Improved media formulations for primary cell cultures of *Botryllus schlosseri* and a morphological characterization case study of thraustochytrids associated with *Botryllus* primary cell cultures

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List of Publications and declaration

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In the presenting work my contribution included: exclusively performed the experiments (organized, documented and managed the observations). Organized and analyzed the data, authored drafts of the paper, prepared the figures and designs and charts.

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In the presenting work my contribution included: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing.

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Abstract

The cultivation of marine invertebrate cells *in vitro* has been of special interest due to the availability of diverse cell types and cellular potentialities in comparison to vertebrates and particularly in response to the high demand for a multitude of scientific applications. While cells in the marine invertebrate urochordate *Botryllus schlosseri* possess extensive potentialities, valuable tools in a variety of biological disciplines, no permanently proliferating cell line has been established in *B. schlosseri*, with results indicating that cell divisions cease 24-72 h from initiation and cultures associated with the opportunistic micro-organism thraustochytrids. Since, previous results showed that *B. schlosseri* cell cultures, have supported the proliferation of thraustochytrid cell types. Consequently, there is a limited knowledge about special requirements to support *Botryllus* cell growth in primary cultures. In a research article, that serves here as the first chapter of my doctoral dissertation (publication 1, chapter 1), I show in details the development of thraustochytrid cell types in primary cultures originated from *B. schlosseri* by employing *Botryllus* cell culture medium, and describing the characterization of these cell types morphologically. To further, extend our understanding and for improved identification of cells (*Botryllus* or thraustochytrid) under *in vitro* conditions, in chapter 2, I made use of confocal microscopy. This allow me to distinguish between thraustochytrid and *Botryllus* cell types based on their natural fluorescences. Compared to mammalian and insect cell cultures, this approach is novel to *Botryllus* primary cell cultures as was used in publication 2 for the first time. The second chapter (publication 2; research article) of the thesis, focuses on the effect of media optimization/variations on the proliferation, cell type composition and viability of primary *B. schlosseri* blood cell cultures up to a culture period of four weeks from initiation. Specifically, publication 2 evaluates the responses of various *B. schlosseri* blood cell types (haemoblasts, macrophage-like cells, granular amoebocytes, morula cells, pigment cells and nephrocytes) under primary cell cultures conditions, using five versions of basic medium. Results reveals that it is possible to maintain *Botryllus* primary cell cultures *in vitro* for up to one month in a consistent and reliable manner. The findings, and the knowledge obtained, of publication 1 and 2 thus improving the *in vitro* conditions required for *B. schlosseri* cells growth. In addition to the first and the second chapters in appendix 1, I show a study that was published as a book chapter demonstrating a general aseptic approach for blood cell extraction of *B. schlosseri*. The essence of this approach is to improve cell yields from a single colony of *B. schlosseri* for *in vitro* applications under aseptic conditions.

Introduction

The capability of cells to live under *in vitro* conditions is an important tool in a variety of scientific disciplines including medical and biological sciences (Butler, 2005; Dietel et al., 1987; Domart-Coulon et al., 1994; Rajala et al., 2007; Joyce, 2007; Saeidnia et al., 2015), which are used as alternative tools for animal experimentation, for biotechnological applications and pathological studies (Saeidnia et al., 2015, Butler, 2005; Kapalczynska et al., 2018; Naganuma et al., 1994). The literature further attests that the preparation of new cell cultures from freshly obtained tissues is still a delicate task requiring differentiated procedures for different types of cells (Alberts et al., 2002). Marine invertebrates may be regarded as a major source for such applications comprising >30% of animal species within the invertebrates (Naganuma et al., 1994; Rinkevich, 1999) and covering more than 20 different phyla which representing a rich source of cell types that significantly differ from one group to another (Rinkevich, 1999; Qarri et al., 2022; Rosner et al., 2021). Thus, different cell types from a variety of marine invertebrates hold a wide range of morpho-genetic potentialities such as multipotency and totipotency (Rinkevich et al., 1994; Rosenfield et al., 1994; Rinkevich and Rabinowitz, 1997; Qarri et al., 2022; Qarri et al., 2023). Consensually, the above direct to high *in vivo* structures of plasticity including cell replacements, proliferation processes and cell lineage in different invertebrate taxa, which may differ significantly even between systematically related groups of organisms (Rinkevich, 1999; Rosner et al., 2021).

During the last decades, many attempts were made for the development of cell cultures from marine invertebrates, primarily within six phyla that include Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata and Urochordata (Rinkevich, 1999, 2011; Rosner et al., 2021; Qarri et al., 2021; Mothersill and Austin, 2000; Munroe et al., 2019; Balakrishnan et al., 2022; Domart-Coulon and Blanchoud, 2022). Although limited success has been achieved and until recently (Hesp et al., 2023), there is yet no single permanent proliferating cell line available from any marine invertebrate (Rinkevich, 1999, 2011; Rosner et al., 2021; Qarri et al., 2022) despite the realization that such cultures are needed for numerous applications (Rinkevich et al., 1994; Bayne, 1998; Rosner et al., 2021; Balakrishnan et al., 2022; Domart-Coulon and Blanchoud, 2022; Hesp et al., 2023). As a matter of fact, it has been repeatedly shown that primary cultures of marine invertebrate cells stop dividing *in vitro* within 72 h from initiation, followed in many cultures with

contaminations of yeast, bacteria, fungi, cyanobacteria and amoebae (Rinkevich, 1998, 2011). In addition, most probably the vast majority of experimental failures are not documented in the refereed literature, leading to disintegrated knowledge on improved *in vitro* protocols for primary cell cultures from marine invertebrates (Grasela, 2012; Domart-Coulon and Blanchoud, 2022), and new research efforts repeatedly recapping former failed approaches and futile experimental protocols (Rinkevich, 1999, 2011). With all the above, a recent progress in sponge cell cultures has highlighted the importance of establishing optimized nutrient media for the development of primary cell cultures (Munroe et al., 2019), research that has led to the succeeded establishment of long lasting cultures from several species of sponges (Conkling et al., 2019; Hesp et al., 2023).

The cosmopolitan colonial urochordate *Botryllus schlosseri* (Chordata, Tunicata, Ascidiacea) belongs to a taxonomic taxon considered as the closest living invertebrates to the Vertebrata (Delsuc et al., 2006) and is used as an important model organism in various scientific disciplines, including stem cells biology (Manni et al., 2019; Ballarin et al., 2021; Voskoboynik et al., 2008), where various cell types from this species possess extensive cellular potentialities such as multipotency and totipotency (Laird et al., 2005; Rinkevich and Rabinowitz, 1994; Rinkevich and Rabinowitz, 1997; Rosner et al., 2009; Rinkevich et al., 2013; Ballarin et al., 2021; Rosner et al., 2021, Vanni et al., 2022), all revealing prospective *in vitro* capabilities. Consequently, these potentialities serve as an important tools in studying a wide range of biological disciplines in this organism such as ecotoxicology (Gregorin et al., 2021; Rosner et al., 2021), immunobiology and allorecognition (Magor et al., 1999; Rinkevich, 2004), developmental biology including colony astogeny (Manni et al., 2019; Rosner et al., 2006; Rosner et al., 2019), regeneration (Voskoboynik et al., 2007), senescence (Rabinowitz and Rinkevich, 2004a; Rinkevich, 2017) and evolutionary biology (Rinkevich, 2002). Colonies of *B. schlosseri* express two tight sexual and asexual (called blastogenesis; Figure 1) modes of reproduction (Manni et al., 2019). The sexual reproduction cycles occur weekly, where following the fertilization of eggs tadpole larvae are released (featuring chordate characteristics that includes striated musculature, neural tube, notochord and tail), swim for a short period of time, attaching to substrates (near the mother colony), losing their tail through apoptosis, and then developing into the first colonial modules, the oozooids that bud the following generations of zooiods (Berrill, 1950; Voskoboynik et al., 2007). Colonies develop from the oozooids through weekly cycles of growth and death (Manni et al., 2019; Rinkevich, 2019) and form several typical star-shaped groups of zooids, each called a system, all embedded

within the tunic, the transparent gelatinous matrix of the colony which also holds the colonial circulatory system connecting all zooids, that is terminated at the periphery of the colony with blind vasculature termini, called ampullae. Each zooid in the colony possesses an oral siphon (branchial siphon/inhalant siphon) and an atrial siphon is shared for all zooids in each system (Berrill, 1950). The weekly blastogenic cycle is composed of four major stages (marked by the letters A to D; Figure 1 a-h; Mukai and Watanabe, 1976), during which sets of primary buds mature to adult zooids in concert with the development of the secondary buds from the body wall of each primary bud concurrently with the resorption (through a massive apoptotic event) of all functional zooids (Lauzon et al., 1993). Thus, the blastogenesis process of somatic self-renewal and high vasculature regeneration capacity suggests an invertebrate model organism that maintain high capacity of stem cell activity throughout life (Qarri et al., 2020; Rinkevich, 2019).

Several studies attempted to develop primary cultures and permanent cell cultures from *B. schlosseri* blood cells (Rinkevich and Rabinowitz, 1994, 1993; Rabinowitz and Rinkevich, 2004; Qarri et al., 2021, 2022). These studies commonly used to extract blood cells that are directly collected from the blood vessels (Qarri et al., 2021, 2022). Other studies used cells originated from epithelial layers that show *de novo* stemness signatures (Rinkevich and Rabinowitz, 1997, Rabinowitz and Rinkevich, 2004, 2005, 2011; Rabinowitz et al., 2009), and cells derived from embryos (Rinkevich and Rabinowitz, 1994). Some of the studies have also focused on the type of medium and medium additives such as growth factors (Rinkevich and Rabinowitz, 1993, 2000). However, these studies described in general the *in vitro* conditions without specifying the cell types compositions of these primary cultures. The above attempts have failed to establish proliferating cell cultures from *Botryllus*, and showed no cell divisions 24-72 h from culture initiation, while in scores of cases cultures were overgrown by opportunistic micro-organisms such as thraustochytrids (Qarri et al., 2021, Rabinowitz et al., 2006, Rinkevich and Rabinowitz, 1993, Rinkevich, 1999, 2011).

Thraustochytrids are an eukaryotic group of unicellular protists, classified into the class Labyrinthulomycetes of the kingdom Chromista, and include >12 genera of which the most studied are *Aplanochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium*, and *Thraustochytrium* (Raghukumar, 2002; Bongiorni, 2011; Nham Tran et al., 2020; Lyu et al., 2021). They are found throughout the world in freshwater, estuarine and marine habitats and feed as saprophores, as

parasites or as bacterivores, while generally found associated with organic detritus, decaying algal and plant material, and in the sediments (Nham Tran et al., 2020; Lyu et al., 2021). Thraustochytrids, produce large quantities of unsaturated fatty acids and *Omega-3* long chain fatty acids (PUFA), docosahexaenoic acid (DHA), squalene and carotenoids (Huang et al., 2003; Burja et al., 2006; Jakobsen et al., 2007; Jakobsen et al., 2008; Nham Tran et al., 2020) which have been recognized by the biotechnology industry as highly valuable for a wide range of health benefits in humans, as for the general wellbeing of adults and infants (Huang et al., 2001; Calder and Yaqoob, 2009; Zarate et al., 2017; Morabito et al., 2019). Hitherto, many yet undisclosed strains of thraustochytrids are found on and within tissues of marine organisms such as sponges, corals, hydroids, bivalves, octopus, squids, nudibranchs, echinoids and tunicates (Cousserans et al., 1974; Raghukumar, 1988; Porter, 1990, Raghukumar and Balasbramanian, 1991; Bower, 1995; Mo et al., 2002; Nham Tran et al., 2020). The literature reveals that some marine invertebrate cell cultures, have supported the proliferation of thraustochytrids originated from sponges, cnidarians, crustaceans, mollusks, echinoderms and tunicates primary cell cultures (Ilan et al., 1996; Blisko, 1998; Frank et al., 1994; Awaji, 1997; Rabinowitz et al., 2006). In primary cultures, these strains thrived up to one month from culture initiation and outnumber the original primary cell cultures (Hsu et al., 1995; Rinkevich and Rabinowitz, 1993, 1994, 1997; Ilan et al., 1996; Blisko, 1998; Frank et al., 1994; Ellis and Bishop, 1989; Awaji, 1997; Kaneko et al., 1995; Ermak and Odintsova, 1996; Kawamura and Fujiwara, 1995; Rabinowitz et al., 2006). For example, records from studies of primary cell cultures originated from urochordates and in particular primary *Botryllus* cultures (from buds and blood cells) where thraustochytrids were developed indicated that in most cases, they appeared >12 days after culture initiation and the authors were unaware of their presence (Rinkevich and Rabinowitz, 1993, 1994, 1997; Sawada et al., 1994; Kawamura and Fujiwara, 1995; Rinkevich, 1999). Furthermore, there are several ways to identify thraustochytrids *in vitro* by carefully observing the cell cultures, but unfortunately, none of them are conclusive since some *Botryllus* and thraustochytrid cell types share the same colors and sizes in cell cultures. Conceivably, these reports on primary cell cultures described the development and the maintenance of thraustochytrids cultures instead of the original invertebrate primary cell cultures, results that point to the necessity of developing aseptic conditions and thraustochytrid detailed cells characterization in such primary cell cultures.

Responding to the above challenges, and in line with the recent progress in marine invertebrate cell culture methodology achieved via the development of optimized nutrient media for primary sponge cell cultures (Munroe et al., 2019), which led to the establishment of long lasting cultures from several species of sponges (Conkling et al., 2019; Hesp et al., 2023) and due to its importance of *B. schlosseri* as a model system in a wide range of biological disciplines and the needs for approved *in vitro* methodologies. The goal of the present thesis is to expand our know-how regarding *in vitro* requirements that better support *B. schlosseri* cell growth. Specifically, in the present publication 1 (chapter 1) by employing *B. schlosseri* cell culture medium, I showed in detail the development of thraustochytrid cell types in primary cultures originated from *Botryllus* blood vessels and further described the characterization of these cell types morphologically. Furthermore, in the present publication 2 (chapter 2) by using confocal microscopy, specific thraustochytrid cell types were distinguished from *Botryllus* cell types. The second chapter of the thesis, focuses on the effect of media optimization/variations on the proliferation, durability, and cell type compositions of primary *B. schlosseri* blood cell cultures up to a culture period of four weeks from initiation.

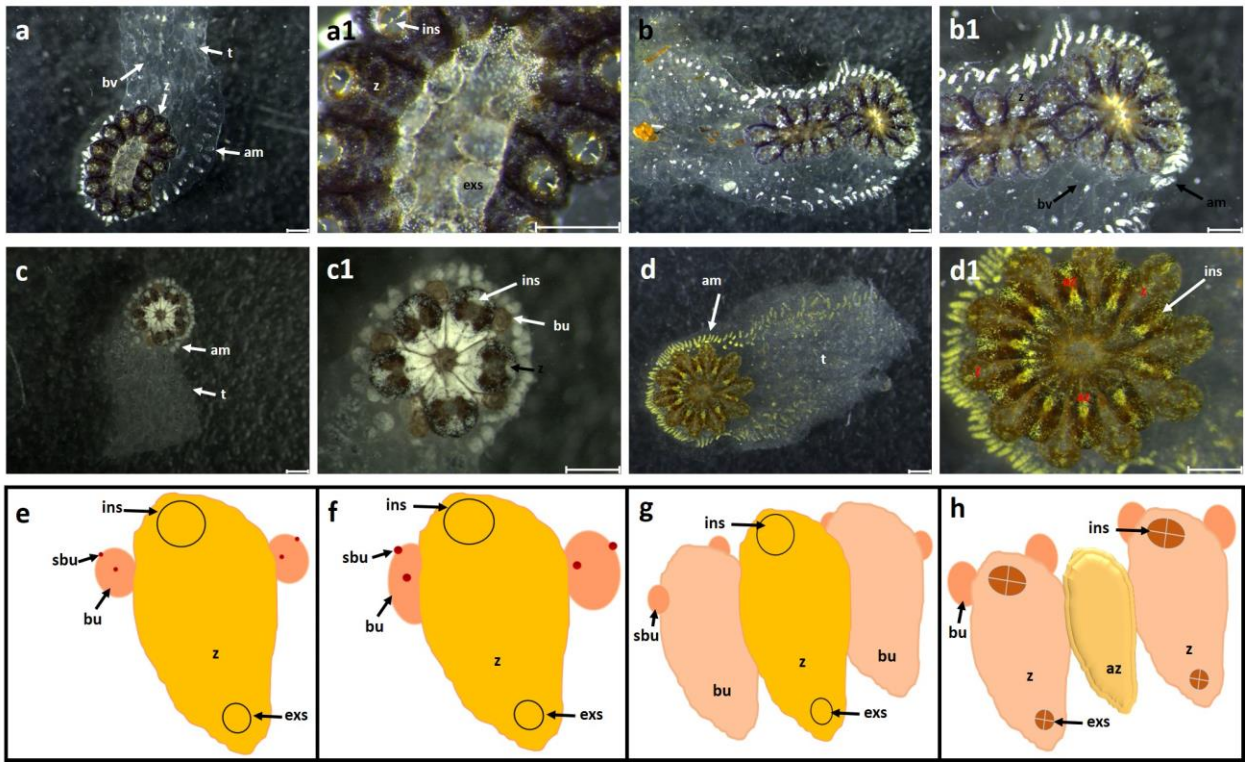

Figure 1: Blastogenesis in *Botryllus schlosseri.* a: a colony at blastogenic stage A with one system containing 14 zooids embedded in the tunic, connected through a network of blood vessels and fringed by a pear-shaped vascular termini (ampullae) extending towards the colony margins. a1: higher magnification of the colony center in part 'a', depicting zooids with open inhalant siphons and exhalant siphon for each zooid. b: a colony at blastogenic stage B with 2 systems and 20 zooids. b1: higher magnification of the systems in part 'b', depicting zooids with open inhalant siphons connected through a network of blood vessels and fringed by a pear-shaped vascular termini (ampullae) extending towards the colony margins. c-c1: a colony at blastogenic stage C compose of one system and containing 5 zooids with open inhalant siphons. The buds approaching the size of the zooids. d: one degenerated system of a colony at blastogenic stage D. d1: higher magnification of the degenerated system in part 'd', depicting 10 partly absorbed zooids with closed inhalant siphons and 10 mature buds which attained the size of the absorbed zooids. e-h: schematic illustration of the four major stages in the weekly blastogenic cycle of *B. schlosseri*. e: blastogenic stage A. An adult zooid with an open siphon, two small primary buds and marked areas (not yet seen) of four budlets (secondary buds, small red dots). f: blastogenic stage B, two days later. Developing buds and clearly depicting budlets connected to the primary buds. g: blastogenic stage C. three days later, further growth of buds that reach almost the size of zooids and fast organogenesis (not seen in the illustration) in the secondary buds. h: blastogenic stage D. Advanced absorption of the functional zooid through apoptosis while the two buds develop to pre-functional zooids, still with close siphons. Secondary buds are further developed, just about the state of primary buds. am = ampulla; az = absorbed zooid; bu = bud; bv = blood vessels; exs = exhalant siphon (black and white circles represent open and close siphons, respectively); ins = inhalant siphon (black and white circles represent open and close siphons, respectively); sbu = secondary bud; t = tunic; z = zooid. Bars = 1mm.

Aims of the thesis

This thesis focuses on the colonial urochordate *Botryllus schlosseri* that is used as an important model organism in various scientific disciplines including *in vitro* applications. *B. schlosseri* represents an invertebrate model system that maintain high capacity of adult stem cell activity, as various circulating blood cell types express multipotent or totipotent phenotypes throughout life. While *in vitro* cultures of isolated blood cells from *Botryllus* may serve as indispensable tools for studying stem cells biology, up to date, there is no single established cell line available from *B. schlosseri*, since these cells stop dividing *in vitro* within 24-72 h after isolation and many of the cultures supported the proliferation of thraustochytrid cell types. Tackling these two major critical obstacles, I applied the *in vitro* methodology on the isolated *B. schlosseri* blood cells to achieve the following aims: 1. Enhance our understanding of thraustochytrid cell types developed in *B. schlosseri* primary cell cultures. 2. To characterize and distinguish between thraustochytrid and *Botryllus* cell types. 3. Enhance our understanding of the *in vitro* conditions required for *Botryllus* cell type's growth, by utilizing five media formulations. 4. To provide an assessment of the dominant cell types, proliferation statuses and viability of the cultured cells in the studied media.

Results

Chapter 1:

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Research Article

Andy Qarri*, Yuval Rinkevich and Baruch Rinkevich

Employing marine invertebrate cell culture media for isolation and cultivation of thraustochytrids

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Abstract: Thraustochytrids, a common group of marine eukaryotic protists, have drawn considerable scientific and industrial interest due to their ability to synthesize high levels of bioactive compounds, including polyunsaturated fatty acids, docosahexaenoic acid, squalene and carotenoids, and their new applications for biofuels. The pharmaceutical and industrial potential of thraustochytrids necessitate effective isolation of new strains and establishment of axenic cultures. To date, existing isolation protocols have used baiting and direct plating methods to generate axenic cultures with varied media compositions that contain peptone and yeast extracts as nitrogen sources, glucose as carbon source, seawater and antibiotics. Here we reveal a new approach for the isolation of thraustochytrids from tissues of marine invertebrates using (a) primary cell cultures in a liquid medium containing basal medium, 50% artificial seawater, vitamins, proteins and antibiotics, and (b) cultivation in the same cell culture medium. Using the colonial tunicate *Botryllus schlosseri* as a model system, thraustochytrid cells thrived in the medium from the day of extraction, grew and proliferated for the next five weeks (five-passages, up to 1.9×10^6 cells ml^{-1} in passage 5; 1.45-fold multiplication $week^{-1}$). This new approach for isolation and cultivation of axenic thraustochytrid cultures enables the isolation of new species with promising bioactive compounds.

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Keywords: Ascidians; cultivation; Labyrinthulomycetes; marine invertebrates; PUFA.

1 Introduction

Polyunsaturated fatty acids (PUFAs) have been recognized as highly valuable for a wide range of health benefits in adults and infants (Calder and Yaqoob 2009; Huang et al. 2001; Morabito et al. 2019; Zárate et al. 2017). The current primary source of essential dietary PUFAs is through the consumption of oily fish, such as salmon, sardines, tuna, and herring, and the ingestion of their processed oils (Calder and Yaqoob 2009), which are ultimately derived throughout the food chain from various microorganisms (Burja et al. 2007; Calder and Yaqoob 2009; Nham Tran et al. 2020). One group of such microorganisms is the thraustochytrids, marine protists that produce large quantities of PUFA, docosahexaenoic acid (DHA), squalene and carotenoids (Burja et al. 2006; Huang et al. 2003; Jakobsen et al. 2007, 2008; Nham Tran et al. 2020). The thraustochytrids have been further explored as new source materials for biofuels and for lipid biofactories, two emerging fields of industrial biotechnology (Gupta et al. 2012; Nham Tran et al. 2020).

Thraustochytrids represent an eukaryotic group of unicellular protists within the class Labyrinthulomycetes of the kingdom Chromista, and include >12 genera of which the most studied are *Aplanochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium*, and *Thraustochytrium* (Bongiorni 2012; Lyu et al. 2021; Nham Tran et al. 2020; Raghukumar 2002). They are found throughout the world in estuarine and marine habitats, and feed as saprobes, as parasites or as bacterivores, while generally found in association with organic detritus, decaying algal and plant material, and in sediments (Lyu et al. 2021; Nham Tran et al. 2020). Traditionally, thraustochytrids were isolated from a variety of marine sources such as sediments, fallen mangrove leaves, seagrasses, algae and coastal waters (Jain et al. 2005; Lyu et al. 2021; Yokochi et al. 2001). Porter (1990) described the baiting isolation approach performed by using sterile pollen grains (from pine or maple)

suspended in a basic liquid nutrient culture medium for several days, followed by the removal of single pollen particles with their attached thraustochytrids and their subsequent inoculation onto an agar medium. The second common approach is the direct isolation of thraustochytrids from substrata such as decaying animal and plant materials, animal mucus, sediments, and water, usually directly plated on agar, and then inoculation of emerged thraustochytrid colonies into various media (Bockelmann et al. 2012; Burgaud et al. 2009; Hinzpeter et al. 2009; Lyu et al. 2021). In parallel, research efforts have focused on the development of basal media for thraustochytrids in solid or liquid forms (Burja et al. 2006; Jakobsen et al. 2007; Lyu et al. 2021). The basic components in all media generally consist of polypeptone (*P*), and yeast extract (*Y*) as nitrogen sources, and agar (*A*) or glucose (*G*) as the carbon source. Any combination of these basal media (PYA or GPY) is supplemented with antibiotics (most commonly streptomycin sulfate, penicillin, ampicillin, kanamycin sulfate and rifampicin), and diluted with sterile water, sterile natural or artificial seawater (ASW) with up to ~50% salinity (Lyu et al. 2021).

Many yet undescribed strains of thraustochytrids were found on and within tissues of marine organisms such as sponges, corals, hydroids, bivalves, octopus, squids, nudibranchs, echinoids and tunicates (Bower 1995; Cousserans et al. 1974; Mo et al. 2002; Nham Tran et al. 2020; Porter 1990; Raghukumar 1988; Raghukumar and Balasbramanian 1991). These strains are generally missed by the baiting and direct plating isolation approaches used, and to date culturing conditions have not been optimized for their continuous growth (Rinkevich 1999). However, some marine invertebrate primary cell cultures have supported the proliferation of thraustochytrids originating from sponges, cnidarians, crustaceans, mollusks, echinoderms and tunicates (Awaji 1997; Blisko 1998; Frank et al. 1994; Ilan et al. 1996; Rabinowitz et al. 2006). In primary cultures, these strains thrived up to one month from inoculation, following the appearance of opportunistic forms like yeast and bacteria (Awaji 1997; Blisko 1998; Ellis and Bishop 1989; Ermak and Odintsova 1996; Frank et al. 1994; Hsu et al. 1995; Ilan et al. 1996; Kawamura and Fujiwara 1995; Kaneko et al. 1995; Rinkevich and Rabinowitz 1993, 1994, 1997; Rabinowitz et al. 2006), results that point to the necessity of developing aseptic conditions in such primary cultures for improved thraustochytrid isolation and cultivation. Here, we present a new approach for the isolation and cultivation of thraustochytrids from marine invertebrate tissues using the model colonial tunicate *Botryllus schlosseri* (Pallas 1766).

2 Materials and methods

2.1 Washing solution

Washing solution was used to reduce bacterial contaminants during cell isolation. Washing solution was made with artificial seawater (ASW) that was prepared as described in Rabinowitz and Rinkevich (2003), autoclaved, sterilized by 0.2 µm filter membrane (Millipore) and stored at room temperature. For each 50 ml washing solution, we used 44 ml of ASW, supplemented with 3 ml of PSA (Biological Industries; Penicillin 10,000 units ml^{-1}, Streptomycin sulphate 10 mg ml^{-1} and Amphotericin B 25 µm ml^{-1}; Cat. 03-033-1B) and 3 ml of Gentamycin Sulfate (Biological Industries; 50 mg ml^{-1}; Cat. 03-035-1).

2.2 Isolation of thraustochytrids

Thraustochytrids were collected aseptically from nine colonies of the colonial ascidian *Botryllus schlosseri*, all at blastogenic stage *C* (detailed description on asexual stages in Voskoboynik et al. 2007 and Manni et al. 2019). Colonies originated from laboratory stocks at the National Institute of Oceanography (Haifa, Israel) and were maintained in aquaria with a running seawater system in a cooled room of 20 °C (12:12 light:dark regimen) as specified (Rinkevich and Shapira 1998). The blood vessels of each colony were punctured under aseptic conditions and the circulating blood cells were collected into 15 ml tubes with large volumes (~5 ml) of washing solution. Prior to culture incubation, the collected *B. schlosseri* blood cells were washed 5 times with washing solution followed by centrifugation (Eppendorf, Hamburg, Germany) of 10 min at 1000 *g* and incubated at 20 °C.

2.3 Medium

Thraustochytrid cultures were maintained in a liquid growth medium containing 21.25 ml DMEM/F-12[HAM] 1:1 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Cat. 01-170-1A), 21.25 ml ASW, 5 ml Fetal Bovine Serum (European grade; Cat. 04-007-1A), 0.5 ml L-Glutamine (Cat. 03-020-1B), 0.5 ml HEPES buffer solution 1 M (Cat. 03-025-1B), 0.5 ml PSA, 0.5 ml Gentamycin Sulfate and 0.5 ml Sodium Pyruvate solution (Cat. 03-042-1B). In addition to sterilized conditions during preparation, the medium was filtered through 0.2 µm membrane (Millipore) and stored at 4 °C. All components, except ASW, were purchased from Biological Industries, Kibbutz Beit-HaEmek, Israel.

2.4 Culture conditions

Attempts were made to maintain pathogen-free conditions during cultivation. In addition to sterilization by filtering (0.2 µm) of the medium before use, glassware was autoclaved and only sterilized plasticware was used. Three experiments were performed and held for 42 days, each containing isolated *B. schlosseri* blood cells originating from three colonies (total nine plates from nine colonies). Each of the isolated *B. schlosseri* blood cell cultures was maintained in a 35 mm Petri dish (Greiner bio-one, CELLSTARR, Petri-dish 35 × 10 mm) containing 3 ml of liquid medium and incubated

(Incubator-Leec; Colwick Industrial Estate, Nottingham NG42AJ, England) at 20 °C under normal atmosphere conditions. The medium was changed every other day. Once an axenic thraustochytrid culture was established, the cells were sub-cultured (passaging), divided into new plates and fed with fresh medium. The contents of each plate were collected into a 15 ml tube, washed 3 times with fresh medium, followed by centrifugation (at 1000 *g* for 10 min) and seeded to new plates with fresh medium.

2.5 Observations, cell counting and fluorescence staining

All cultures were observed once every other day where cells were counted using a hemocytometer and photographed under the microscope (Olympus inverted system microscope, model Ix70, equipped with DP73 camera). Cell viability was determined using Trypan Blue Solution (Biological Industries; Cat. 03-102-1B). In order to confirm the presence of thraustochytrids, cultures were sampled and cells were stained with fluorescent dye Hoechst 33342 (cat: 62249, Thermo Fisher) for nuclei staining. The stained cells were observed and photographed under an epifluorescence microscope with UV excitation filter (360–370 nm). Viable cell fold increase was calculated at the end of each specific passage (average of three plates ± SD) divided by the viable cell number at onset of that passage (average of three plates ± SD).

2.6 DNA extraction and PCR conditions

Cell cultures were centrifuged at 1000 *g* for 10 min at room temperature and DNA extracted as described by Mo and Rinkevich (2001). DNA samples were subjected to a polymerase chain reaction (PCR) protocol optimized for the recovery of thraustochytrid 18S rDNA profiles using three sets of primers (A1, A2 and A3) following Mo et al. (2002). PCR amplifications were carried out using an Eppendorf Mastercycler gradient (Germany) and products visualized on 1.5% agarose gel.

3 Results

3.1 Characteristics of cell culture initiation

Blood cells were collected from nine *Botryllus schlosseri* colonies (Figure 1a and b). At onset (day 0), heterogeneous cell populations ($1.16 \times 10^6 \pm 0.18$ cells ml^{-1}) were obtained, revealing *B. schlosseri* and clearly visible thraustochytrid cells growing side by side in suspension with *Botryllus* cells (Figure 1c and d), followed by an increase in thraustochytrid cell numbers by 24 h from onset. At 8 d from onset, thraustochytrid cells dominated the cultures (Figure 1d and f), leaving debris of dead *B. schlosseri* cells. The thraustochytrid cell types revealed both vegetative and amoeboid cell forms (Lyu et al. 2021; Morabito et al. 2019). The vegetative cells included mononucleated cells (5–10 µm; Figure 2a and b), multinucleated cells (2 up to 30 nuclei cell^{-1} 10–50 µm; Figure 2c and d) and sporangia (20–200 µm; Figure 2e and f), while the amoeboid mononucleated cells appeared as cell clusters, each composed of at least 3 cells, reaching sizes of up to 200 µm cluster^{-1} (Figure 2a). Zoospores or gametes were not observed. During this period *B. schlosseri* cells decreased by >95%, from $1.16 \times 10^6 \pm 0.2$ cells ml^{-1} at onset to $0.05 \times 10^6 \pm 0.04$ cells ml^{-1} at day 8 (Figure 3a), and the number of thraustochytrid aggregates that could be accurately counted increased from 7.2 ± 3.9 at onset to 72.8 ± 9.1 at day 8 (Figure 3b). At day 8, all cultures contained thraustochytrid aggregates and single cells including mononucleated, multinucleated and sporangia (Figure 1f).

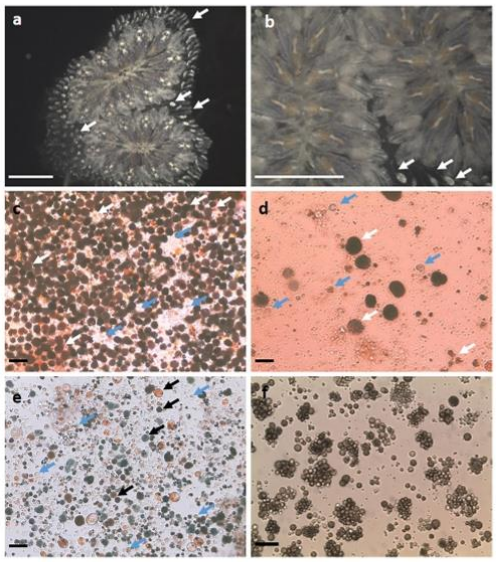

Figure 1: Primary cell cultures from *Botryllus schlosseri* circulating cells. a, b: Anterior and posterior views, respectively, of a *B. schlosseri* colony from which the cells were obtained. Peripheral blood vessels (white arrows) were punctured and the cells were collected aseptically for cultivation. Scale bars = 1 mm. c, d: Cultures at onset; heterogeneous and mixed cell suspensions with *B. schlosseri* blood cells (white arrows) and few thraustochytrid cells (blue arrows). e: 24 h later, cultures show debris of dead *B. schlosseri* cells (black arrows) and thraustochytrid cells (blue arrows). f: Eight days from initiation, thraustochytrid cells (singles and aggregates) outnumbered the *Botryllus* cells, taking over the cultures. Scale bars for c–f = 100 µm.

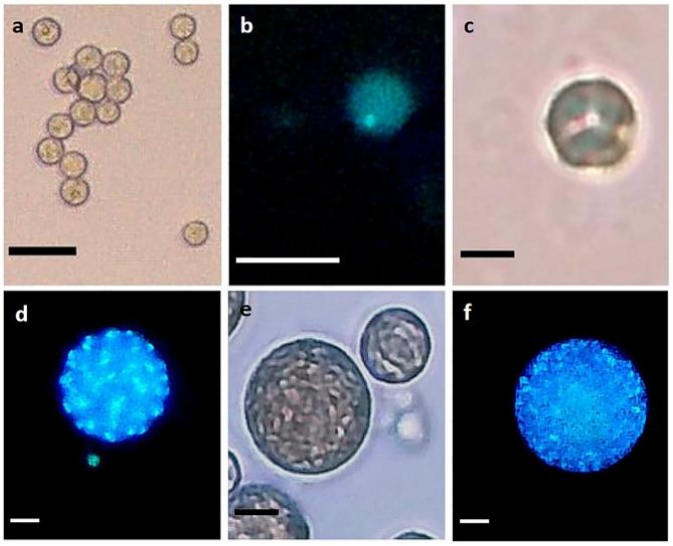

Figure 2: Thraustochytrid cell morphologies in developing cultures. a: A typical cell aggregation with 12 mononucleated cells, surrounded by two single cells and a single bi-cell aggregation. b: Single mono-nucleated cell stained with Hoechst for nucleus staining. c: A single multinucleated cell containing four nuclei. d: A single multinucleated cell stained with Hoechst, revealing 30 nuclei. e: Small and large sporangia. f: A single sporangium cell stained with Hoechst. Scale bars = 20 µm.

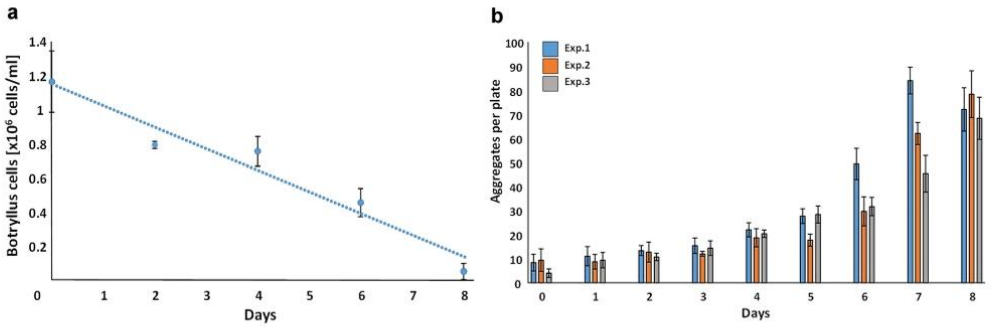

Figure 3: *Botryllus schlosseri* and thraustochytrid cells in primary cultures during the first 8 days from initiation. a: *B. schlosseri* viable cells ($\times 10^6$ cells ml^{-1}). Points are averages (±SD) of three experiments. Trend line has equation $y = -0.1278 \times 1.1493$ with $R^2 = 0.9491$. b: Thraustochytrid aggregates per plate for three experiments. Each column is average of three plates (±SD).

3.2 Molecular identification

PCR amplifications of 18S rDNA of the thraustochytrid axenic cultures (day 18; $n = 3$) were performed using three sets of primers. The amplifications revealed the existence of the undescribed strain Thraustochytriidae sp. BS2 (100% identity; GenBank accession number AF257315.2) (Mo et al. 2002).

3.3 Thraustochytrid axenic subculture

Axenic cultures of thraustochytrid cells (n = 9) were established (91.4–97.1% viable cells) after 8 days post cell extraction. Then, the cultures were split on a weekly basis, while the medium was replaced every other day. During the subsequent 5 weeks, five passages (P1–P5) were performed. Starting from P1 (9–14 days from onset) the thraustochytrid cells grew in suspensions as single cells or aggregates, each 10–150 µm in size (Figure 4a and b). The aggregates were composed of sporangia and mononucleated cells and the single cells were composed of sporangia, mononucleated and multinucleated cells (Figure 4b). Thraustochytrid cells for P1 plates ($0.12 \times 10^6 \pm 0.12$ cells ml^{-1}; Figure 4k) were counted on days 10, 12 and 14, revealing $0.05 \times 10^6 \pm 0.01$, $0.07 \times 10^6 \pm 0.01$ and $0.25 \times 10^6 \pm 0.13$ cells ml^{-1}, respectively (Figure 4l). At P2 (15–21 days from onset; $0.64 \times 10^6 \pm 0.17$ cells ml^{-1}; Figure 4k), the thraustochytrid

suspension was composed of single cells (mononucleated, multinucleated and sporangia) and aggregates (varied 10–300 µm; Figure 4c) of sporangia and mononucleated cells (Figure 4c and d). The thraustochytrid cells for P2 plates were counted on days 16, 18 and 20, revealing $0.43 \times 10^6 \pm 0.09$, $0.72 \times 10^6 \pm 0.06$ and $0.76 \times 10^6 \pm 0.08$ cells ml^{-1}, respectively (Figure 4l). At P3 (22–28 days from onset; $0.92 \times 10^6 \pm 0.11$ cells ml^{-1}; Figure 4k), the suspensions were composed of single cells and aggregates (different sizes that varied between 100 to about 500 µm; Figure 4e) of sporangia and mononucleated cells (Figure 4e and f). The single sporangia reached sizes of up to 200 µm (Figure 4f). Thraustochytrid cells for P3 plates were counted on days 22, 24, 26 and 28, revealing $0.84 \times 10^6 \pm 0.09$, $0.89 \times 10^6 \pm 0.05$, $0.89 \times 10^6 \pm 0.05$ and $1.06 \times 10^6 \pm 0.12$ cells ml^{-1}, respectively (Figure 4l). At P4 (29–35 days from onset; $1.28 \times 10^6 \pm 0.2$ cells ml^{-1}; Figure 4k), the suspensions were made of single cells (mononucleated, multinucleated and sporangia) and few aggregates (Figure 4g and h). The pentagonal, hexagonal, heptagonal and octagonal single cell shapes (Figure 4h) were probably the results of high cell densities. Thraustochytrid cells for P4 plates were counted on days 30, 32 and 34, revealing $1.06 \times 10^6 \pm 0.15$, $1.3 \times 10^6 \pm 0.03$ and $1.5 \times 10^6 \pm 0.06$ cells ml^{-1}, respectively (Figure 4l). At P5 (36–42 days from onset; $1.67 \times 10^6 \pm 0.12$ cells ml^{-1}; Figure 4k), the suspensions were made of single cells (mononucleated and sporangia; Figure 4i) and few aggregates (Figure 4i and j). The single cells appeared as pentagonal, hexagonal, heptagonal and octagonal shapes, as in P4 (Figure 4j). Thraustochytrid cells for P5 plates were counted on days 36, 38, 40 and 42, revealing $1.48 \times 10^6 \pm 0.02$, $1.62 \times 10^6 \pm 0.02$, $1.67 \times 10^6 \pm 0.03$ and $1.90 \times 10^6 \pm 0.01$ cells ml^{-1}, respectively (Figure 4l).

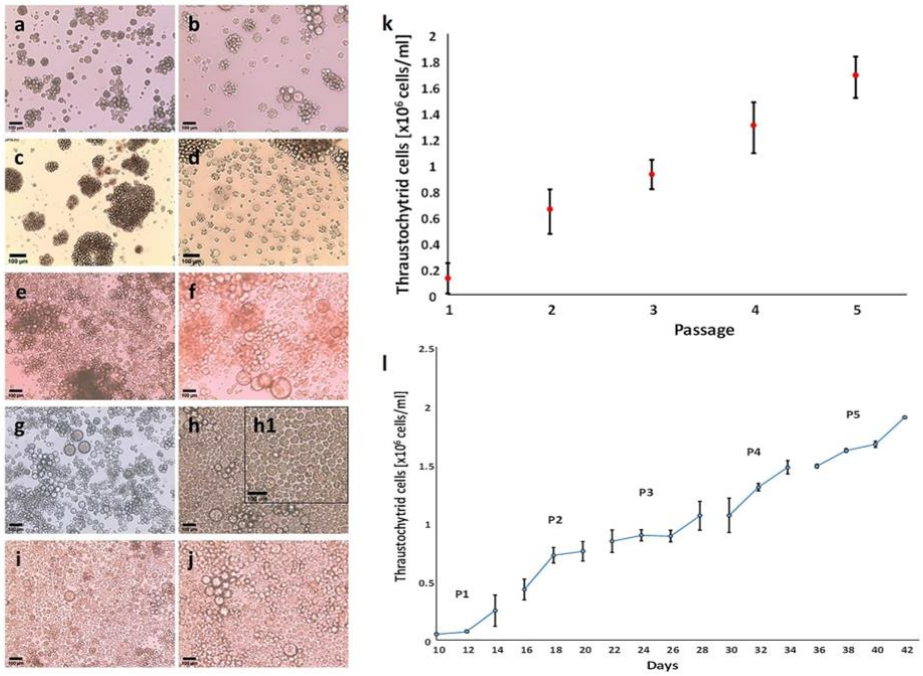

Figure 4: Five weeks cultivation of thraustochytrid axenic cultures, including five passages (P1–P5). a, b: Cell cultures at day 12 from initiation, P1. Thraustochytrid cells phenotypes appear as single cells and aggregates. c, d: Day 18, P2. Single cells and aggregates. e, f: Day 26, P3. Single cells and aggregates. g, h: Day 32, P4. Pentagonal, hexagonal, heptagonal and octagonal shapes of single cells. Aggregates observed as well but not counted. h1: Enlargement of Figure 4h, showing details of cell structures. i, j: Day 40, P5. Single cells with circular, pentagonal, hexagonal, heptagonal and octagonal shapes. Aggregates observed as well but not counted. k, l: Five weeks growth pattern of thraustochytrids under the current conditions. k: Thraustochytrid cells ($\times 10^6$ cells ml^{-1}) at the five weekly passages. Data are averages ± SD for a specific passage (3–4 counts per passage). l: Cell numbers ($\times 10^6$ cells ml^{-1}) for thraustochytrid cultures, 10–42 days from initiation. P1–P5 represent passage numbers. Data are averages of three experiments ± SD Scale bars for a–j = 100 µm.

3.4 Long term cultivation

Fold increase of thraustochytrid cells was evaluated for the five passages (P1–P5) to establish preferred long-term culture conditions for thraustochytrid growth. Fold increase values were: 4.75 ± 2.25 for P1 and 1.82 ± 0.5, 1.28 ± 0.3, 1.41 ± 0.2 and 1.3 ± 0.01, for P2, P3, P4 and P5, respectively, summarizing a weekly average increase of 1.45 fold.

4 Discussion

Over the past two decades, isolation methods such as direct plating and pollen baiting (Mariana Rosa et al. 2011) have helped to identify marine thraustochytrids that are capable of synthesizing large quantities of PUFAs and other compounds, making them of considerable industrial and scientific interest (Burja et al. 2006; FioRito et al. 2016; Huang et al. 2003; Jain et al. 2005; Jakobsen et al. 2007, 2008; Lyu et al. 2021; Nham Tran et al. 2020; Yokochi et al. 2001). The direct plating method for thraustochytrid isolation has been successfully applied on sediment samples (Gupta et al. 2012), seawater (Ueda et al. 2015) and decaying mangrove leaves and debris (Fan et al. 2002), and is based on direct plating of thraustochytrids onto solid nutrient media, supplemented with antibiotics (Lyu et al. 2021; Mariana Rosa et al. 2011). These solid media contain mostly glucose (carbon source), yeast extract and peptone (nitrogen sources), agar and seawater. The pollen baiting methodology was used for isolation of thraustochytrids from seaweeds, mangroves and plant debris (Damare 2015; Gao et al. 2013; Mariana Rosa et al. 2011; Wang et al. 2019; Wilkens and Maas 2012) and is based on collections of the pollen baits with the attached thraustochytrids (one of the few marine microorganisms that is able to feed on pollen nutrients) all inserted in nutrient free seawater, transplanting them into media for cultivation.

Here we developed and tested a new approach for the isolation and cultivation of thraustochytrids from tissues of marine invertebrates via the establishment of primary host cell cultures. This approach is further based on previous studies revealing the spontaneous proliferation of thraustochytrids in primary cell cultures from a wide range of marine organisms (sponges, corals and tunicates), where many long-term cultures were contaminated by thraustochytrids (Grasela et al. 2012; Rabinowitz et al. 2006; Rinkevich 1999). These primary cell cultures were supported by a medium containing a basic medium supplemented with vitamins, proteins and antibiotics. Working on primary cultures from extirpated buds of *B. schlosseri,* Rabinowitz et al. (2006) recorded a lag period of 1–4 weeks from culture initiation until thraustochytrids became discernable in cultures, a significantly longer period than observed in this study. While in the former study the media were replaced once a week, and the initial density of animal cells was approximately 10^4 cells $well^{-1}$, we show here that, by changing the medium every other day and by initiating the cultures with about two orders of magnitude higher numbers of extracted cells (10^5–10^6 cells ml^{-1}), thraustochytrids were observed as of onset and multiplied by 1.45 folds/week, at least for the first 5 weeks following initiation.

The current study thus demonstrates that improved cell culture media can be used for thraustochytrid isolation from tissues of marine organisms and thereafter, can be used to sustain proliferating thraustochytrid cultures. This new approach, tested on *B. schlosseri* primary cell cultures may increase the thraustochytrid strains/species available for research into various industrial and pharmaceutical applications.

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Bionotes

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Chapter 2:

cells

MDPI

Article

Improved Media Formulations for Primary Cell Cultures Derived from a Colonial Urochordate

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Abstract: The cultivation of marine invertebrate cells in vitro has garnered significant attention due to the availability of diverse cell types and cellular potentialities in comparison to vertebrates and particularly in response to the demand for a multitude of applications. While cells in the colonial urochordate *Botryllus schlosseri* have a very high potential for omnipotent differentiation, no proliferating cell line has been established in *Botryllus*, with results indicating that cell divisions cease 24–72 h post initiation. This research assessed how various *Botryllus* blood cell types respond to in vitro conditions by utilizing five different refinements of cell culture media (TGM1–TGM5). During the initial week of culture, there was a noticeable medium-dependent increase in the proliferation and viability of distinct blood cell types. Within less than one month from initiation, we developed medium-specific primary cultures, a discovery that supports larger efforts to develop cell type-specific cultures. Specific cell types were easily distinguished and classified based on their natural fluorescence properties using confocal microscopy. These results are in agreement with recent advances in marine invertebrate cell cultures, demonstrating the significance of optimized nutrient media for cell culture development and for cell selection.

Keywords: *Botryllus schlosseri*; primary cultures; blood cells; cell culture media; cell proliferation; cell viability

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1. Introduction

Countless attempts have been made to develop cell cultures from marine invertebrates. Since the 1960s, over 500 peer-reviewed publications have been published on this topic, with the main focus being on six phyla, which are Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata, and Urochordata [1–7]. However, until recently [8], not a single continuous cell line was established for any aquatic invertebrate taxon, as all efforts to obtain lasting proliferating cultures from marine invertebrates have inexplicably failed [1,2,9], despite the increasing demand of these cultures for a wide range of applications [5–8,10,11]. Further, it has been repeatedly shown that primary cultures of marine invertebrate cells cease dividing 24–72 h from onset [1,2]. It is worth noting that a substantial number of unsuccessful attempts to establish primary cell cultures from marine invertebrates are not reported in the scientific literature, resulting in incomplete or fragmented knowledge of effective in vitro protocols [7,12], and researchers often end up revisiting unsuccessful methods and futile experimental protocols [1,2]. Considering the above discussion, a recent advancement in sponge cell culture has emphasized the significance of developing optimized nutrient media for the development of primary cell cultures [4–13], a research approach that has led to the succeeded establishment of long-lasting cultures from several sponge species [8,13].

Botryllus schlosseri (Chordata, Tunicata, Ascidiacea), a colonial urochordate of cosmopolitan distribution, serves as a valuable model organism in various scientific disciplines, including stem cell biology [14,15], which has garnered significant attention due to the exceptional cellular potentialities exhibited by various cell types from this species, such as multipotency and totipotency [5,16–22], all of which hold great promise for in vitro applications. Colonies of *B. schlosseri* exhibit both tight asexual (referred to as blastogenesis) and sexual modes of reproduction [14,15]. After fertilization of the eggs, tadpole larvae are released and swim for approximately half an hour before attaching to substrates, metamorphosing and developing into the first colonial modules, known as oozooids, that bud the subsequent generations of zooids [23,24]. In each *B. schlosseri* colony, the zooids create typical star-shaped systems, all embedded within the colony's tunic, a transparent gelatinous matrix of the colony that also holds the colonial circulatory system, connecting all zooids and terminating at the periphery of the colony with blind vasculature termini, called ampullae. *B. schlosseri* colonies develop through the asexual mode of reproduction by undergoing weekly blastogenic cycles, which involve series of growth and death of the zooids. Each weekly blastogenic cycle consists of four major stages (A to D; sensu [25]), during which sets of primary buds mature to adult zooids, while secondary buds emerge from the body walls of primary buds. Concurrently, all functional zooids undergo resorption through massive apoptotic and phagocytosis events of all functional zooids [26], with properties indicating a high capacity for stem cell activity throughout life [27,28].

Several studies have attempted to develop primary cultures from various cell types of *B. schlosseri*, including blood cells [10,29–32], cells originated from epithelial layers that exhibit de novo stemness signatures [18,30,33–35], and cells originating from embryos [17]. Some of these studies have also focused on optimizing the type of medium and media additives such as growth factors [29,36]. Despite these attempts, no proliferating primary cell cultures from *Botryllus* have been established, with no cell divisions observed beyond 72 h after culture initiation. In numerous cases, cultures were also overgrown by opportunistic microorganisms, including bacteria and protists [1,2,17,29,31,32,37]. In response to the above challenges, and in line with the approach taken by Munroe et al. [4] and Conkling et al. [13], the present study aimed to (a) enhance our understanding of the in vitro conditions that promote *B. schlosseri* cell growth, by utilizing five media formulations, and (b) provide an assessment of the proliferation status of cultured cells in the studied media.

2. Materials and Methods

2.1. B. schlosseri Husbandry

B. schlosseri colonies were collected from the rocky intertidal zone at Helgoland Island (Germany), tied on 5 × 7.5 cm glass slides and reared for 3 days at the Biological Station Helgoland of the Alfred Wegener Institute, transferred to the Institute of Regenerative Biology and Medicine, Munich, and kept vertically in slots of glass staining racks, in 5 L plastic tanks (22.5 °C) supplied with a standing artificial sea water system (ASW, [31,32]) and aerated by air stones. Colonies were fed daily with dried algae powder and commercial food (Reef energy plus, Red Sea, Ltd., London, UK). ASW was changed three times a week, and colonies were gently cleaned twice a week using small, soft brushes to remove trapped food particles, fouling organisms, and debris. All experimental colonies were in good health and well adapted to their maintenance conditions.

2.2. B. schlosseri Cell Extraction under Aseptic Conditions

Colonies were first taken out from aquaria and photographed under a stereomicroscope (Leica M50 equipped with a camera Leica DFC310 FX, Leica Microsystems, Wetzlar, Germany), and cells were extracted from *B. schlosseri* as described [32], under aseptic conditions using washing solution (WS), Merck Millipore, Darmstadt, Germany. WS [32] was made with autoclaved ASW, 0.2 µm filtered (Corning; Cat. No. 431218), supplemented with a penicillin streptomycin mixture (Gibco; 15070-063) or with penicillin, streptomycin,

amphotericin b, 10,000 IU/mL, 10 mg/mL, 25 μg/mL (MP Biomedicals; Cat. No. 1674049), and gentamicin (50 mg/mL; Gibco; 15750-037) and stored at room temperature. Three WS variations were prepared containing ASW and antibiotic stock solutions: WS1 contained 88% ASW and 12% penicillin streptomycin; WS2 contained 88% ASW, 6% penicillin, streptomycin, amphotericin b, and 6% gentamicin; WS3 contained 88% ASW and 12% gentamicin.

2.3. Blood Cell Observations

Primary blood cell cultures were observed once every other day, where cells were counted using a hemocytometer and photographed under the microscope (Primo Vert, Zeiss inverted system microscope, equipped with a camera). Cell viability was determined using Trypan Blue Solution (Gibco; Cat. No. 15250-061, Thermo Fisher Scientific, Waltham, MA, USA). Contamination by thraustochytrids [1,31], single-celled saprotrophic eukaryotes, was monitored once every other day by carefully observing culture samples under the microscope, while the presence of yeast and fungi was detected by Calcofluor White Stain (Sigma-Aldrich; 18909, St. Lois, MI, USA), according to the manufacturer's instructions. Samples were visualized under a fluorescent microscope with an emission range of 300 to 440 nm.

2.4. Media

B. schlosseri cultures were maintained in a liquid growth medium (tunicate growth medium; TGM) containing ASW, basal media (DMEM/F-12[HAM] 1:1, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Gibco, Cat. No. 11320-074; RPMI medium 1640, Gibco, Cat. No. 21875-034), foetal bovine serum (FBS qualified HI, Brazil, Gibco, Cat. No. 10500-064), antibiotics, l-glutamine solution 200 mM (Sigma-Aldrich, 59202C), sodium pyruvate solution (Sigma-Aldrich, S8636), and HEPES buffer solution 1M (Sigma-Aldrich, H0887). Five medium variants (Table S1) were then prepared: (1) medium TGM1 (pH = 7.21) contained 1% of l-glutamine solution 200 mM, 1% of HEPES buffer solution 1M, 1% of penicillin streptomycin, 1% of sodium pyruvate solution, 10% of foetal bovine serum, and 86% of DMEM/F-12[HAM] 1:1; (2) medium TGM2 (pH = 7.13) contained 1% of l-glutamine solution 200 mM, 1% of HEPES buffer solution 1M, 1% of penicillin streptomycin, 1% of sodium pyruvate solution, 10% of foetal bovine serum, and 86% of RPMI medium 1640; (3) medium TGM3 (pH = 6.76) contained 1% of l-glutamine solution 200 mM, 1% of HEPES buffer solution 1M, 1% of penicillin streptomycin amphotericin b, 1% of gentamicin, 1% of sodium pyruvate solution, 10% of foetal bovine serum, and 85% ASW; (4) medium TGM4 (pH = 7.01) contained 1% of l-glutamine solution 200 mM, 1% of HEPES buffer solution 1M, 1% of gentamicin, 1% of penicillin streptomycin, 1% of sodium pyruvate solution, 10% of foetal bovine serum, 63% of DMEM/F-12[HAM], and 22% of ASW; (5) medium TGM5 (pH = 7.14) contained 1% of l-glutamine solution 200 mM, 1% of HEPES buffer solution 1M, 1% of penicillin streptomycin, 1% of sodium pyruvate solution, 20% of foetal bovine serum, 38% of DMEM/F-12[HAM], and 38% of ASW. All media were stored at 4 °C and were used within five days.

2.5. General Culture Conditions

Media were filtered (0.2 µm) before use, all glassware was autoclaved, and only sterilized plasticware was used. Fifteen experiments on primary cultures (cells growing in suspension) were performed and kept for 2 to 4 weeks, with each containing lumped *B. schlosseri* blood cells from 2–4 colonies (total 40 colonies) in blastogenic stages A–D. There were no discernible differences between cell types extracted from various blastogenic states, as shown in a previous study [32]. Extracted cells were evenly distributed into multiple 35 mm Petri dishes (Thermo Fisher Scientific Nunc, 171099; 0.5×10^6 cells/mL, 1.5×10^6 cells/dish) containing 3 mL of liquid medium and were incubated (Innova 42) at 20 °C under normal atmosphere conditions. The medium was changed every other day, where the contents of the dishes were collected into a 15 mL tube, washed 3 times with fresh medium, followed by centrifugation (1000× *g*, 10 min), and seeded to new dishes

with fresh medium. The criterion for experiment termination was $\leq$ 50% viability of total recovered cells.

2.6. Blood Cell Characterization

We used 12 colonies in blastogenic stages A–D (A = 3, B = 3, C = 3, and D = 3). Prior to cell extraction, each colony was photographed under a fluorescent microscope (Leica M205 FCA equipped with a camera Leica DFC9000 GT) using four channels (blue: Ex 325–378 nm, Em 438–485 nm; green: Ex 450–490 nm, Em 500–550 nm; red: Ex 540–580 nm, Em 593–667 nm; and far red: Ex 590–650 nm, Em 663–737 nm). Images were obtained using Leica software (LAS X version: 3.6.0.20104). Post cell extractions, the blood cells in ASW were imaged in 35 mm glass-bottom dishes (Ibidi, Cat. No. 81218) using a laser scanning confocal microscope (Zeiss LSM710) with four channels (blue: Ex 405 nm, Em 453 nm; green: Ex 488 nm, Em 536 nm; red: Ex 561 nm, Em 607 nm; and far red: Ex 633 nm, Em 697 nm), and images were obtained using Zen 2.3. The intensity of the fluorescent signal per cell was calculated using Fiji software (http://imagej.nih.gov/ij (accessed on 2 May 2023)). Each image was inverted to a grey scale followed by the measurement of the light signal emitted by cells in relative fluorescent units (RFU). For cell identification in cultures, primary cultures were sampled, and cells were stained with fluorescent dye Hoechst 33342 (Thermo Fisher, Cat. No. H3570). Then, cell pellets were washed with ASW (X3), followed by centrifugation at 1000$\times$ *g* for 10 min, and stained with DiD (Vybrant, V22887) for membrane labeling. Cell pellets were washed (three times) with ASW and centrifuged (1000$\times$ *g*, 10 min). The cells were then seeded on glass coverslips (coated with 0.01% Poly L Lysine solution, Sigma-Aldrich P4707, according to the manufacturer's instructions), and inserted at the bottoms of 12 well plates, left to adhere for approximately 4 h. Then, each well containing stained cells was fixed for 30 min with 4% paraformaldehyde (PFA; Thermo Fisher Scientific, Cat. No. 043368.9M) at room temperature, coverslips were mounted on cover glass slides, and cells were observed and photographed under an epifluorescence microscope (Zeiss AxioImager2, Zeiss, Oberkochen, Germany). Images were obtained using Zen 2.3.

2.7. Hematoxylin and Eosin (H&E) Staining

The H&E staining protocol was performed on blood cell samples that were seeded on glass coverslips (coated with 0.01% Poly L Lysine solution) at the bottoms of 12 well plates and left to adhere. Then, each well was fixed with 4% PFA. Mayer's Hematoxylin solution (Sigma; Cat. No. MHS32) and 0.5% Eosin Y-solution (Sigma, Cat. No. 1.09844.1000) were employed according to the manufacturer's instructions. Coverslips were mounted on cover glass slides, and cells were observed and photographed under an epifluorescence microscope (Zeiss AxioImager2) with bright field. Images were obtained using Zen 2.3.

2.8. Immunofluorescence Staining

Blood cell samples were seeded on glass coverslips that were inserted at the bottom of 12 well plates and coated with 0.01% Poly L Lysine solution and left to adhere. Each well was then fixed with 4% PFA. Then, samples were washed two times with PBS, were permeabilized in 0.1% Triton x-100 in PBS for 10 min at 4 °C, and were re-washed two times with 0.02% Tween-20 in PBS. Nonspecific binding sites were blocked by incubation in 5% bovine serum albumin diluted in 0.02% Tween-20 in PBS for 60 min at 4 °C. The samples were incubated overnight at 4 °C with primary anti PCNA (Proliferating Cell Nuclear Antigen) antibody developed against rabbit (Abcam, ab18197, 1:1000), washed with 0.02% Tween-20 in PBS (2 $\times$ 10 min), and incubated with secondary antibodies (Invitrogen, Alexa fluor 568 donkey anti rabbit, Cat. No. 10042, 1:1000) for 120 min at 4 °C. The samples were then washed twice with PBS, and the coverslips were mounted on slides using Fluoromount-G with DAPI (Invitrogen, Cat. No. 00-4959-52, Waltham, MA, USA). Negative controls for each experiment were established on coverslips that were incubated with blocking solution, lacking primary antibodies, and were exposed only to secondary

antibodies. Cover slides were photographed under an epifluorescence microscope (Zeiss AxioImager2). Images were obtained using Zen 2.3. The counting of total stained cells was conducted using Fiji software (http://imagej.nih.gov/ij (accessed on 2 May 2023)).

2.9. Statistics

Statistical analyses were conducted on the cell types using SPSS V16. A one-way ANOVA test using a post hoc comparison (Bonferroni and Tukey HSD) was applied on the cell types, including $PCNA^+$ cells, at four time points (day 0, 24 h, days 3, 8).

3. Results

3.1. Identification of B. schlosseri Blood Cell Populations

Twelve *B. schlosseri* colonies (blastogenic stages: A = 3, B = 3, C = 3, D = 3) were used. Prior to cell extractions, the natural auto-fluorescence of the blood cell emissions was evaluated by capturing images of each colony using blue, green, red, and far-red channels under a fluorescent microscope. The *B. schlosseri* tunic matrix did not autofluoresce. However, distinct blood cell populations emitted varying levels of natural fluorescence (Figure 1). This prompted us to use confocal microscopy to examine in detail the extracted blood cells (Figure 2) and to calculate fluorescence intensity. The results showed that haemoblasts (n = 12 cells; Figure 2(a1–5)) exhibited fluorescence intensity values of 0, 32.6 ± 16.4, 59.7 ± 14.07, and 141.16 ± 32.3 [RFU] for blue, green, red, and far-red channels, respectively. Morula cells (n = 28; Figure 2(b1–5)) showed fluorescence intensity values of 2.1 ± 1.02, 17.01 ± 20.7, 46.7 ± 19.8, and 61.3 ± 34.8 RFU for blue, green, red, and far-red channels, respectively. Pigment cells (n = 15; Figure 2(c1–5)) showed fluorescence intensity values of 4.3 ± 1.3, 22.4 ± 10.2, 19.3 ± 4.6, and 40.92 ± 12.01 RFU for blue, green, red, and far-red channels, respectively. Nephrocytes (n = 17; Figure 2(d1–5)) exhibited fluorescence intensity values of 0.75 ± 0.9, 23.5 ± 9.95, 12.9 ± 4.3, and 45.3 ± 14.13 RFU for blue, green, red, and far-red channels respectively. In addition, we observed the following two life stages of thraustochytrid cells that were present in the cultures: mononucleated cells (n = 4; Figure 2(e1–5)) that showed fluorescence intensity values of 4.46 ± 5.1, 4.11 ± 6.45, 22.28 ± 7.97, and 2.93 ± 3.51 RFU for blue, green, red, and far-red channels, respectively, and sporangia cells (n = 9; Figure 2(f1–5)) that showed fluorescence intensity values of 4.6 ± 3.85, 29.5 ± 14.35, 18.25 ± 5.04, and 9.3 ± 10.54 RFU for blue, green, red, and far-red channels, respectively.

Isolated haemocytes of *B. schlosseri* (from blastogenic A–D colonies) and thraustochytrids were subsequently identified using histological (H&E), nuclear (Hoechst), and membrane (DiD) staining (Figures 3–5). The haemoblasts (4–6 μm; Figure 3a,b) are small, brown, spherical blood cells. They contain a circular nucleus positioned in the middle of the cell and a nucleolus (often non-recognizable) stained (Figure 4a) in blue while surrounded by a thin layer of basophilic cytoplasm (Figure 5a–d). The macrophage-like cells (10–20 μm) are large ameboid phagocytes, marked (using H&E) as orange, red, pink, and blue colored cells, with an elliptical nucleus at the periphery of the cell and recognizable brown, yellow, and black vacuoles (Figure 3c,d) that occupy most of the cells' volumes (Figures 4b and 5e–h). Morula cells (7–11 μm) are spherical in shape, with a barely recognizable small nucleus (2 μm) situated at the periphery of the cell, possessing diverse brown, yellow, and black vacuoles (Figure 3e,f). H&E stained these cells in orange, pink, and blue colors, with recognizable vacuoles that occupy most of the cell volume (Figures 4c and 5i–l). Granular amoebocyte cells (7–15 μm) are oval shaped, containing a small nucleus (2 μm) and micro/macro granules, with vacuoles stained with orange, pink, red, and blue colors (Figures 4d and 5m–p). The pigment cells (7–20 μm) are spherical and granular in appearance and contain brown granules of varying sizes, with colors diverged from dark blue or brown to black that exhibit Brownian motion (Figure 3g,h). These cells were stained (H&E) as pink, red, and blue colors, and the round nucleus appeared in different positions within the cell (Figures 4e and 5q–t). The nephrocytes (7–20 μm) share similar staining character-

istics as pigment cells. These cells also contain brown and yellow granules (Figure 3i,j), exhibiting (H&E) pink and blue colors (Figures 4f and 5u–x).

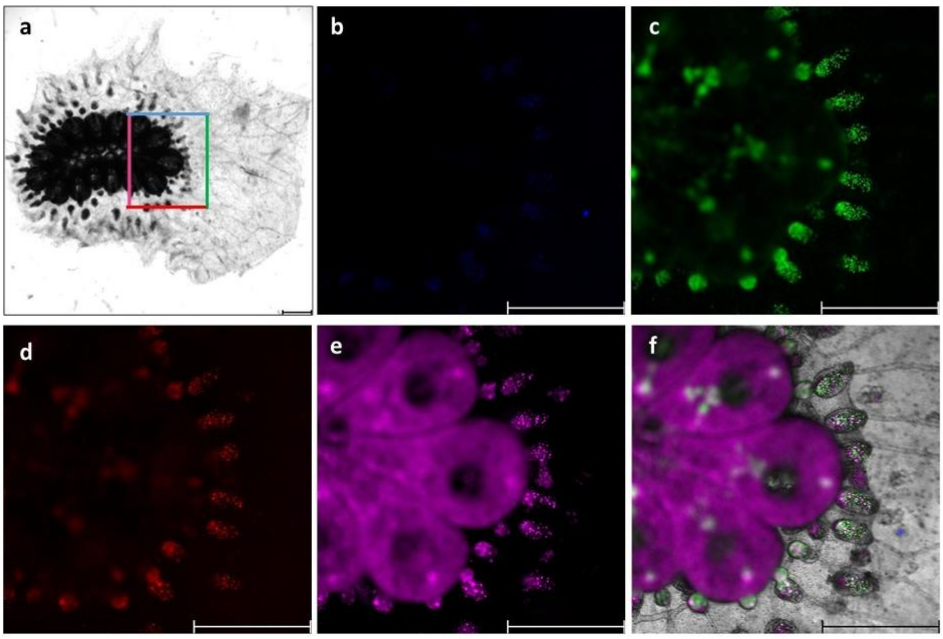

Figure 1. *B. schlosseri* whole-colony autofluorescence (**a**–**f**) performed on a single system with 15 zooids from a colony at blastogenic stage A: (**a**) Bright field image: the colored rectangle indicates the same highly magnified colonial area imaged under four channels (**b**–**e**); (**b**) the blue channel; (**c**) the green channel; (**d**) the red channel; (**e**) the far-red channel; (**f**) a merged high magnification area of a bright field image with the four channels. Scale bars: 1 mm.

We further observed two distinct life stages of thraustochytrid cells, which may appear as cell clusters after nine days in vitro. The mononucleated cells (4–10 µm) are spherical and smooth, containing a small, hardly recognizable nucleus (1–2 µm). These cells appeared in clusters (up to 200 µm) composed of at least 3 cells, each. H&E staining marked the cell cytoplasm as pink, and the membranes surrounding the cell were stained with a thin blue line (Figure 4g). The sporangia cells (10–200 µm) are dark brown, spherical cells with a grainy texture and a barely recognizable nucleus. Stained (H&E) cells appeared pink with patterns of blue (Figure 4i).

3.2. Cultivation of B. schlosseri Blood Cells

We used 40 *B. schlosseri* colonies (7–15 zooids per colony) in blastogenic stages A–D (Table 1). Blood cells were extracted from the marginal ampullae and washed with one of three types of washing solutions (WS1, WS2, WS3; Table 1), were subjected to specific antibiotic combinations, and were then cultured in suspensions of one of five medium variants (TGM1- TGM5). In total, 15 experiments were carried out with high cell viability observed at onset (87.08–94.65%; Table 1). Cultures were observed every second day for up to 26 days (Figure 6), and four time points (0, 24 h, days 3, 8) were specifically studied by counting cell types (Figure 7) and assessing the proliferative status using PCNA (Figures 8 and S1–S4).

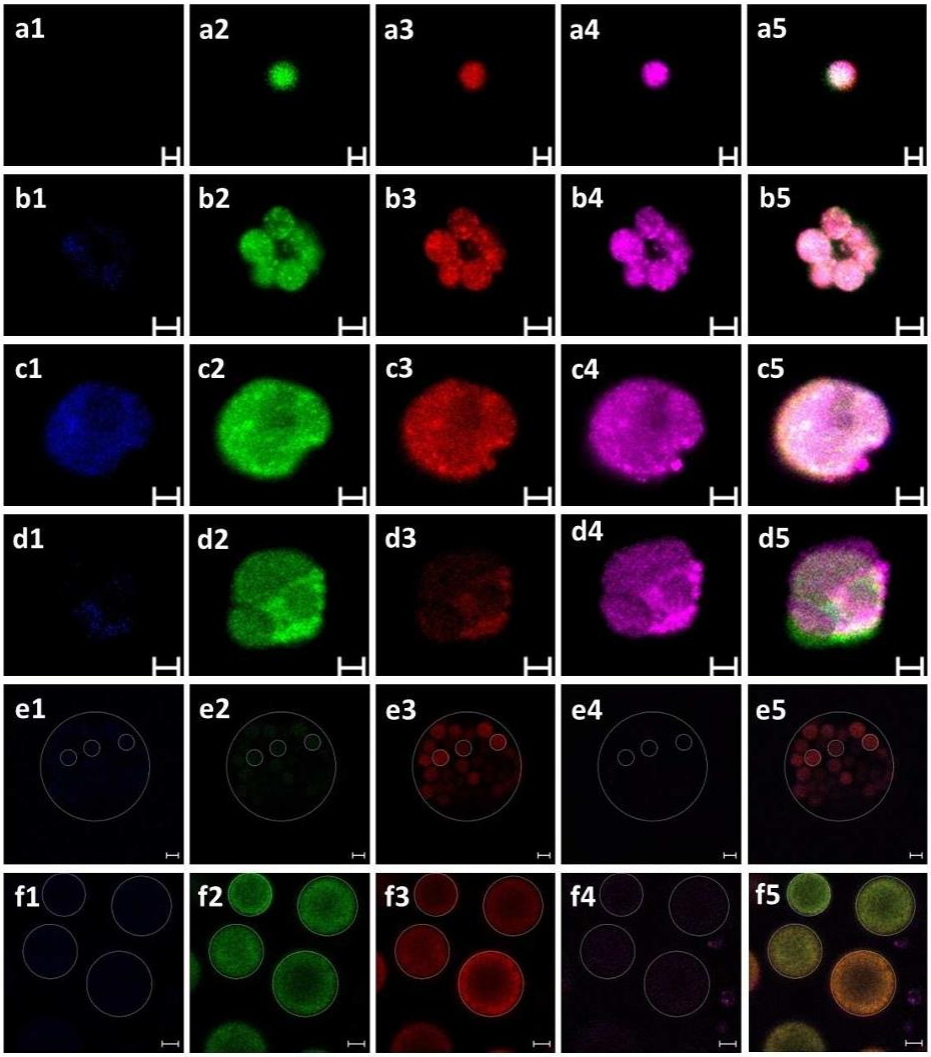

Figure 2. Confocal autofluorescence images in *B. schlosseri* and thraustochytrid cells at various wavelengths. (**a1**–**a5**) A hemoblast, an undifferentiated cell: blue (**a1**), green (**a2**), red (**a3**), and far-red (**a4**). (**a5**) Merged image of four channels. (**b1**–**b5**) A morula cell (immunocyte cell, belongs to the cytotoxic lineage): blue (**b1**), green (**b2**), red (**b3**), and far-red (**b4**). (**b5**) Merged image of four channels. (**c**–**d**) Storage cells. (**c1**–**c5**) Pigment cell: blue (**c1**), green (**c2**), red (**c3**), and far-red (**c4**). (**c5**) Merged image of four channels. (**d1**–**d5**) A nephrocyte cell: blue (**d1**), green (**d2**), red (**d3**), and far-red (**d4**). (**d5**) Merged image of four channels. (**e**,**f**) Thraustochytrid cells. (**e1**–**e5**) Clump of single multinucleated cells (dashed circle): blue (**e1**), green (**e2**), red (**e3**), and far-red (**e4**). (**e5**) Merged image of four channels. (**f1**–**f5**) Sporangia cells (dashed circles): blue (**f1**), green (**f2**), red (**f3**), and far-red (**f4**). (**f5**) Merged image of four channels. Scale bars: 2 μm in a, 5 μm in (**b**–**d**), 20 μm in (**e**,**f**).

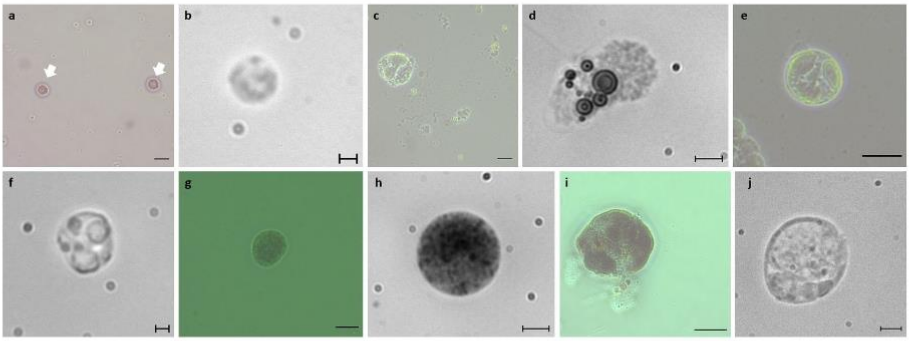

Figure 3. *B. schlosseri* live circulating blood cells under the microscope. (**a**,**b**) Haemoblasts: (**a**) two haemoblasts (arrows) under a bright field (**b**). (**c**,**d**) Macrophage-like cells: macrophage-like cell (**c**) under bright field (**d**). (**e**,**f**) Morula cells: morula cell (**e**) under a bright field (**f**). (**g**,**h**) Pigment cell: pigment cell imaged under the microscope (**g**) with a bright field (**h**). (**i**,**j**) Nephrocyte cell: nephrocyte cell imaged under the microscope (**i**) with a bright field (**j**). Scale bars: 2 μm in (**b**,**f**), 5 μm in (**d**,**h**,**j**), 10 μm in (**a**,**c**,**e**,**g**,**i**).

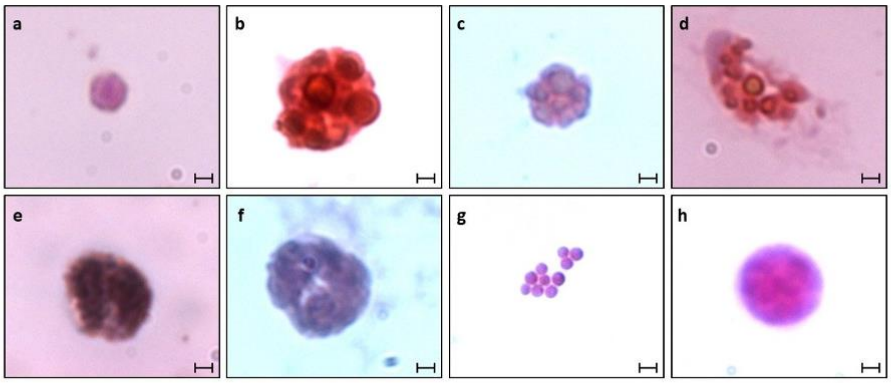

Figure 4. Haemocytes of *B. schlosseri* and thraustochytrid cells fixed and then stained with H&E: (**a**) hemoblast cell; (**b**) macrophage-like cell; (**c**) morula cell; (**d**) granular amebocyte cell; (**e**) pigment cell; (**f**) nephrocyte cell; (**g**,**h**) thraustochytrid cells, (**g**) mononucleated cell cluster composed of ten individual cells and (**h**) sporangium cell. Scale bars: 2 μm in (**a**–**f**,**h**), 5 μm in (**g**).

3.2.1. Primary Cultures: TGM1 Medium

Cell cultures were studied for 18 days (Figure 6a) with minimal contamination events (just two [fungi] out of 17 plates, on days 12 and 14; Figure S5f). At the beginning of the study (day 0; Figure S5a), one-way ANOVA revealed significant differences ($p < 0.001$) in the distributions of cell types, which formed three distinct groups (morula; macrophage-like and pigment cells; granular amoebocyte cells; Figure 7a) and four distinct groups of $PCNA^+$ cell dispersals (haemoblasts and macrophage-like cells; morula and pigment cells; nephrocyte cells; granular amoebocyte cells; Figures 8a and S1a). The same three groups of cell types ($p < 0.001$; Figure 7f) were recorded at the 24 h, 3 day, and 8 day time points (Figures 7k,p and S5b), while non-significant differences ($p > 0.05$) were recorded in $PCNA^+$ cell distributions at the 24 h time point (Figure S2a), $PCNA^+$ haemoblasts and macrophage-like cells (36.8 ± 0.58% and 28.9 ± 3.3%, respectively; Figure 8f) outnumbered other cell types. At day three (Figure S5c), we recorded different ($p < 0.001$) $PCNA^+$ cell distributions (haemoblasts and macrophage-like cells; nephrocyte cells; pigment cells; gran-

ular amoebocyte cells; Figures 8k and S3a). At day 8 (Figure S5d), only two distinct groups of $PCNA^+$ cells were recorded ($p < 0.05$; haemoblasts and nephrocyte cells, respectively; Figures 8p and S4a). Cell viability was reduced from >90% to 84% at days 4–6 (Figure 6f), with cell populations dominated by macrophage-like cells, morula cells, and pigment cells. From day 10 to 18, cell numbers further decreased from $1.99 \times 10^6 \pm 0.14$ to $0.83 \times 10^6 \pm 0.16$ cells/mL^{-1}, respectively, and a sharp decline of viability was observed from 78.17% ± 1.5 to 48.4% ± 0.8, respectively (Figures 6a,f and S5e).

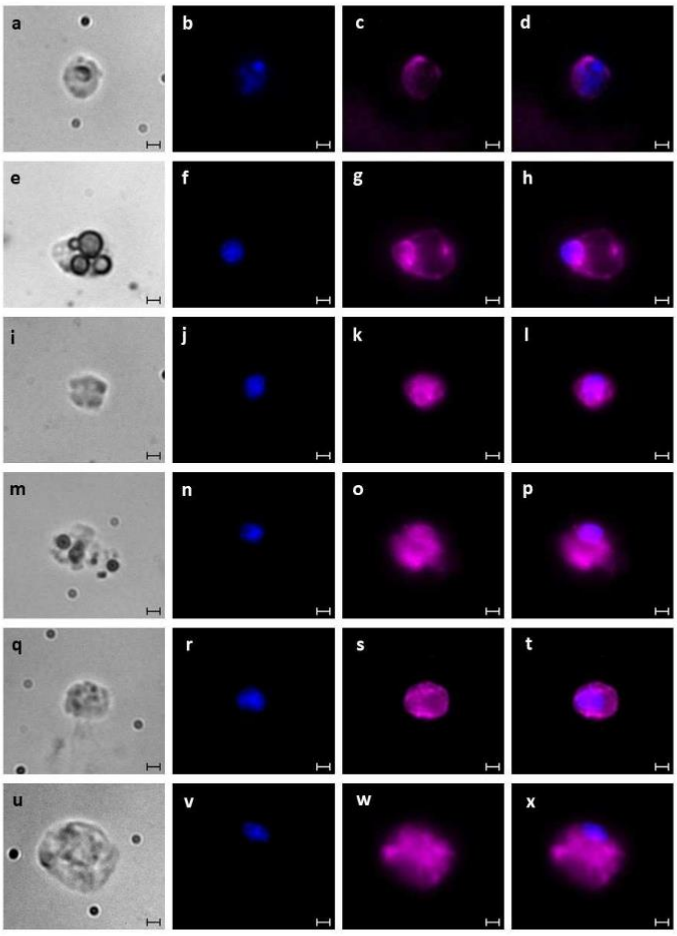

Figure 5. Haemocytes of *B. schlosseri* stained with Hoechst and DiD upon fixation. (**a**–**d**) Haemoblast cell imaged with bright field channel (**a**) stained with Hoechst (**b**) and DiD (**c**); (**d**) merged image. (**e**–**h**) Macrophage-like cell imaged with bright field channel (**e**) stained with Hoechst (**f**) and DiD (**g**); (**h**) merged image. (**i**–**l**) Morula cell imaged with a bright field channel (**i**) stained with Hoechst (**j**) and DiD (**k**); (**l**) merged image. (**m**–**p**) Granular amoebocyte cell imaged with a bright field channel (**m**) stained with Hoechst (**n**) and DiD (**o**); (**p**) merged image. (**q**–**t**) Pigment cell imaged with a bright field channel (**q**) stained with Hoechst (**r**) and DiD (**s**); (**t**) merged image. (**u**–**x**) Nephrocyte cell imaged with a bright field channel (**u**) stained with Hoechst (**v**) and DiD (**w**); (**x**) merged image. Scale bars = 2 μm.

Table 1. Yields of *B. schlosseri* blood cell extractions and cell viability in the five media formulations. WS = washing solution.

Medium	Exp. No.	No. of Colonies	Blastogenic Stage	No. of Zooids	WS Type	Cell Numbers/Exp n ± STD ($\times 10^6$)	Viability ± STD (%)
TGM1	1	2	A, D	7, 11	WS1	3.1 ± 1.1	92.1 ± 1.3
	2	3	B, C, D	12, 8, 8		2.9 ± 1.4	92.3 ± 2.7
	3	4	A, C, C, D	15, 10, 8, 12		2.7 ± 0.7	90.4 ± 1.7
TGM2	1	3	B, C, D	10, 11, 9	WS1	2.6 ± 0.6	88.6 ± 2.9
	2	3	C, C, D	8, 8, 12		2.1 ± 0.9	91.6 ± 4.9
TGM3	1	2	A, D	14, 11	WS2	1.3 ± 0.33	94.6 ± 2.0
	2	2	B, D	8, 10		2.4 ± 0.9	87.1 ± 6.4
	3	3	B, C, D	7, 8, 12		2.1 ± 1.2	90.8 ± 2.9
TGM4	1	2	A, C	11, 12	WS3	2.8 ± 0.9	91.1 ± 1.4
	2	2	B, C	10, 7		2.3 ± 0.5	92.8 ± 1.3
	3	2	C, D	15, 10		3.2 ± 0.9	90.7 ± 6.7
	4	3	C, C, D	11, 8, 12		2.8 ± 1.0	89.9 ± 6.2
TGM5	1	3	C, C, D	7, 8, 10	WS1	1.6 ± 0.6	94 ± 3.4
	2	3	B, C, D	10, 12, 15		2.6 ± 1.4	91.9 ± 4.0
	3	3	A, D, D	11, 8, 7		2.2 ± 1.0	94.3 ± 1.6

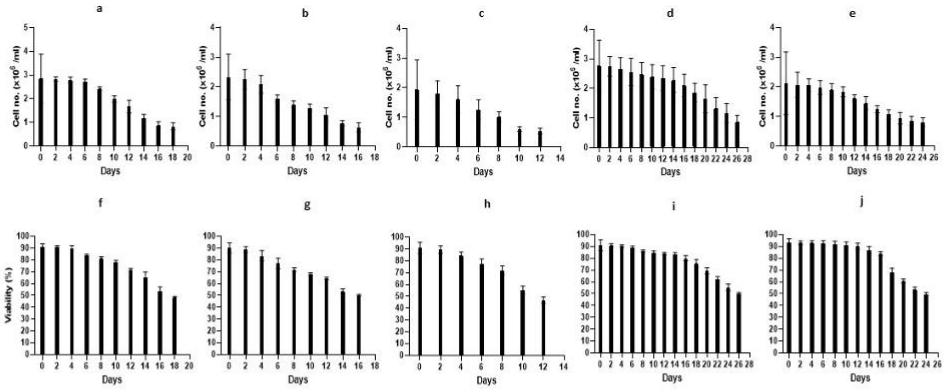

Figure 6. Cell numbers (10^6 cells/mL) and cell viability (%) of primary cultures in the five media versions, up to 26 days from onset. Cell numbers in medium TGM1 (**a**), TGM2 (**b**), TGM3 (**c**), TGM4 (**d**), and TGM5 (**e**). Cell viabilities in medium TGM1 (**f**), TGM2 (**g**), TGM3 (**h**), TGM4 (**i**), and TGM5 (**j**).

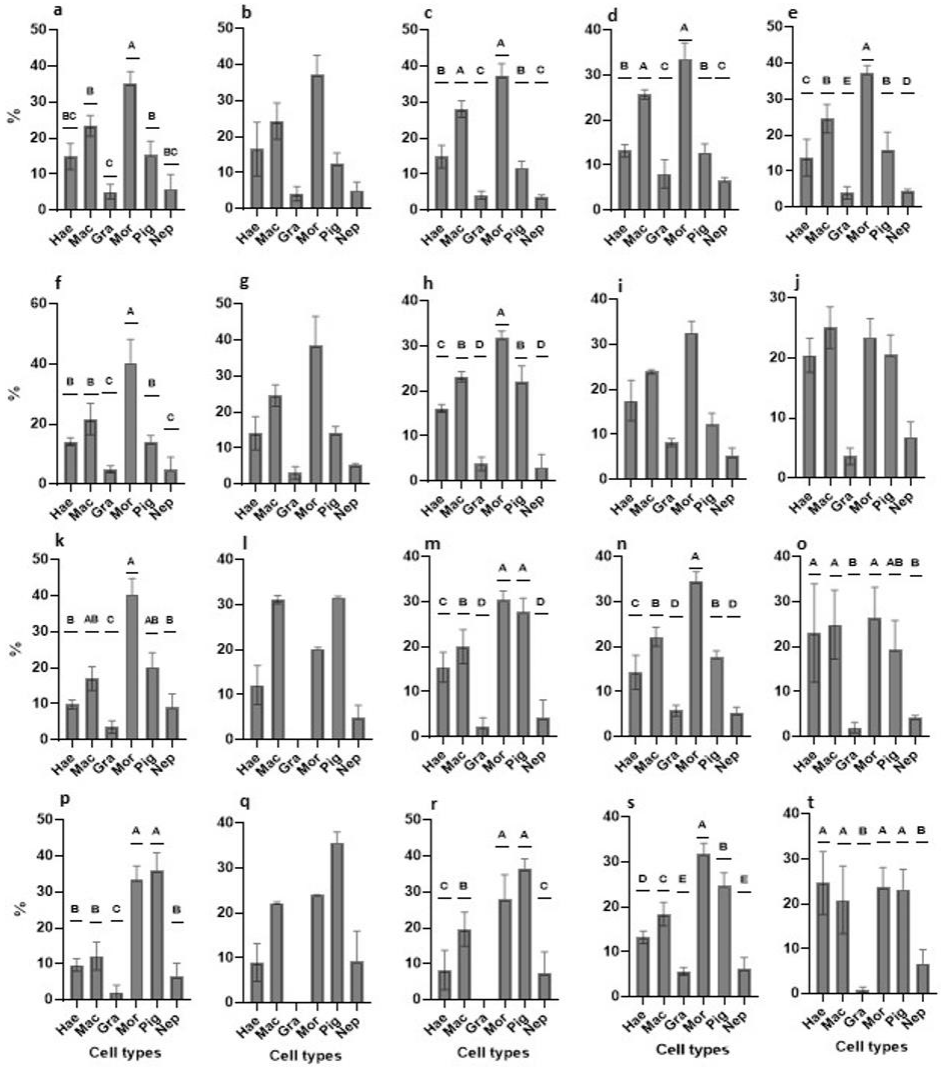

Figure 7. Distributions (%) of cell types in *B. schlosseri* primary cultures under five media types at onset, 24 h, 3 days, and 8 days from initiation. (**a**–**e**) At onset in medium TGM1 (**a**), TGM2 (**b**), TGM3 (**c**), TGM4 (**d**), and TGM5 (**e**). (**f**–**j**) At 24 h in medium TGM1 (**f**), TGM2 (**g**), TGM3 (**h**), TGM4 (**i**), and TGM5 (**j**). (**k**–**o**) at day 3 in medium TGM1 (**k**), TGM2 (**l**), TGM3 (**m**), TGM4 (**n**), and TGM5 (**o**). (**p**–**t**) At day 8 in medium TGM1 (**p**), TGM2 (**q**), TGM3 (**r**), TGM4 (**s**), and TGM5 (**t**). Hae, haemoblasts; Mac, macrophage-like cells; Gra, granular amoebocytes; Mor, morula cells; Pig, pigment cells; Nep, nephrocytes. Capital letters above bars represent statistically different groups ($p < 0.001$, $p < 0.05$; one-way ANOVA using post hoc comparison Bonferroni and Tukey HSD).

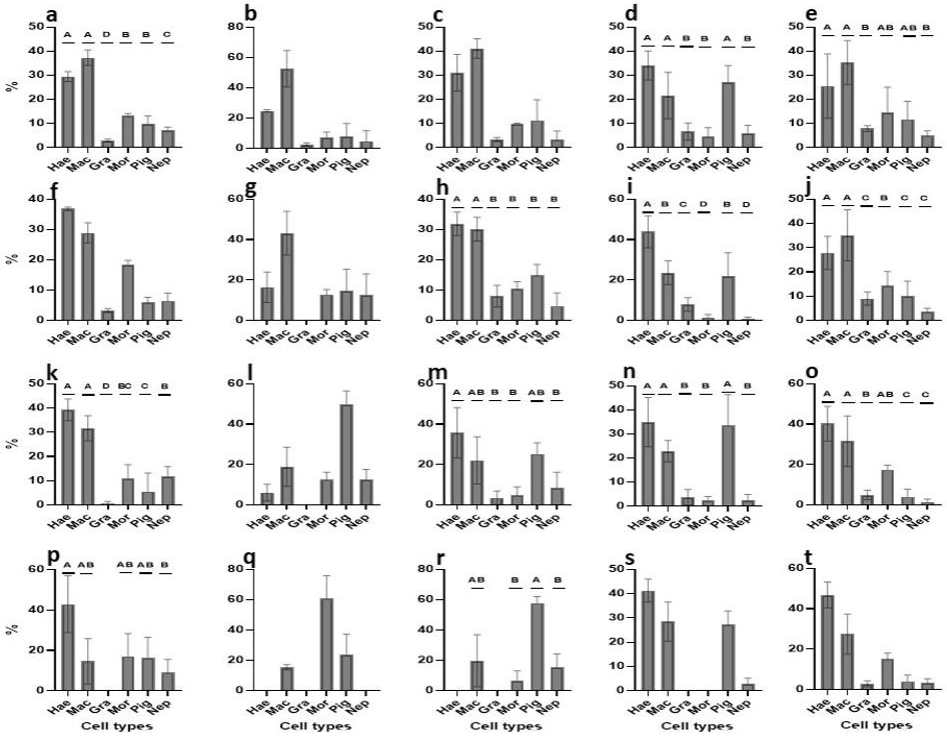

Figure 8. Distributions (%) of PCNA$^+$ cells in *B. schlosseri* primary cultures under five media conditions at onset, 24 h, 3 days, and 8 days from initiation. (**a**–**e**) At onset in medium TGM1 (**a**), TGM2 (**b**), TGM3 (**c**), TGM4 (**d**), and TGM5 (**e**). (**f**–**j**) At 24 h in medium TGM1 (**f**), TGM2 (**g**), TGM3 (**h**), TGM4 (**i**), and TGM5 (**j**). (**k**–**o**) At day 3 in medium TGM1 (**k**), TGM2 (**l**), TGM3 (**m**), TGM4 (**n**), and TGM5 (**o**). (**p**–**t**) At day 8 in medium TGM1 (**p**), TGM2 (**q**), TGM3 (**r**), TGM4 (**s**), and TGM5 (**t**). Hae, haemoblasts; Mac, macrophage-like cells; Gra, granular amoebocytes; Mor, morula cells; Pig, pigment cells; Nep, nephrocytes. Capital letters above bars refer to statistically different groups ($p < 0.001$, $p < 0.05$; one-way ANOVA using post hoc comparison Bonferroni and Tukey HSD).

3.2.2. Primary Cultures: TGM2 Medium

Cell cultures were studied for 16 days (Figure 6b), with minimal contamination events (three [×2 thraustochytrids, yeasts] out of nine plates, on days 10 and 14; Figure S6f,g). No significant difference ($p > 0.05$; one-way ANOVA) was recorded at the beginning (day 0; Figure S6a) in the distributions of cell types with macrophage-like cells and morula cells (24.4 ± 5.05% and 37.3 ± 5.3%, respectively), comprising >61% of total cell numbers (Figure 7b) and macrophage-like cells portrayed 52.56 ± 11.9% of all PCNA$^+$ cells ($p > 0.05$, one-way ANOVA; Figures 8b and S1b). At the 24 h time point, morula cells and macrophage-like cells continued as the most abundant (>64%) cell types (24.6 ± 3% and 38.6 ± 8%, respectively; Figure 7g; $p > 0.05$, Figure S6b), while macrophage-like cells were the most abundant cell type, with a reduction from onset (43.2 ± 10.9%) of all PCNA$^+$ cells ($p > 0.05$; Figures 8g and S2b). At day three, macrophage-like cells, morula cells, and pigment cells (31.15 ± 0.95%, 20.23 ± 0.3%, and 31.62 ± 0.28%, respectively; $p > 0.05$, Figure S6c) comprised >83% of total cell numbers (Figure 7l), while macrophage-like cells, morula cells, and pigment cells (18.9 ± 9.7%, 12.4 ± 3.9%, and 49.9 ± 6.6%, respectively) comprised >81% of all PCNA$^+$ cells ($p > 0.05$; Figures 8l and S3b). At day 8

($p > 0.05$; one way ANOVA; Figure S6d), macrophage-like cells, morula cells, and pigment cells (22.19 ± 0.27%, 23.94 ± 0.1%, and 35.66 ± 2.3%, respectively) comprised >81% of total cell numbers (Figure 7q), and macrophage-like cells, morula cells, and pigment cells (15.48 ± 1.7%, 60.71 ± 15.2%, and 23.8 ± 13.47%, respectively) comprised >99% of all $PCNA^+$ cells ($p > 0.05$, one-way ANOVA; Figures 8q and S4b). Cell viability values were reduced from >83% to 77% at days 4–6 (Figure 6g), with cell populations dominated by haemoblasts, macrophage-like cells, morula cells, and pigment cells. From days 10 to 16, cell numbers further decreased from $1.29 \times 10^6 \pm 0.13$ to $0.63 \times 10^6 \pm 0.15$ cells/mL^{-1}, and a sharp decline of viability was observed, from 68.15% ± 1.1 to 50.2% ± 0.7 (Figures 6b,g and S6e).

3.2.3. Primary Cultures: TGM3 Medium

Cell cultures were studied for 12 days (Figure 6c) with no contamination. At day 0 (Figure S7a), significant differences ($p < 0.001$; one-way ANOVA) were recorded in cell type distributions that formed three distinct groups (macrophage-like cells and morula cells; haemoblasts and pigment cells; nephrocytes and granular amoebocyte cells; Figure 7c), while non-significant differences ($p > 0.05$) obtained for $PCNA^+$ cells with haemoblasts and macrophage-like cells (31.06 ± 7.7% and 41.11 ± 4.02%, respectively; Figures 8c and S1c). At the 24 h time point (Figure S7b), cell type distributions formed four distinct groups ($p < 0.001$; morula cells; macrophage-like and pigment cells; haemoblasts; granular amoebocyte and nephrocyte cells; Figure 7h) and two distinct groups of $PCNA^+$ cells (haemoblasts and macrophage-like cells; granular amoebocyte, morula, pigment, and nephrocyte cells; Figures 8h and S2c). At day three (Figure S7c), we recorded four distinct cell groups ($p < 0.001$; morula and pigment cells; macrophage-like cells; haemoblasts; granular amoebocyte and nephrocyte cells; Figure 7m) and two distinct $PCNA^+$ cell groups (haemoblasts; granular amoebocyte cells, morula cells, and nephrocyte cells; Figures 8m and S3c). At day 8 (Figure S7d), we recorded three cell type groups (morula and pigment cells; macrophage-like cells; haemoblasts and nephrocyte cells; $p < 0.001$; Figure 7r) and two distinct $PCNA^+$ groups (pigment cells; morula and nephrocyte cells; $p < 0.05$; Figures 8r and S4c). Along this period, the only observed change in cellular morphologies was of storage cells (pigment and nephrocyte cells) that transformed from oval to elongate structures, which is consistent with the results of Rinkevich and Rabinowitz [29]. Cell viability was reduced from >84% to 77% at days 4–6 (Figure 6h), with cell populations dominated by haemoblasts, macrophage-like cells, morula cells, and pigment cells. From day 10 to 12, cell numbers further decreased from $0.6 \times 10^6 \pm 0.1$ to $0.53 \times 10^6 \pm 0.1$ cells/mL^{-1}, and a sharp decline of viability was observed from 55.3% ± 3.4 to 46.8% ± 2.7 (Figures 6c,h and S7e).

3.2.4. Primary Cultures: TGM4 Medium

Cell cultures were studied for 26 days (Figure 6d) with no contamination. At day 0 (Figure S8a), we recorded three distinct cell groups ($p < 0.001$; macrophage-like cells and morula cells; haemoblasts and pigment cells; granular amoebocyte and nephrocyte cells; Figure 7d) and two $PCNA^+$ cell groups ($p < 0.001$; haemoblasts, macrophage-like cells, and pigment cells; granular amoebocyte, morula, and nephrocyte cells; Figures 8d and S1d). At the 24 h time point (Figure S8b), no difference ($p > 0.05$) was recorded in cell type distributions, with haemoblasts, macrophage-like cells, and morula cells (17.58 ± 4.5%, 23.9 ± 0.5%, and 32.6 ± 2.5% respectively) comprising >74% of total cell numbers (Figure 7i), and four significant ($p < 0.001$) $PCNA^+$ cell groups (haemoblasts; macrophage-like cells and pigment cells; granular amoebocyte, morula, and nephrocyte cells; Figures 8i and S2d) were recorded. Day three results (Figure S8c) revealed a significant difference ($p < 0.001$) in cell type distributions compared to the onset and in $PCNA^+$ cells when compared to 24 h. Four distinct cell groups (morula cells; macrophage-like cells and pigment cells; haemoblasts; granular amoebocyte and nephrocyte cells; Figure 7n) and two distinct $PCNA^+$ groups (haemoblasts, macrophage-like cells, and pigment cells; granular amoebocyte, morula, and nephrocyte cells; Figures 8n and S3d) were present. At day 8 (Figure S8d),

five distinct ($p < 0.001$) cell type distributions were recorded (morula cells; pigment cells; macrophage-like cells; haemoblasts; granular amoebocyte and nephrocyte cells; Figure 7s), while there was no significant difference in $PCNA^+$ cell distributions. Haemoblasts, macrophage-like cells, and pigment cells (41.3 ± 4.7%, 28.5 ± 8.1%, and 27.2 ± 5.6%, respectively) comprised 97% of all cell types (Figures 8s and S4d). Cell viability remained stable (>90% to 89%) at days 4–6 (Figure 6i), with cell populations dominated by macrophage-like cells, morula cells, and pigment cells. From day 10 to 14, cell numbers and viability remained stable with values of $2.4 \times 10^6 \pm 0.42$, $2.3 \times 10^6 \pm 0.4$ cells/mL^{-1}, 84.9% ± 1.4, and 83.4% ± 1.3 (Figure 6d,i), and cell populations were dominated by haemoblasts, macrophage-like cells, morula cells, and pigment cells. From day 16 to 21, cell numbers decreased from $2.11 \times 10^6 \pm 0.4$ to $1.65 \times 10^6 \pm 0.5$ cells/mL^{-1}, accompanied by a sharp decrease of viability from 79.98% ± 2.3 to 69.4% ± 2.6 (Figure 6d,i). Cell populations were dominated by haemoblasts and pigment cells (Figure S8e). From day 22 to 26 (the fourth week), cell numbers further declined from $1.33 \times 10^6 \pm 0.4$ to $0.87 \times 10^6 \pm 0.2$ cells/mL^{-1}, accompanied by a sharp decrease of viability from 62.6% ± 1.8 to 50. 5% ± 0.8 (Figure 6d,i). Cell populations were dominated mostly by pigment cells (Figure S8f).

3.2.5. Primary Cultures: TGM5 Medium

Cell cultures were studied for 24 days (Figure 6e) with no contamination. At onset (day 0; Figure S9a), five distinct cell type groups ($p < 0.001$) were recorded (morula cells; macrophage-like cells, and pigment cells; haemoblasts; nephrocyte cells; granular amoebocyte cells; Figure 7e), as well as two distinct ($p < 0.001$) $PCNA^+$ cell groups (haemoblasts and macrophage-like cells; granular amoebocyte and nephrocyte cells; Figures 8e and S1e). After 24 h (Figure S9b), no distinct cell type distributions ($p > 0.05$) were recorded, with haemoblasts, macrophage-like cells, morula cells, and pigment cells (20.5 ± 2.8%, 25.1 ± 3.5%, 23.5 ± 3.1%, and 20.5 ± 3.3%, respectively) comprising >89% of the total cell types (Figure 7j), while three distinct groups ($p < 0.001$; haemoblasts and macrophage-like cells; morula cells; granular amoebocyte, pigment and nephrocyte cells; Figures 8j and S2e) were recorded in $PCNA^+$ cell distributions. At day three (Figure S9c), we recorded two distinct groups in cell distributions (haemoblasts, macrophage-like cells, and morula cells; granular amoebocyte and nephrocyte cells; $p < 0.05$; Figure 7o) and three distinct groups (haemoblasts and macrophage-like cells; granular amoebocyte cells; pigment and nephrocyte cells; $p < 0.001$; Figures 8o and S3e) in $PCNA^+$ cell distributions. At day 8 (Figure S9d), two significant groups (haemoblasts, macrophage-like cells, morula cells, and pigment cells; granular amoebocyte and nephrocyte cells; $p < 0.001$; Figure 7t) were found in cell distributions, and no significant difference ($p > 0.05$) in the distributions of $PCNA^+$ cells was observed, with haemoblasts, macrophage-like cells, and morula cells (46.9 ± 6.4%, 27.5 ± 9.9%, and 15.1 ± 3%, respectively) covering >89% of the total cells (Figures 8t and S4e). Cell viability values were stable (>93% to 92%) at days 4–6 (Figure 6j), with cell populations dominated by haemoblasts, macrophage-like cells, morula cells, and pigment cells. Cell numbers slightly decreased from day 10 to 14 ($1.83 \times 10^6 \pm 0.2$ to $1.5 \times 10^6 \pm 0.2$ cells/mL^{-1}), in concordance with a decrease in viability from 91.3% ± 2.5 to 86.9% ± 3.2 (Figure 6e,j), where cell populations were dominated by haemoblasts and pigment cells. From day 16 to 21, cell numbers further decreased from $1.24 \times 10^6 \pm 0.1$ to $0.94 \times 10^6 \pm 0.2$ cells/mL^{-1}, and a sharp decrease of viability was observed from 83.8% ± 2 to 60.7% ± 1.8 (Figure 6e,j), dominated by haemoblasts and pigment cells (Figure S9e). From day 22 to 24, cell numbers remained stable ($0.8-0.9 \times 10^6$ cells/mL^{-1}), with a decrease in cell viability from 53.4% to 49.6% (Figure 6e,j), dominated mostly by pigment cells (Figure S9f).

3.2.6. Cell Types Changes in Primary Cultures—An Overview

The distribution of cell types varied among the five media and changed over time (Figure 7, Table 2). Initially (day 0), cell type distribution profiles exhibited a high degree of similarity, with macrophage-like cells and morula cells being the predominant cell types in

all media. After 24 h, macrophage-like cells and morula cells remained the most prevalent cell types, but the presence of pigment cells (TGM3, TGM5) and haemoblasts (TGM5) was also observed. By day three, alterations in the distribution of cell types were noticed, with morula and pigment cells being more prominent in the TGM1 medium and macrophage-like cells and pigment cells being more prevalent in the TGM2 medium. However, the abundance profiles of TGM3, TGM4, and TGM5 media remained similar to that of the 24 h time point. By day 8, no further changes in cell type distribution were detected, and the abundance profiles of all five media were almost identical to those of day three.

Table 2. Major findings for primary cultures (cell type abundance and PCNA$^+$ cells) across the five media versions, ranging from the onset to 8-day-old cultures. Hae, haemoblasts; mac, macrophage-like cells; mor, morula cells; pig, pigment cells.

Medium	Major Outcomes	Onset	24-h	Day-3	Day-8
TGM1	abundant cell types	mac, mor	mac, mor	mor	mor, pig
	PCNA$^+$ cells	hae, mac	hae, mac, mor	hae, mac	hae
TGM2	abundant cell types	mac, mor	mac, mor	mac, mor, pig	mac, mor, pig
	PCNA$^+$ cells	hae, mac	mac	pig	mor
TGM3	abundant cell types	mac, mor	mac, mor, pig	hae, mac, mor, pig	mac, mor, pig
	PCNA$^+$ cells	hae, mac	hae, mac	hae, mac, pig	pig
TGM4	abundant cell types	mac, mor	hae, mac, mor	mac, mor, pig	hae, mac, mor, pig
	PCNA$^+$ cells	hae, mac, pig	mac, mor, pig	hae, mac, pig	hae, mac, pig
TGM5	abundant cell types	mac, mor	hae, mac, mor, pig	hae, mac, mor, pig	hae, mac, mor, pig
	PCNA$^+$ cells	hae, mac	hae, mac	hae, mac, mor	hae, mac, mor

3.2.7. Proliferation of Primary Cultures—An Overview

PCNA$^+$ activity varied among the five media and changed over time (Figure 8, Table 2). Initially, haemoblasts and macrophage-like cells were the most actively proliferating cell types in all media, with an additional case of PCNA$^+$ activity in pigment cells in the TGM4 medium. After 24 h, we observed PCNA$^+$ cell activity in haemoblasts, macrophage-like cells, and morula cells in TGM1 medium; macrophage-like cells in TGM2; haemoblasts and macrophage-like cells in TGM3; haemoblasts, macrophage-like cells, and pigment cells in TGM4; and haemoblasts and macrophage-like cells in TGM5. On day three, the most actively proliferating cell types varied by medium: haemoblasts and macrophage-like cells in TGM1; pigment cells in TGM2; haemoblasts in TGM3; haemoblasts and pigment cells inTGM4; and haemoblasts and macrophage-like cells in TGM5. By day 8, haemoblasts were the most actively proliferating cell types in TGM1, TGM4, and TGM5 media, while morula cells and pigment cells were the most actively proliferating in TGM2 and TGM3, respectively.

4. Discussion

The world's oceans and seas host hundreds of thousands of animal species, primarily invertebrates, with many types of cells that exhibit a wide range of cellular potentialities [38] and offer endless applications. Yet, while numerous cell lines have been commonly derived from vertebrates and terrestrial invertebrate taxa, e.g., insects and arachnids, until just recently [8], all efforts to develop cell lines from marine invertebrates have failed, despite many attempts made on a wide range of species over the past decades [1–3,6,7,9]. However, to establish a sustainable pharmaceutical industry based on the "blue economy", the most viable approach is (a) to cultivate cells under controlled conditions by creating widespread "cell factories", still an unaccomplished objective, and (b) to scale up cell cultures from marine organisms in bioreactors. These evolving stages not only allow the production of large supplies for any needed bioactive material, but they further make available the

manufacturing of a diverse range of novel industrial bioproducts. The recent advances in marine invertebrate cell culture methodology supported by the development of optimized nutrient media for primary sponge cell cultures [4,13] have led the authors to establish long-lasting cultures from several species of sponges [8,13], revealing the importance of fine-tuning the medium for successful outcomes.

Following the importance of *B. schlosseri* as a model system in a wide range of biological disciplines [14,15,39–41] and the need to develop approved in vitro methodologies for its research (general approach in Rinkevich and Rabinowitz [36]), several research endeavors have centered around obtaining high cell yields; identifying appropriate factors for proper cell adhesion and attachment [30,32]; initiating primary cell cultures from embryos, epithelial cells, and circulating blood cells [10,17,18,29]; revealing impacts of media additives on primary cultures, such as growth factors [29]; establishing a defined medium for circulatory blood cells [29]; and evaluating the in vitro delayed stemness of extirpated colonial organs, including the emerged stemness signatures in epithelial monolayers [30,34,35]. All attempts to establish a proliferating cell line from *Botryllus* cells have been unsuccessful, with cells dividing for a brief period of 24–72 h post extraction [1,2,17,29]. To address these difficulties, the present study aimed to investigate the response of various types of *B. schlosseri* blood cells (haemoblasts, macrophage-like cells, granular amoebocytes, morula cells, pigment cells, and nephrocytes; [42,43]) under in vitro conditions. Specifically, we assessed cell type-specific responses using five versions of a basic medium during the initial few crucial weeks after initiation. To aid in cell identification, we utilized confocal microscopy and the differential autofluorescence of various cells, which provided new insights. For example, our findings indicated that haemoblasts were the only *B. schlosseri* cell type to exhibit a high signal in the far-red channel, which distinguishes them from thraustochytrid cells, and they lacked blue channel fluorescence, supporting Rinkevich and Rabinowitz's [29] previous findings.

The different media variations (TGM1-TGM5) had distinct impacts on primary cultures of *B. schlosseri* blood cell types, reflected as changes in cell proliferation, viability, and dominant cell types. With regards to cell proliferation, we showed that the medium may stimulate the proliferation of distinct circulating cell types at different time points (onset, 24 h, 3 days, and 8 days). During this period, abundant cell types (haemoblasts, macrophage-like cells, morula cells, and pigment cells) exhibited varying activity patterns in different media that were formulated with varying proportions of basal media and ASW. For instance, TGM1 and TGM2 contained DMEM F12/HAM and RPMI, respectively, without ASW, whereas TGM3 contained ASW without DMEM F12/HAM or RPMI. In this study, cell proliferation was observed for at least 5 days, exceeding the documented 3 days post isolation in vitro barrier [2,29,32,37].

Similar to cell proliferation, differences in the viability of the cells were observed among the five media versions, resulting in variations in culture longevities. When setting 50% survivorship as a cut-off value, the TGM1, TGM2, and TGM3 cultures lasted for 18, 16, and 12 days, respectively, while TGM4 and TGM5 allowed for longer cultivation periods of 26 and 24 days, respectively. These findings align with those of [44], who conducted a short-term in vitro study on coral cells and found that cell viability decreased from 70% to 30% within the first week, as well as with Rinkevich and Rabinowitz [29], who studied *Botryllus* blood cell cultivation and observed a decrease in viability within four weeks. Rabinowitz and Rinkevich [30] reported shorter viability (7–9, 5–13, and 6–8 days) for *Botryllus* epithelial monolayers cultured with DMEM, RPMI, and HAM F12 on coated collagen 1 substrate. Regarding cellular morphology, this culturing criterion remained largely unchanged, except for the storage cells (pigment and nephrocyte cells) cultured in TGM3 medium (containing 85% ASW), which transformed from oval to elongated structures. This observation aligns with Rinkevich and Rabinowitz's [29] findings that pigment cells undergo changes in shape when exposed to high salt concentrations in the culture medium.

The distribution of cell types varied among the five media and underwent alterations over time, similar to cell proliferation and viability. In the first 24 h, macrophage-like cells and morula cells were the most abundant cell types in all tested media. By day 3, while the abundance profiles of TGM3 and TGM4 media remained similar to that of 24 h, morula and pigment cells were more prominent in TGM1 medium. At day 8, no further changes in cell type distribution were observed, and the abundance profiles of all five media were almost identical to those of day three. As mentioned above, to the best of our knowledge, no study has yet revealed the distribution of *Botryllus* blood cell types under prolonged in vitro conditions. Our results show that, at onset, macrophage-like cells, granular amoebocyte cells, and morula cells varied between 23 and 28% each, while granular amoebocyte and morula cells varied between 40 and42% each. These results are in line with the literature [29,43] regarding the distributions of granular amoebocyte and morula cells but not for macrophage-like cells.

The findings of this study demonstrate that altering the basic culture medium can cause varying growth and proliferation rates among different types of cells, as observed in our study of *Botryllus* blood cells. These findings align with a recent study on the cultivation of sponge cells [4] that followed up with studies documenting the enhancement of the quantity and viability of sponge cells [8,13]. The results of the present study thus demonstrate that it is possible to culture *Botryllus* blood cells in vitro for up to one month in a consistent and reliable manner. During the initial week of culture, there was a noticeable medium-dependent increase in the proliferation of distinct blood cell types (could be further supported by increased mortality in other cell types), which eventually led, within less than one month from initiation, to the development of medium-specific primary cultures. This discovery may pave the way for the creation of various cell cultures, each consisting of distinct cell types. Further, the aforementioned outcomes were reinforced by the ease with which cell types could be identified and classified based on their natural fluorescence patterns using confocal microscopy, an additional tool, for improved cell type identification in the development of cell cultures from *B. schlosseri* circulating blood cells. Compared to mammalian and insect cultures, this approach is novel to marine invertebrate cell cultures.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12131709/s1, Figure S1: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at onset; Figure S2: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 24 h from initiation; Figure S3: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 3 days from initiation; Figure S4: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 8 days from initiation; Figure S5: *B. schlosseri* primary blood cell cultures in medium TGM1; Figure S6: *B. schlosseri* primary blood cell cultures in medium TGM2; Figure S7: *B. schlosseri* primary blood cell cultures in medium TGM3; Figure S8: *B. schlosseri* primary blood cell cultures in medium TGM4; Figure S9: *B. schlosseri* primary blood cell cultures in medium TGM5; Table S1: Comparisons of media TGM1, TGM2, TGM3, TGM4, and TGM5 compositions.

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Supplementary Materials: Improved media formulations for primary cell cultures derived from a colonial urochordate

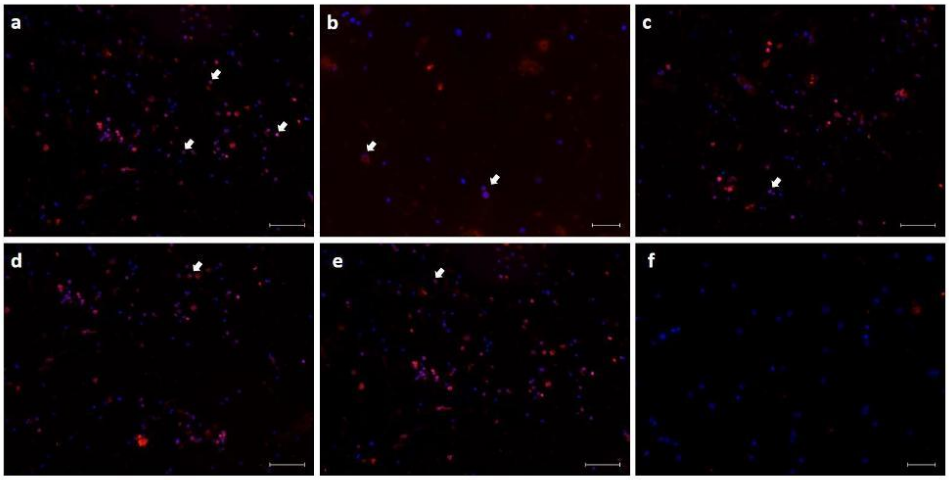

Figure S1: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at onset. a: TGM1. b: TGM2. c: TGM3. d: TGM4. e: TGM5. Blue dots are DAPI stained cell nuclei, red dots indicate PCNA$^+$ stained nuclei and pink cells (some marked with arrows) indicating PCNA$^+$ cells. f: negative control depict DAPI stained cell nuclei and unspecific staining of PCNA antibody. Scale bars: 20 μm in b and f, 50 μm in a, c, d and e.

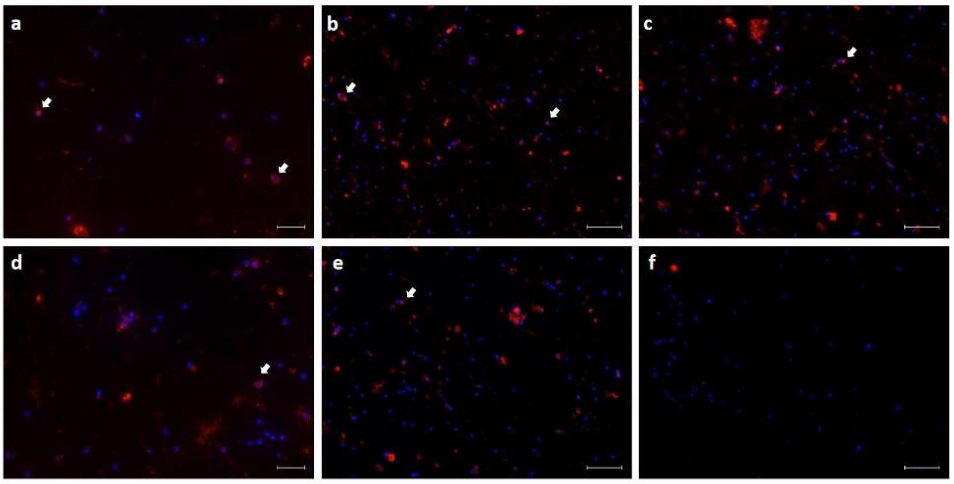

Figure S2: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 24 h from initiation. a: TGM1. b: TGM2. c: TGM3. d: TGM4. e: TGM5. Blue dots are DAPI stained cell nuclei, red dots indicate PCNA$^+$ stained nuclei and pink cells (some marked with arrows) indicating PCNA$^+$ cells. f: negative control depict

DAPI stained cell nuclei and unspecific staining of PCNA antibody. Scale bars: 20 μm in a and d, 50 μm in b, c, e and f.

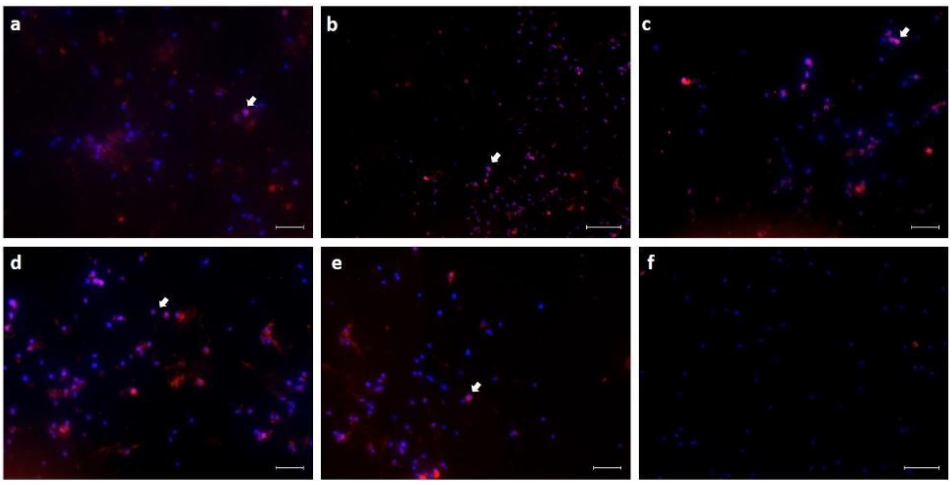

Figure S3: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 3 days from initiation. a: TGM1. b: TGM2. c: TGM3. d: TGM4. e: TGM5. Blue dots are DAPI stained cell nuclei, red dots indicate $PCNA^+$ stained nuclei and pink cells (some marked with arrows) indicating $PCNA^+$ cells. f: negative control depict DAPI stained cell nuclei and unspecific staining of PCNA antibody. Scale bars: 20 μm in a, c, d and e, 50 μm in b and f.

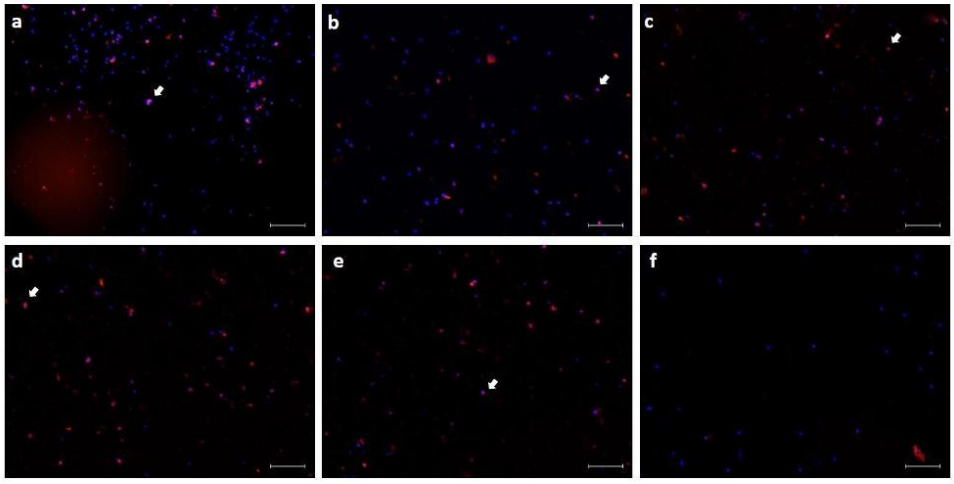

Figure S4: Immunofluorescence staining of *B. schlosseri* primary cultures under five media conditions at 8 days from initiation. a: TGM1. b: TGM2. c: TGM3. d: TGM4. e: TGM5. Blue dots are DAPI stained cell nuclei, red dots

indicate PCNA$^+$ stained nuclei and pink cells (some marked with arrows) indicating PCNA$^+$ cells. f: negative control depict DAPI stained cell nuclei and unspecific staining of PCNA antibody. Scale bars = 50 µm.

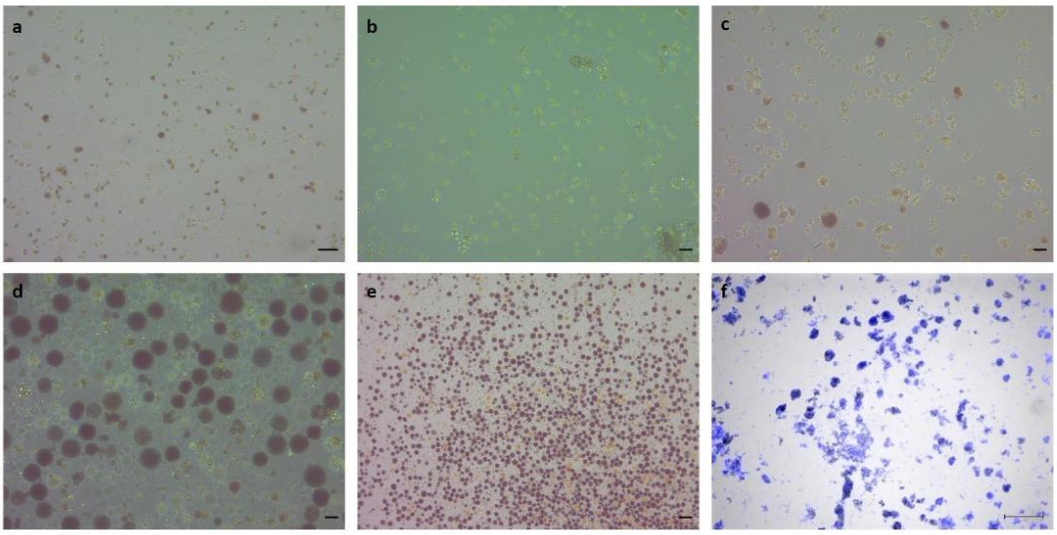

Figure S5: *B. schlosseri* primary blood cell cultures in medium TGM1: at onset (a), 24 h (b), days 3 (c), 8 (d) and 15 (e). f: fungi contamination at day 14 detected by calcofluor white staining. Scale bars: 10 µm in b, c and d, 20 µm in a, 80 µm in e, 100 µm in f.

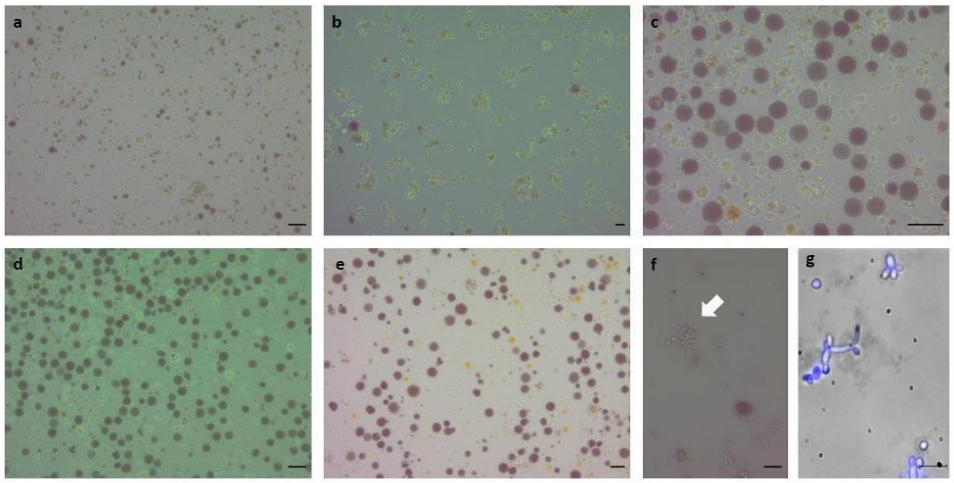

Figure S6: *B. schlosseri* primary blood cell cultures in medium TGM2: at onset (a), 24 h (b), days 3 (c), 8 (d) and 12 (e). Thraustochytrids contamination (arrow) at day 10 (f). g: yeast contamination at day 14 detected by calcofluor white staining. Scale bars: 10 μm in b, f and g, 20 μm in a and e, 40 μm in c and d.

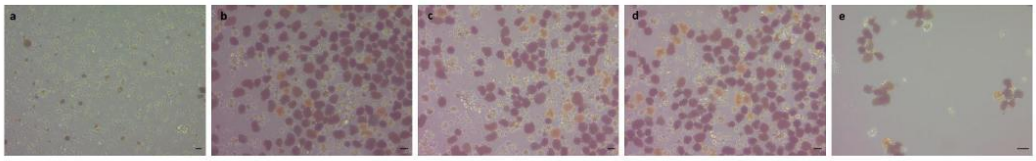

Figure S7: *B. schlosseri* primary blood cell cultures in medium TGM3: at onset (a), 24 h (b), days 3 (c), 8 (d) and 10 (e). Scale bars: 10 μm.

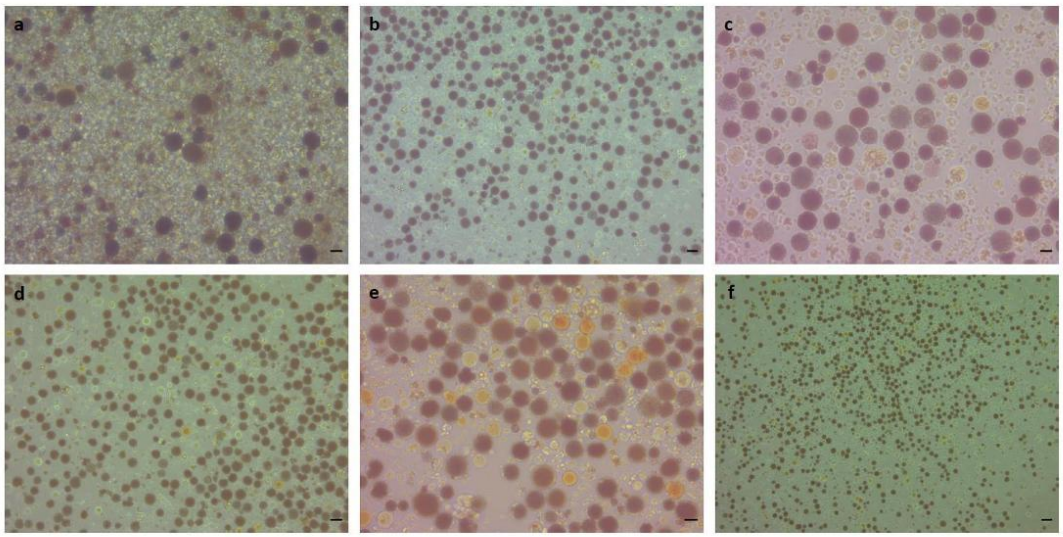

Figure S8: *B. schlosseri* primary blood cell cultures in medium TGM4: at onset (a), 24 h (b), days 3 (c), 8 (d), 17 (e) and 25 (f). Scale bars: 10 μm in b-d, 20 μm in a, e and f.

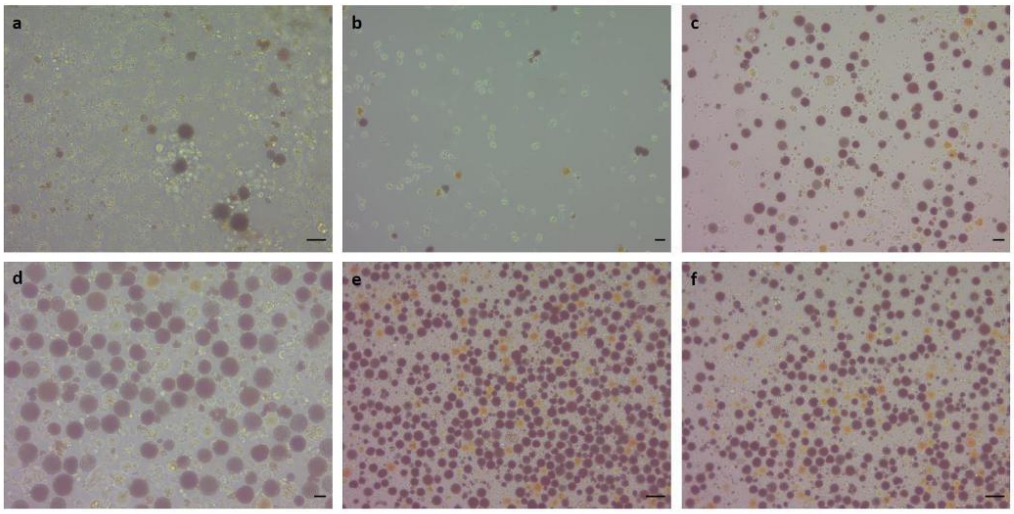

Figure S9: *B. schlosseri* primary blood cell cultures in medium TGM5: at onset (a), 24 h (b), days 3 (c), 8 (d), 17 (e) and 23 (f). Scale bars: 10 μm in a-f, 20 μm in f.

Table S1: Comparisons of media TGM1, TGM2, TGM3, TGM4 and TGM5 compositions. L-Glu - L-Glutamine, Hep - HEPES buffer, PSA - Penicillin Streptomycin Amphotericin b, Genta – Gentamicin, PS - Penicillin Streptomycin, SP - Sodium Pyruvate, FBS - Fetal Bovine Serum, DMEM - DMEM/F-12[HAM] 1:1 basal medium, RPMI - RPMI basal medium 1640, ASW – artificial sea water. +/- indicates the presence or absence of components in each medium.

Medium/Component	L-Glu	Hep	PSA	Genta	PS	SP	FBS	DMEM	RPMI	ASW
TGM1	+	+	-	-	+	+	+	+	-	-
TGM2	+	+	-	-	+	+	+	-	+	-
TGM3	+	+	+	+	-	+	+	-	-	+
TGM4	+	+	-	+	+	+	+	+	-	+
TGM5	+	+	-	-	+	+	+	+	-	+

Discussion

The world's oceans and seas host a variety of invertebrates with multiple types of cells revealing a wide range of cellular potentialities that offer endless applications (Rinkevich et al., 2022). Nevertheless, while several cell lines have been commonly derived from terrestrial invertebrate taxa, e.g. insects and arachnids, until just recently (Hesp et al., 2023) all attempts to develop cell lines from marine invertebrates have failed, despite many efforts made on a wide range of species over the past decades (Rinkevich, 1999, 2005, 2011; Mothersill and Austin, 2000; Balakrishnan et al., 2022; Domart-Coulon and Blanchoud, 2022). The recent advances in marine invertebrate cell culture methodology that has been made by the development of optimized nutrient media for primary cultures (Munroe et al., 2019; Conkling et al., 2019), have led to the establishment of long lasting cultures from several species of sponges (Conkling et al., 2019, Hesp et al., 2023), revealing the importance of media for successful outcomes. Because of the importance of the marine invertebrate *B. schlosseri* as a model system in a wide range of biological disciplines (Rinkevich, 2017; Manni et al., 2019; Rinkevich, 2005) and the need to develop approved *in vitro* methodologies for its research (Rinkevich and Rabinowitz, 2000; Qarri et al., 2022), several research activities have focused on cell yields, distinguishing suitable factors for proper cell adhesion and attachment (Rabinowitz and Rinkevich, 2004; Qarri et al., 2022), initiating primary cell cultures derived from embryos, epithelial cells and circulating blood cells (Rinkevich and Rabinowitz, 1993, 1994, 1997; Rinkevich et al., 1994). Other studies focused on revealing the impacts of media additives on primary cultures, such as growth factors (Rinkevich and Rabinowitz, 1993), establishing medium for circulatory blood cells (Rinkevich and Rabinowitz, 1993) and evaluating *in vitro* stemness signatures in epithelial monolayers (Rabinowitz and Rinkevich, 2004, 2011; Rabinowitz et al., 2009). The above attempts have failed to establish proliferating cell cultures from *Botryllus*, and showed no cell divisions 24-72 h from culture initiation, while in scores of cases cultures were overgrown by opportunistic micro-organisms such as thraustochytrids (Qarri et al., 2021, Rabinowitz et al., 2006, Rinkevich and Rabinowitz, 1993, Rinkevich, 1999, 2011). Consequently, the literature reveals that there is a limited knowledge about the characterization of cell types including the development of thraustochytrids, proliferation statuses, viability and special requirements needed to support *Botryllus* cell growth in primary cultures.

In response to the above challenges, chapter 1 (Qarri et al., 2021) focus on thraustochytrids developed in primary cultures of *B. schlosseri* colonies (at blastogenic stage C) using *Botryllus* cell culture medium. As of 24 h from *Botryllus* culture initiation (onset), the undescribed thraustochytrid strain BS2 (Thraustochytriidae sp. BS2; GeneBank accession number AF257315.2) developed and by day 8 thraustochytrid cells grew and dominated the cultures. This strain confirms previous results on *Botryllus* primary cultures (Mo et al., 2002). Thus, in order to improve our understanding of thraustochytrid cell types developed in *Botryllus* primary cultures, these were described and characterized morphologically using nuclei (Hoechst) staining. Results revealed the existence of three thraustochytrid cell types including mononucleated cells (5-10 µm), multinucleated cells (consisting of 2-30 nuclei per cell; 10-50 µm) and sporangia (20-200 µm). The mononucleated cells, also appeared as cell clusters composing at least 3 cells and reaching sizes of up to 200 µm per cluster. At day 8 and thereafter, all cultures contained thraustochytrid aggregates and single cells which included mononucleated, multinucleated cells and sporangia. Chapter 1 thus, enhance our understanding regarding the cell morphologies of thraustochytrid cell types developed in *B. schlosseri* cultures and further confirming previous documentation of their cell morphologies (Lyu et al., 2021; Morabito et al., 2019). Since the literature is lack of information and in many cases previous studies described the thraustochytrid cell types as aggregates or single cells that dominated *Botryllus* primary cultures without providing a sufficient documentation (Rinkevich and Rabinowitz, 1993, 1994).

Chapter 2 (Qarri et al., 2023) investigates how different types of *B. schlosseri* blood cell (haemoblasts, macrophage-like cells, granular amoebocytes, morula cells, pigment cells and nephrocytes) respond under *in vitro* conditions. Specifically, their responses were assessed using five versions of a basic medium (TGM1- TGM5) during the initial few crucial weeks from onset. To enhance cell identification under *in vitro* conditions confocal microscopy have been used, for the first time, and the differential autofluorescence of various cells, revealing novel results. For example, haemoblasts were found as the only cell type in *B. schlosseri* to exhibit a high signal in the far-red channel, which distinguishes them from thraustochytrid cells as well, and they did not exhibit blue channel florescence like other blood cells, further supporting the finding of Rinkevich and Rabinowitz (1993) as described above. Moreover, the isolated haemocytes and the developed thraustochytrid cell types in primary cultures, were consequently identified using histological (H&E), nuclear (Hoechst) and membrane (DiD) staining, which resulted with further identification

of the cultured cells. Cellular morphology of all cell types, remained similar from onset except for storage cells (pigment and nephrocyte cells) which transformed from oval to elongated shapes structures in TGM3 medium, confirming past results of storage cells cultured in similar medium (Rinkevich and Rabinowitz, 1993).

The primary cultures have been affected by changes in cell proliferation, viability, and dominant cell types due to the different versions of media used (TGM1, TGM2, TGM3, TGM4 and TGM5). As for cell proliferation, the basic condition of the medium can stimulate significant proliferation of various circulating cell types at different time points (onset, 24 h, 3 and 8 days). For instance, the most abundant cell types (haemoblasts, macrophage-like cells, morula and pigment cells) exhibited varying patterns of blood cells activity in different media versions from onset to day 8. The above media, were formulated with varying proportions of basal media (DMEM F12/HAM or RPMI) and artificial sea water (ASW) whereas, TGM1 and TGM2 contained basal media DMEM F12/HAM and RPMI respectively, without ASW, and TGM3 consisted of ASW without basal media. There are no scientific evidence culturing *Botryllus* cells without ASW or with only basal media. However, various levels of cell proliferation observed for haemoblasts, macrophage-like cells and pigment cells in these media versions. Therefore, it is reasonable to assume that the three media version (TGM1, TGM2 and TGM3) are still "inappropriate" or lacking essential factors, such as basal media or ASW. Despite this, cell proliferation was achieved even though *Botryllus* cells typically stop dividing within 3 days of isolation *in vitro* (Qarri et al., 2022; Rabinowitz et al., 2006; Rinkevich and Rabinowitz, 1993; Rinkevich, 2011).

As with cell proliferation, the viability of the cells also showed variation among the five media versions, leading to differences in the longevity of the cultures. The cultures initiated with TGM1, TGM2 and TGM3 lasted between two to three weeks (18, 16 and 12 days respectively), while longer cultivation periods of four weeks were observed with TGM4 and TGM5 (26 and 24 days respectively). The above confirming previous results (Domart-Coulon et al., 2004) that showed a short term *in vitro* study on coral cells and found that viability decreased from 70% to 30% within the first week of culture initiation as well as with Rinkevich and Rabinowitz, 1993 who observed decrease in *Botryllus* blood cells viability within four weeks from onset.

Similar to cell proliferation and viability, the distribution of cell types also showed variation among the five media versions. At 24 h from onset, macrophage-like cells and morula cells being the

predominant cell types in all tested media and by day 3, while the abundance profiles of TGM3 and TGM4 media remained like that of 24 h, morula and pigment cells were more prominent in TGM1 medium. At day 8, no further changes in cell type distribution observed, and the abundance profiles of all five media were almost identical as day three. As mentioned above, there are no scientific evidence revealing the distribution of *Botryllus* blood cell types under prolonged *in vitro* conditions. The findings of chapter 2, illustrate that it is possible for *Botryllus* blood cells to grow and multiply when the culture medium is altered, which corroborates the suggestions put forth by recent research on the cultivation of sponge cells (Munroe et al., 2019). These recommendations have led to an enhancement in both the quantity and viability of cells in the present study and to the development of proliferating cell cultures in sponges (Conkling et al., 2019, Hesp et al., 2023). The above results demonstrating the possibility of culturing *Botryllus* blood cells *in vitro* for a short period of time (up to one month) in a consistent and reliable manner.

To conclude, the novel findings and the knowledge obtained, of publication 1 and 2 (chapter 1 and 2 respectively) thus improving the *in vitro* conditions required for *B. schlosseri* cell growth and may lead to the creation of various cell cultures, each consisting of distinct cell types.

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Appendices

Publication and declaration

Appendix Publication 1:

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In the presenting work my contribution included: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing.

Paper declaration: This paper reports on original research I conducted during the period of my Higher Degree and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Appendix Publication 1

Improving the Yields of Blood Cell Extractions from *Botryllus schlosseri* Vasculature

Andy Qarri, Yuval Rinkevich and Baruch Rinkevich

Abstract: The tunicate *Botryllus schlosseri* belongs to the Vertebrata's closest living invertebrate group. This colonial species represents an invertebrate model system that maintain high capacity of adult stem cell activity, where various blood cell types, expressing multipotent or totipotent phenotypes, circulate in vasculature throughout life. While isolated *Botryllus* blood cells may serve as indispensable tools for studying stem cells biology, up to date, no single cell line is available. The major bottle-necks for established cultures include the lack of cell division under in vitro conditions as from 24 to 72 h post isolation and enhanced contami-nation rates by bacteria and protists. Moreover, low yields of blood cells are of significant hindrance to the development of long-term cultures since lower numbers of cells eventually lead to poor results. Tackling these two critical technical obstacles, we present here methodologies for improved aseptic conditions and for higher yields of cells extracted from colonial vasculature. This study was performed on two colonial stocks (Israel, laboratory stocks; Helgoland, Germany—field collected stocks) which resulted with a significant difference in the numbers of cell extrac-tions between the two stocks and significantly different blood cell yields between various blastogenic stages (laboratory stocks), further revealing differences between field/laboratory-maintained colonies.

1. Introduction

The cosmopolitan tunicate *Botryllus schlosseri* belongs to a taxonomic taxon considered as the closest living invertebrates to the Vertebrata (Delsuc et al. 2006) and is used as an important model species in a wide range of biological disciplines (Ben-Hamo and Rinkevich 2021), such as ecotoxicology (Gregorin et al. 2021; Rosner et al. 2021), immunobiology and allorecognition (Magor et al. 1999; Rinkevich 2004), developmental biology including colony astogeny (Manni et al. 2019; Rosner et al. 2006; Rosner et al. 2019), regeneration (Voskoboynik et al. 2007), senescence (Rabinowitz and Rinkevich 2004a; Rinkevich 2017), evolutionary biology (Rinkevich 2002) and above all—stem cell biology (Ballarin et al. 2021; Voskoboynik et al. 2008). *B. schlosseri* colonies express two modes of reproduction, sexual and asexual (Manni et al. 2019). Sexual reproduction cycles occur weekly, each starting with the

fertilization of eggs and progressing through embryonic stages into a tadpole larva featuring chordate characteristics that includes striated musculature, neural tube, notochord and tail (Voskoboynik et al. 2007). The tadpole larva swims for a short period of time and then attaches to a substrate near the mother colony, loses the tail through apoptosis, and then develops into the first zooid (the colonial module), called an oozooid (Berrill 1950). Colonies develop from the oozooids through weekly cycles of growth and death (Manni et al. 2019; Rinkevich 2019) and form several typical star-shaped groups of zooids, each called a system, that are embedded within the tunic, the transparent gelatinous extra cellular matrix (ECM) of the colony which contains cellulose cross-linked with proteins as well as the colonial circulatory system (Figure 1). Colonial systems are connected to each other via common blood vessels, which carry at the periphery of the colony sets of blind vasculature termini, called ampullae (spherical to elongate in structure). Each zooid in the colony possesses an oral siphon (branchial siphon) and an atrial siphon is shared for all zooids in each system (Berrill 1950).

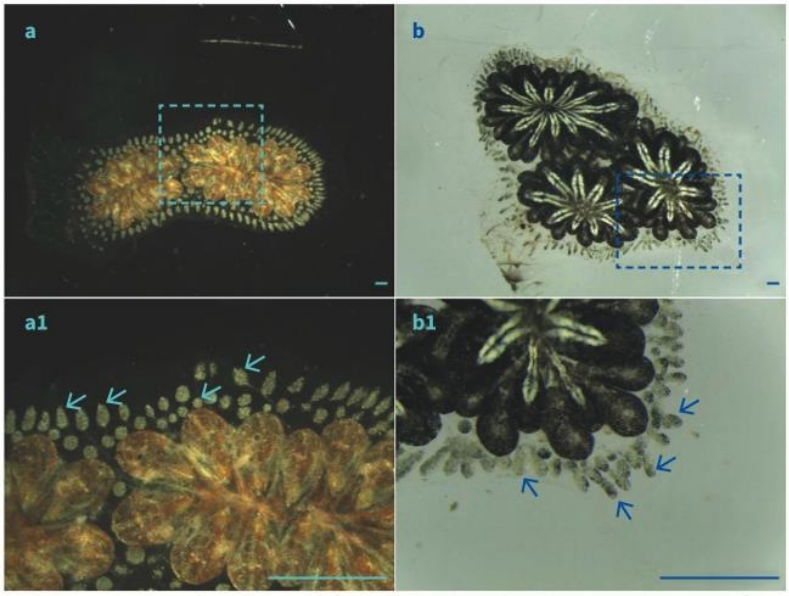

Figure 1. A *B. schlosseri* colony originated from the Israeli stock at the National Institute of Oceanography, Haifa (**a**,**a1**), at blastogenetic stage A, and (**b**,**b1**) a colony from Helgoland Island, Germany in blastogenetic stage C. Cells extracted from the marginal ampullae (arrows in a1, **b1**) of the colonies. Dotted squares represent the enlarged area of a1 and b1, respectively. Bars = 0.1 mm (**a**,**b**) and 1 mm (**a1**,**b1**). Source: Graphic by authors.

The asexual mode of development in *B. schlosseri* is expressed as weekly developmental cycles called blastogenesis, where each blastogenic cycle is composed of four major stages (marked by the letters A to D (Mukai and Watanabe 1976), during which the primary buds mature to adult zooids in concert with the development of the secondary buds from the body wall of each primary bud. A massive apoptotic event concludes each blastogenic cycle with the morphological resorption of all parental zooids, concurrently followed with the development of primary buds to functioning zooids (Lauzon et al. 1993). Thus, the blastogenesis process can be characterized by somatic self-renewal and vasculature regeneration, which demonstrate a model organism that carry out continuous somatic proliferation throughout the organism life span. In other words, the weekly budding process of somatic self-renewal and high vasculature regeneration capacity suggests an invertebrate model organism that maintain high capacity of stem cell activity throughout life (Ben-Hamo and Rinkevich 2021; Qarri et al. 2020; Rinkevich 2019).

Blood cell isolation and culturing are essential tools in the study of stem cells and regeneration in this model organism. Various cell types from *B. schlosseri* possess extensive potentialities such as multipotency and totipotency (Laird et al. 2005; Rinkevich and Rabinowitz 1994; Rinkevich and Rabinowitz 1997; Rosner et al. 2009; Rosner et al. 2021) and may serve as important tools in studying immunology, developmental biology, apoptosis and regeneration (Ballarin et al. 1994; Lauzon et al. 1993; Rosner et al. 2009; Rosner et al. 2021; Voskoboynik et al. 2007). Studies that attempted to develop primary cultures and permanent cell cultures from *B. schlosseri*, commonly used to extract blood cells that are directly collected from the blood vessels (Ballarin et al. 2008; Rinkevich and Rabinowitz 1993). Other studies used cells originated from epithelial layers (Rinkevich and Rabinowitz 1997), which show de novo stemness signatures (Rabinowitz et al. 2009; Rabinowitz and Rinkevich 2011) and cells originating from embryos (Rinkevich and Rabinowitz 1994). However to date, no single *Botryllus* cell line is available and it has repeatedly shown that extracted cells stop dividing in vitro within 24–72 h after their isolation. Moreover, many of the cultures are contaminated with opportunistic organisms including bacteria and protists, such as thraustochytrids (Qarri et al. 2021; Rabinowitz et al. 2006; Rinkevich and Rabinowitz 1993; Rinkevich and Rabinowitz 1994; Rinkevich 1999; Rinkevich 2011.)

The above studies indicate that, in order to establish long-term cell cultures, attempts should approach two critical technical statuses prior cell cultivation, (a) approved methodologies for aseptic conditions and (b) high yields of cell extraction. High yields of cells are of significant importance since lower numbers of cells eventually lead to poor results and fast senescence of extracted cells, primarily when dealing with blood cells that represent a rapid turnover and survival of only several weeks (Raftos et al. 1990; Rinkevich 1999; Rinkevich 2011). These

limitations have led to attempts of pooling of blood cells originated from several colonies. For example, Ballarin et al. (2008) extracted 10^6 cells from more than three colonies, and Kamer and Rinkevich (2002) obtained the same cell concentrations (10^6 cells) by cutting the tunic matrix and the zooids without specifying the number of used colonies.

Responding to the above challenges, here we present a general aseptic approach with higher yield for blood cell (including stem cells) extractions employed on *B. schlosseri* colonies originating from two colonial stocks, the long-term established laboratory colonial cultures from Israel and from newly collected colonies originated from Helgoland Island, Germany. The essence of this approach is to improve blood cells yields from a single colony for in vitro applications.

2. Materials and Methods

2.1. Botryllus schlosseri Husbandry

Twenty-two colonies originated from Israeli cultures (long-term cultures maintained at the National Institute of Oceanography, Haifa, Israel) and freshly collected colonies from Helgoland Island, Germany, were selected for cell extraction experiments. Thirteen colonies (blastogenic stages A = 4, B = 3, C = 2 and D = 4) were derived from laboratory stocks reared in the Israeli facility for several years and originated from several USA west coast marinas (Monterey, Half Moon Bay and Moss Landing, California), as from Nelson Marina, New Zealand. The colonies were kept vertically on 5 × 7.5 cm^2 glass slides in slots of glass staining racks at 20 °C, in a 21-Liter plastic tank under a 12:12 h light:dark regimen, in a standing seawater system, as described (Rinkevich and Shapira 1998). Air stones were continuously used and the seawater was changed twice a week. Colonies were fed daily with freeze-dried rotifers, green unicellular algae and commercial powdered plankton. Nine colonies (blastogenic stages A = 5, B = 2 and C = 2) were collected from the rocky intertidal zone in Helgoland. These colonies were reared at the Biological Institute Helgoland (BAH) of the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research—house C and maintained as the Israeli stock colonies under running seawater system and temperatures between 15.7 and 23.4 °C. Colonies were fed daily with dried algae powder. Colonies of both stocks were gently cleaned twice a week using small and soft brushes to remove trapped food particles, fouling organisms and debris. All experimental colonies were in good health and well adapted to their maintenance conditions.

2.2. Aseptic Solution, Instruments and Environment

Washing solution (WS) was used in order to reduce contaminations during the process of cell extraction. Artificial seawater (ASW) was prepared as described in

Rabinowitz and Rinkevich (Rabinowitz and Rinkevich 2004b), autoclaved, sterilized by a 0.2 μm filter membrane (Millipore) and stored at room temperature. For each 50 mL of WS, we used 44 mL of ASW, supplemented with 3 mL of PSA (Biological Industries; Penicillin 10,000 units/mL, Streptomycin sulphate 10 mg/mL and Amphotericin B 25 μm/mL; Cat. 03-033-1B. MP Biomedicals; Cat. 091674049) and 3 mL of Gentamycin Sulfate (Biological Industries; 50 mg/mL; Cat. 03-035-1. Gibco; 50 mg/mL; Cat. 15750037). Only sterilized plasticware was used. In addition to ASW sterilization, glassware was routinely autoclaved. Cell extraction protocols were carefully observed to maintain pathogen-free conditions. Additionally, prior to cell isolation colonies were kept under sterile conditions in a biosafety cabinet within a 20 °C cool room (Israel) and in an incubator of 20 °C (Helgoland).

2.3. Cell Extraction Approach under Aseptic Conditions

Before cell extraction procedure (Figure 2) colonies were taken out from aquaria and photographed under stereomicroscope (SMZ1000, Nikon equipped with DeltaPix digital camera Invenio 3SII, S/N: 3648213012. Leica M125 equipped with a camera Leica IC80 HD). Then colonies were meticulously cleaned by soft brushes, and the glass slides on which they were grown were comprehensively cleaned and wiped with 70% ethanol. Using razor blades under a biosafety cabinet, the colonies were carefully pulled off from the slides and placed in the centers of sterile 60 mm Petri dishes (Greiner bio-one, CELLSTARR 628160, Petri-dish 60 × 15 mm^2) for approximately 20 min in a humidity chamber, containing ASW, to actively attach to the dish substrates (detailed procedure in Rinkevich and Weissman (1987)). Then, 6 mL of WS (Washing solution) was added, and animals were left under sterile conditions for 48 h without food (starvation with antibiotic supplements significantly reduced contaminations of cell cultures; Rinkevich and Rabinowitz 1993). Then, the WS was changed 12 times (every hour for the first 6 h, left for 12 h and then, from the 18th hour, the WS changes protocol was repeated for the next 6 h; total of 12 washes). Following the above, all peripheral ampullae of each colony in a plate were punctured with an insulin syringe needle (28-Gauge) and the WS containing *B. schlosseri* blood cells was dropped into a 15 mL tube, pursued by centrifugation (2000 rpm for 10 min) using Eppendorf (Hamburg, Germany). The plates were then supplemented with WS and left for an addition 1.5 h in a biosafety cabinet, following which the WS from each plate was collected into a tube and cell extraction procedure (described above) was performed again. *B. schlosseri* blood cells in the tubes were centrifuged and the pellets were suspended in 1 mL of WS for further investigations.

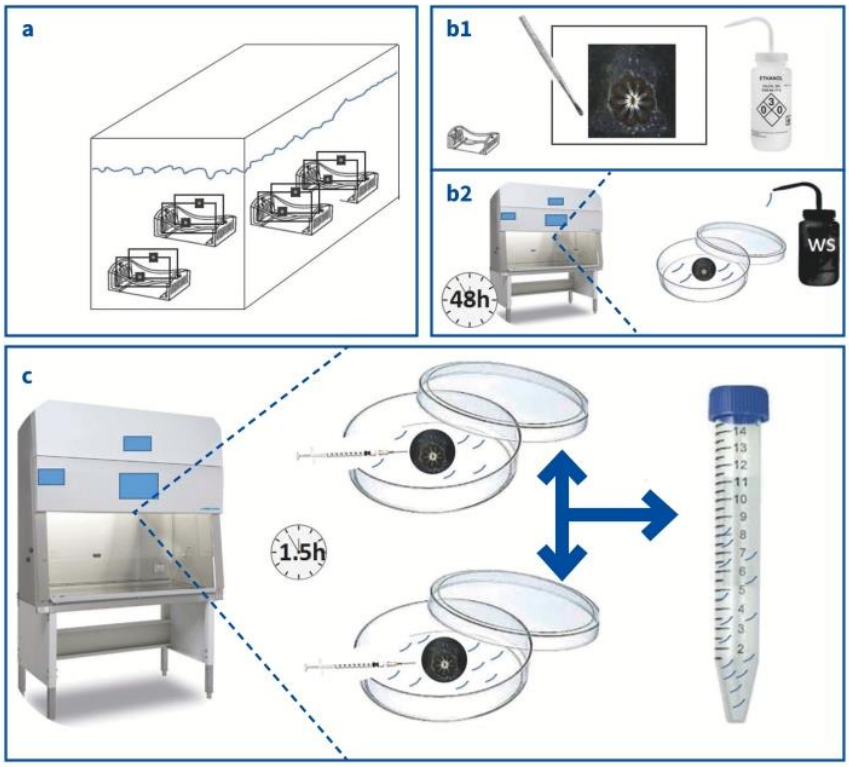

Figure 2. Schematic illustration of the cell extraction approach. (**a**) Colonies maintained under laboratory conditions, in tanks. (**b1**) Prior to cell extraction, colonies with their glass substrates are removed from their growth system, cleaned meticulously by soft brushes and the glass slides are wiped with 70% ethanol. (**b2**) Working in biosafety cabinet, each colony is removed, using a razor blade, from the glass substrate and is transferred to a sterile 60 mm Petri dish until actively attached. Then the Petri dish is being filled with 6 mL of WS. The plates are left in a biosafety cabinet for 48 h following 12 changes of WS. (**c**) Cell extraction is performed within a biosafety cabinet by puncturing the ampullae of each colony with the insulin syringe needle. This procedure is repeated after 1.5 h. Then the WS containing *B. schlosseri* blood cells are collected into a tube for further investigation. Source: Graphic by authors.

2.4. Cell Observation and Counting

All cell extractions were counted, using a hemocytometer, and photographed under the microscope (Olympus inverted system microscope, model I × 70, equipped with DP73 camera. Leica ICC50 HD). Cell viability was determined using Trypan

Blue solution (Biological Industries; Cat. 03-102-1B. Gibco; Cat. 15250061). Obtained values of *B. schlosseri* blood viable cells were between 93.6 and 98.5%.

2.5. Statistical Analyses

Statistical analyses were applied on extracts of two *B. schlosseri* colonial stocks originated from Israeli laboratory cultures and Helgoland Island, Germany using an SPSS V16. An independent-samples T test was performed on two stock cell yields. One-way ANOVA test using post hoc comparison (Bonferroni and Tukey HSD) was applied on blastogenesis of cell extracts of each *B. schlosseri* colonial stocks. Pearson correlation test was performed on zooid numbers of each colony with respect to cell yields.

3. Results

Cell Yields

The cell extraction protocol was performed on the 22 *Botryllus schlosseri* colonies originating from the Israeli stock of colonies (Figure 1a; 13 colonies in blastogenic stages A-D) and from Helgoland, Germany newly established stock (Figure 1b; 9 colonies in blastogenic stages A–C). Zooid numbers of the two stocks varied between 8 and 32 per colony for the Israeli colonies and 8 and 31 for the Helgoland colonies, yet no correlation (Figure 3) was recorded between the number of zooids per colony (of the colonial sizes used in this experiment) and cell yields for each stock ($r_{pearson} = -0.097$, $p > 0.05$; $r_{pearson} = 0.503$, $p > 0.05$; for Israeli/Helgoland stocks, respectively). Cells were extracted (Figure 4) from the marginal ampullae (Figure 1(a1,b1)) and numbers of cells and viability were studied on yields upon cell collections with respect to donors' blastogenic stages. Comparing between the two stocks of colonies, the results revealed a significant difference ($p < 0.0001$; independent-sample T test) in the numbers of cell extractions between the two stocks, where more cells were extracted from the freshly collected Helgoland stock. Within stock analyses revealed significant blastogenic-associated differences in cell yields from the Israeli stock colonies ($p < 0.05$; one-way ANOVA) but not in the freshly collected colonies from Helgoland ($p > 0.05$; one way ANOVA).

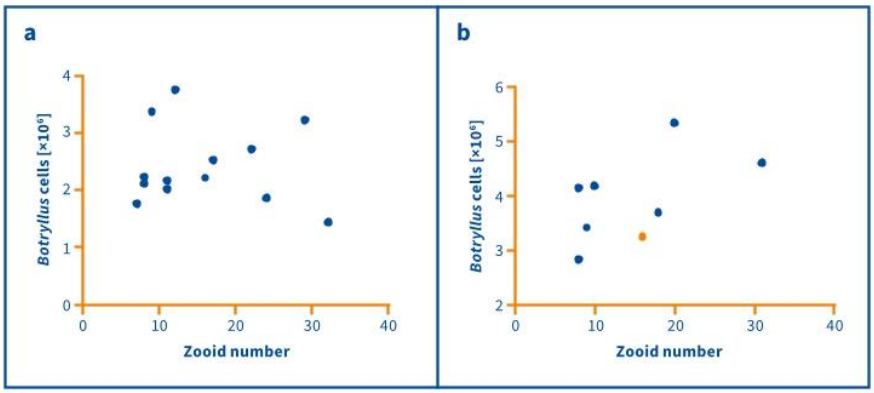

Figure 3. The correlation for cell yields vs. zooid numbers. (**a**) Cell yields vs. zooid numbers for the Israeli stock. (**b**) Cell yields vs. zooid numbers for the Helgoland stock. The red dot in b represents two different colonies with 16 numbers of zooids and cell yields of 3.28 and 3.27 $\times$ 10^6 cells. Source: Graphic by authors.

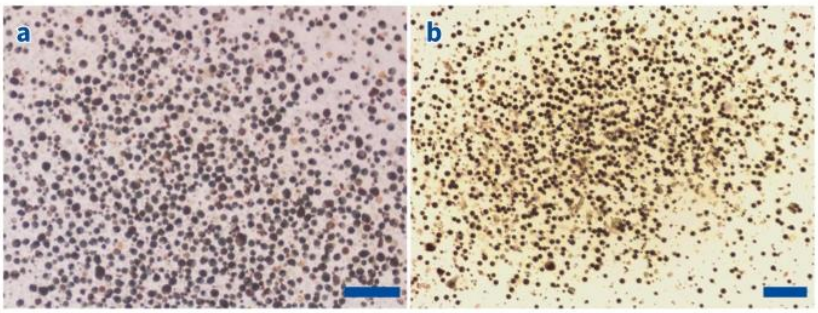

Figure 4. *B. schlosseri* cells under in vitro conditions. (**a**) A primary culture of blood cells from a blastogenesis stage B colony originated from the Israeli stock. (**b**) A primary culture of blood cells from a blastogenesis stage A colony from Helgoland, Germany. Bars = 100 μm. Source: Graphic by authors.

Cell yields from the Israeli blastogenic stages A and C colonies (Figure 5) composed of two significant groups, shared by the cell yields of blastogenic stages B and D colonies (Tukey HSD comparison). Cell yields from blastogenic stage A colonies (n = 4) varied between (2.12 $\pm$ 0.3) $\times$ 10^6 and (3.75 $\pm$ 0.2) $\times$ 10^6 ($p < 0.05$) in the Israeli stocks. For Helgoland colonies (n = 5), cell numbers varied between (2.9 $\pm$ 0.08) $\times$ 10^6 and (4.6 $\pm$ 0.69) $\times$ 10^6 ($p > 0.05$). Cell yields from blastogenic stage B colonies (n = 3) varied between (2.2 $\pm$ 0.13) $\times$ 10^6 and (2.7 $\pm$ 0.3) $\times$ 10^6 ($p > 0.05$) in the Israeli stocks, and for Helgoland colonies (n = 2) cell numbers varied between (2.2 $\pm$ 0.21) $\times$ 10^6 and (5.35 $\pm$ 0.3) $\times$ 10^6 ($p > 0.05$). Cell yields from blastogenic stage

C colonies (n = 2) varied between (1.76 ± 0.12) × 10^6 and (1.87 ± 0.34) × 10^6 ($p < 0.05$) in the Israeli stocks, and for Helgoland colonies (n = 2) cell numbers varied between (3.28 ± 0.08) × 10^6 and (3.72 ± 0.83) × 10^6 ($p > 0.05$). Cell yields from blastogenic stage D colonies ($n = 4$) varied between (1.44 ± 0.11) × 10^6 and (2.17 ± 0.48) × 10^6 ($p < 0.05$) in the Israeli stocks.

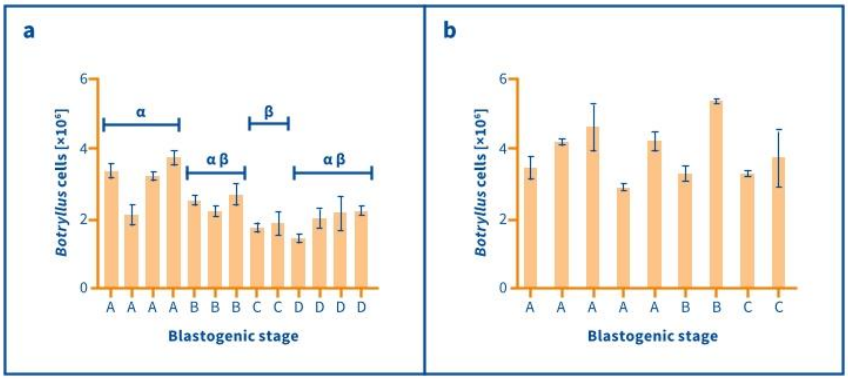

Figure 5. *B. schlosseri* cell yields. (**a**) Cell yields (×10^6) of colonies at blastogenic stages A–D originated from the Israeli stock. (**b**) Cell yields (×10^6) of colonies at blastogenic stages A–C originated from Helgoland Island. Each column represents an average of two extractions per colony (±S.D.). α and β symbolize statistical group differences between the tested blastogenesis stages obtained by Tukey HSD. Source: Graphic by authors.

4. Discussion

The literature reveals that primary cell cultures originated from *B. schlosseri* vasculature stop dividing 24–72 h post isolation (Rinkevich 1999) and further indicates low yields of cells per colony (Table 1) with high contamination rates (Rinkevich 1999; Rinkevich 2011.)

Table 1. Studies on *B. schlosseri* that refer to blood cell yields under laboratory conditions. Abbreviations: AC—Aseptic conditions; C—a colony; LS—laboratory stocks; NS—not specified; ABX—antibiotics; FSW—filtered seawater; FASW—filtered artificial seawater; ?= unknown number of colonies.

No of Colonies	Colony Origin	Cell Extraction under AC	Use of: FSW FASW/ABX	Cell Yields Colony^{-1}	Reference
>3	Italy (Venice)	No	FSW	3×10^5/C	(Ballarin et al. 2008)
NS	Italy (Venice)	No	FSW	5×10^6/?	(Menin and Ballarin 2008)
NS	LS	No	FASW	1×10^6/?	(Kamer and Rinkevich 2002)
NS	Italy (Venice)	No	FSW	8–10 $\times 10^6$/?	(Ballarin et al. 1994)
NS	LS	No	FSW	8–10 $\times 10^6$/?	(Ballarin et al. 2011)
NS	Italy (Venice)	No	FSW	1×10^7/?	(Ballarin and Cima 2005)
NS	LS	No	FSW/ABX	1×10^5/?	(Rinkevich and Rabinowitz 1993)
1	Helgoland	Yes	FASW/ABX	5.3–5.4 $\times 10^6$/C	This study
1	Israel LS	Yes	FASW/ABX	3.6–3.9 $\times 10^6$/C	This study

Source: By the authors.

Here we present an improved approach for blood cell extractions from *B. schlosseri* vasculature, performed under our aseptic conditions, which showed reduced contamination rates as compared to former outcomes. Yet, this issue was not analyzed in the present study. We used two *B. schlosseri* colonial stocks originated from a long-term laboratory cultures (from Israel) and colonies freshly collected from Helgoland Island, Germany. While at the colonial sizes used in this experiment there was no differences between the blood cell numbers obtained per colony, the results of this study clearly revealed, (1) a significant difference ($p < 0.0001$) in the number of blood cells obtained between the two disparate stocks and (2) changes in the numbers of blood cells obtained from various blastogenic stages (recorded only for the Israeli stock colonies). We obtained two significantly different blood cell yields between colonies at blastogenic stage A vs. stage C colonies. These results point to possible differences in numbers of total blood cells between freshly collected colonies from the field and colonies from established stocks, a result which should be taken into consideration when cell yields are an important component in structuring a research. This is also an interesting result regarding the *B. schlosseri* blood cell (and potentially stem cells) biology that should be studied in further experimentation.

The present study is the first that focuses on cell yields from *B. schlosseri* colonies. The literature (Table 1) reveals that past studies used undefined numbers of colonies in the research, or that the yield was lower than levels detailed in this study. Thus, our approach demonstrates potential for improving the extracting of circulating cells, including stem cells, under aseptic conditions, for any in vitro application, without pooling cells from different genotypes, augmenting the importance of *B. schlosseri* as a model organism in the field of cell biology (Ballarin et al. 2011; Ben-Hamo and Rinkevich 2021; Frizzo et al. 2000; Rosner et al. 2021). As a final point, the recognition of this model organism in the field of cell biology and stem cells biology is associated with its circulating blood cells that hold potentialities such as multipotency and totipotency (Ballarin et al. 2021; Laird et al. 2005; Rosner et al. 2009; Rosner et al. 2021).

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