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Fifteen years of coral cryopreservation

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Abstract

Over the past decade, coral cryopreservation research has advanced rapidly due to the field's inter-disciplinary approach, whereby concepts and technologies from cryogenic biology, nano-engineering, and cellular biochemistry have been combined. Cryopreservation of coral gametes, larvae, and their dinoflagellate endosymbionts has been under continuous development, and the resulting publications have gathered the attention of conservationists across the globe. Herein we have reviewed progress in this field, with a particular emphasis on 1) understanding the factors required for successful cryopreservation and 2) optimizing cryopreservation protocols for different cell/tissue types. We advocate, at a minimum, 1) reducing cryo-injury (i.e., ice crystal formation) and 2) optimizing freezing techniques, for developing successful cryopreservation protocols for coral gametes, tissues, larvae, and the endosymbiotic dinoflagellates that reside within the tissues of all reef-building corals.

Keywords: Coral reef, Conservation, Freezing, Symbiodiniaceae, Cryobank

Introduction

Coral reefs (Fig. 1) are unique, high-biodiversity ecosystems that are defined by both their biological ("coral") and geological ("reef") components (Buddemeier et al., 2004). The framework-building corals owe their success to the photosynthetic dinoflagellates (family Symbiodiniaceae) that inhabit their gastrodermal cells. The endosymbiotic relationship between corals and dinoflagellates is mutually beneficial, as the former provide shelter and nutrients to their endosymbionts, while the dinoflagellates nourish their hosts with photosynthetically fixed carbon. Coral reefs are valuable not only to the plethora of marine organisms that call reefs home, but also to humans, as they provide nurseries for commercially important fish,

Fig. 1. A healthy coral reef near Bandaneira, Maluku, Indonesia. Photo credit: Anderson Mayfield.

shelter coastlines from wave damage, are sources of pharmaceuticals, and are common tourist destinations. Indeed, the economies of many small island nations, as well as the welfare of their citizens, are intrinsically linked with healthy coral reefs. Unfortunately, climate change and more localized human activities (e.g., seawater pollution) have resulted in mass coral decline across the globe (Hoegh-Guldberg et al., 2007). Climate change-associated temperature increases have been causing mass coral "bleaching" events since the 1980s (reviewed in Donner et al., 2017), with bleaching events now occurring annually in many parts of the world. Such breakdowns occur because most coral-dinoflagellate endosymbioses exist near the upper threshold of their thermotolerance (Mayfield & Gates, 2007). This dramatic loss of coral necessitates proactive conservation and preservation strategies (Gattuso et al., 2015), including (non-exhaustively) environmental and

climate mitigation, coral transplantation, coral stress-hardening, assisted evolution, and even cryopreservation for when/if the aforementioned approaches fail (Tsai et al., 2010b).

Most studies on marine invertebrate cryopreservation have focused on oysters (Hughes, 1973; Yankson & Movse, 1991; Tervit et al., 2005) and sea urchins (Adams et al., 2003, 2006). Studies on the cryopreservation of sea cucumbers (Shao et al., 2006), shrimp (Tsai and Lin, 2009; Lin et al., 2013a), and mollusks (Odintsova et al., 2001, 2009) have also been conducted. In contrast, coral cryopreservation began in earnest only 10-15 years ago (Hagedorn et al., 2006a; Tsai et al., 2010b); our knowledge of coral cryobiology is consequently in its infancy. Since then, coral sperm (Hagedorn et al., 2012, 2017; Viyakarn et al., 2017), oocytes (Fig. 2; Tsai et al., 2011, 2016; Tsai & Lin, 2012), larvae (Daly et al., 2018; Cirino et al., 2019), "tissue balls" (coral tissues that have detached from their calcium carbonate exoskeletons; Feuillassier et al., 2014a), and Symbiodiniaceae (Chong et al., 2016c; Lin et al., 2019a; Di Genio et al., 2020) have all been studied, with cryopreservation effects documented by monitoring mitochondrial DNA content (Tsai et al., 2014a, 2015a), gene expression (Chong et al., 2017), protein structure (Lu. 2019), and other means (discussed in detail below). Different coral tissues and life history stages require their own optimized freezing protocols, and several such

Fig. 2. A stony coral (*Hydnophora rigida*) spawning in Nanwan Bay, Taiwan. Photo credit=Fuwen Kuo.

methods developed to date have been described below. Since cryopreservation can damage cells, tissues, and DNA (Kopeika et al., 2014), coral cryobiology studies must ensure high viability of thawed biological material. This topic is also discussed in this review.

Cryo-injury

Cryopreservation is a long-term storage technique conducted to preserve structurally intact living cells and tissues for extended periods of time at very low temperatures (Tsai & Lin, 2012). Several factors affect the success of coral cryopreservation, most notably the limiting of, or even avoiding, cryo-injury, which is typically brought on by intracellular ice crystal formation. Chilling stress can damage cellular structures and reduce viability (even killing cells; Benson, 2008). It is therefore necessary to reduce chilling sensitivity to minimize the damage caused by cryopreservation. The degree of chilling stress increases with cooling rate (Morris, 1981), and coral oocytes with high lipid content generally sustain less chilling stress (Lin et al., 2011; Tsai & Lin, 2012); this could be due to coinciding reductions in lipid peroxidation (Darin-Bennett & White, 1977; Lin et al., 2012, 2013b).

Ice crystals **(**Fig. 3**)** can form intracellularly or extracellularly and result in a loss of water from a cell (Muldrew & McGann, 1990), as well as damage to cellular structures and macromolecules. However, ice crystal formation can be

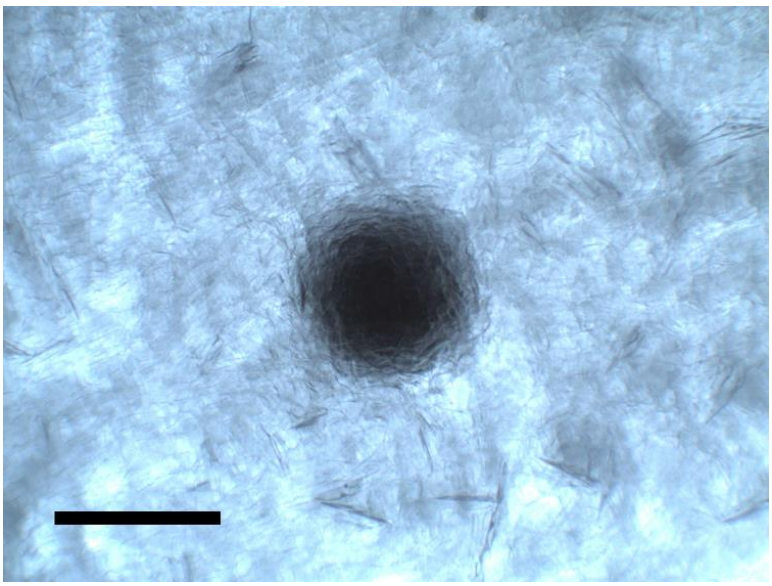

Fig. 3. Extracellular ice formation around a coral oocyte of *Junceela fragilis* undergoing freezing. Scale bar=500µm. Photo credit=Chiahsin Lin.

prevented by controlling the rate of cooling (Mazur et al., 1972); rapid cooling and slow warming generally cause more ice crystal-related injuries than slow cooling and rapid warming. This finding was corroborated by Hagedorn et al. (2006a) in a study in which coral larvae underwent slow freezing in two steps to allow the larvae sufficient time to dehydrate, thereby preventing ice crystal formation. Vitrification can also prevent ice crystal formation and has been applied to coral oocytes and larvae (Tsai et al., 2015b, 2016; Daly et al., 2018; Cirino et al., 2019). In addition to chilling stress and ice crystal formation, cells may be damaged by the solution itself when the cooling rate is too low (Chong, 2015). This generally occurs when cells are exposed to a high concentration of solute for an excessive period, leading to cellular dehydration. This weakens the complex structures of proteins and results in loss of lipids and phospholipids (Chong, 2015). Weakened membranes are more permeable to electrolytes and are thus easily ruptured when cells revert to the isotonic state (Mazur et al., 1972). The solution effect can be reduced by gradually adding and removing cryoprotectants (CPAs) to prevent excessive osmotic pressure changes (Adams et al., 2003).

State-of-the-art freezing techniques

Different types of cryopreservation techniques are currently being used for coral cryopreservation. The most common are slow-freezing (Fig. 4), two-step freezing, and vitrification. Each technique has different features, as well as different pros and cons. Hence, it is necessary to understand the details and limitations of each when developing protocols. Slow-freezing is a process in which cells are slowly cooled below their freezing points. The resulting water efflux rate reduces dehydration and ice crystallisation impacts. "Controlled slow-freezing" involves a series of steps in which the sample is pre-cultured or acclimated and then cryoprotected (Benson, 2008). Several studies have used controlled, programmable freezing (Fig. 5) with oocytes of gorgonian corals (e.g., *Junceella juncea* & *J. fragilis*; Goh, 2019) and their Symbiodiniaceae endosymbionts (*Gerakladium*; Zhao, 2017). Short-term, low-temperature preservation and slow-freezing have also been used for the cryopreservation of oocytes of 1) *Echinopora sp.* (stony coral; Tsai & Lin, 2012), and 2) *Ellisella robusta* (gorgonian; Lin et al., 2014; Goh, 2019).

Two-step freezing is a method in which cells are frozen at low temperatures by immersing samples first in liquid

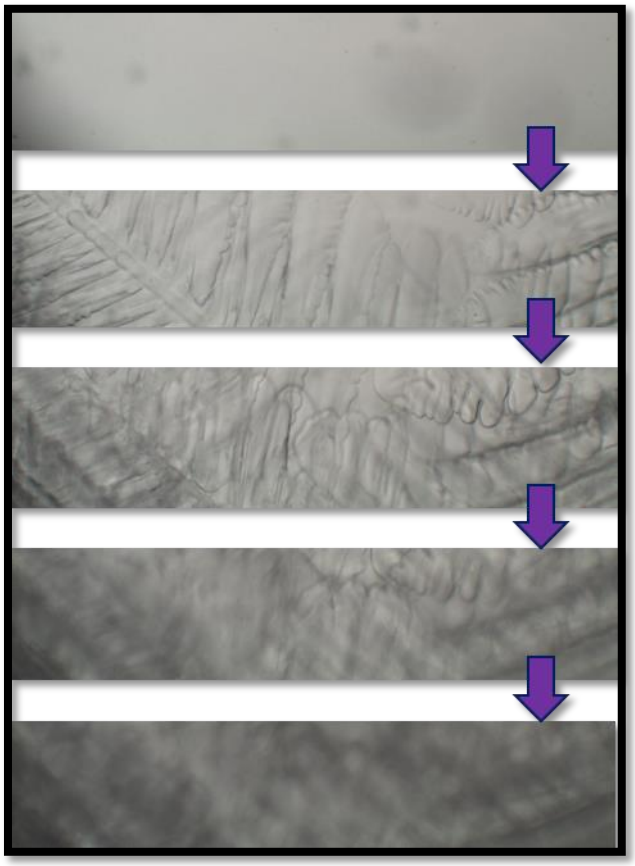

Fig. 4. A sequence of images depicting ice crystal formation during controlled, slow freezing. Photo credit=Chiahsin Lin.

nitrogen vapor or dry ice and then at cryogenic temperatures for storage. Unlike slow-freezing, two-step freezing does not require any specialised or controlled cooling equipment, and generally low CPA concentrations are used (Chong *et al*., 2016c). It also allows the sample to undergo osmosis with the CPA and applies a slow cooling rate to minimise the formation of intracellular ice crystals (Tsai & Lin, 2012). Following complete dehydration, cells are immersed in liquid nitrogen for rapid freezing and then stored. Several experiments have been conducted using two-step freezing with Symbiodiniaceae (Chong et al., 2016c; Lin et al., 2019b; Di Genio et al., 2020), coral sperm (*Acropora humilis*; Viyakarn et al., 2017), and the primary cells of other marine invertebrates (Odintsova et al., 2001). Cooling rates of 40°C/min for *Acropora digitifera* (Ohki et al., 2014), 41.7°C/min for *A. humilis* (Viyakarn *et al*., 2017), and 20-30°C/min for *A. tenuis*, *A.*

Fig. 5. Programmable cooling freezes samples by gradually cooling them at a controlled rate in a computerized freezer. Photo credit=Chiahsin Lin.

millepora, *A. palmata*, and *Fungia scutaria* (Hagedorn et al., 2012, 2017) have been used.

Vitrification is a rapid cooling method in which cells directly enter a "glass" state through the use of a vitrification solution that thwarts ice crystal formation (Chong et al., 2016c). *J. juncea* oocytes (Tsai et al., 2015b, 2016), Symbiodiniaceae cells (Hagedorn & Carter, 2015; Zhao, 2017), and coral larvae (Daly et al., 2018) have all been successfully cryopreserved via vitrification. Cirino et al. (2019) actually managed to cryopreserve larvae housing endosymbiotic dinoflagellates by vitrified cryopreservation; following nano-gold laser thawing, the larvae successfully grew, settled, and developed. Vitrification has also been used for *P. damicornis* tissue balls (Feuillassier et al., 2015).

Factors affecting successful cryopreservation

Successful cryopreservation requires a suitable freezing protocol that limits biomolecular damage, thereby resulting in high sample viability post-thawing. Cooling rate, CPA composition and concentration, equilibrium time, and thawing time can all be optimized to improve cell viability. The cooling rate is critical for successful cryopreservation, as it determines whether cells remain in equilibrium with their extracellular environment or become progressively supercooled (in which case there is an increasing possibility of ice formation;

Kumar et al., 2003). Cooling rates that are excessively low or excessively high will compromise the survival of cells after cryopreservation. Tsai et al. (2010a) recommended a slow cooling rate to permit a sufficient degree of cellular dehydration; however, an excessively slow rate may lead to CPA toxicity within oocytes in particular and can result in extracellular ice formation; the latter phenomenon then creates an osmotic gradient across the cell membrane that draws water out (Tsai & Lin, 2012). This consequently limits the amount of cellular water that can crystallize. For coral sperm, oocytes, and larvae, as well as *in hospite* and cultured Symbiodiniaceae, the optimal rate of cooling can be determined by factoring in the 1) permeability of the cell membrane to water, 2) surface-to-volume ratio of the cell, 3) type and concentration of CPA(s), 4) CPA infiltration rate, and other factors (Tsai et al., 2010b, 2011; Lin & Tsai 2012; Viyakarn et al., 2017; Cirino et al., 2019; Di Genio et al., 2020).

CPAs protect cells from freezing and initially cause cells to shrink, later swelling to a new equilibrium volume as the CPAs disperse throughout the cell (Adams et al., 2003). There are two types of CPAs: 1) membrane-permeating and 2) non-membrane-permeating; each plays a different role during cryopreservation. Permeating CPAs are low molecular weight, can diffuse freely across membrane (Tsai et al., 2011), and include glycerol (Gly), ethylene glycol (EG), propylene glycol (PG), methanol (MeOH), and dimethyl sulfoxide (DMSO). They have been used in numerous experiments; for instance, EG was used to vitrify coral oocytes and larvae (Tsai et al., 2015b; Cirino et al., 2019), and MeOH was used to cryopreserve coral oocytes and algae (Tsai & Lin, 2012; Chong et al., 2016c). DMSO has been used to cryopreserve *A. humilis* sperm (Viyakarn et al., 2017). Non-permeating CPAs have much higher molecular weights and cannot cross cell membranes; they include sugars (Tsai et al., 2018), lipids, and polyvinylpyrrolidone (Pedro et al., 2005; Shaluei et al., 2013). During cryopreservation, non-permeating CPAs are typically combined with permeating CPAs: MeOH+sucrose for sperm *A. digitifera* (Ohki et al., 2014) and EG+sucrose for tissue ball *P. damicornis* (Feuillassier et al., 2014a).

Equilibration time refers to the period during which cells are mixed with CPAs before undergoing cryopreservation. This enables osmosis to occur without affecting cell viability. The equilibrium time normally depends on the type of cells and CPAs used and can be adjusted to enhance osmosis. For example, the equilibrium time should be shorter when high-

permeability CPAs are used (Chong, 2015); only 2 min of equilibration time were required to prepare coral oocytes and larvae for vitrification (Tsai et al., 2015b, 2016) and cryopreservation (Cirino et al., 2019). For *A. humilis* sperm and Symbiodiniaceae, times of 15 (Viyakarn et al., 2017) and >30 min (Di Genio et al., 2020) were required, respectively.

Thawing is the last stage of the cryopreservation process and represents the period during which cells are revived. Various thawing methods can be employed: room temperature thawing (Bougrier & Rabenomanana, 1986), water bath thawing (Tsai et al., 2016), and laser warming (Cirino et al., 2019). However, the thawing time must be precise and accurate. For instance, rapid thawing can improve the survival of coral samples (Tsai et al., 2016) since it restricts the re-growth of ice crystals. However, rapid thawing can also be lethal to some frozen samples because of the resulting osmotic stress. Symbiodiniaceae (Chong et al., 2016c), *J. juncea* and *J. fragillis* (Lin et al., 2011), and *Echinopora* sp. (Tsai & Lin, 2012) cells have been thawed in water baths. Cirino et al. (2019) utilized gold nanorods to absorb and then emit laser-derived energy to rapidly and uniformly rewarm samples.

To determine whether cells have survived cryopreservation, post-thaw viability must be assessed. Mitochondria play a vital role in cell energy metabolism by providing ATP. However, the use of CPAs during freezing disrupts coral mitochondrial distribution and membrane potential, which then lowers ATP production (Tsai et al., 2014a). In a low-temperature study of *Echinopora* sp., mitochondria DNA copy numbers were used as a proxy of coral oocyte health after treatment with different CPA concentrations (Tsai et al., 2015a). Vital stains can also be used to assess viability since only intact membranes will bar their entry (Mazur, 1970). Both fluorescein diacetate (FDA) and propidium iodide (PI) have been used in coral cryopreservation research (Tsai et al., 2010b; Lin *et al*., 2011); the former requires cellular esterase activity and an intact membrane whereas PI is a more traditional vital stain (Jones & Senft, 1985; Tsai et al., 2008). FDA+PI were used in conjunction in gorgonian coral oocytes, though the data were spurious given the unique membrane characteristics of these soft corals (Tsai et al., 2011).

Optimizing cryopreservation protocols for different cell/tissue types

The cryopreservation of coral sperm, oocytes, larvae, embryos, fragments, tissue balls, and Symbiodiniaceae

(including cultured samples) requires different freezing techniques and CPAs since differing cell/tissue types respond uniquely to dehydration, CPAs, chilling, and ice crystals. We now discuss the current protocols in use for coral cryopreservation.

Progress in sperm cryopreservation

The cryopreservation of sperm plays an important role in preserving paternal genetic diversity and has benefited the aquaculture industry since a source of viable sperm ensures that the life cycle can be completed *ex situ* (Martinez-Paramo et al., 2016). Banking frozen invertebrate semen also has important implications for hybridization, selective breeding, gynogenesis, domestication, and stock conservation, and cryopreserved gametes can be used for seeding shrinking populations; the gametes also 1) provide easy and inexpensive transport of genetic material among living populations, 2) extend generation intervals, and 3) serve as a source material for research (Ballou, 1992; Wildt et al., 1997; Holt & Pickard, 1999). Sperm cryopreservation is especially important now that it is harder for corals to survive *in situ* due to climate change and other anthropogenic impacts (discussed in more below).

Most coral sperm cryopreservation studies have used DMSO as the major CPA (Hagedorn et al., 2012; Tsai et al., 2014b; Viyakarn et al., 2017; Tab. 1). Although most studies have shown that sperm frozen
with DMSO have higher viability than other CPAs (e.g., EG & PG; Hagedorn et al., 2006b, 2012, 2017), DMSO is highly

Tab. 1. Cryopreservation of coral sperm. CPA=cryoprotectant. NOEC="No observed effect" concentration.

Species	Experimental or cryopreservation method	Cryoprotectant	Reference
Acropora humilis	Two-step freezing	2 M DMSO	Viyakarn et al. (2017)
Acropora tenuis *Acropora millepora*	Two-step freezing	10% DMSO	Hagedorn et al. (2017)
Junceella juncea *Junceella fragillis*	CPA effect	NOEC up to 3 M for MeOH or DMSO	Tsai et al. (2014b)
Acropora digitifera	Two-step freezing	0.9 M sucrose with 20% MeOH	Ohki et al. (2014)
Acropora palmata *F. scutaria*	Two-step freezing	10% DMSO	Hagedorn et al. (2012)
Fungia scutaria	Slow freezing	10% DMSO	Hagedorn et al. (2006b)

toxic to coral sperm and can lead to 1) the immediate swelling of their tails (Hagedorn et al., 2006b) and 2) post-thaw osmotic stress; we recommend, then, that researchers consider alternatives. Ohki et al. (2014) successfully used 0.9 M sucrose with 20% methanol with *A. digitifera* sperm. This high viability may be attributed to the fact that sugars are compatible osmolytes that protect cells against osmotic shock during extracellular water depletion caused by ice formation. They also preserve the structural and functional integrity of membranes at low temperatures (Crowe et al., 1990).

Oocyte cryopreservation

Coral oocyte cryopreservation (Tab. 2) was first developed in 2010b by Tsai et al. though remains a challenge due to their high sensitivity to CPAs and chilling, as well as their low membrane permeabilities (Lin et al., 2011, 2012, 2014; Tsai et al., 2010b, 2016). Furthermore, the high lipid content of oocytes results in low surface area-to-volume ratios, which are associated with freezing and thawing difficulties (Lin et al., 2013b). The limited time available to collect coral gametes also imposes a limitation. Broadcast spawning corals generally release gametes (Fig. 2) only during the spring or summer full moon periods, and for < 1 h on just 2-3 nights. According to Tsai & Lin (2012), coral oocytes have substantial chilling tolerance at 0 and 5°C, but not at -5°C; for this reason, we recommend vitrification (Tsai et al., 2015a, 2016) since it limits chilling injury. That being said, oocyte susceptibility to cryopreservation varies dramatically across taxa. For instance, *J. fragillis* has higher tolerance towards CPAs compared to other gorgonians, while *Echinopora robusta* demonstrates higher tolerance to chilling compared to other hermatypic corals (Tsai et al., 2016). The latter observation has been attributed to high lipid content (Lin et al., 2012); elevated concentrations of phosphatidylethanolamines and fatty acids are linked to high membrane fluidity, which protects cells from cold-temperature stress (Lin et al., 2012, 2013b). High membrane fluidity reduces the amount of CPAs needed during cryopreservation, which thereby limits CPA toxicity issues (Lin et al., 2014). The most effective CPA for the cryopreservation of coral oocytes to date is MeOH, which protects cells by permeating through the cell membrane and reducing the formation of ice crystals (Tsai et al., 2014a). However, MeOH is a poor vitrificant; EG is instead the CPA of choice when cryopreserving coral oocytes via vitrification (Tsai et al., 2015a, 2016).

Tab. 2. Cryopreservation of coral oocytes. CPA=cryoprotectant. Mt=mitochondrial.

Species	Experimental or cryopreservation method	CPA(s)	Reference
Echinopora gemmacea *Oxypora lacera*	Physiological assessment	---	Lin et al. (2019c)
E. gemmacea *O. lacera*	Physiological assessment	---	Lin et al. (2018)
O. lacera *E. gemmacea* *Montipora incrassata* *M. hispida* *Galaxea fascicularis* *Merulina ampliata*	Ultrastructural observations	---	Tsai et al. (2016)
Junceella fragilis *Ellisella robusta*	Vitrification	3.5 M PG+1.5 M EG+ 2 M MeOH	Tsai et al. (2016)
Junceella juncea	Vitrification	3.5 M PG+1.5 M EG+ 2 M MeOH	Tsai et al. (2015a)
E. gemmacea	Mt DNA content	0.5 M MeOH	Tsai et al. (2015b)
J. juncea *J. fragillis*	Ultrastructural observations	---	Tsai et al. (2014c)
J. juncea *J. fragillis*	Lipid phase transition	---	Lin et al. (2014)
E. gemmacea	Mt DNA content	1 M MeOH	Tsai et al. (2014a)
Platygyra daedalea *E. gemmacea* *Echinophyllia aspera* *O. lacera* *Astreopora expansa*	Lipid content & composition	---	Lin et al. (2013b)
J. juncea *J. fragillis*	Lipid content & composition	---	Lin et al. (2012)
E. gemmacea	Chilling	0.5 M MeOH	Tsai & Lin (2012)
J. juncea *J. fragillis*	Chilling	1 M MeOH	Lin et al. (2011)
J. juncea *J. fragillis*	CPA effects	MeOH<EG< PG<DMSO	Tsai et al. (2011)
E. gemmacea	CPA effects	MeOH<DMSO<PG=EG	Tsai et al. (2010b)

Coral larvae and adult tissue cryopreservation

Corals can reproduce both sexually and asexually. Therefore, preserving sexually derived cells such as oocytes and sperm can preserve biodiversity and even provide a biological platform for crossing different genotypes to potentially increase resilience (Hagedorn et al., 2012). Recently, both asymbiotic (Daly et al., 2018) and symbiotic (Cirino et al., 2019) coral larvae have been successfully

cryopreserved by vitrification and nano-laser warming. In the latter study, 55% of thawed *Seriatopora caliendrum* larvae survived treatment, and 9% were able to settle. It is hope that more endosymbiotic coral species will be cryopreserved in the near future, though both vitrification and laser warming conditions must be optimized for each new species test.

Compared with coral sperm and oocytes, studies on the cryopreservation of coral embryos are non-existent, and adult coral cryopreservation is in its infancy (Tab. 3.). This is due to the complex, dual-compartmental nature of coral gastrodermal cells, as well as their atypical lipid composition, both of which increase cryopreservation difficulty (Feuillassier et al., 2014a). Furthermore, the calcium carbonate exoskeleton of adult corals thwarts cryopreservation success (Feuillassier et al., 2014b). DMSO, MeOH,

Tab. 3. Cryopreservation of adult and larval corals. CPA=cryoprotectant.

Species	**Material**	**Experimental or** cryopreservation method	**CPA(s)**	**Reference**
Seriatopora caliendrum	Larvae	Vitrification & laser warming	2M EG+1M PG	Cirino et al. (2019)
Fungia scutaria	Larvae	Vitrification & laser warming	10% PG+5%DMSO+1 M trehalose	Daly et al. (2018)
Pocillopora damicornis	Tissue ball	Vitrification	1.5 M EG+1.5 M Gly+1.5 M DMSO	Feuillassier et al. (2015)
P. damicornis	Apex	CPA effects	EG>MeOH>DMSO>Gly	Feuillassier et al. (2014b)
P. damicornis	Tissue ball	CPA effects	≤ 4 M EG, MeOH, Gly, or DMSO	Feuillassier et al. (2014a)
P. damicornis	Adult fragment	Chilling	≤ 1.5 M DMSO	Hagedorn et al. (2013)
F. scutaria	Larva	Analysis of internal osmolality	---	Hagedorn et al. (2010b)
P. damicornis *F. scutaria*	Larvae	CPA effects, ice nucleation temperature, & permeability	PG	Hagedorn et al. (2006a)

and Gly have all been used as CPAs in cryopreservation experiments of adult coral fragments (Hagedorn et al., 2012) and tissue balls (Feuillassier et al., 2014a), with the non-permeating CPAs EG+sucrose tested with adult coral branches (Feuillassier et al., 2014b) and tissue balls (Feuillassier et al., 2015). Although alcohol+sugar CPA cocktails may reduce the toxicity of the alcohol and limit cellular damage (Renard, 1991), such was not the case with *P. damicornis* tissues (Feuillassier et al., 2014a), in which immersion in 0.2 M sucrose+0.75 M DMSO+0.75 M MeOH+0.75 M EG did not confer any survival benefit after cryopreservation.

Symbiodinaceae cryopreservation

Corals owe their evolutionary success to their mutualistic relationship with dinoflagellates of the family Symbiodiniaceae; these endosymbionts supply nutrients and energy to their host corals and are consequently obligatory for all reef-building corals (Whitehead & Douglas, 2003; Russell, 2013). Given the concerns with climate change-induced bleaching raised above, Symbiodiniaceae cryopreservation will play a critical role in the future restoration of coral populations (Chong et al., 2016a). Unfortunately, this field is in its nascent stages (Tab. 4), with only several of the nine Symbiodiniaceae genera successfully cryopreserved to date. It will be critical to cryopreserve all such types because some, such as *Durusdinium*, are known to have higher thermal tolerance (Stat et al., 2009).

Different freezing methods have been used in Symbiodiniaceae cryopreservation studies. For instance, two-step freezing with EG and PGs as the CPAs was used for *Durusdinium* and *Gerakladium* (Lin et al., 2019b; Chong et al., 2016c), though vitrification with DMSO as the CPA was superior for *Cladocopium* (Hagedorn & Carter, 2015). However, because different CPAs were used in studies with different endosymbiont types, it is currently not possible whether to know whether differential cryopreservation success is due to the type of Symbiodiniaceae, the CPA used, or the freezing method. With respect to the freezing method, vitrification and two-step freezing have both been executed successfully (Zhao, 2017; Lin et al., 2019b). In the former, all freezable water is removed from the cells after sugar addition, and the highly concentrated internal aqueous compartment vitrifies as described above. During two-step freezing, Symbiodiniaceae cells are permeabilized with CPAs before being vapor-frozen in LN_2 (Santiago-Vázquez et al., 2007; Chong et al., 2016c; Lin et al., 2019b; Thongpoo et al., 2019). In both processes,

Tab. 4. Cryopreservation of Symbiodiniaceae. Mt=mitochondrial. CPA=cryoprotectant.

Species/host	Experimental or cryopreservation method	CPA(s)	Reference
Symbiodinium (cultured) *Cladocopium* (cultured) *Fugacium* (cultured)	Two-step freezing	Various CPAs at different concentrations	Di Genio et al. (2020)
Gerakladium/Junceella fragilis	Luminometry & flow cytometry	MeOH=DMSO<EG<PG< Gly	Lin et al. (2019a)
Durusdinium (cultured)	Mt DNA content	2M PG	Thongpoo et al. (2019)
Durusdinium (cultured)	Two-step freezing	2M PG+2M EG	Lin et al. (2019b)
Gerakladium/J. fragilis	Gene expression	1 M MeOH+0.4 M sucrose	Chong et al. (2017)
Gerakladium/J. fragilis	Gene expression	1 M MeOH+0.4 M sucrose	Chong et al. (2017)
Gerakladium/J. fragilis	Vitrification & controlled slow-cooling	1 M Gly	Zhao, (2017)
Gerakladium/J. fragilis	Two-step freezing	1 M MeOH+0.4 M sucrose	Chong et al. (2016c)
Cladocopium/Fungia scutaria /Pocillopora damicornis /Porites compressa	Vitrification	10% DMSO+5 % MeOH+0.5 M trehalose	Hagedorn & Carter (2015)
Cladocopium/F. scutaria, /P. damicornis, /P. compressa	Chilling	---	Hagedorn et al. (2010a)
Breviolum/Pseudopterogorgia elisabethae	Two-step freezing	20% ethanol or 20% MeOH	Santiago-Vazquez et al. (2007)

dehydration followed by extracellular freezing prevent intracellular ice crystal formation.

In addition to the ATP assays discussed above, numerous methods have been described for evaluating the viability of Symbiodiniaceae. The aforementioned vital staining dyes penetrate cells with damaged membranes though can be labor-intensive unless flow cytometry is employed. Furthermore, they can overestimate the number of viable Symbiodiniaceae (Lin et al., 2019a) because dead cells do not always have ruptured and permeable membranes (Altman et al., 1993). Additionally, it can be hard to distinguish SYTOX® from chlorophyll fluorescence (Lebaron et al., 1998; Zetsche & Meysman, 2012). A preferable approach may be the CellTiter-Blue assay, in which the metabolic activity of cells is assessed based on their ability to convert the redox dye resazurin into the fluorescent end product resorufin. Santiago-Vázquez et al. (2007) successfully used this approach assess the viability of *Breviolum*.

Pulse amplitude-modulated (PAM) fluorometry, which measures the maximum dark-adapted yield of

photosystem II (Fv/Fm), has been used as a proxy for photosynthetic efficiency in corals, and Hagedorn et al. (2015) used Fv/Fm values as a proxy for health of cryopreserved *Cladocopium*. Since cryopreservation experiments can generate a large number of samples to be analyzed by any such viability assay, the ideal approach may well be one that can be conducted quickly. In the end, cell survival and growth are the easiest-to-interpret, and likely most relevant, indicators of the viability of cryopreserved and thawed cells.

Coral cryo-banking

Gene banks preserve genetic material from different species in different forms and for varying purposes, including scientific studies, breeding efforts, and conservation endeavors. One bank, Genome 10K, aims to sequence at least one species from each genus of vertebrates in the animal kingdom for research purposes (Koepfli et al., 2015). Cryobanks are one form of gene bank, and many exist across the globe. They are particularly important for aquaculture and biopreservation (both discussed above), and the Frozen Ark Project (Clarke, 2009) aims to preserve samples for both research and preservation. National Museum of Marine Biology and Aquarium (NMMBA) has been involved with the Frozen Ark project since 2015. If the oceans become too marginalized for coral survival, their genetic material can be cryopreserved until a point in time in which coral propagation is once again viable *in situ* (Mayfield et al., 2019). Different genotypes could even be crossed to increase genetic diversity and potentially yield more environmentally resilient populations. Thawed coral specimens could be reared in captivity to reduce aquarium trade impacts on in situ populations. Currently, coral cryobanks can be found at Taiwan's NMMBA (Mayfield et al., 2019; Di Genio et al., 2020; Fig. 6), the Hawaii Institute of Marine Biology (USA), and the Taronga Western Plains Zoo (Australia; Hagedorn and Spindler, 2014).

Conclusions

The general cryopreservation protocol involves 1) the initial freezing of the samples, followed by 2) long-term storage, and finally 3) thawing for subsequent culture or fertilization in the case of gametes. Future efforts should seek to cryopreserve increasingly more complex tissue types, in which case more advanced diagnostic approaches may be needed to assess health post-thaw (e.g., ultrastructural observations and molecular biotechnology). Not only will these data allow us to understand the physiological implications of extreme low-temperature exposure on coral gametes, embryos,

Fig. 6. Cryopreserved coral cells, larvae, and Symbiodiniaceae are deposited in cryobanks for long-term storage. Researchers from Taiwan's National Museum of Marine Biology and Aquarium an are actively collecting and cryopreserving coral cells to be used for research (e.g., genetic modification & gene mapping projects) and as a backup source of genetic material for future reef restoration endeavours. Photo credit=Chiahsin Lin.

larvae, and, but they will be critical to optimizing protocols that give thawed samples the best chance at survival in the new, and ideally improved, milieu in which they will ultimately reside.

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