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1 Article

# 2 Cnidarian cell cryopreservation: A powerful tool for 3 cultivation and functional assays

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## 15 Running Title: Cryopreservation of Cnidarian Cell Cultures

16 **Abstract:** Cnidarian primary cell cultures have a strong potential to become a universal tool to  
17 assess stress-response mechanisms at the cellular level. However, primary cell cultures are  
18 time-consuming regarding their establishment and maintenance. Cryopreservation is a commonly  
19 used approach to provide stable cell stocks for experiments, but it is yet to be established for  
20 Cnidarian cell cultures. The aim of this study was therefore to design a cryopreservation protocol  
21 for primary cell cultures of the Cnidarian *Anemonia viridis*, using dimethyl sulfoxide (DMSO) as a  
22 cryoprotectant, enriched or not with foetal bovine serum (FBS). We determined that DMSO 5%  
23 with 25% FBS was an efficient cryosolution, resulting in 70% of post-thaw cell survival. The success  
24 of this protocol was first confirmed by a constant post-thaw survival independently of the cell  
25 culture age (up to 45 days old) and the storage period (up to 87 days). Finally, cryopreserved cells  
26 displayed a long-term recovery with a maintenance of the primary cell culture parameters and  
27 cellular functions: formation of cell aggregates, high viability and constant cell growth, and  
28 unchanged intrinsic resistance to hyperthermal stress. These results will further bring new  
29 opportunities for the scientific community interested in molecular, cellular and biochemical aspects  
30 of cnidarian biology.

31 **Keywords:** primary cell culture; sea anemone; *Anemonia viridis*; DMSO; marine invertebrate;  
32 post-thaw recovery

## 33 1. Introduction

34 *In vitro* cell cultures are important tools for research in many fields, including development,  
35 virology, cancer research, toxicity testing, biotechnology, biomedicine as well as for environmental  
36 research [1–3]. Mammalian cells lines are well established and commonly used since decades,  
37 followed by other vertebrates (e.g. zebrafish; for review Vallone et al. 2007 [4]) and insect cell lines  
38 (for review Lynn, 2001 [5]). Despite much effort devoted since the 1970s (see for reviews Rinkevich,  
39 2005 [6], 2011 [7] and Cai and Zhang, 2014 [8]) marine invertebrate cell cultures are not as advanced.  
40 While marine invertebrate cell lines (i.e. permanently established cell cultures) are yet to be  
41 available, recent reports on the establishment of primary cell cultures are encouraging with  
42 maintenance and/or growing from a couple of days to weeks [9–14].

43 Once protocols for reproducibly initiating primary cell cultures are established, the next  
44 important obstacle to overcome is the development of preservation procedures in order to outreach

45 primary cell cultures limitations, notably their limited lifespan. Indeed, such a preservation tool will  
46 reduce the frequency of primary cell culture establishment and will minimize wastage of a valuable  
47 resource at reseedings by creating cell stocks for as long as a primary cell culture is healthy. Among  
48 preservation procedures, cryopreservation is considered to be the optimal long-term storage method  
49 for maintaining a variety of biological materials, including cell cultures, in a state of metabolic arrest  
50 for considerable periods of time [16].

51 To date, for marine invertebrates, spermatozoa, oocytes, embryos and different larval stages  
52 have been successfully cryopreserved mostly from Mollusk and Echinoderm species, but also from  
53 Arthropod and Cnidarian species (see for reviews Odintsova and Boroda, 2012 [17] and Paredes,  
54 2015 [18]). Other biomaterials have been studied in a cryopreservation context, such as coral  
55 fragments [19] and primmorphs from sponge cells [20]. The tolerance to various cryoprotectants of  
56 tissue balls from corals was also investigated by Feuillassier et al. (2015) [21]. However, considering  
57 the limited advancements in marine invertebrate cell cultures, cryopreservation of marine  
58 invertebrate dissociated cells is seldom reported [18]. If they are, they largely focus on Mollusks  
59 [22–30], with some other studies conducted on Echinoderms (e.g. dissociated cells from sea urchins  
60 larvae [24,28]), on sponge cells [31–34] and on coral cells dissociated from embryos or larvae [35,36].  
61 Besides, none of these studies, except those on sponge cells, maintained cryopreserved cells in  
62 cultures for more than a few days nor use cryopreserved cells for subsequent experiments.

63 One of the major factors that determines the success of a cryopreservation protocol is the type of  
64 cryoprotecting agents (CPAs) used [16]. CPAs prevent damage to the cells from changes in osmotic  
65 pressure and intracellular ice crystal formation. Among the various CPAs, dimethyl sulfoxide  
66 (DMSO), a penetrating CPA, is the most common and widely used cryoprotectant to maintain frozen  
67 cell lines. The precise mechanism by which it protects cells remains unclear; it has been suggested  
68 that DMSO depresses the freezing point of cryosolutions [37], and that it can modulate the water  
69 network hydrating the membrane hence reducing the stress induced by the volume changes of  
70 water during freeze-thaw [38]. Penetrating CPAs could however induce some cytotoxicity due to the  
71 disruption of intracellular signalling which results in cell death [39–41]. For marine invertebrate  
72 studies, Paredes (2015) [18] reported in her review that DMSO was the most effective CPA for 70% of  
73 the published work on germ cells, embryos and larvae compared to others CPAs such as glycerol.  
74 This trend is also found for marine invertebrate dissociated cells [22,35,21,33]. In addition, in marine  
75 invertebrate studies, DMSO was found as an efficient CPA on its own [22,29,35] but more frequently  
76 in combination with other CPAs or with proteins, vitamins or sugar cocktails [18,24, 26,27,33].  
77 Indeed, the preservative capacity of DMSO was long known to be increased when serum, such as  
78 foetal bovine serum (containing cocktail of proteins), is added to the cryosolution [42,43].

79 We have previously reported the establishment of primary cell cultures of a soft-body  
80 cnidarian, the temperate sea anemone *Anemonia viridis* [9]. The established cell culture protocol  
81 resulted in the maintenance of primary cell cultures with gastrodermal signature [15]. These cell  
82 cultures were successfully used to assess the cellular response (e.g. viability) to environmental stress  
83 [15] thus creating new perspectives for further fundamental, environmental and biotechnological  
84 questions. An efficient cryopreservation procedure would therefore be an essential and powerful  
85 tool for facilitating research in deciphering molecular mechanisms and cellular events in cnidarian  
86 cells.

87 The aim of this study was therefore to design a cryopreservation protocol for primary  
88 gastrodermal *A. viridis* cell cultures in order to ensure a high post-thaw cell survival, preserving  
89 long-term recovery: cell viability, cell growth and physiological responses. All these advances will  
90 participate to raise the cnidarian cell cultures as a model system for marine invertebrate research  
91 perspectives.

## 92 2. Material and Methods

### 93 2.1. Biological Material

94 Five individuals of *Anemonia viridis* (Forskål 1775) were collected (prefectural authorization  
95 n°107 ; 02/28/2019) from 'Plage des ondes', Antibes, France, (43°33'17"N, 7°07'17.7"E), and  
96 maintained in a closed-circuit aquarium with artificial seawater (ASW) at 36-38 ‰ with Prodigio  
97 Expert Reef Salt, at 18.0 ± 0.5 °C with weekly water changes. A LED bar (450 nm – Deckey LED  
98 aquarium) provided light at a constant saturating irradiance of 100 µmol m<sup>-2</sup>s<sup>-1</sup> (measured using a  
99 special sensor QSL-100, Biospherical Instruments Inc.) on a 12h:12h (light:dark) photoperiod. Sea  
100 anemones were fed once a week with oysters.

### 101 2.2. Primary cell cultures

102 From each *A. viridis* individual, an independent primary cell culture was obtained and  
103 maintained as described in Ventura et al. (2018) [15]. Briefly, cell dissociation was performed  
104 enzymatically with 0.15% collagenase type I (Sigma-Aldrich). Cells were cultured at 20.0 ± 0.5 °C and  
105 in the dark, in an optimized culture medium (CM) consisted of : 20% GMIM (Gibco), 5% foetal  
106 bovine serum (FBS; PAA/GE Healthcare), 1% kanamycin (100 µg/ml, Sigma-Aldrich), 1%  
107 amphotericin B (2.5 µg/ml; Interchim), 1% antibiotic antimycotic solution (Sigma-Aldrich), 1% L-  
108 glutamate (Sigma-Aldrich) and 71% of filtered ASW. The CM was adapted in respect to the  
109 Mediterranean Seawater characteristics (i.e. salinity 40 ppt and pH 8.1). From day 3, culture medium  
110 was replaced weekly and cells were seeded at 250,000 cells/ml in 12 well-plates.

### 111 2.3. Cryopreservation protocol

112 As cryoprotectant, DMSO (Sigma-Aldrich) was tested at two concentrations in the final CPA  
113 solution: 5% or 10% (following Munroe et al., 2018 [33]). DMSO was dissolved in the CM or in the  
114 CM enriched with foetal bovine serum (FBS) at 25% final. Control conditions without DMSO were  
115 also tested using CM enriched or not with FBS (i.e. 'CM' or 'CM + 25% FBS').

116 From day 17 after dissociation, the primary cell cultures were established with reliable cellular  
117 parameters [15]. By consequence, the cultivated cells were cryopreserved at different time points,  
118 from day 17 to 45 after cell dissociation. Each cryopreserved material contained 2 million cells that  
119 were placed in a cryotube containing 1 ml of the tested solution. Cryotubes were directly placed in a  
120 -80°C freezer (Ultra-Low Temperature VIP series, SANYO) and kept there for 8 to 87 days.

121 For thawing, cryotubes were removed from the -80°C freezer after the defined period and  
122 immediately transferred for 1-2 min into a water bath, pre-warmed at 20°C.

123 For seeding the cryopreserved cells, the cryotubes were centrifuged for 5 min at 1500 rpm. The  
124 supernatant was then removed, the cell pellets resuspended in the cell culture medium and seeded  
125 at 250,000 cells/ml in 12 well-plates [15].

### 126 2.4. Cell survival, cell viability, cell growth rate and cell size assessment

127 Cell survival was measured right after thawing cryopreserved cells, before reseeding. It was  
128 determined as the percentage of viable cells relative to the 2 million cells initially cryopreserved. To  
129 assess the number of viable cells, a sub-sample (100 µl) of cryopreserved cells was harvested after  
130 the thawing phase. Cell viability was assessed by evaluating the membrane integrity thanks to the  
131 Evans blue method. Therefore, viable cells (unstained) and dead cells (stained) were identified and  
132 counted. on a Neubauer improved haemocytometer (Sigma-Aldrich) using an optic microscope  
133 (Zeiss Axio Imager Z1).

134 Cell viability was measured every week to monitor the cell culture health state overtime. A  
135 sub-sample (100 µL) of cultivated cells was harvested weekly and using Evans blue method, viable  
136 cells (unstained) and dead cells (stained) were identified and counted. The cell viability was defined  
137 as the percentage of viable cells relative to total cells (i.e. viable and dead cells). In addition, two  
138 complementary methods for cell viability assessment, i.e. overall enzymatic activity using the

139 fluorescein diacetate (FDA) staining combined with a non-vital dye (Hoechst) and cell metabolic  
140 activity with 2-(4, 5-dimethyl-2-thiazolyl)-3, 5-diphenyl-2H tetrazolium bromide (MTT) assay, were  
141 also conducted (see details in Supplementary Material and Methods).

142 Cell growth rate was also assessed every week using the previous viable cells counts with  
143 Evans blue method. The following formula was then used to calculate the 7-day averaged daily  
144 growth rate:

$$145 \text{ Cell daily growth rate} = \frac{(\text{Viable cells}_{d+7} - \text{Viable cells}_d) / \text{Viable cells}_d}{7}, (d = \text{day})$$

146 Cell growth rate and cell viability were monitored for each cell culture before and after  
147 cryopreservation. The monitoring of these factors for cryopreserved cells was done by considering  
148 that the age of the cells at the thawing time is the same age they were at the freezing time.

149 Before and after cryopreservation, during the cell counts under optic microscope (objective  
150 x20), cells were measured, and cell sizes were scored with Zeiss microscope software Zen 2 (blue  
151 edition).

## 152 2.5. Hyperthermal stress experiment

153 In order to assess the maintenance of cryopreserved cells functionality, the response of  
154 cryopreserved cells to a controlled stress experiment was investigated. The cultivated *A. viridis* cells  
155 response to hyperthermal stress was assessed following the protocol published by Ventura et al.  
156 (2018) [15]. Hyperthermal stress was induced in 12-well plates exposed to two different  
157 temperatures: 20°C (control) and 28°C (hyperthermal condition), for 7 days. This experiment was  
158 conducted either with non-cryopreserved or cryopreserved cells, 7 days after thawing. At least four  
159 independent experiments were conducted from four primary cell cultures. For each assay, we  
160 analyzed 3 wells as technical replicates.

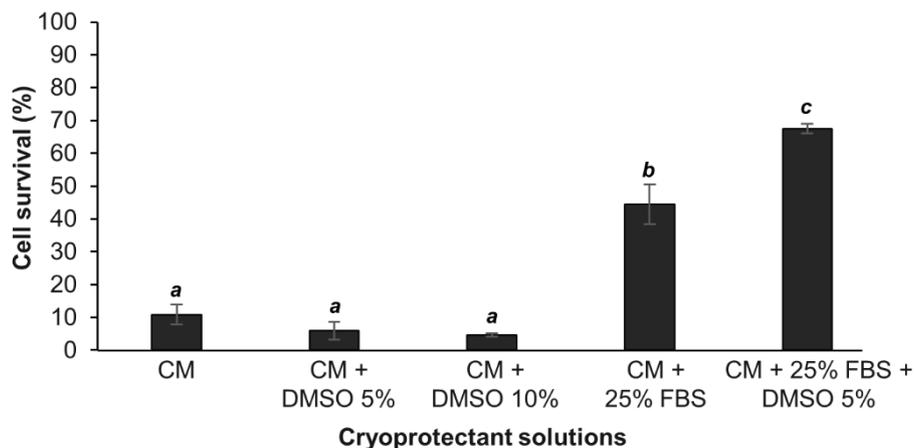
## 161 2.6. Statistical analyses

162 All statistical analyses were conducted using the R v.3.6.0 software[44]. In order to assess the  
163 effect of the cryosolutions on cell survival, to compare global viability and growth between  
164 non-cryopreserved and cryopreserved cells, and to compare viability and growth rate values after  
165 hyperthermal stress, either one-way ANOVA analyses were performed when parametric analyses  
166 were possible (under normality and variance equality assumptions), or Kruskal-wallis when  
167 non-parametric analyses were required. These analyses were followed, if necessary, by the  
168 appropriate post-hoc, i.e. Tukey for ANOVA analyses, and Dunn for Kruskal-wallis analyses. Then,  
169 to investigate the effect of the storage duration and the cell culture age on the cell survival,  
170 correlation tests with linear regression model were conducted. Finally, repeated measures ANOVA  
171 were conducted to compare cell viability and growth through time of non-cryopreserved and  
172 cryopreserved cultures.

### 173 3. Results and Discussion

#### 174 3.1. Success in set up of cryopreservation protocol on cell survival

175 The efficiency of the cryopreservation solutions was first evaluated with the percentage of cells  
 176 that survive a cryopreservation period of  $10 \pm 1$  days and the subsequent thawing process. When  
 177 cells were cryopreserved in the culture medium (CM) or in CM with DMSO 5 or 10% the mean  
 178 percentage of cells that survived at  $-80^{\circ}\text{C}$ , was around 7%. There were no significant differences  
 179 between these conditions (ANOVA;  $p > 0.05$ ) (Figure 1). However, when cells were cryopreserved in  
 180 the two conditions containing an FBS supplementation, we observed a significant higher percentage  
 181 of cell survival compared to non-enriched medium conditions ( $p < 0.01$  and  $p < 0.0001$  respectively for  
 182 the CM+25% FBS and for the DMSO 5% in the CM+ 25%FBS). Compared to FBS enriched CM alone  
 183 ( $\pm 45\%$  survival rate), adding 5% of DMSO to the FBS enriched CM significantly enhanced the  
 184 survival rate of the cells to 67% ( $p < 0.05$ ; Figure 1). Thus, the latter constituted the optimal  
 185 cryosolution among those tested for cryopreserving cnidarian cells.



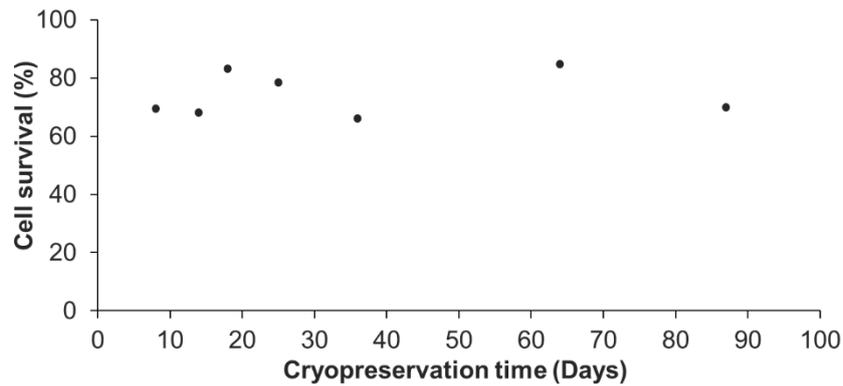
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187 **Figure 1.** Percentage of cell survival following  $10 \pm 1$  days of cryopreservation and thawing. For each  
 188 tested cryopreservation solution, the number of viable cells were compared to the 2 million cells  
 189 initially cryopreserved. Mean values and standard errors are represented, with  $n \geq 3$  biological  
 190 replicates per condition. The ANOVA revealed significant differences between data ( $p = 1.14 \cdot 10^{-10}$ ) and  
 191 the results from the Tukey post-hoc analysis are represented with letters:  $a \neq b$  ( $p < 0.01$ ),  $a \neq c$   
 192 ( $p < 0.0001$ ),  $b \neq c$  ( $p < 0.05$ ).

193 As it was observed in most of marine invertebrate studies [18,24,26,27,33], DMSO was found to  
 194 be an efficient CPA for *A. viridis* cultivated cells when it combined with serum supplementation. The  
 195 reason could be that carbohydrates, lipids and proteins present in serums act as membrane  
 196 stabilizers, therefore they may help preventing membrane damage during the freezing process  
 197 [45–48].

198 The survival rate of the designed cryopreservation protocol for *A. viridis* cultivated cells with  
 199 the optimal cryosolution is comparable to those determined for dissociated cells [26,33] or other  
 200 biomaterials [18] from marine invertebrates, as well as the ones from vertebrate *in vitro* cells [49].

201 Since the optimal cryopreservation solution, among those tested in this study, was found to be  
 202 the DMSO 5% in FBS enriched culture medium, this solution was reused for different  
 203 cryopreservation durations in order to determine the influence of the cryopreservation time on cell  
 204 survival after thawing. The representative results for a *A. viridis* cell culture shown in Figure 2  
 205 demonstrated that there was no influence of the cryopreservation duration on the cell survival.  
 206 Indeed, from 8 to 87 days at  $-80^{\circ}\text{C}$ , the percentage of cells that survived cryopreservation and  
 207 thawing, did not vary significantly (linear regression model; coefficient not statistically significantly  
 208 different from 0,  $p > 0.05$ ). The statistical analyses done on all available data (at least 3 biological  
 209 replicates) confirmed that there were no significant differences between the cryopreservation  
 210 durations ( $p > 0.05$ ; data not shown).



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**Figure 2.** Percentage of cell survival following different cryopreservation durations and thawing. For each tested cryopreservation duration, the number of viable cells were compared to the 2 million initially cryopreserved cells. These representative results were obtained from one primary cell culture cryopreserved at 24 days after initial seeding in 7 cryotubes, each cryotube being analyzed at the end of a given cryopreservation duration (from 8 to 87 days). No significant differences between times were found (linear regression model;  $r^2=0.037$ ;  $p=0.443$ ).

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Cells could therefore be cryopreserved for almost 3 months without any impact on the post-thaw survival compared to short-term cryopreservation, further validating the efficiency of the designed protocol. This is a major and significant progress for marine invertebrate cell cryopreservation. In fact, although few studies evaluated the cryopreservation efficiency after a storage of several weeks [22,24,25,28] and a maximum of 12 months [30], the majority only cryopreserved cells for a few hours to a few days [23,26,27,35,36,29,33].

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Cryopreservation of vertebrate cell lines is well-advanced, and protocols allow to keep cryopreserved cells for years [16]. Therefore, additional experiments are required to assess if longer cryopreservation durations are possible and/or to determine the maximal duration using the protocol developed in this study. If a duration limitation were to be found, a long-term storage in liquid nitrogen, or  $-150^{\circ}\text{C}$  freezers following the initial  $-80^{\circ}\text{C}$  freezing should be envisioned.

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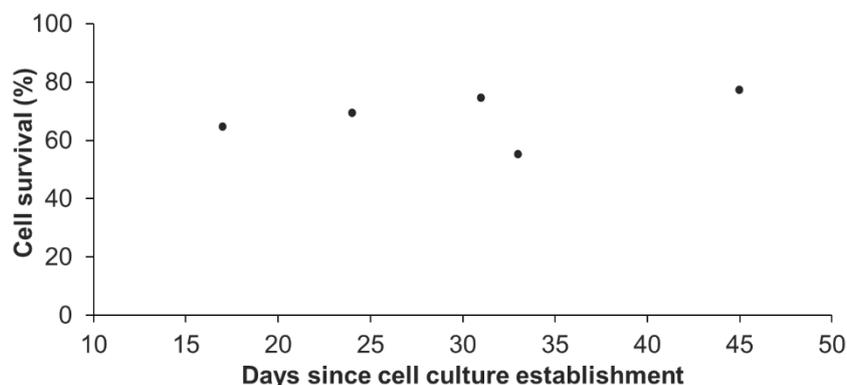
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In addition, we also assessed the influence of the age (time after initial seeding) of the primary cell culture on the survival at thawing. Representative results of one primary cell culture, cryopreserved at five different ages (from day 17 to day 45, Figure 3) revealed no significant differences (linear regression model; coefficient not statistically significantly different from 0,  $p>0.05$ ). The statistical analyses done on all available data (3 biological replicates) confirmed that there was no influence of the culture age on the cell survival ( $p>0.05$ ; data not shown). One primary cell culture can therefore be cryopreserved at each reseeding, as long as the cellular parameters previously defined for “healthy” *A. viridis* primary cell culture are maintained, i.e. cell aggregates formation, high viability and constant growth rate [15]. Being able to do so, considerable amounts of cell stocks for each cell culture could be created.



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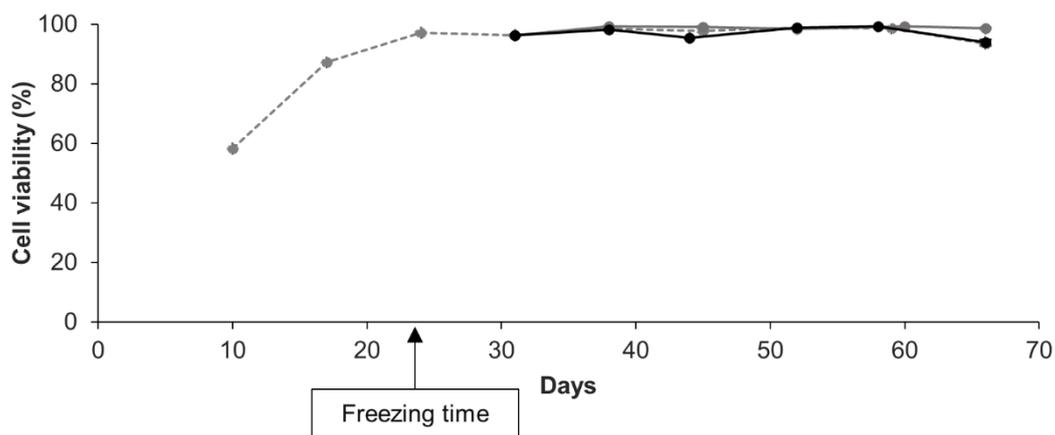
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**Figure 3.** Percentage of cell survival after thawing in function of the age of the cell culture. Representative results were obtained from one primary cell culture cryopreserved at different times

242 since its initial seeding (from day 17 to day 45). At each age tested, one cryotube was analyzed. No  
 243 significant differences between ages were found (linear regression model;  $r^2=0.15$ ;  $p=0.512$ ).

### 244 3.2. Absence of cryopreservation impact on cell recovery and functional parameters

245 To assess long-term cell recovery, we measured weekly, at each reseeding, the cell viability of  
 246 all primary cell cultures cryopreserved. Viability of cryopreserved cells was monitored for a period  
 247 going from 6 to 12 weeks after thawing and was compared to the viability of the corresponding  
 248 non-cryopreserved cell culture. Results of cell viability monitoring for 6 weeks after thawing for one  
 249 primary cell culture cryopreserved at day 24 for 8 or 36 days are presented in Figure 4. The data  
 250 show that cryopreserved cells were stably viable after thawing through time (>90% viability) with a  
 251 cell viability equivalent to that of the corresponding non-cryopreserved cell culture (repeated  
 252 measures ANOVA;  $p>0.05$ ). Repeated measures ANOVA conducted on all biological replicates,  
 253 confirmed no differences in the cell viability over time between non-cryopreserved and  
 254 cryopreserved cells nor with the cryopreservation storage period ( $p>0.05$ ; see Figure S1 and S2). The  
 255 mean of viability, over time, was maintained at  $95 \pm 1.9\%$  and at  $96 \pm 0.99\%$ , respectively in the  
 256 different non-cryopreserved and cryopreserved cell cultures monitored (ANOVA;  $p>0.05$ ; see Figure  
 257 S3a). In addition, data obtained with the two complementary cell viability assays performed on  
 258 non-cryopreserved and cryopreserved cells at different time points of the kinetics confirmed all  
 259 these results, i.e. no differences in cell viability between non-cryopreserved and cryopreserved cells  
 260 and a maintenance over time of the cell viability (ANOVA ;  $p>0.05$ ; see Figure S4).



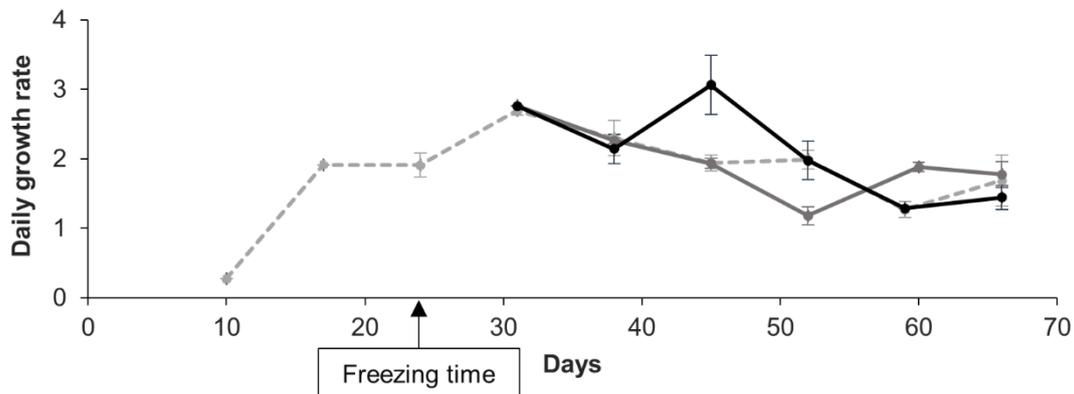
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262 **Figure 4.** Over time cell viability of a representative cell culture, comparing cryopreserved and  
 263 non-cryopreserved cells. Non-cryopreserved culture in grey dotted line, the same culture  
 264 cryopreserved at 24 days since initial seeding and thawed after 8 days of storage in grey solid line  
 265 and after 36 days in black solid line. The age of the cryopreserved cells at thawing is considered the  
 266 same as at the freezing time. Mean values of three technical replicates are shown with standard error  
 267 bars (although not visible because smaller than the data point symbols). Repeated measures ANOVA  
 268 revealed no significant differences in cell viability at each time between the non-cryopreserved  
 269 culture and the cryopreserved ones ( $p=0.582$ ).

270 As a first functional parameter, the long-term cell growth was assessed. Indeed, we considered  
 271 the resumption of cell cycle after cryopreservation as an essential functional parameter to explore.  
 272 Non-cryopreserved and cryopreserved cells displayed a similar daily growth rate over time and  
 273 independently of the cryopreservation duration (8 or 36 days) as shown by a representative cell  
 274 culture in Figure 5 (repeated measures ANOVA;  $p>0.05$ ).

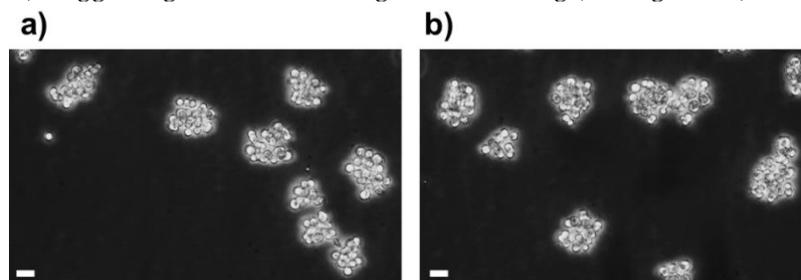
275 The same result was obtained for all cell cultures tested in this study (see Figure S1 and S2).  
 276 Interestingly, the cryopreserved cells stored for 79 days (Figure S2d) displayed a low initial growth  
 277 rate one week after thawing and reseeding, suggesting that cryopreserved cells may need a longer  
 278 time (between one and two weeks after thawing) to fully recover after such cryopreservation storage  
 279 duration.

280 Furthermore, the mean of the daily growth rate was maintained at  $1.78 \pm 0.39$  and at  $1.73 \pm 0.33$ ,  
 281 respectively in the different non-cryopreserved cell cultures and cryopreserved cell cultures  
 282 monitored, with no significant differences between these two conditions (ANOVA;  $p > 0.05$ ; see  
 283 Figure S3b).



284 **Figure 5.** Over time daily growth rate of a representative cell culture, comparing cryopreserved and  
 285 non-cryopreserved cells. Non-cryopreserved cell culture in grey dotted line, the same culture  
 286 cryopreserved at 24 days and thawed after either 8 days of storage in grey solid line or after 36 days  
 287 in black solid line. Mean values of three technical replicates and standard error bars are shown.  
 288 Repeated measures ANOVA revealed no significant differences in cell growth trend between the  
 289 non-cryopreserved culture and the cryopreserved ones ( $p = 0.722$ ).  
 290

291 Long-term cell viability and growth rate monitoring of cryopreserved vs non-cryopreserved  
 292 cell cultures corroborated the healthy state of cryopreserved cells. Therefore, these analyses were  
 293 completed with weekly microscope observations in order to assess the cell culture behavior. In the  
 294 Figure 6, the comparison of a 31-day old non cryopreserved culture with the corresponding  
 295 cryopreserved culture (considering the thawing age is equal to the freezing age) showed that  
 296 cryopreserved cells, as the non-cryopreserved cells, form adherent cell aggregates, which is the  
 297 characteristic architecture of *A. viridis* primary cell cultures [9,15]. Besides, cryopreserved cells in  
 298 culture presented the same mean size ( $5.2 \pm 0.49 \mu\text{m}$ ) that non-cryopreserved cells ( $5.17 \pm 0.54 \mu\text{m}$ ;  
 299 ANOVA;  $p = 0.957$ ), suggesting no volume change after thawing (see Figure S5).

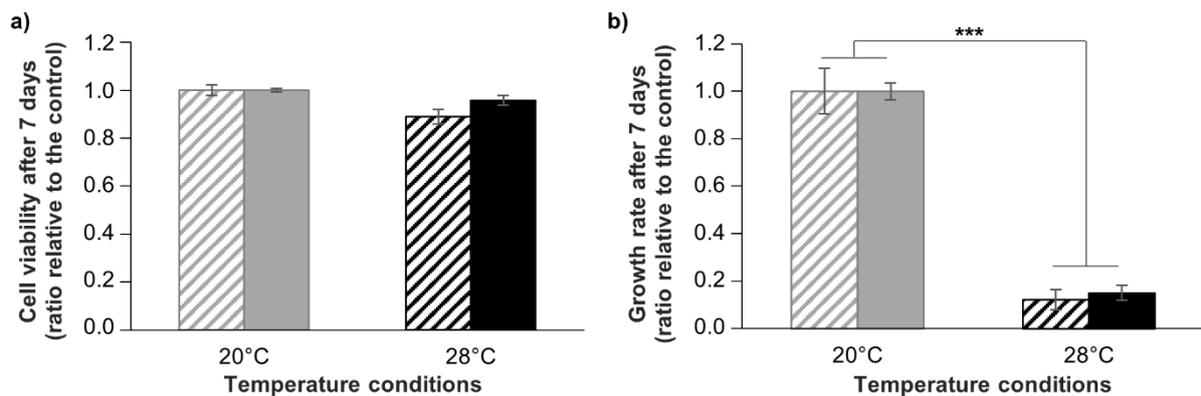


300 **Figure 6.** Observation of aggregates of *A. viridis* gastrodermal cells in culture before and after  
 301 cryopreservation; **a)** Cell culture at day 31 since its establishment, and **b)** the same culture  
 302 cryopreserved at day 24 for 79 days, 7 days after thawing. Phase contrast microscopy (objective x20),  
 303 scale bars =  $10 \mu\text{m}$ .  
 304

305 Therefore, cryopreserved cells maintained through time identical viability, growth and shape to  
 306 the corresponding non-cryopreserved cell culture, and this independently of the cryopreservation  
 307 duration. These results indicate that the cryopreservation designed protocol stored cells in a healthy  
 308 state allowing them to fully recover after thawing and to behave like their origin culture. This  
 309 monitoring constitutes an essential part in validating the storage protocol for further use of  
 310 cryopreserved cells and represents a major strength of this study. Indeed, the reseeded of thawed  
 311 cells was rarely done on previous marine invertebrate studies, and cells are usually maintained only

312 for a few hours to a few days [22–24,28,25,27], with a maximum of 15 days for bivalve cells in Dessai  
 313 (2018) [30] and around 40 days for sponge cells [32,34].

314 As a second parameter of the cell functionality after cryopreservation, we investigated the  
 315 response of cryopreserved cells to a controlled stress experiment. In Cnidarians, and more  
 316 particularly in our research model, *A. viridis*, hyperthermal stress is well known to induce oxidative  
 317 damages and cell death, i.e. apoptosis [50,51]. Using *A. viridis* primary cell cultures, we previously  
 318 reported that hyperthermia (+8°C) didn't induce any oxidative damage or impact on survival but  
 319 provoked a drastic decrease of cell growth [15]. Thus, in this study, we compared the response of  
 320 cryopreserved and non-cryopreserved cells submitted to the same hyperthermal stress, in terms of  
 321 viability and growth. The results show that hyperthermal stress didn't change significantly the cell  
 322 viability neither for non-cryopreserved cells nor for cryopreserved cells (ANOVA;  $p>0.05$ ) (Figure  
 323 7a). Moreover, cell growth rate was drastically decreased by around 80% after 7 days at 28°C  
 324 compared to 20°C condition (Kruskal-wallis;  $p<0.001$ ), without any significant differences between  
 325 non-cryopreserved and cryopreserved cells (Kruskal-wallis;  $p>0.05$ ) (Figure 7b). These results are in  
 326 line with data from Ventura et al. (2018) [15] and show that cryopreserved cells displayed identical  
 327 resistance to non-cryopreserved cells, strongly corroborating the non-alteration of cell functionality.  
 328 Therefore, not only *A. viridis* cells *in vitro* can be successfully cryopreserved and reseeded, but they  
 329 can be reliably used for further experiments, like it is done for mammalian cells. Although functional  
 330 analyses are sometimes conducted for marine invertebrate cells, through metabolic and enzymatic  
 331 activities [23,26,24,28,25,30], only some sponge cells studies conducted experiments using  
 332 cryopreserved cells [31,34]. However, this is a fundamental assessment in order to validate the  
 333 cryopreservation protocol for creating reliable models.



334

335 **Figure 7.** Comparison of cryopreserved and non-cryopreserved cells in response to hyperthermia.  
 336 Assessment of cell viability (a) and growth (b) of *A. viridis* cells in response to a hyperthermal stress  
 337 of +8°C (black bars) for 7 days, for non-cryopreserved cultures (striped bars) and for cryopreserved  
 338 cultures (filled bars). Cell viability and growth are expressed relative to control condition (20°C –  
 339 grey bars). Mean values with standard error bars are shown, biological replicates  $n\geq 4$ . The asterisks  
 340 represent the significant differences between control and stress conditions (\*\*\*) Kruskal-Wallis:  
 341  $p<0.001$ ).

#### 342 4. Conclusions

343 In this study we succeeded to design an easy and rapid cryopreservation procedure for  
 344 *Anemonia viridis* primary cell cultures. The established protocol enabled us to obtain high cell  
 345 survival after thawing and a full long-term recovery of the cell culture behavior. The development of  
 346 cryopreservation in cnidarian primary cell cultures enables us to preserve stable cell stocks available  
 347 shortly after thawing for experimental procedures and sharing with the scientific community. This  
 348 new tool will be an important asset to raise *A. viridis* primary cell cultures as a powerful model for  
 349 studying and understanding the cnidarian properties (i.e. symbiosis lifestyle, response to stress,  
 350 aging), difficult to study in most cnidarian models at the molecular and cellular levels.

351 **Supplementary Materials**

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Supplementary Material and Methods

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Complementary cell viability assays

355

FDA (Fluorescein Diacetate, 4 µg/mL; Sigma-Aldrich) and Hoechst 33342 (5 µg/mL; Sigma-Aldrich) were

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added and incubated with cells during 15 minutes at 20°C in the dark. Viable cells (fluorescent in green)

357

and dead cells (fluorescent in blue) were identified and counted on a Neubauer improved

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haemocytometer (Sigma-Aldrich) using a fluorescence microscope (Zeiss Axio Imager Z1). The cell

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viability was defined as the percentage of viable cells relative to total cells (i.e. viable and dead cells).

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MTT assay was performed following manufacturer instructions with slight modifications. Briefly, prior the

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assay 60 000 cells were seeded in triplicate in 96-well plate in 100 µL of culture medium and incubated for 24h.

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20 µL of 5mg/mL MTT solution (Sigma-Aldrich) is then added to each well and incubated 5h at 20°C in the

363

dark. Then, the supernatant is removed, and the yielded formazan was dissolved in the suitable detergent

364

(isopropanol) for 15 minutes. Subsequently, the plates' light absorption (OD) is read at wavelength 590 nm on

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spectrofluorometer (SAFAS, Monaco). The cell viability was expressed as follow: Viability % = (cryopreserved

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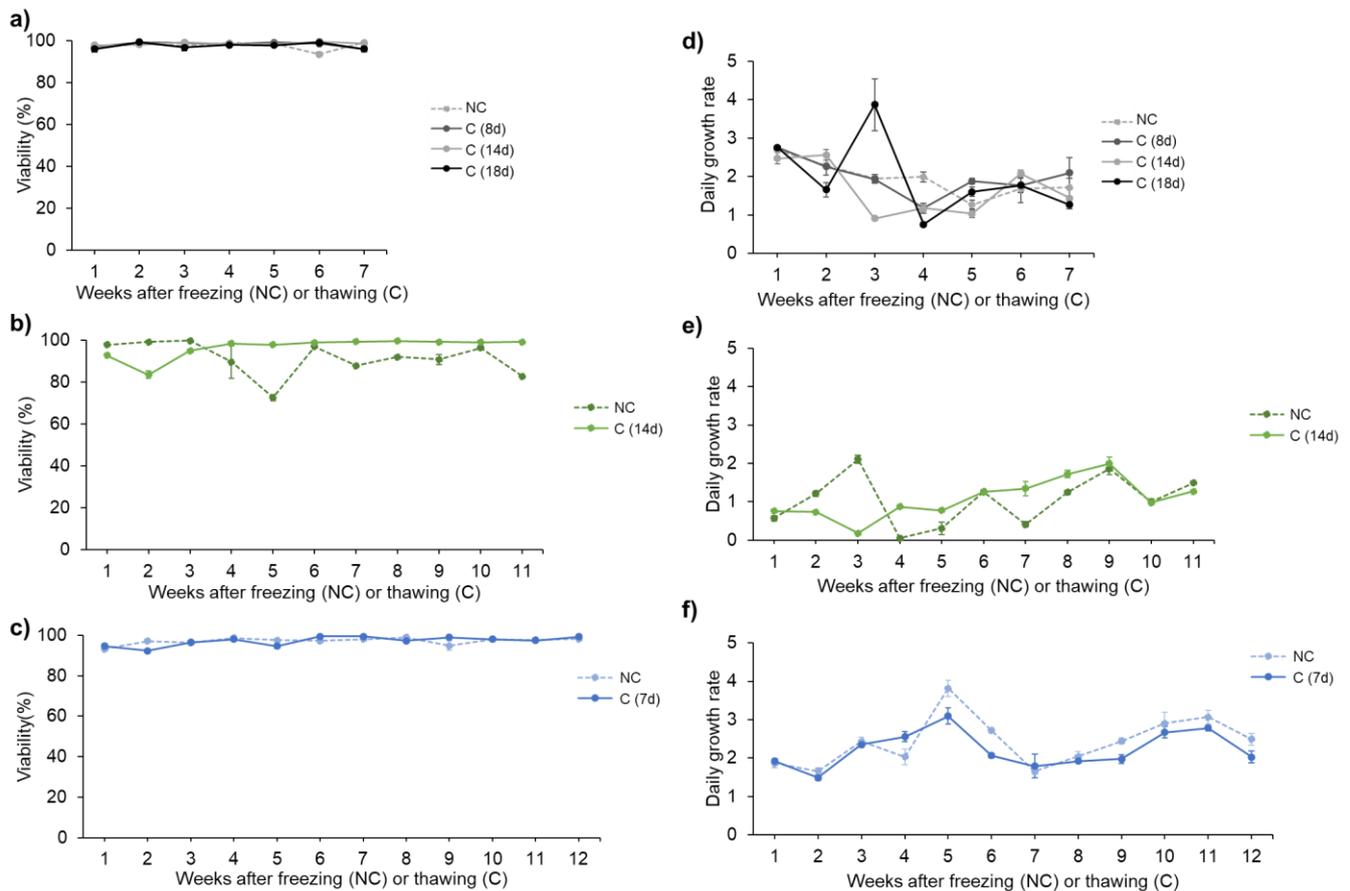
cells OD /non-cryopreserved cells OD) ×100

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Supplementary Figures

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**Figure S1.** Over time viability (a, b, c) and daily growth rate (d, e, f) of three primary cell cultures,

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comparing non-cryopreserved cells (NC) and cells cryopreserved for a short storage period (C). The

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cryopreservation storage period is mentioned in the legend, it goes from 8 days ('8d') to 18 days

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('18d'). Each graph per variable measured represent one primary cell culture and its corresponding

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cryopreserved cell culture(s). Time points are mentioned as "Week after freezing (NC) or thawing

376

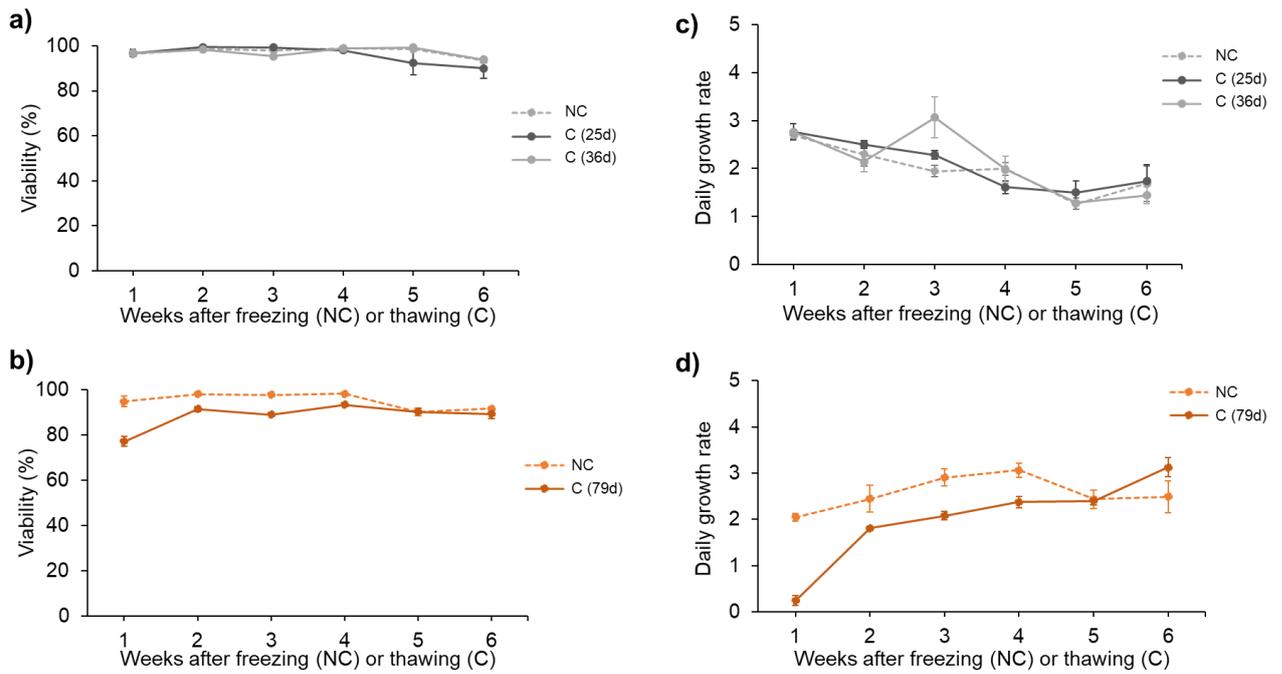
(C) time" i.e. the monitoring presented here for the non-cryopreserved cultures begins after the

377

freezing time, and the one for cryopreserved cells is done after thawing, thus considering the age at

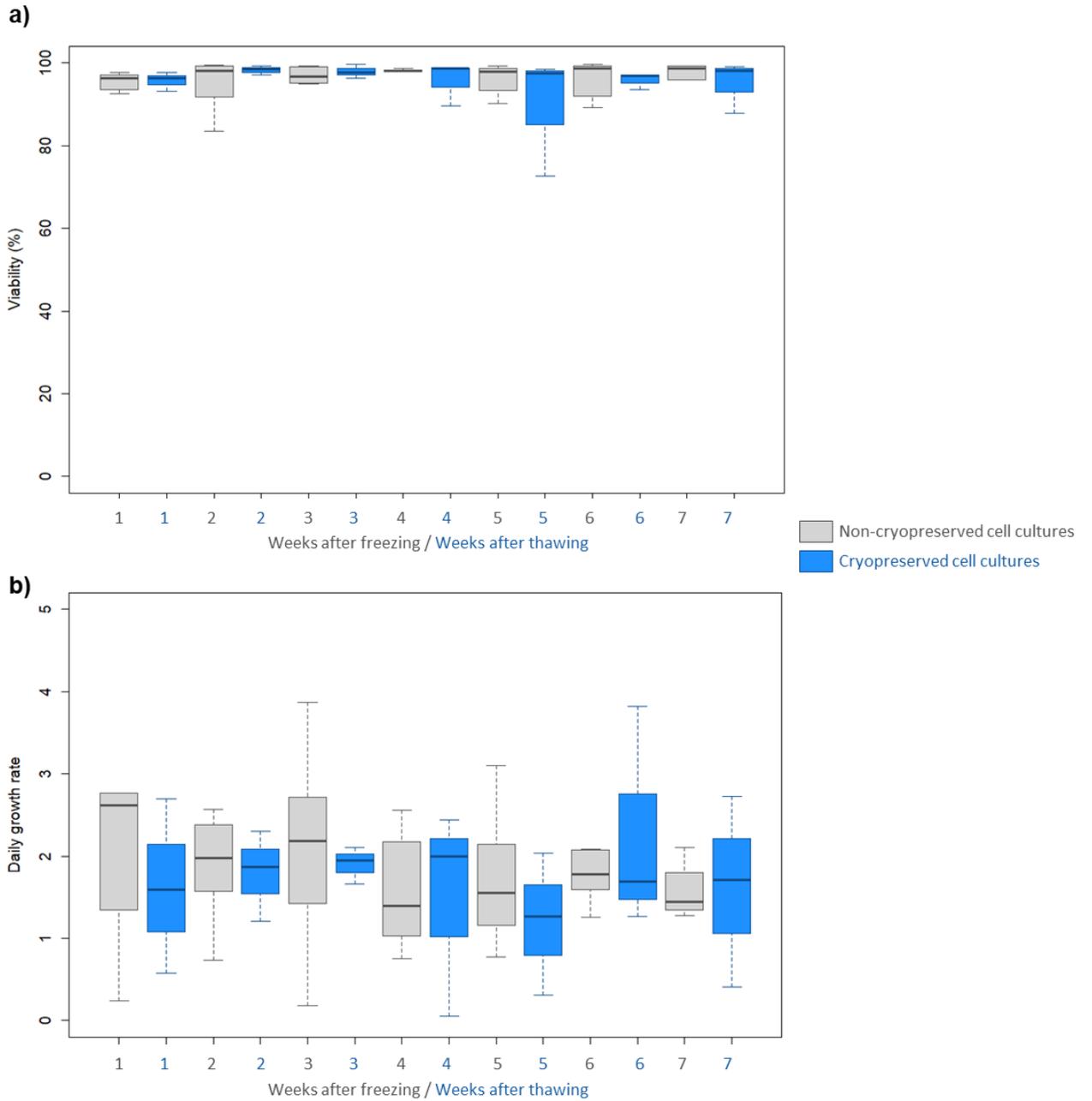
378

that time is the same age as at the freezing time.



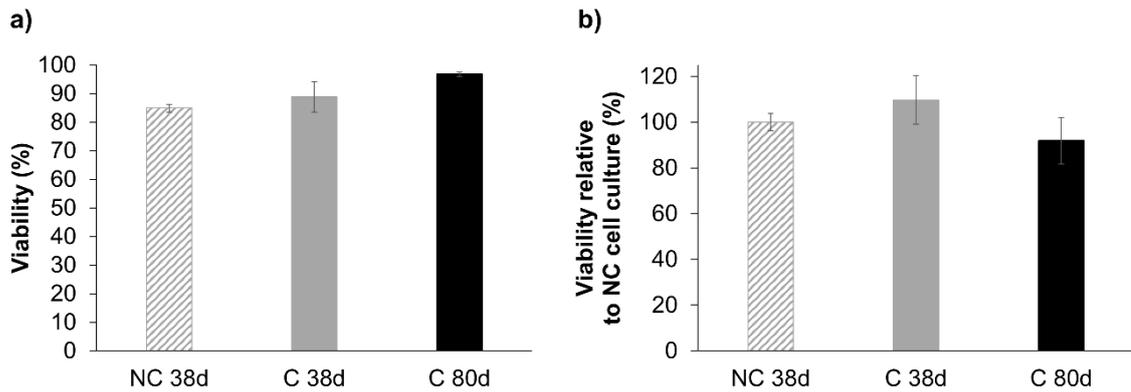
379

380 **Figure S2.** Over time viability (a, b) and daily growth rate (c, d) of two primary cell cultures, comparing  
 381 non-cryopreserved cells (NC) and cells cryopreserved for a long storage period (C). The cryopreservation  
 382 storage period is mentioned in the legend, it goes from 25 days ('25d') to 79 days ('79d'). Each graph per  
 383 variable measured represent one primary cell culture and its corresponding cryopreserved cell culture(s).  
 384 Time points are mentioned as "Week after freezing (NC) or thawing (C) time" i.e. the monitoring  
 385 presented here for the non-cryopreserved cultures begins after the freezing time, and the one for  
 386 cryopreserved cells is done after thawing, thus considering the age at that time is the same age as at the  
 387 freezing time.



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**Figure S3.** Box plots of over time viability (a) and daily growth rate (b) of all cell cultures monitored (non-cryopreserved in gray and cryopreserved in blue). Time points are identical to the ones present in Fig. S1 and S2 and are here going from 1 to 7 weeks, which represent the time points after freezing (for non-cryopreserved cells) or after thawing (for cryopreserved cells) where at least 3 biological replicates were monitored.



394

395 **Figure S4.** Comparison of cell viability between 38-day old non-cryopreserved cell cultures ('NC  
 396 38d'), and cryopreserved cell cultures, either 38-day old ('C 38d') or 80-day old ('C 80d'). Cell  
 397 viability was measured with (a) FDA/Hoechst staining and with (b) MTT assay. Mean values with  
 398 standard error bars are shown ( $n \geq 3$ ). ANOVA analyses revealed no significant differences in the  
 399 viability values between non-cryopreserved and cryopreserved cells, and over time between  
 400 cryopreserved cells ( $p=0.45$  for panel a;  $p=0.378$  for panel b).

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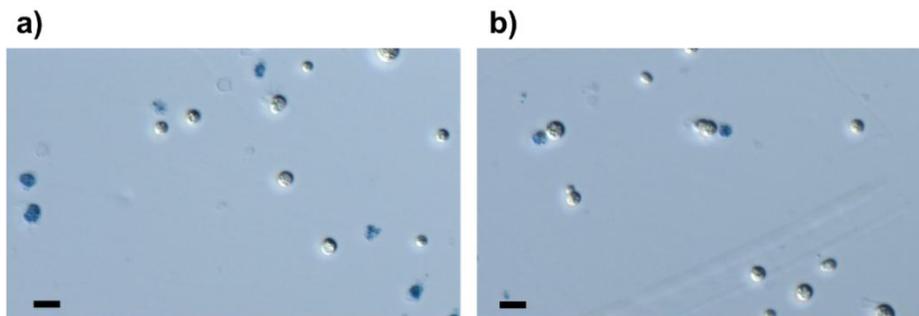
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407 **Figure S5.** Observation, on Neubauer improved haemocytometer under optic microscope (objective  
 408  $\times 20$ ), of the same *A. viridis* gastrodermal cell culture (a) before and (b) after cryopreservation and  
 409 stained with Evans blue (i.e. the dead cells stained in blue) (scale bar = 10  $\mu\text{m}$ ).

410

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418 S.B-V. reviewed the manuscript.

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