on the visual observation of the two channels, it was quite clear that dyes exist in both channels and when observing the intensity results gained from tracking analysis, there are two intensities measured. Of course, intensity values responded to the channels indicating that when measuring the green channel values (channel 1), the intensity peaks are higher with acridine orange and nuclei. Pearson's correlation, which is an indicator for the correlation between the channels, varied significantly between the test animals (Fig. 25).

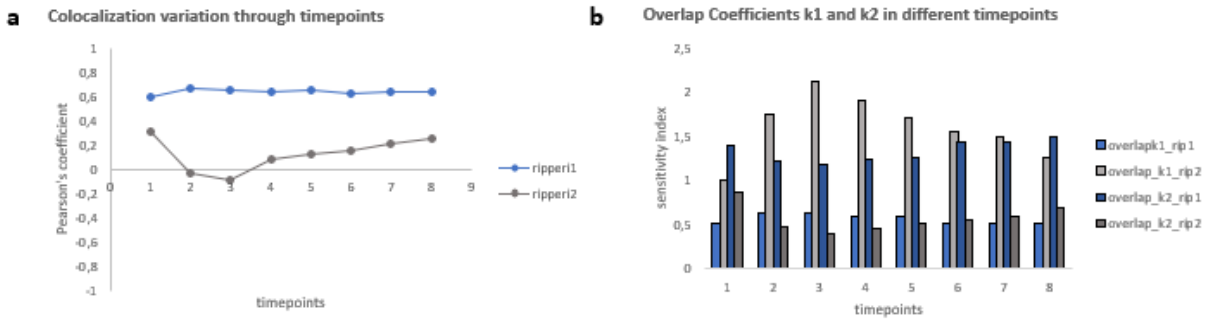

Figure 26. In figure A, the Pearson's coefficients are compared between the test animals and as it was shown, ripperi2 has quite dwindling numbers indicating that there was little correlation (close to 0, random values). However, in ripperi1 the correlation values were kept quite steady through the timepoints. In figure B, interesting phenomenon between the k1 and k2 values (which indicate the sensitivity to the changes red/green intensities): this explained how much individual animal can differ from each other, how successful the dyeing was and illustrated the poor reliability for this specific experiments.

To conclude the results: cellular structures and compartments were visualized. The long soaking time (overnight) enabled penetration through the cuticle; however, dyes also attached the cuticular layers. This led to a blurry outcome but more importantly the use of two dyes, which are already theoretically overlapping, did indeed overlap. This does not affect visualization of the data or the observations of cellular movement, but statistical and quantitative analysis suffer from these types

of issues. Therefore, this study does not provide high accuracy data of image analysis but highlights the fact that during anoxybiosis there is cellular reorganization and morphometric changes.

4.4. Discussion

Tardigrades are extremely unique animals due to their stress tolerance. Understanding the mechanisms that are responsible for cryptobiotic states and survival are the key factors that can place tardigrades among the more well-known model organisms. This project showed that using fluorescent microscopy to study these animals can be used but there are several issues that still need to be tackled.

As it was stated in several research articles, anoxybiosis causes the animal to swell and become immobilized. Besides the morphological changes, this behavior is very species specific as stated in Nelson's article (2002) of tardigrade evolution and ecology[14]. All this was observed during our experiments, i.e., differences in anoxia tolerance of *M. ripperi* and *P. fairbanksi*. However, compared to anhydrobiosis, anoxybiosis is less studied and based on research related to anhydrobiotic behavior, it is shown that several mechanisms are involved. Boothby has conducted various research of molecular responses to lack of water and emphasized the role of unique tardigrade proteins[35,37,43]. Therefore, it can be estimated that during anoxybiosis there are responses happening at molecular level. Understanding these phenomena is not involved in our study, but the aim was to create protocol for general fluorescent microscopy and benefit from it in further studies which can lead to deeper insight of the anoxia tolerance.

The old dogma introduced a few decades ago that supported the role of metabolic factors is now put aside[55,55]. Anoxia tolerance is a combination of metabolic activity by shutting down the energy consumption and lowering MDR close to 0, there is however, a complex interaction of molecules that regulate the induction of different proteins and/or activation/inhibition of signaling pathways[76]. Krivoruchko (2010) connected the mammalian oxygen related diseases and overall cell survival in anoxic conditions to important feature in understanding the core of multiple pathological conditions related to either hypoxia or oxidative stress[76]. Within the past 5 years there has been intriguing research conducted by tardigradologists about survival and unique mechanisms that are vastly different and astonishing compared to studies related to other organisms. Either

understanding tardigrades or applying the current knowledge of tardigrades to other more well-known models, can provide new insights in many fields of science.

Dyed cellular components, such as cell membranes and nuclei, revealed movement and reorganization of the cells during anoxia. To support the findings of this project, we compared the results to a few imaging research conducted on tardigrades. X-ray imaging performed by Gross *et al.* (2019), reported on the distribution of volume of storage cells in *Hypsibius exemplaris* and Czerneková and Jönsson (2018) conducted extremely detailed study on storage mitosis including general information of tardigrade storage cells; and these two studies can support our investigation of fluorescent response in tardigrade cellular structures[46,62]. Even though each study used different species, in our main experiment with *M. ripperi*, the results can reveal similarities and considering further research, point out protocols that need to be improved. However, tardigrade cuticle is complex, and evaluating the results achieved by soaking is therefore quite challenging. Previous research of tardigrade physiology indicated the number of cells to be from around 4000 up to 40000 cells and more precisely the number of larger storage cells to be around 1000[6,46]. These numbers were used as frames in this study to evaluate the dyeing accuracy and success. As mentioned in the paper of tardigrade cuticle by Czerneková (2021), the flocculent coat (containing i.e., lipids) is thickest on the limbs which can lead to accumulation of dyes, thus strong intensity peak and uneven distribution, in worst case phototoxicity or saturation[12]. These distinctive features need to be considered when designing an experiment. There are individual differences that are linked to the age or sex of the animal among other variables such as the number of storage cells. This can vary from species to species, and even within the same species among individuals. Therefore, planning and creating step by step workflow for fluorescent imaging is important. Variation between the animals was highlighted during the experiments and if using several animals, the variation from animal to animal can cause statistical difficulties. The uniformity of animals involved in the experiments is now proven to be one step to consider when planning to do fluorescent imaging. The developmental stage of the animal, sex of the animal and even fasting before experiment could improve uniformity for a more reliable quantitative assessment of cell movement patterns.

This study focused on visualizing the cellular movement, and as parameters, the displacement of objects (cells) and tracks were enough to prove the set hypotheses. We were able to visualize cellular relocation of the storage cells dyed with NR. Besides storage cells, smaller compartments (nuclei) dyed with AO were observed to be displaced. The overall workflow was simplified and easy

to run through leading to the fact that tardigrades can be dyed without harming them, imaged with LSFM, and even recovered from these experiments. The fundamental issues and improvement steps are i) evenly distributed dye with less variation among the animals and ii) image analysis (one dye methods, segmentation, and lighter pipeline for large datasets).

As there is only little research done on the fluorescent imaging of tardigrades and therefore not much to compare these results with, it is not wise to draw conclusions without any supportive background. This research created more open questions for further studies which can focus on developing high throughput pipeline for segmentation for such large amount of data. If working with other related topics, this project revealed that the workload associated with image analysis step is massive. Besides the image analysis, the optimization of “the best outcome” is still on-going process including reduction of overlapping between dyes/channels if used two-dyes protocol and smaller statistical variation between samples (finding optimized parameters and variables). Besides these features, the mounting and handling of the animals require improvement: unmounting the animals after LSFM imaging was exceedingly difficult and often lethal. The aim of the project was to see the responses of anoxia on living animals. Agarose did not dissolve into water after 24 h, so the animals had to be freed manually and mechanical force was the cause of death.

Finally, this study provided an important contribution to the study of the stress tolerance mechanisms using fluorescent dyes and live specimens. Our results indicated animals tolerate well the imaging and anesthesia and yet we obtained numbered data of anoxybiosis. With similar experiments it is possible to learn more of the responses that are triggered from environmental hazards.

4.5. Conclusions

This project aimed to investigate if tardigrades can be used as part of fluorescence microscopy studies and if staining cellular structures is possible. This aim was achieved. It was shown that during the anoxybiosis, the animal did not just swell or immobilize, but that the body also contracted and then relaxed. Transition into anoxybiosis was achieved quickly, varying between 15 and 20 minutes across animals. It was easy to observe cellular movement, however, to put the obtained data into visual graph illustrating the movement was extremely hard. This was because there were such huge variations between individuals leading to quite different outcomes of the single trial. It is assumed

that the dyeing solution is partially absorbed through the cuticle or on the other hand the dye enters the animal through the digestive tract. Overnight staining gave satisfactory results and clear cellular staining was obtained. After creating a protocol for dyeing, the desired results were obtained. However, this study did not conclude any deeper investigation of molecular interactions during cryptobiotic state, but the outcome was to state that the anoxybiosis is morphogenetic cellular movement.

As 3D visualization is becoming a basic procedure in biomedical and biological imaging and new methods are developed to deal with large data sets, there are several steps and features still to improve. This project utilized arivis 4D Vision, which is an easy platform to analyze even large light sheet data sets, but based on gained results, there are issues with the consistency of segmentation. Segmentation is considered the most difficult part of image analysis and in this specific study the cuticle and live unfixed sample created slightly suspicious outcome. Next aim is to create more simplified and therefore lighter pipeline for segmentation for LSFM image sets. To consider, the image quality varied throughout the experiments due the individual differences of the animals and caused issues both in the image quality consistency and segmentation, but overall, the benefits of using fluorescent microscopy with tardigrades was proven to be worthwhile. Especially that the animals can be kept alive during the imaging, deeper insights into tardigrade survival strategies can be obtained with appropriate fluorescent dyes and light sheet microscopy.

Ethics statement

The experiments in this study did not require an approval by an ethics committee. Both species are in their natural habitat, they are not genetically modified. All procedures performed herein complied with institutional guidelines.

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