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Elucidating the role of apoptosis during cyclical body regeneration in Botryllus schlosseri

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By

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Dedicated in loving memory of Ravi Thackurdeen (1992-2012)

ABSTRACT

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Apoptosis is the principal form of programmed cell death by which multicellular animals rid themselves of old cells in order to allow new cells to grow. The colonial ascidian *Botryllus schlosseri* is a model system for homeostatic cell death and regeneration. During its unique, weekly budding cycle known as blastogenesis, new buds are produced asexually and eventually replace the old adult zooid generation through resorption via circulating phagocytes. Using polyester wax-embedded tissue sections, we have shown that TUNEL-positive (terminal deoxynucleotidyl transferase nick end-labeling), apoptotic cells are observed within circulating phagocytes in all stages of the colony's death phase (takeover), including the final stage during which the primary bud undergoes a growth surge and becomes a functional adult zooid. Suggested explanations of these intriguing results yield three potential theories: (1) the growing primary bud is a site of cell corpse elimination for the dying generation, (2) the phagocytosed materials of the dying generation are recycled and reincorporated into the new adult generation, or (3) the apoptotic cells of the dying adult zooid generation release mitogenic factors and stimulate proliferation in neighboring cell populations. Our findings are consistent with the idea that dying cells are trophic in nature, promoting growth and differentiation during whole body regeneration.

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Introduction

Apoptosis, also known as programmed cell death (PCD), is a fundamental process found in all living metazoans (Danial and Korsmeyer, 2004; Elmore, 2007). First described by Kerr, Wyllie, and Curie in 1972, apoptosis has come to be defined as the evolutionarily conserved, intracellular death program, which is the primary mechanism of genetically programmed cell suicide and elimination (Lauzon *et al.*, 2007; Kerr, Fuchs and Steller, 2011). Apoptosis is critical to the survival of the organism and its physiological roles include organogenesis, organ remodeling, tissue homeostasis, and wound healing, as well as the culling of the initial over-production of cells and deletion of unwanted structures during embryonic development (Elmore, 2007; Fuchs and Steller, 2011; Greenhalgh, 1998; Lindsten *et al.*, 2000; Jacobson *et al.*, 1997). Additionally, apoptosis acts as a complement to mitosis in regulating cell population size in adult tissues (Fuchs and Steller, 2011). Programmed cell death is also used as a host defense mechanism; it is employed during immune reactions and is necessary for removal of cells that have been damaged by disease, infection, or toxic agents (Fuchs and Steller, 2011; Norbury and Hickson, 2001). Abnormalities in cell death regulation—both insufficient and excessive apoptosis—can have severe and often dangerous consequences for the organism; pathological apoptosis can be manifested in cancer, autoimmune disorders, infertility, ischemic damage, and neurodegenerative diseases (Danial and Korsmeyer, 2004; Elmore, 2007; King and Cidlowski, 1998).

Previous studies have indicated that there exist three main pathways of apoptosis: the extrinsic pathway, the intrinsic pathway, and the perforin/granzyme pathway (Elmore,

2007). In the extrinsic pathway (death receptor pathway), apoptosis is triggered via trans-membrane receptor-mediated interactions, while in the intrinsic pathway (mitochondrial pathway), non-receptor-mediated, mitochondrial-initiated stimuli produce intracellular signals that act directly on targets within the cell to signal destruction (Elmore, 2007). The perforin/granzyme pathway, in comparison, involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell (Elmore, 2007). All three pathways converge on the same terminal pathway, the execution pathway, resulting in the final cyto-morphological changes observed in cells undergoing apoptosis (Elmore, 2007). Caspases, enzymes in the cysteine protease family, are the central executioners of the process (Hengartner, 2000). They are expressed ubiquitously as inactive zymogens, and are activated via cleavage at specific aspartic residues in response to death-inducing stimuli in apoptotic cells (Hengartner, 2000; Thornberry and Lazebnik, 1998; Fuchs and Steller, 2011). Caspases are highly conserved throughout evolution and perform a number of various functions, including the activation of the nuclease that leads to the distinctive nucleosomal ladder (Hengartner, 2000; Wyllie, 1980).

Apoptosis is characterized by a number of clear cyto-morphological changes. During the early stages of the process, cells begin to shrink, and pyknosis can be observed using light microscopy (Kerr *et al.*, 1972). During this cellular shrinkage, the cytoplasm of the cell increases in density and the organelles become closer in proximity to one another (Häcker, 2000). The condensed chromatin then fragments at the linker region of DNA between nucleosomes as nuclear fragmentation occurs (karyorrhexis), resulting in free 3′ hydroxyl groups at the ends of the fragments (Elmore, 2007; Jänicke *et al.*, 1998; Majno and Joris, 1995). The cell membrane subsequently begins to bleb and

pinches off into apoptotic bodies that separate from the cell, which often contain pyknotic nuclear fragments (Elmore, 2007; Manjo, 1995). In the final stage of the process, apoptotic bodies are rapidly engulfed by phagocytes; this engulfment is triggered by the presentation of specific intracellular molecules on the cell surface, the most well-characterized of which being phosphatidylserine (Fan and Bergmann, 2008). Cell fragments are then transferred from the phagocyte to the lysosome, where they are broken down into their basic building blocks (amino acids, nucleotides, monosaccharides, and fatty acids) (Fuchs and Steller, 2011).

Apoptosis, however, is not an isolated cellular event. Cell-cell signaling is one of the main mechanisms by which the self-destruction of a cell is induced, but programmed cell death is by no means silent. Recent studies have shown that apoptotic cells can have diverse effects on neighboring cells as well, the most relevant for this study being apoptosis-induced compensatory cell proliferation, in which apoptotic cells release mitogenic factors to neighboring stem or progenitor cells, inducing cell division (Jäger and Fearnhead, 2012; Fan and Bergmann, 2008). Thus, apoptosis may have an even more pronounced role in homeostasis and regeneration than previously thought.

Given the crucial role of apoptosis in both the life and death of all multicellular organisms, a thorough understanding of the mechanisms and intricacies of this process is of utmost importance. Colonial ascidians of the subfamily Botryllinae are ideal organisms for studying programmed cell death, as they exhibit an extraordinary capability of regulating apoptosis and its complement, mitosis, in a coordinated and cyclical fashion (Lauzon *et al.*, 2007). These organisms are of the subphylum Tunicata; this branch of the phylum Chordata that has been recently shown using phylogenomic analysis to replace

the subphylum Cephalochordata as the closest invertebrate ancestor of modern-day vertebrates (Delsuc *et al*., 2006; Ballarin *et al.*, 2008). The specific animal used in this study is *Botryllus schlosseri*, a colonial ascidian native to coastal regions of the Mediterranean Sea (Berrill, 1950). Today, *B. schlosseri* has achieved a cosmopolitan distribution, as it is found in shallow waters and harbors throughout the world (Berrill, 1950).

B. schlosseri colonies originate from a single, sexually produced larval tadpole, which displays a chordate body plan including a notochord, dorsal nerve tube, segmented musculature, and gills slits (Lauzon *et al.*, 2002; Ruppert *et al.*, 2004). After hatching from the maternal colony, the free-swimming tadpole soon migrates to a solid, subtidal surface where it undergoes metamorphosis into the next life cycle stage, and becomes an oozooid (Lauzon *et al.*, 2007; Milkman, 1967). At this stage, all chordate features have been lost except for the pharyngeal slits, and the sessile, filter-feeding oozooid begins to asexually generate buds as outgrowths of its lateral wall, near the anteroventral end (Milkman, 1967; Sabbadin, 1969). Buds begin as hollow, blastula-like spheres, and eventually differentiate into three layers: the outer epidermis, a layer of circulating blood cells, and the inner atrial epithelium (Brien, 1968; Sabbadin, 1969). After approximately one week, the oozooid regresses and the first generation of asexual buds mature into adult filter-feeding zooids (Sabbadin, 1969). As this process continues, the number of adult zooids increase, and a star-shaped system eventually forms (Sabbadin, 1969). For the remainder of the colony's life span, two generations of asexual buds are continually produced: primary buds, offshoots of the adult zooid, and secondary buds, offshoots of the primary buds (Lauzon *et al.*, 2007). Buds are connected to each other, the parent

zooids, and the rest of the colony through a vast network of extracorporeal vasculature (Burighel and Brunetti, 1971). The entire colony is embedded in a transparent, gelatinous, cellulose-rich tunic, throughout which the blood vessels also transverse and eventually terminate, forming the vascular ampullae at the periphery of the colony (Zaniolo, 1981).

It is its unique budding cycle, however, which makes *B. schlosseri* such a pertinent model system for this study. At any point in the colony's lifecycle, three generations are present: the adult zooid, the primary bud, and the secondary bud (Watanabe, 1953; Sabbadin, 1969). All three generations develop in concert through the week-long budding cycle and thus function as a single, synchronous physiological unit (Watanabe, 1953). This budding cycle is known as blastogenesis and consists of four main phases, A through D. Phase A represents the onset of the new blastogenic cycle; the oral and excurrent siphons of the zooid open, and the secondary bud begins to evaginate from the lateral wall of the primary bud and skews towards the anterior hemisphere of the adult zooid (Lauzon *et al.*, 2002). In phase B, the heartbeat begins in the primary bud, and the secondary bud grows to form a closed double-layered vesicle (Lauzon *et al.*, 2002). In phase C, organogenesis begins in the secondary bud, and pigment cells become visible in the outer epithelium of the primary bud (Lauzon *et al.*, 2002). Throughout these first three phases, the adult zooid is feeding through its pulsating open siphon, and its size remains relatively constant (Lauzon *et al.*, 2002).

The final phase of blastogenesis and the focus of this particular study, phase D, is often known as takeover (Lauzon *et al.*, 2002). This phase involves massive physiological change through the simultaneous processes of programmed cell death and organismal regeneration (Lauzon *et al.*, 1992). In the first sub-stage of takeover, stage

D1, periodic contractions of the zooids are observed; however, by the end of this sub-stage, the siphons have shut down, but are still responsive to mechanical stimulus (Lauzon *et al.*, 1996). In stage D2, contractions have terminated and the zooid is no longer responsive when probed with a mechanical stimulus (Lauzon *et al.*, 1996). Stage D3 is characterized by the shrinkage of the adult zooid, loss of pigmentation, appearance of circulating phagocytes and simultaneous resorption of zooid organs and tissues (Lauzon *et al.*, 1996). Circulating phagocytes play an integral role in this process; they have been shown to engulf cell corpses present during takeover, therefore functioning in programmed cell clearance following massive cell death in the tissues and organs of the adult zooid generation (Burighel and Brunetti, 1971; Lauzon *et al.*, 1993; Voskoboynik *et al.*, 2004; Lauzon *et al.*, 2007). During stage D3, the heart is still beating, which presumably facilitates removal of cell corpses via phagocytes through the lacunae and vascular sinuses of the dying zooid (Lauzon *et al.*, 2002). In the final sub-stage, stage D4, the zooid is reduced to the size of a small vesicle, in which the heart has completely stopped beating (Lauzon *et al.*, 1996). Throughout this process, the primary bud has grown increasingly larger to become the pre-zooid, and by the onset of the next cycle, has developed into the new adult zooid generation (Sabbadin, 1969). Similarly, the secondary bud has grown in concert to become the new primary bud, off of which a new secondary bud will invaginate (Sabbadin, 1969). The takeover phase of blastogenesis is successfully completed within 24-36 hours.

Given the fundamental role of apoptosis during blastogenesis, as well as the sheer number of cells that undergo programmed cell death during this process, we have focused our present study on understanding the spatial distribution and fate of apoptotic cells in

this model organism. We have narrowed our scope to examine solely the final phase of blastogenesis (takeover), during which the most drastic physiological changes occur in the regenerative process. We hypothesized that the greatest apoptotic activity would be located in the dying adult zooid, while lower levels of apoptotic activity would be detected in both the primary and secondary buds, where mitosis is the main process driving their development into the next functional generation of adults. Furthermore, we hypothesized that high levels of apoptosis would be detected within the blood vessels, where circulating phagocytes are known to play a critical role in cell corpse clearance during asexual regeneration (Burighel and Brunetti, 1971; Lauzon *et al.*, 1993; Voskoboynik *et al.*, 2004; Lauzon *et al.*, 2007).

In order to determine the distribution of apoptotic cells in *B. schlosseri* colonies, tissues sections from each sub-stage of takeover were examined using the terminal deoxynucleotidyl transferase (TdT) dUTP nick end-labeling (TUNEL) assay. TdT is an enzyme that catalyzes the addition of bromolated deoxyuridine triphosphate nucleotides (Br-dUTPs) to the free 3´ hydroxyl groups that are formed as genomic DNA fragments during apoptosis (Otsuki *et al.*, 2003). Fluorescently-labeled antibodies specifically directed against Br-dUTPs allow for the observation of such DNA fragments using fluorescence microscopy. Thus, the selective detection and localization of apoptotic cells can be achieved.

While the underlying mechanisms involved in programmed cell death and whole-body, mitotic regeneration observed in *B. schlosseri* are largely unknown, it is evident that a large degree of crosstalk must take place, as these processes are observed to occur in concert with one ùanother. The goal of this study was to elucidate the location of

apoptotic cells and role that apoptosis plays in this regeneration in an attempt to better understand the relationship between these processes.

Methods

Specimen preparation

Animal Collection and Maintenance

Wild *Botryllus schlosseri* colonies were collected on algal blades of floating docks in Eel Pond in Woods Hole, MA in September 2012. After being carefully scraped off so as to prevent damage, the colonies were allowed to attach to 5 x 7.5 cm glass microscope slides (Fisher Scientific Research, Philadelphia, PA) and subsequently placed in sea water tanks for transport to the Union College Biology Department in Schenectady, NY, where all further experiments were performed. For feeding, the colonies were transferred to smaller containers of seawater from Woods Hole, and fed a diluted solution of aqua-cultured plankton daily (10 drops Phytoplex, 5 drops Chromaplex in 5 mL distilled water, distributed equally between five small containers) (Kent Marine, Franklin, WI).

Developmental Staging

Four healthy colonies were selected for imaging each of the developmental stages of blastogenesis. Glass microscope slides with adhered *Botryllus schlosseri* colonies were submerged in seawater on a slide holder in a plastic petri dish. Whole-body imaging was performed using a Leica DFC 450C stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were taken daily; the four colonies were followed for two complete blastogenetic cycles. Morphological characteristics of the adult zooid, primary bud, and secondary bud were used to assign the appropriate developmental stage, as previously described (Lauzon *et al.*, 2002).

Fixation and Embedding

Two to three colonies were chosen in each substage of stage D (D1 through D4) as representative samples for experimentation. Colonies were anaesthetized in liquid scintillation vials using MS222 (tricaine methanesulfonate) for 5 minutes in filtered sea water, and then fixed in fresh 4% paraformaldehyde/PBS (pH = 7.4/7.5) for two hours at 4°C. Samples were washed four times with 5mL of PBS buffer at this same temperature, with each wash lasting 10 minutes. Colonies were then dehydrated using a graded ethanol sequence (50%, 70%, 85%, 95%) for 10 minutes each on a shaker at 4°C. The final wash in 100% ethanol for 10 minutes was repeated three times.

Colonies were partitioned into groups of one or two zooid systems using a razor blade and then infiltrated in molten polyester wax (Electron Microscopy Sciences, Fort Washington, PA) at 42°C for 2 hours in a shaking hybridized oven (Robbins Scientific, Sunnyvale, CA). The wax was then removed and infiltration with fresh molten polyester wax was repeated. These specimens were incubated overnight at 42°C in the oven so as to complete the infiltration process.

Plastic square microtome embedding molds (VWR scientific, Radnor, PA) were pre-warmed on a slide warmer at 42°C and a zooid system was placed in the center of each mold, with the dorsal plane facing down. The molds were then infiltrated with fresh molten polyester wax on the slide warmer. Once the zooid systems had been correctly positioned, the molds were removed from the heat and placed overnight in a 4°C chamber to allow the wax to solidify.

Sectioning

Forty-eight hours prior to sectioning, polyester wax molds were placed in an enclosed container on a bed of calcium chloride pellets so as to prevent the hydration of the wax from water vapor in the air. After being removed from the dessication chamber, the edges of the wax blocks were shallowly shaved with a razor blade, creating a trapezoidal flat section in the center of the block, which contained the embedded specimen. The block was then loaded into an LKB Bromma 2218 Historange Microtome containing a disposable, high profile stainless steel blade (VMR Scientific, Radnor, PA), and 6 μm sections were cut. As soon as section curling began to occur, the microtome blade was replaced prior to making additional sections.

Once deep enough into the wax block so as to reach the ventral side of the organism, the sections were immediately collected and placed in a 30°C water bath in order to facilitate section uncurling. The sections were then placed on Fisherbrand SuperFrost Plus glass microscope slides (VMR Scientific, Radnor, PA) and left at room temperature overnight, so as allow them to dry and adhere to the glass. Each slide contained six sections, which were ordered into three groups of pairs. Slides were then stored in a slide box at room temperature until needed.

<u>TdT dUTP nick end labeling (TUNEL) assay</u>

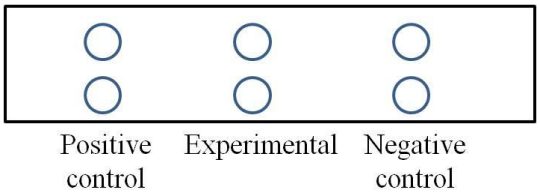
Dewaxing and Rehydration

Glass slides containing serial sections were submerged in Coplin jars containing a graded ethanol series for three minutes each, beginning with 100% ethanol, and following

with 95%, 85%, 70%, and 50% ethanol solutions. Slides were then submerged in 0.85% NaCl for 5 minutes, followed by a submersion in PBS (pH=7.4) for 5 minutes. This process served to remove the polyester wax and rehydrate the tissues. The slides were then allowed to air-dry. After all of the liquid had evaporated, the slides were checked under an SV8 Zeiss stereomicroscope (Carl Zeiss Group, Oberkochen, Germany) for leftover residue; if any was present, slides were incubated in water for five minutes, or until the residue was no longer visible. Slides were again allowed to air-dry.

Permeabilization

Three pairs of tissue sections per slide were isolated using a PAP pen (Electron Microscopy Sciences, Hatfield, PA); from left to right, pairs were labeled as the positive control, experimental, negative control.



Each pair was then washed with 100μL of phosphate buffered saline (PBS) (pH=7.4, stored at 4°C) so that the entire sample was submerged. The PBS was then removed after 3 minutes of incubation, followed by two additional identical PBS washes.

Tissue section pairs were incubated for 5 minutes in 100μL of a freshly prepared solution of proteinase K (20 μg/mL, stored at -20°C). Subsequently, a "quick wash" of 100μL of PBS (PBS flooded onto sample, and then immediately removed), followed by

three 100μL PBS washes on each pair, each lasting 3 minutes in order to remove excess proteinase K.

Positive Control

DNase I (Deoxyribonuclease I) buffer solution (Invitrogen, Carlsbad, CA) was prepared by mixing 90 μL sterile milli-Q water with 10 μL of 10x DNase I buffer (stored at -20°C) per pair. The experimental pair and the negative control pair were each incubated with 100 μL of the DNase I buffer solution. The positive control pair was incubated with 100 μL DNase I solution, which contained 80 μL of milli-Q water, 10 μL of 10x DNase I buffer, and 10 μL of DNase I (stored at -20°C). Slides were incubated for 20 minutes at 37°C in a moisture chamber (Tupperware container lined with wet paper towels) so as to create a humidified environment in order to minimize evaporative loss of fluid on the tissue sections. Solution was then removed from the slides, followed by one quick wash with 100 μL PBS per pair. Three 3-minute incubations of 100 μL PBS per pair were then performed on the slides.

TUNEL Staining

Tissue section pairs were washed twice with 100 μL Wash Buffer (Biovision, Milpitas, CA) for five minutes each. The positive control pair and experimental pair were each incubated with 51μL DNA Labeling Solution, which contained the following: 32.25 μL distilled water, 10 μL TdT Reaction Buffer, 8 μL Br-dUTP, and 0.75 μL TdT Enzyme (Biovision, Milpitas, CA). The negative control pair was incubated with 51 μL DNA

Labeling Solution lacking TdT Enzyme, therefore yielding the following mixture: 33 μL distilled water, 10 μL TdT Reaction Buffer, and 8 μL Br-dUTP (Biovision, Milpitas, CA). All reagents were stored at -20°C and kept on ice until immediately prior to use. The slides were then incubated in the dark for 1 hour at 37°C in the same moisture chamber as previously described. Given bromine's sensitivity to light, all subsequent steps were carried out in the least amount of light possible.

After incubation, the solutions were removed from the slides, followed by a quick wash of 100 μL PBS, and three 3-minute incubations of 100 μL PBS. All tissue pairs were then incubated with 100 μL Antibody Solution (95 μL Rinse Buffer and 5 μL Anti-BrdU-FITC, both stored at 4°C) (Biovision, Milpitas, CA) in the moisture chamber for 30 minutes at room temperature in the dark. The solution was then removed, followed by a quick wash with 100 μL of PBS. Slides were then submerged for 5 minutes in a Coplin jar of distilled water, and subsequently allowed to air-dry in a dark chamber.

Sections were then each mounted in a drop of Fluoro-Gel antifading reagent (Electron Microscopy Sciences, Hatfield, PA) and covered with a 24 x 50 mm glass coverslip (Premium Cover Glass 12-544-14, thickness no. 1) (Fisher Scientific Research, Philadelphia, PA). Results were observed using an Olympus BX60 epifluorescence microscope (Olympus Corporation, Center Valley, PA) and images were obtained using a SPOT 3CCD T60C camera (Diagnostic Instruments, Mahwah, NJ). When not in use, slides were stored in a slide box at 4°C.

Results

Developmental staging of B. schlosseri blastogenesis

Images taken using a Leica DFC 450C stereomicroscope and camera of the ventral side of a designated *B. schlosseri* colony tracked the development of the three asexual generations at various stages during blastogenesis. Stage A1 represents the onset of the new blastogenetic cycle; the oral and excurrent siphons of the zooid are open, and the secondary bud (not visible at this magnitude) has begun to evaginate from the lateral wall of the primary bud (Fig. 1). In stage B1, the heartbeat is observed in the primary bud, and the secondary bud will soon grow to form a closed double-layered vesicle (Fig. 1). In stage C1, organogenesis begins in the secondary bud, and pigment cells are visible in the outer epithelium of the primary bud (Fig. 1). Throughout these first three stages, the adult zooid is feeding through its siphons, and its size remains relatively constant. In stage D1, periodic contractions of the zooids are still seen and are still responsive to mechanical stimulus (Fig. 2). The primary bud grows increasingly larger next to its parental adult zooid. In stages D2 and D3, contractions have terminated and the zooid is no longer responsive when probed with a mechanical stimulus (Fig. 2). The adult zooid begins to contract in size and pigmentation is lost, though the heart is still beating. The primary bud grows to become the pre-zooid. In stage D4 leading to stage A1 of the new blastogenesis cycle, the adult zooid heart has completely stopped beating, and the zooid is reduced to the size of a small vesicle, which will eventually be resorbed (Fig. 2). By the end of stage D4, the pre-zooid will mature to a fully functioning new adult zooid, and the budding cycle will repeat. Though not visible at this magnitude and resolution, the

secondary bud will grow in concert throughout takeover to become the new primary bud by the end of stage D4, and in the next cycle of A1, a new secondary bud will evaginate.

Immunofluorescent localization of apoptotic cells in B. schlosseri tissues using terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assay

Green and red fluorescent cells were observed in various tissues of all three generations of *B. schlosseri* during takeover (stages D1 through D4)*;* red cells indicated the presence of nuclei (with intact or fragmented genomic DNA), while green fluorescent cells (TUNEL-positive) specifically indicated fragmented DNA, which was used as a marker of apoptotic cells (Figures 3-6). Tissues observed included the intestine, stomach, endostyle, blood space/blood vessels, peribranchial cavity, and tunic.

In the dying adult zooid, TUNEL-positive cells were observed to appear in the earliest stage of takeover (D1), and were noted to be most prevalent in the stomach, intestine, and tunic of the animal (Fig. 3). The abundance of TUNEL-positive cells in the adult zooid increased in a stage-dependent fashion throughout takeover; stages D2 and D3 were observed to have a greater presence of TUNEL-positive cells concentrated in these same tissues and organs. At these stages, phagocytes were also interspersed with the TUNEL-positive cells. Phagocytes were slightly larger than normal cells in the sample, and with the correct magnification and resolution, red fluorescent nuclei were observed within them, indicating that the phagocytes had engulfed cells whose DNA has yet to be fragmented (Fig. 3).

In the new generation (the primary bud developing into the new adult zooid), the same pattern of TUNEL-positive staining was observed (Fig. 4). During stage D2, green

fluorescence was observed primarily in the blood space of the new generation, with small numbers of TUNEL-positive cells present in the stomach and the intestine. In stage D3, more TUNEL-positive cells were observed to accumulate in the stomach and the intestine, and the presence of phagocytes in the surrounding structures became evident. Finally, in the last stage of takeover (D4), the greatest numbers of TUNEL-positive cells were observed in the new generation, and were largely concentrated in the stomach and intestine.

In the newly forming primary bud, few (if any) TUNEL positive cells were observed at any point during takeover (Fig. 5). TUNEL-positive cells and phagocytes were observed to accumulate in the tunic, and were especially evident in stages D3 and D4 (Fig. 6). The blood vessels of the colony were also observed to contain circulating phagocytes and cell corpses during all stages of takeover (Fig. 6).

Positive and negative controls were performed for each experiment, and experimental results were only analyzed if the controls had been successful. Negative controls contained no TUNEL-positive cells; only red fluorescent nuclei were observed (Fig. 6). All cells of the positive controls were labeled green, as they had been chemically treated with DNase I, and thus all contained fragmented DNA (Fig. 6).

Discussion

The goal of this study was to localize the patterns of apoptotic programmed cell death during the takeover phase of *B. schlosseri* blastogenesis using the TdT dUTP nick end-labeling (TUNEL) assay with the intention of further understanding the fate of apoptotic cells and their role in this unique budding cycle. The presence of apoptotic cells was observed to increase in a stage-dependent fashion in both the dying adult zooid and new generation pre-functional zooid, while no indication of apoptosis was detected in the new primary bud. The tunic was also observed to be a site of apoptotic programmed cell death, with the highest levels of apoptotic cells occurring in the finals stages of takeover. Blood vessels were seen to contain circulating apoptotic cells, as well as blood phagocytes with engulfed cell fragments.

Three theories to explain apoptosis localization

The results of this study indicate that blastogenesis is a complex, multi-faceted physiological and biochemical event, in which apoptotic programmed cell death plays a crucial role. The observed stage-dependent increase of apoptotic cells in the adult zooid during takeover is consistent with past studies and supports our hypothesis and logical conclusion (Voskoboynik *et al.*, 2004; Tiozzo *et al.*, 2006; Lauzon *et al.*, 2002 Gilman, unpublished observations, 2012;). As the adult zooid is observed to contract, regress, and eventually die during takeover, one would expect high levels of apoptosis to be present at this stage. Indeed, this is exactly what was observed in this study; the greatest concentration of apoptotic cells in the dying adult zooid occurred in stage D3, just before

the structures completely regressed. Apoptotic programmed cell death is thus the primary mechanism by which the organism succeeds in terminating the eldest generation.

On the other hand, the observed results of the pre-functional zooid during takeover are contrary to that which was hypothesized at the beginning of the study. Instead of finding low levels of apoptosis during the growth and differentiation of this generation, the same pattern of stage-dependent, increasingly high levels of apoptosis was observed in the pre-functional zooid as in the dying adult zooid. These results were consistent with a previous study performed localizing apoptotic cells in *B. schlosseri* pre-functional zooids during blastogenesis (Tiozzo *et a*, 2006). At the onset of takeover, apoptotic cells appeared to home selectively to the primary bud (soon-to-be pre-functional zooid) and increase in concentration thereafter; the secondary bud, on the other hand, was almost entirely TUNEL-negative. More specifically, the apoptotic cells were shown to concentrate pronouncedly in the stomach and the intestine in the primary bud/pre-functional zooid. These results were unanticipated given that this generation is growing and has been observed to undergo mitotic cell division in order to achieve this growth (Brown, unpublished observations, 2011; Kawamura *et al.*, 2008). Mitotic cells can be selectively observed using an antibody against the phosphorylated version of Histone H3 protein. This protein is part of the octamer core in chromatin and is phosphorylated at serine 10 (ser10) during chromosome condensation of mitosis, and thus, is an ideal marker to detect mitotic cells (Goto *et al.,* 1999). Observations from both Brown (unpublished observations, 2011) and Kawamura *et al.* (2008) indicate that mitotic activity is present in the primary bud, with high numbers of mitotic cells observed along the organs of the digestive tract, including those of the pre-functional zooid. In our

study, it was peculiar to detect apoptosis in the pre-functional zooid; from an energy conservation perspective, it makes little sense for the organism invest energy in generating new cells, only for them to die soon thereafter. We have therefore proposed three potential theories to explain this observed phenomenon. It is important to note that these theories are neither exhaustive nor mutually exclusive, but we believe they offer potential insight into the observations recorded in this study.

The first potential explanation is the expulsion theory. In this scenario, apoptotic cells of the dying adult zooid generation are transported to the gut of the pre-functional zooid in order to be expunged from the body into the sea water column. In this sense, the apoptotic cells function as a form of cellular waste products, of which the accumulation is detrimental to optimal functioning of the organism. The gut, having a direct conduit to the external sea water environment, would be an ideal homing location for these apoptotic cells, as their expulsion could be accomplished relatively quickly. The results observed in this study are consistent with this theory, as there is a notable concentration of apoptotic cells observed to accumulate in the gut of the pre-functional zooid. This theory is also supported by the findings of Tiozzo and colleagues (2006) in which transmission electron microscopy images show degenerating cells accumulating in the gastric and esophagus epitheliums of the pre-functional zooid. Our findings also suggest that the tunic could be a site of cell corpse clearance, as it is in direct contact with the external seawater as well. Apoptotic cells were present in the tunic at high concentrations during the later stages of takeover, and most likely move through the carbohydrate matrix in order to be expunged. As TUNEL-positive phagocytes were observed to be prevalent in the tunic during the final stages of takeover, it is possible that the elimination of cell

corpses occurs via these phagocytes, which could potentially emigrate across the blood vessel walls into the tunic, and eventually move out into the external environment. Transmission electron microscopy carried out at this stage of blastogenesis has revealed numerous phagocytes with engulfed cell corpses infiltrating the extravascular tunic, and is consistent with this scenario (Lauzon, unpublished observations).

In both of these cases, the colonial vasculature would play an integral role in the trans-generational movement of cell corpses for elimination. The blood vessels traverse the tunic and connect all three asexual generations to one another; therefore, these vessels would most likely be responsible for transporting the dying apoptotic cells from the adult zooid generation to these expulsion sites (Lauzon *et al.*, 2002). Phagocytes were observed within the blood vessels in this study, further supporting this notion. In addition, there is evidence that the heart is very important in cell clearance of the dying adult zooid; it continues to beat for 20 – 24 hours after the zooid begins to regress, and is ultimately the last organ to die (Lauzon *et al.*, 2002). It is hypothesized that this continued blood flood through the colony assists in the clearance of engulfed cell corpses that are either free in the vessels or carried by blood phagocytes (Lauzon *et al.*, 2002).

The second theory that may potentially explain the unusual accumulation of apoptotic cells in the pre-functional zooid during takeover is the recycling theory. This explanation suggests that the dying adult zooid cells do not die just to make room for the new generation to come to maturation, but instead, such cells are broken down into biomolecules and serve as nutrients necessary for the growth of the new generation. According to this scenario, cell corpses (either free or engulfed) may move from the regressing adult zooid via the vasculature, and eventually arrive at the gut of the new

generation, where they could be further digested and absorbed. The gut would be a logical place for this absorption to happen, as this is the site of nutrient absorption that is attained via the external seawater and filtered through the siphons.

Several past studies support this theory. Microsurgeries performed by Lauzon and colleagues (2002), in which all adult zooids were removed before the onset of takeover (zooidectomy), yielded much smaller functional zooids than sham-operated or control colonies with adult zooids left intact. This significant decrease in pre-functional/adult zooid length is certainly consistent with the recycling theory underlying blastogenesis; if there are no adult zooids cells present to undergo apoptosis and migrate to the gut of the new generation, consequently, there should be no biomolecules to be harvested and a lack of nutrients to assist in the growth of the new generation. This absence of recycled nutrients could explain the resulting small size of the pre-functional zooid generation in zooidectomized colonies.

In this same study, Lauzon *et al.* (2002) carried out budectomies, in which primary buds (soon-to-be pre-functional zooids) were microsurgically removed prior to takeover. As the colony then progressed through blastogenesis, the remaining primary buds grew to be much larger than those observed in any of the control organisms (Lauzon *et al.*, 2002). The size of the new adult generation was inversely proportional to the number of primary buds that remained after the microsurgery (Lauzon *et al.*, 2002). In budectomies in which all but one primary bud was removed prior to takeover, this lone primary bud was observed to grow to an unprecedented size, and became a “superzooid” upon maturation (Lauzon *et al.*, 2002).

The appearance of a superzooid is also consistent with the recycling theory. Since all but one of the primary buds had been removed, if the cells of the dying adult zooid are indeed then broken down into biomolecules serving as food for the next asexual generation, the single remaining primary bud would be the only recipient. Therefore, one would expect that the lone primary bud would grow to become larger than expected and ultimately become a functional superzooid, as observed in this previous study (Lauzon *et al.*, 2002). If this theory is valid, the vasculature also must play an essential role in transport of biomolecules. In order for the developmental synchrony of blastogenesis to be retained in these microsurgically-altered organisms, the blood vessels had to be left intact (Lauzon *et al.*, 2002). This indicates that if the dying adult zooid is a nutrient source for the new blastogenetic generation, the biomolecules travel trans-generationally via the colonial vasculature.

The third potential explanation aligns with a theory that has emerged recently called apoptosis-induced compensatory cell proliferation (AICCP), which attempts to establish a functional link between apoptotic cell signaling and the growth of neighboring cells within tissues and organs of living organisms. The concept of AICCP hypothesizes that apoptotic cells release mitogenic factors during the process of their own demise, which in turn, induce neighboring stem or progenitor cells to proliferate (Jäger and Fearnhead, 2012; Fan and Bergmann, 2008; Chera and Ghila, 2009). This phenomenon has been observed in many different organisms in the animal kingdom such as *Drosophila*, *Xenopus*, planaria, *Hydra*, and mice (Fan and Bergmann, 2008; Chera and Ghila, 2009; Li *et al.*, 2010; Hwang *et al.*, 2004; Tseng *et al.*, 2007). As exemplified in these studies, AICCP can serve different purposes in different organisms. For example,

Hydra uses AICCP to promote head regeneration, while *Drosophilia* uses it for homeostatic maintenance in imaginal discs in the eye (Fan and Bergmann, 2008; Chera and Ghila, 2009). In mice, on the other hand, AICCP was shown to play a role in skin wound healing (Li *et al.*, 2010). In all of these cases, apoptosis is no longer considered an isolated event, but rather, it serves an integrated and more complex purpose.

The results observed in this study are certainly consistent with the theory of apoptosis-induced compensatory cell proliferation. Since apoptosis and proliferation occur in tandem during takeover in *B. schlosseri*, it is possible that the apoptotic cells of the adult zooid release mitogenic factors that then either move via the vasculature to the primary bud and induce it to grow and differentiate into the new generation adult zooid, or in situ as those cell corpses are reabsorbed by tissues of the new asexual generation. If this hypothesis is valid, apoptosis in *B. schlosseri* can be understood as a multi-faceted method of regeneration, serving to both rid the organism of the oldest generation, but also ensuring that the new generation grows to take its place. Testing this hypothesis would ideally involve carrying out TUNEL staining and phospho Histone H3 antibody staining simultaneously on slides in order to determine whether mitosis occurs alongside apoptotic cell corpses in the new generation. However, this approach is impracticable as TUNEL staining involves treating the slides with proteinase K, which is a broad-spectrum serine protease, and would digest the Histone H3 necessary for mitotic labeling (Ebeling *et al.*, 1974).

Phagocytes assist in cell corpse clearance

It is clear that phagocytes play an important role in the process of zooid resorption (Lauzon *et al.*, 2002; Lauzon *et al.*, 2013; Ballarin *et al.*, 1993; Ballarin and Cima, 2005). In this study, we observed that TUNEL-positive phagocytes circulate in the colonial vasculature at all stages of takeover; their TUNEL-positive status is likely the result of cell corpses that they have engulfed and are beginning to break down. Intact nuclei were also observed within phagocytes. The phagocytes then travel through the colonial vasculature to achieve trans-generational movement, propelled by the beating of the adult zooid heart, which continues to pump blood late into takeover (Lauzon *et al.*, 2002). These findings indicate that the phagocytes function to engulf the cells of the dying adult zooid and clear the cell corpses from the area via the vasculature in order to allow for resorption of this structure. Thus, such phagocytes are an essential part of the asexual regeneration and homeostatic maintenance of the colony.

Circulating phagocytes may also undergo apoptotic cell death during takeover. Lauzon and colleagues (2013) have identified two major populations of phagocytes present in *B. schlosseri*: a static population positioned along the lining of the circulatory system epithelia, and a mobile population that continuously circulates throughout the colony and exhibits selective homing to mesenchymal niches called ventral islands. These ventral islands function as sites of phagocyte turnover, as ventral island phagocytes were observed to engulf one another at these locations during and following takeover (Lauzon *et al.*, 2013). Our findings also suggest that TUNEL-positive phagocytes accumulate in the extravascular tunic during the later stages of takeover. Although direct demonstration of trans-endothelial migration of phagocytes from blood into tunic is

lacking as of this writing, our observations suggest that elimination of dying adult zooid cells is accomplished in part by the tunic, as it could function to expel the apoptotic cells into the surrounding seawater.

Future studies

The results obtained in this study are meant to serve as building blocks for further elucidation of the role of apoptosis during asexual regeneration of *B. schlosseri*. Now that the patterns of apoptosis have been localized, it is possible to continue to understand why such patterns are present and what function they serve for organismal regeneration within the colony. The three possible theories (expulsion, recycling, and AICCP) represent the beginning stages of this process, but in order to determine the validity of each, additional studies must be carried out.

TUNEL staining on budectomized and zooidectomized colonies represents an important step in elucidating the true purpose of the apoptotic cell migration to the new generation. For example, if the recycling theory is accurate, one would expect to observe a high concentration of TUNEL-positive cells in the superzooid as a result of the breakdown of cells it absorbs for food. However, this theory would be weakened if the pre-functional zooids of zooidectomized organisms contained TUNEL-positive cells. Since the adult zooids of these organisms would have been removed during the zooidectomy, this would indicate that the apoptotic cells must have come from within the pre-functional zooids themselves. As previously mentioned, an effective way to simultaneously visualize apoptotic and mitotic cells must be developed in order to support the AICCP theory. With the current methods, the TUNEL staining proteinase K

step would inhibit the phospho Histone H3 antibody staining. One possibility would be to carry out TUNEL and phospho Histone H3 labeling on separate serial sections.

In addition, transmission electron microscopy must be utilized in order to obtain a fine structural detail and understanding of the fate of the apoptotic cells during the takeover phase of blastogenesis. If the elimination theory were to be correct, this approach would allow for direct observation of cell corpse expulsion in the gut and tunic. Resorption of cell fragments and biomolecules by the new generation cells would be able to be observed if the recycling theory were accurate; similarly, the AICCP theory would be supported if the movement of apoptotic cells could be tracked at various time points during takeover, and if this movement was found to be spatially and temporally linked with the onset of mitotic activity in the new generation. Therefore, using these combined approaches of antibody staining and transmission electron microscopy, it will be possible to further confirm where the fate of apoptotic cells and what influence they have on the regenerative nature of a *B. schlosseri* colony.

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References

Ballarin, L., Cima, F., Sabbadin, A. (1993). Histoenzymatic staining and characterization of the colonial ascidian *Botryllus schlosseri* hematocytes. *Italian Journal of Zoology*. 60, 19-24.

Ballarin, L., Cima, F. (2005). Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-funcitonal characterization. *European Journal of Histochemistry*. 49, 225-264.

Ballarin, L., Burighel P., Cima F. (2008). A tale of death and life: natural apoptosis in the colonial ascidian *Botryllus schlosseri* (Urochordata, Ascidiacea). *Current Pharmaceutical Design.* 14(2), 138-147.

Berrill, N. (1950). *The Tunicata, with an account of the British species.* Ray Society, London.

Brien, P. (1968). Blastogenesis and morphogenesis. *Advances in Morphogenesis.* 7, 151-203.

Brown, C. (2011). Spatiotemporal patterning of mitosis during cyclical blastogenesis of the marine protochordate *Botryllus schlosseri.* Undergraduate senior thesis, Union College.

Burighel, P., Brunetti, R. (1971). The circulatory system in the blastozooid of the colonial ascidian *Botryllus schlosseri*. *Italian Journal of Zoology.* 38(3), 273-289.

Chera, S., Ghila, K. (2009). Apoptotic cells provide an unexpected source of Wnt3 signaling todrive hydra head regeneration. *Developmental Cell.* 17(2), 279-289.

Danial, N., Korsmeyer S. (2004). Cell death: critical control points. *Cell*. 116(2), 205-219.

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature.* 439(7079), 965-968.

Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H., Lang, H. (1974). Proteinase K from *Tritirachium album* limber. *European Journal of Biochemistry*. 47, 91-97.

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology.* 35(4), 495-516.

Fan, Y., Bergmann, A. (2008). Distinct Mechanisms of Apoptosis-Induced Compensatory Proliferation in Proliferating and Differentiating Tissues in *Drosophilia* Eye. *Developmental Cell.* 14(3), 399-410.

Fuchs, Y., Steller, H. (2011). Programmed Cell Death in Animal Development and Disease. *Cell.*147(4), 742-758.

Gilman, J. (2012). Spatio-temporal patterns of apoptosis and mitosis during organismal regeneration in a basal chordate. Undergraduate senior thesis, Union College.

Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., Sakurai, M., Okawa, K., Iwamatsu, A., Okigaki, K., Takahashi, T., Inagaki, M. (1993). Identification of a novel phosphorylation site on Histone H3 coupled with mitotic chromosome condensation. *The Journal of Biological Chemistry.* 274, 25543-25549.

Greenhalgh. D. (1998). The role of apoptosis in would healing. *The International Journal of Biochemistry &Cell Biology.* 30(9), 1019-1030.

Häcker, G. (2000). The morphology of apoptosis. *Cell and Tissue Research.* 301(1), 5-17.

Hengartner, M. (2000). The biochemistry of apoptosis. *Nature.* 407, 770-776.

Hwang, J., Kobayashi, K., Agata, K., Ikeo, K., Gojobori, T. (2004). Detection of apoptosis during planarian regeneration by the expression of apoptosis-related genes and TUNEL assay. *Gene.* 333, 15-25.

Jacobson, M., Weil, M., Raff, M. (1997). Programmed cell death in animal development. *Cell.*88(3), 347-54.

Jäger, R., Fearnhead, H. (2012). "Dead cells talking": The silent form of cell death is not so quiet. *Biochemistry Research International*.

Jänicke, R., Sprengart, M., Wati, M., Porter, A. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. 273(16), 9357-9360.

Kawamura, K., Tachibana, M., Sunanaga, T. Cell proliferation dynamics of somatic and germline tissues during zooid life span in the colonial tunicate *Botryllus primigenus. Developmental Dynamics.* 237, 1812-1825.

Kerr, J., Wyllie, A., Currie, A. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer.* 26:239-257.

King, K., Cidlowski, J. (1998). Cell cycle regulation and apoptosis. *Annual Review of Physiology.* 60, 601-617.

Lauzon, R., Ishizuka, K., Weissman I. (1992). A cyclical, developmentally regulated death phenomenon in a colonial urochordate. *Developmental Dynamics.* 174, 71-83.

Lauzon, R., Patton, C., Weissman, I. (1993). A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri* (Tunicata, Ascidiacea). *Cell & Tissue Research.* 272(1), 115-127.

Lauzon, R., Chang, W., and Dewing, L. (1996). Evidence for transcriptional modulation but not acid phosphatse expression during programmed cell death in the colonial tunicate *Botryllus schlosseri*. *Microscopy Research and Technique.* 34: 218-227 (1996).

Lauzon, R., Ishizuka, K., Weissman, I. (2002). Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: a model for development and regeneration. *Developmental Biology.* 249(2), 333-348.

Lauzon, R., Kidder S., Long P. (2007). Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate. *Developmental Biology.* 301(1), 92-105.

Lauzon R., Brown, C., Kerr, L., Tiozzo, S. (2013). Phagocyte dynamics in a highly regenerative urochordate: insights into development and host defense. *Developmental Biology.* 374(2), 357-373.

Li, F., Huang, J., Chen, Y., Peng, D., Roop, J., Bedford, C., Li, C. Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. (2010). *Science Signaling.* 3(110), ra13.

Lindsten, T., Ross, A., King, A., Zong, W., Rathmell, J., Shiels, H., Ulrich, E., Waymire, K., Mahar, P., Frauwirth, K., Chem, Y., Wei, M., Eng, V., Adelman, D., Simon, M., Ma, A., Golden, J., Korsmeyer, S., MacGregor, G., Thompson, C. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Molecular Cell.* 6(6), 1389-1399.

Majno, G., Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *American Journal of Pathology.* 146(1), 3-15.

Milkman, R., (1967). Genetic and developmental studies on *Botryllus schlosseri*. *The Biological Bulletin.* 132(2), 229-243.

Norbury, C., Hickson, I. (2001). Cellular responses to DNA damage. *Annual Review of Pharmacology and Toxicology.* 41, 367-401.

Otsuki, Y., Li, Z., Shibata, M. (2003). Apoptotic detection methods – from morphology to gene. *Progress in Histochemistry and Cytochemistry.* 38(3), 275-339.

Ruppert, E., Fox, R., Barnes, R. (2004). Urochordata, invertebrate zoology: a functional evolutionary approach. Thomas Brooks/Cole, Belmont, CA. 940-963.

Sabbadin, A. (1969). The compound ascidian *Botryllus schlosseri* in the field and in the laboratory. *Stazione Zoologica.* 37, 62-72.

Sanamyan, K.; Monniot, C. (2013). *Botryllus schlosseri* (Pallas, 1766). In: Shenkar, N.; Gittenberger, A.; Lambert, G.; Rius, M.; Moreira Da Rocha, R.; Swalla, B.J.; Turron, X. (2013) Ascidiacea World Database. Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=103862.

Thornberry, N., Lazebnik, Y. (1998). Caspases: enemies within. *Science.* 281(5381), 1312-1316.

Tiozzo, S., Ballarin, L., Burighel, P., Zaniolo, G. (2006). Programmed cell death in vegetative development: apoptosis during the colonial life cycle of the ascidian *Botryllus schlosseri*. *Tissue and Cell.* 38(3), 193-201.

Tseng, A., Adams, D., Qui, D., Koustubhan, P., Levin, M. (2007). Apoptosis is required during early stages of tail regeneration in *Xenospus laevis*. *Developmental Biology.* 301(1), 62-69.

Voskoboynik, A., Rinkevich, B., Weiss, A., Moiseeva, E., Reznick, A. (2004). Macrophage involvement for successful degernation of apoptotic organs in the colonial urochordate *Botryllus schlosseri. The Journal of Experimental Biology.* 207, 2409-2416.

Watanabe, H. (1953). Studies on the regulation in fused colonies in *Botryllus primigenus* (Ascidiae compositae). *Science Reports of Tokyo Bunrika Daigaku*. Section B7, 183-198.

Wyllie, A. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature.* 284, 555-556.

Zaniolo, G. (1981). Histology of the ascidian *Botryllus schlosseri* tunic: in particular, the test cells. *Italian Journal of Zoology.* 48, 169-178.

Appendix

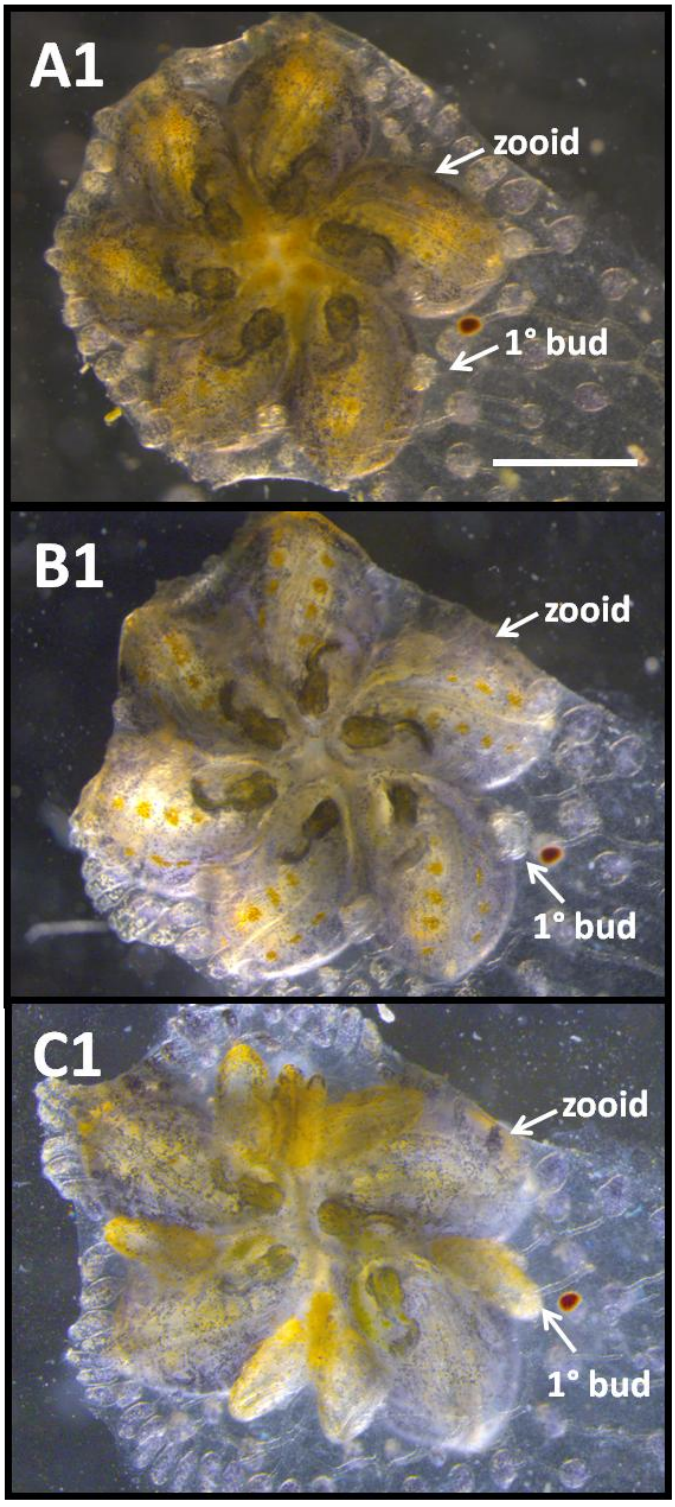

Figure 1: Developmental staging of *B. schlosseri* blastogenesis in phases A through C. Images were taken using a Leica DFC 450C stereomicroscope and camera of a designated *B. schlosseri* colony at various stages during its lifecycle in order to track the development of the three asexual generations present in the colony: the adult zooid, the primary bud, and the secondary bud. The three images were taken approximately 48 hours apart. Panels A1, B1 and C1 represent ventral views of a single system colony. Scale bar = 1 mm.

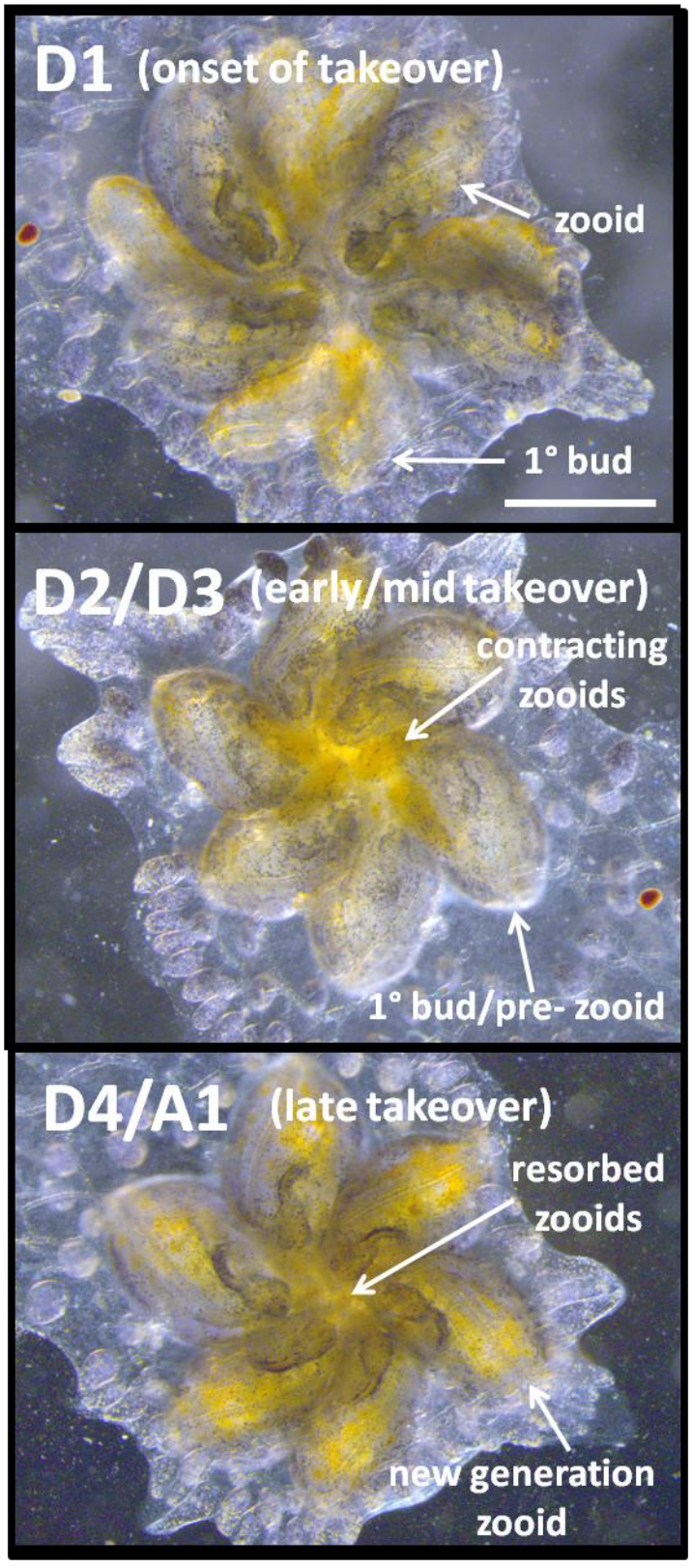
D1 (onset of takeover)
zooid
1° bud
D2/D3 (early/mid takeover)
contracting zooids
1° bud/pre- zooid
D4/A1 (late takeover)
resorbed zooids
new generation zooid

Figure 2: Developmental staging of *B. schlosseri* during the takeover phase of blastogenesis (stages D1-D4). Images were taken using a Leica DFC 450C stereomicroscope and camera of a designated *B. schlosseri* colony at each of the stages of takeover in order to track the regenerative metamorphosis of the three asexual generations present in the colony: the adult zooid, the primary bud, and the secondary bud. Panels D1, D2/3 and D4/A1 represent ventral views of a single system colony. Scale bar = 1 mm.

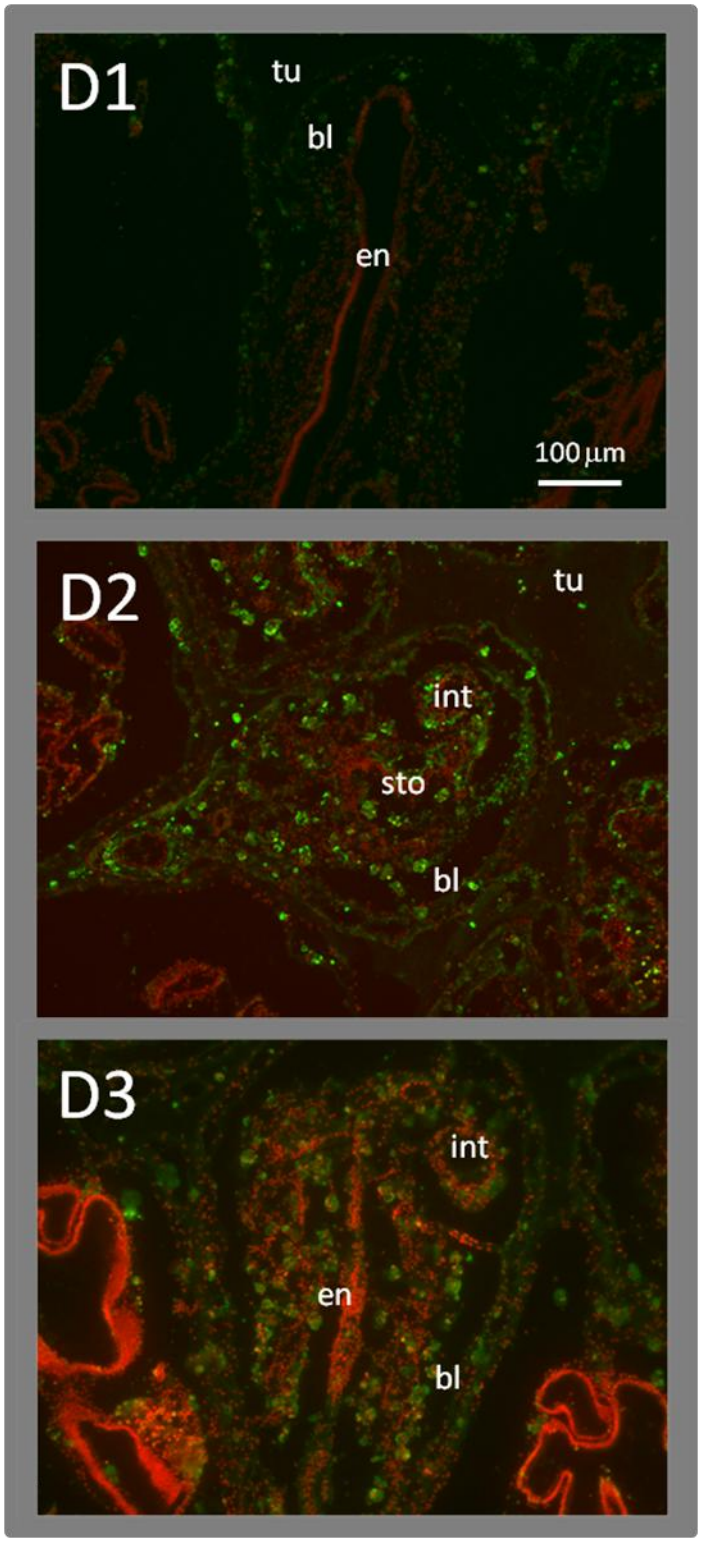
D1
tu
bl
en
100 µm
D2
tu
int
sto
bl
D3
int
en
bl

Figure 3: Immunohistochemistry of TUNEL positive cells in dying adult zooid during stages D1 through D3 of blastogenesis in *B. schlosseri*. Serial tissue sections of the ventral axis of the adult zooid were stained with TdT dUTP nick end labeling (TUNEL) assay in order to mark fragmented DNA (green fluorescence), indicative of apoptotic cells. Red fluorescence indicate any cell nuclei (apoptotic or not) as a result of the propidium iodide counterstain. TUNEL-positive cells were most prevalent in the stomach, intestine, and tunic during all stages. The number of apoptotic cells increased in a stage-dependent fashion during takeover. Stage D4 is not shown, as in this phase, the adult zooid is resorbed.

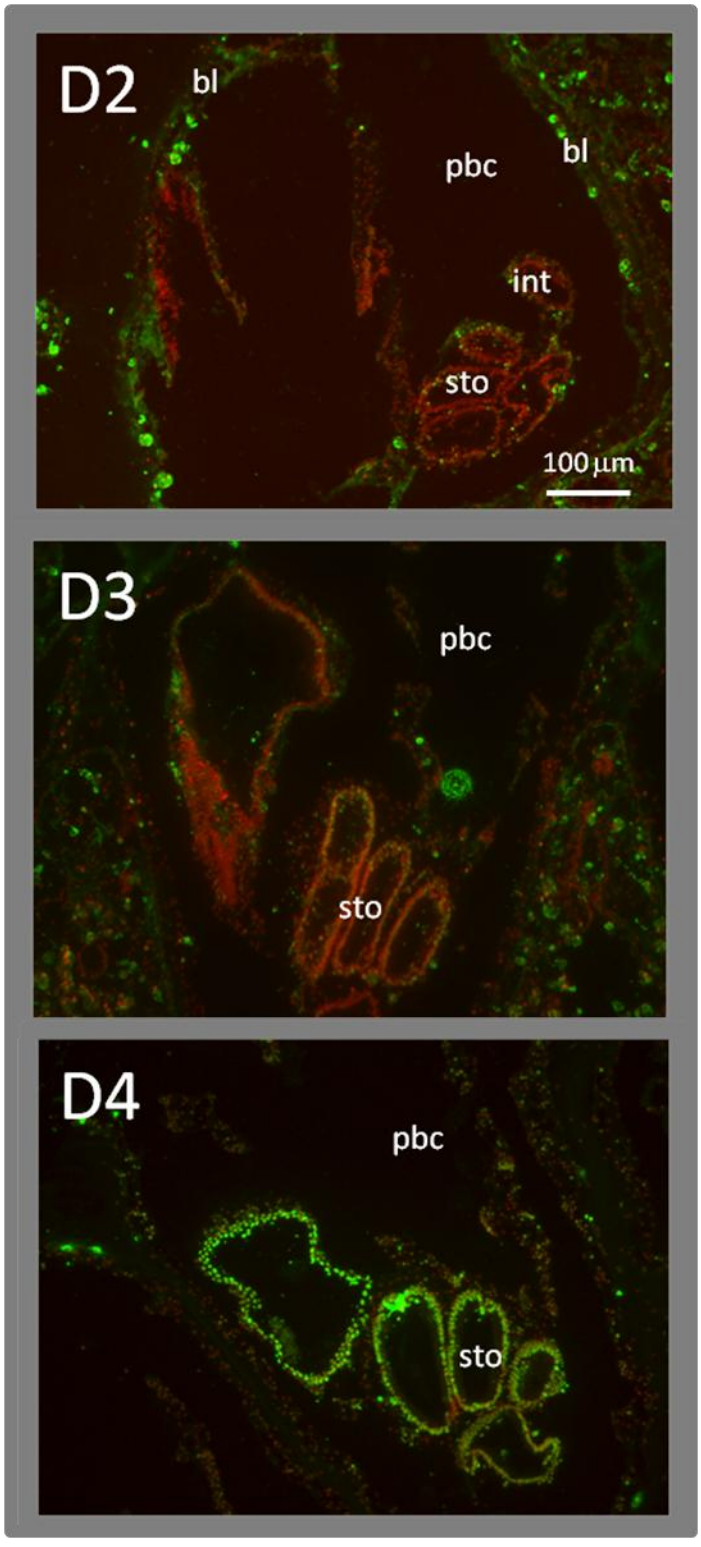
D2
bl
pbc
bl
int
sto
100 µm
D3
pbc
sto
D4
pbc
sto

Figure 4: Immunohistochemistry of TUNEL positive cells in the new generation of pre-functional zooids during stages D2 through D4 of blastogenesis in *B. schlosseri*. Serial tissue sections of the ventral axis of the pre-functional zooid (former primary bud) were stained with TdT dUTP nick end labeling (TUNEL) assay in order to mark fragmented DNA (green fluorescence), indicative of apoptotic cells. Red fluorescence indicate any cell nuclei (apoptotic or not) as a result of the propidium iodide counterstain. TUNEL-positive cell prevalence increased, specifically in the stomach and intestine, as the primary bud grew and differentiated into the new adult zooid generation.

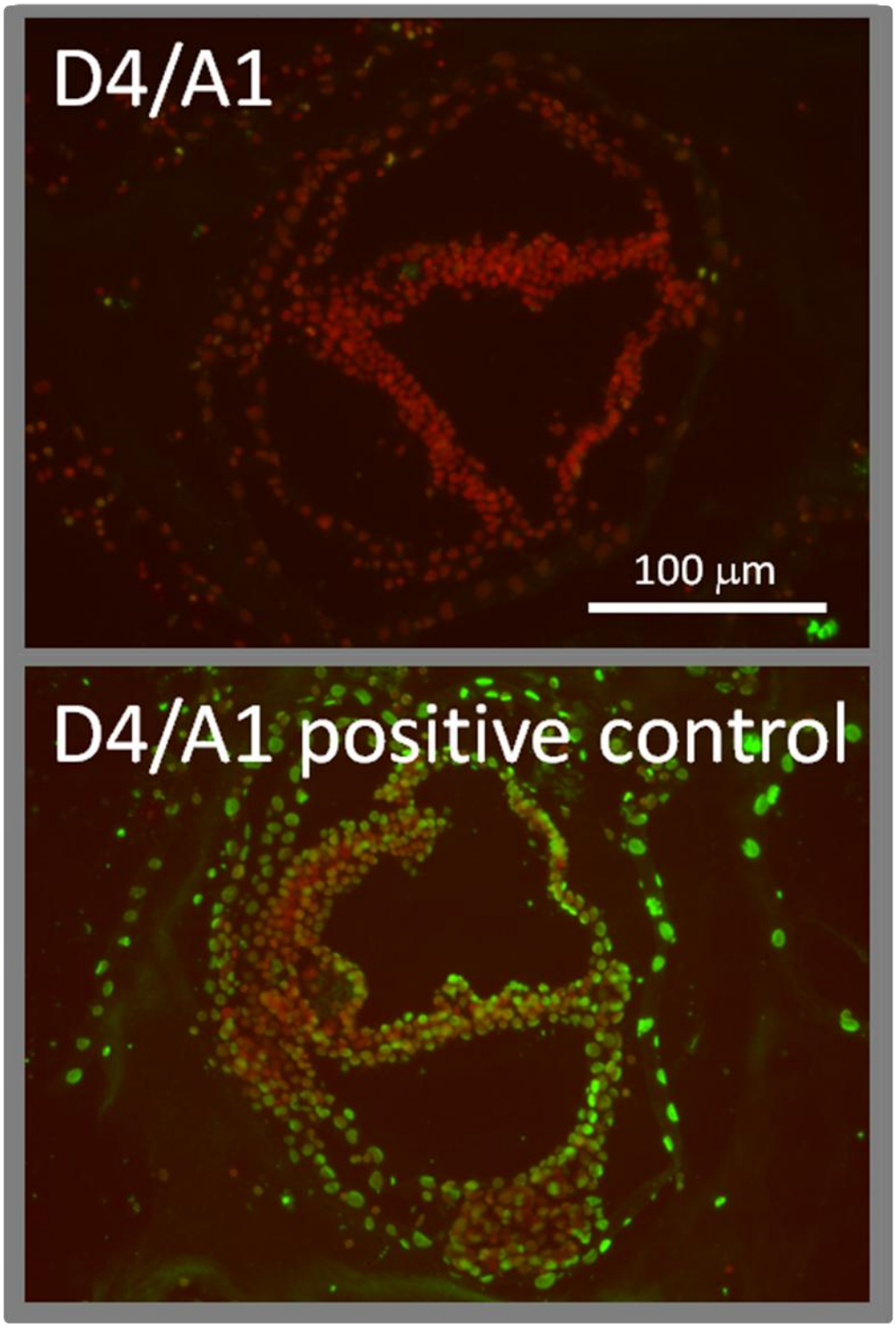
D4/A1
100 μm
D4/A1 positive control

Figure 5: Immunohistochemistry of TUNEL positive cells in newly developing primary bud during stages D4/A1 of blastogenesis in *B. schlosseri*. Serial tissue sections of the ventral axis of the newly developing primary bud (former secondary bud) were stained with TdT dUTP nick end labeling (TUNEL) assay in order to mark fragmented DNA (green fluorescence), indicative of apoptotic cells. Red fluorescence indicate any cell nuclei (apoptotic or not) as a result of the propidium iodide counterstain. In the experimental sections, few TUNEL-positive cells were identified in any organs of the newly developing primary bud. The positive control shown below the experimental verifies the validity of the assay.

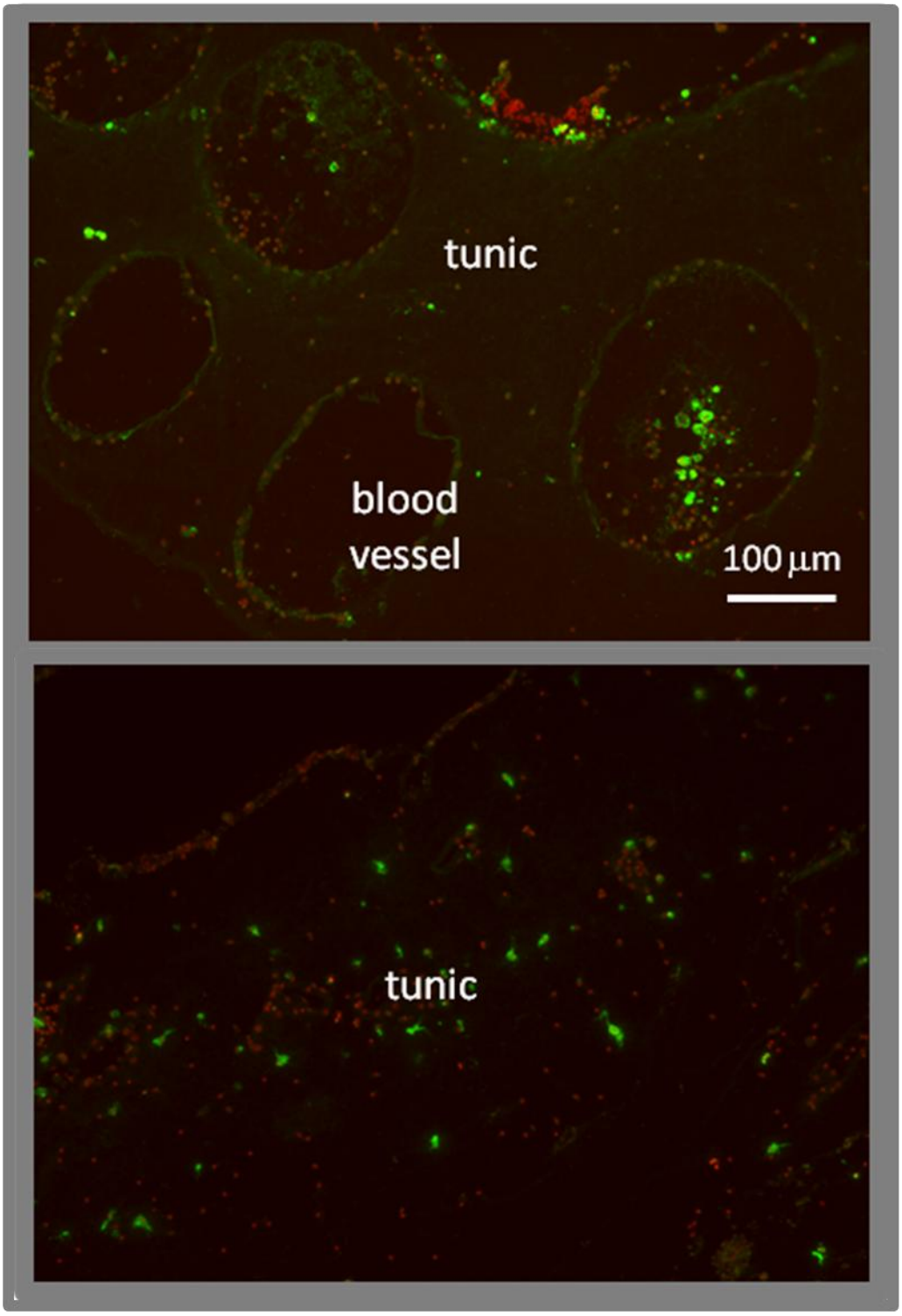
tunic
blood
vessel
100 μm
tunic

Figure 6: Immunohistochemistry of TUNEL positive cells in tunic and blood vessels during stages D4/A1 of blastogenesis in *B. schlosseri*. Serial tissue sections of the ventral axis of the tunic and blood vessels were stained with TdT dUTP nick end labeling (TUNEL) assay in order to mark fragmented DNA (green fluorescence), indicative of apoptotic cells. Red fluorescence indicate any cell nuclei (apoptotic or not) as a result of the propidium iodide counterstain. TUNEL-positive cells were present in the tunic throughout takeover. In addition blood vessels were observed to contain circulating phagocytes, many of which contained engulfed cell corpses.

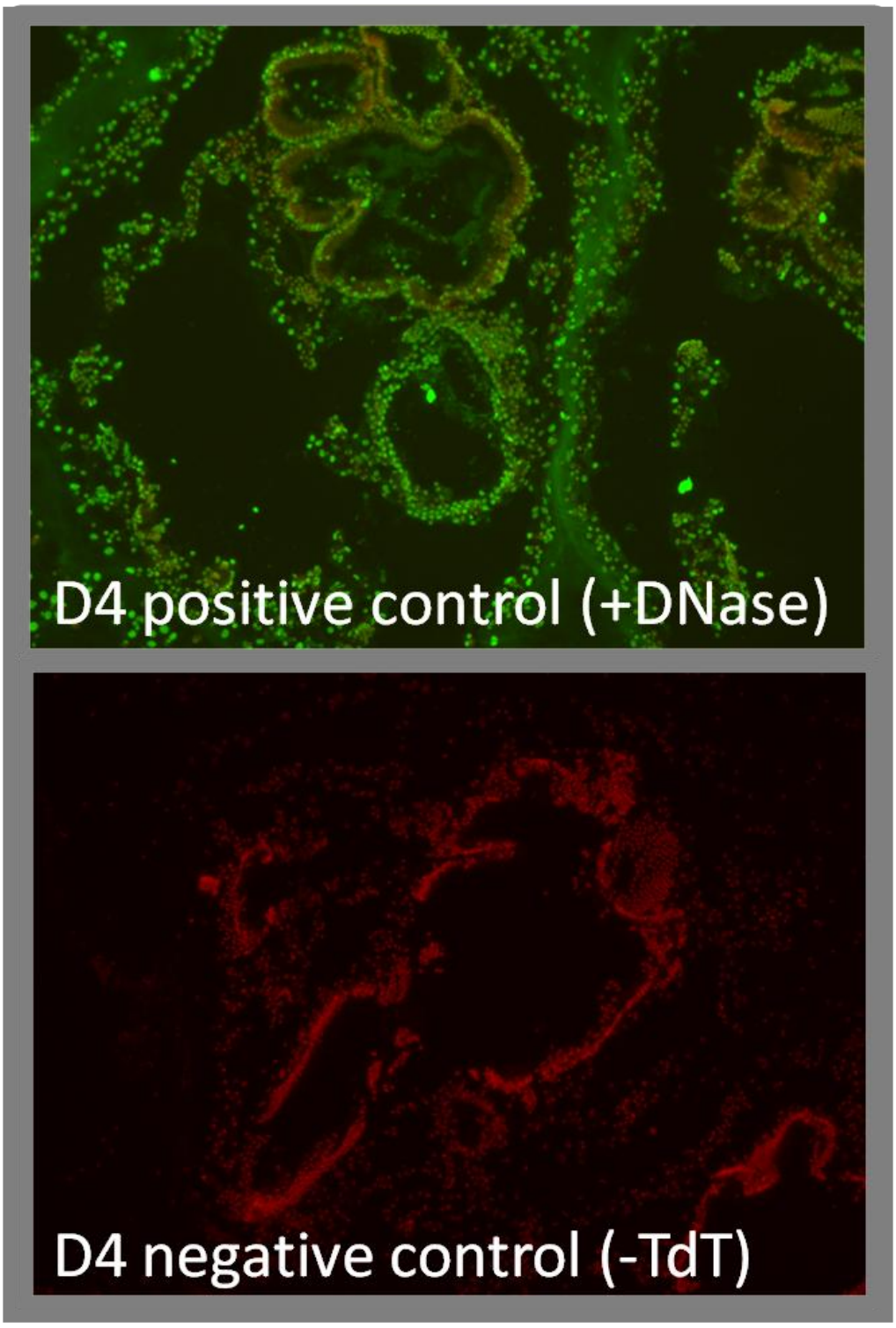
D4 positive control (+DNase)
D4 negative control (-TdT)

Figure 7: Immunohistochemistry of TUNEL assay positive and negative controls of adult zooid in a stage D4 colony. Serial tissue sections of the ventral axis of the tunic and blood vessels were stained with modified TUNEL assay reagents so as to yield positive and negative controls. The positive control was treated with DNase, so as to fragment all nuclear DNA; thus, all cells appear TUNEL-positive. The negative control lacked TdT enzyme, and therefore catalysis of the addition reaction of Br-dUTP to 3′-OH of the fragmented DNA in apoptotic cells was unable to occur. Thus, all cells appear TUNEL-negative.