PROTOCOL DESIGNS TO OPTIMIZE CRYOPRESERVATION OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

by

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Abstract

Cryopreserving cells for human health therapeutics is a multidisciplinary process with great complexity. Cryopreservation of human umbilical vein endothelial cells (HUVECs) has facilitated vascular biology research since they were first isolated in 1973 [59]; however identifying key variables to optimize HUVEC cryopreservation has been a challenge.

It is hypothesized that through the use of interrupted cooling protocols, key variables to optimize cryopreservation of HUVECs can be determined. Interrupted cooling protocols, which have been well-characterized using human erythroleukemia TF-1 cells [150], [151], [152], were previously used to study HUVEC cryopreservation conditions [147]. Mazur proposed the two-factor hypothesis to explain observations of optimal cooling, which is affected by cell characteristics and solution characteristics [101]. Hydraulic conductivity, a cell characteristic related to water flow across the cell membrane [31], is very low for HUVECs [149], suggesting that slower cooling protocols may be better than rapid cooling protocols. Cryoprotectants are useful if optimal cooling is insufficient to maintain high viability; however neither DMSO nor hydroxyethyl starch (HES) have previously been used in interrupted cooling studies of HUVEC suspensions.

Several methods were carefully considered to ensure high experiment repeatability: *i)* HUVEC culture and cell preparation methods, *ii)* fluorescence microscopy methods, and *iii)* flow cytometry methods. The HUVEC culturing and cell preparation methods that contributed directly to high experiment repeatability and high membrane integrity include: *i)* growing to ≤ 15 population doublings, *ii)* growing to 50% – 80% culture flask coverage, *iii)* maintaining HUVECs on ice for 2 – 4 hours prior to experiments, and *iv)* consistent thawing followed by

immediate measurement of membrane integrity. Fluorescence microscopy image capture methods were optimized using the Viability3 cell counting program (version 3.2, Great Canadian Computer Company, Spruce Grove, Alberta, Canada) as a reference. Flow cytometry methods were optimized to record fluorescence emissions from different fluorochromes separately, resulting in separation of membrane-intact cells, membrane-damaged cells and background light scatter.

Two interrupted cooling protocols were used: *i)* graded freezing [107], an interrupted slow cooling protocol [152], and *ii)* two-step freezing [108], an interrupted rapid cooling protocol. Interrupted cooling protocols were used to determine key variables to optimize HUVEC cryopreservation, namely: *i)* flow cytometry versus fluorescence microscopy membrane integrity measurements, *ii)* cooling protocol, *iii)* cooling rate, *iv)* cryoprotectant addition procedures, *v)* cryoprotectant composition, and *vi)* plunge temperature. Flow cytometry, either in the presence or absence of cryoprotectant, was determined to be the more stringent method to measure membrane integrity. Higher membrane integrities were attained after the interrupted slow cooling protocol than the interrupted rapid cooling protocol. A cooling rate of 1.0 °C/min resulted in better survival of HUVECs than a 0.2 °C/min cooling rate. It was predicted that a previously designed cryoprotectant addition procedure would be optimal [138]; however adding 20% DMSO directly to HUVECs to a final concentration of 10% DMSO for 15 minutes at 0 °C resulted in membrane integrities that were similar. Increasing the cryoprotectant concentration from 10% to 20% DMSO did not provide additional protection to HUVECs. However, adding HES with 10% DMSO was beneficial for HUVEC survival. The optimal cryopreservation procedure obtained in this work involved cooling HUVECs in suspension in the presence of 10% DMSO and 8% HES at 1.0 °C/min to −45 °C before cryogenic storage. A significant

improvement in HUVEC survival was measured compared to the 64.8% ± 2.2% membrane integrity of supplied HUVECs measured in this work and the 69.2% ± 2.3% reported in the literature for the standard protocol using good manufacturing practices [140]. Importantly, we describe detailed procedures to ensure reproducible results and explore the effects of key variables required to optimize the cryopreservation of HUVECs. The careful attention to methods and the use of interrupted cooling protocols can be used to design studies to improve routine cryopreservation of HUVECs and other types of cells.

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Glossary and Abbreviations

Culture flask coverage – Estimation of the amount of culture surface area covered by cells and converted to a percentage

DMSO – Dimethyl sulfoxide

EGM – Endothelial growth medium, product CC-3162 from Lonza

FC – Measurement of membrane integrity using flow cytometry

FC Dbl – Measurement of membrane integrity using flow cytometry, counting fluorescence measurements in the Dbl region as membrane-intact cells

FCS – Foetal calf serum

FM – Measurement of membrane integrity using fluorescence microscopy

FS – Forward light scatter

HES – Hydroxyethyl starch

HUVEC – Human umbilical vein endothelial cell

%MI – Percent membrane integrity

S.D. – Standard deviation

SEM – Standard error of the mean

SS – Side light scatter

SYTOEB – SYTO 13 and ethidium bromide solution for membrane integrity assessment

SYTOPI – SYTO 13 and propidium iodide solution for membrane integrity assessment

SYTOGelRed – SYTO 13 and GelRed solution for membrane integrity assessment

%Recov – percent recovery

Chapter One: Thesis Background

1.1 Introduction

Human umbilical vein endothelial cells (HUVECs) are a model system for vascular biology research since they were first successfully cultured in 1973 [59]. HUVECs are vascular endothelial cells that line the interior surface of veins in human umbilical cords. These were the first type of endothelial cells to be cultured and their availability has been facilitated for many purposes through routine cryopreservation procedures.

HUVECs are identified based on their: *i)* cobblestone morphology, *ii)* presence of von Willebrand factor VIII, *iii)* CD31 and CD105 expression and *iv)* acetylated low density lipoprotein uptake [85]. HUVECs are used to study physiology and pathophysiology of vascular disorders [22] but also for study of biomaterials in tissue engineering [21], [75], [131], [175], and drug delivery systems [84], [129], [162], [200]. Investigations and applications include: vasoregulation [122], [139], [161], [180], coagulation [17], [24], [62], [202], fibrinolysis [41], [43], [76], [156], atherosclerosis [12], [18], [26], [157], vasculogenesis and angiogenesis [114], [155], [179], [183]. Their benefit in research can also be as a healthy, quiescent counterpart to dysfunctional endothelial cells [183]. Research into HUVEC cryopreservation was originally adapted from corneal cells [77], [78], [135], [138], [177], but despite substantial research on HUVECs, their cryopreservation has not been characterized or optimized. Identifying the key variables is an important first step to optimize HUVEC cryopreservation. Methods to cryopreserve HUVEC suspensions can serve as a model to optimize cryopreservation of cells in suspension and tissues with endothelial layers (e.g. corneas).

1.2 Cell Response to Freeze-Thaw Stress

Measuring cell response to freeze-thaw stress is an important first step to investigate cryopreservation of cells. Many cell responses can be measured, but the plasma membrane is of particular interest in cryobiology [97]. "*The plasma membrane is the principal interface between the extracellular medium and the cytosol, and acts as a semipermeable barrier allowing for the*

efflux/influx of water during the freeze/thaw cycle while also serving as a barrier to preclude seeding of the intracellular solution by extracellular ice" [167]. Therefore, a fundamental measure of cellular response to freeze-thaw stress is membrane integrity.

Membrane integrity is a measure of the proportion of cells in the population with intact membranes. When the membrane is intact, osmotic regulation can occur. Supercooling, or undercooling, is the phenomenon in which solutions remain in an unfrozen state below their freezing point [99]. Supercooling can exist both inside and outside the cell. Ice formation is one mechanism to relieve supercooling. Extracellular ice formation can be induced [123] or it can occur spontaneously. Cells can relieve intracellular supercooling either by undergoing intracellular ice formation or by becoming sufficiently dehydrated [96]. The mechanism by which intracellular ice formation occurs has been linked directly to membrane damage, with the proposition that intracellular ice is a result rather than a cause of damage [127]. Ice excludes solutes to the unfrozen fraction [72], thus increasing solute concentration. Therefore, for cells to reduce supercooling by dehydration, a sufficient amount of ice must be present in the extracellular solution and cells must be allowed a sufficient amount of time to dehydrate.

Mazur developed the two-factor hypothesis of freezing injury to explain observations of optimal cooling rates [101]. Cooling cells slower than the optimal rate in the presence of ice results in cell death by excessive dehydration and solute toxicity [98], [136] while cooling cells faster than optimally results in cell death by intracellular ice formation. During optimal cooling, cells experience a hypertonic environment and have time to sufficiently dehydrate before cryogenic storage.

Depending on cooling rate, the thawing rate can also have an impact on cell survival. Studies with fibroblasts, yeast and red blood cells show that if cooling is slow, cells are less sensitive to thawing rate [39], [101], [104], [118], [137]. However, if cooling is rapid, cells are more sensitive to thawing rate because ice nuclei which formed inside the cell during cooling subsequently grow and cause damage during slow thawing [101]. Many types of cells which are rapidly cooled can be saved from freezing injury by rapid thawing [109]. There are exceptions with mouse embryos [79], but in general, rapid thawing results in a higher survival compared to slow thawing. Cryoprotectants also mitigate slow cooling damage and enable survival of cells at lower cooling rates.

1.3 Cryoprotection

A sufficient number of cells may not survive even with optimal cooling, so cryoprotectants can offer cells protection from freeze-thaw stress. Lovelock showed that as a 0.9% aqueous sodium chloride solution is frozen, high concentrations of sodium chloride develop which cause damage to human red blood cells during freezing and thawing [86]. Cryoprotectants, such as glycerol [142], can reduce the harmful effects of solutes at a given sub-zero temperature during freezing by: reducing the amount of ice, increasing osmolality, and reducing cell shrinkage [86], [136].

A critical temperature range between −3 °C and −40 °C exists where solute concentrations increase and cryoprotectants have been shown to mitigate damage of human red blood cells [87], bull semen [90], and chick skin [176]. Dimethyl sulfone ($DMSO_2$) in water is ineffective as a cryoprotectant due to its reduced solubility below −1.6 °C [111]. The criteria for selecting cryoprotectants is that they have a high solubility and are non-toxic at high concentrations [115].

Cryoprotectants can be classified based on their ability to permeate cell membranes [116]. Permeating cryoprotectants protect cells by: *i)* increasing intracellular and extracellular osmolality [35], [107], *ii)* depressing the freezing temperature which reduces the amount of ice formed and therefore the damaging effects of ice and solute concentration at a given temperature [38], [39], [86],[107], *iii)* reducing the extent of cell shrinkage [35], and i*v)* raising the vitrification temperature [117]. Dimethyl sulfoxide (DMSO) is a permeating cryoprotectant which can pass through cell membranes and was first demonstrated for human and bovine red blood cells and bull spermatozoa [88]. DMSO has a high solubility in water [111] and reduces ice formation [145], making it effective as a permeating cryoprotectant.

Another type of cryoprotectant is the non-permeating cryoprotectant, which is not capable of diffusing through intact cell membranes. Non-permeating cryoprotectants can protect cells by increasing extracellular osmolality, causing cells to dehydrate which reduces the likelihood of intracellular ice formation during cooling [106]. These cryoprotectants also protect cells from freeze-thaw stress by reducing the amount of ice formed [73]. Extracellular polymeric solutes are common non-permeating cryoprotectants whose protective effects have been attributed in part to the solution glass transition temperature [173]. HES was first demonstrated as a cryoprotectant at a final concentration of 15% for erythrocytes [71]. HES is a complex carbohydrate with a wide

distribution of molecular weights and hydroxyethylations [172], which in some cases has made cryopreservation studies with HES difficult to compare [164], [166]. Those forms of HES which are well characterized have available osmotic virial coefficients, based on HES specifications for weight average molecular weights, hydroxyethyl substitutions and HES:NaCl mass ratios [20]. In cryopreservation of fibroblasts, HES alone has shown cryoprotective capabilities at concentrations greater than 5% [106], [107], but in combination with 5% DMSO, HES did not improve survival [106]. Pentastarch, a low molecular weight HES is used in Pentaspan, a plasma volume expander [13]. Its use in clinical settings makes it ideal to use as a cryoprotectant for human health therapeutics.

Cryoprotectants, although beneficial, can introduce stress during addition and removal. Volume excursions can be damaging to cell membranes [136]. Upon addition, both permeating and non-permeating cryoprotectants increase extracellular osmolality, which causes cells to dehydrate and decrease in cell volume. After a certain time, permeating cryoprotectants enter the cell, which can result in a transient change in cell volume. The amount by which cell volumes change depends on: *i)* hydraulic conductivity, a membrane characteristic used to describe water diffusion across the cell membrane [31], *ii)* solute permeability, a membrane characteristic used to describe solute diffusion across the cell membrane [30], [32], [56], [57], [192], and *iii)* intracellular solution osmotic virial coefficients, used to describe changes in intracellular osmolality as a function of solute concentration [153]. In studying porcine aortic endothelial cells, it was observed that cells could withstand a reduction to one third of their isotonic volume upon DMSO addition; it was also observed that cells could withstand an expansion to three times their isotonic volume upon DMSO removal [192]. The cryoprotectant addition and removal can damage cells through these volume excursions.

1.3.1 Cryoprotectant Toxicity

Depending on concentration, cryoprotectants can be toxic which can cause greater damage than osmotic stress [33], [44]. To maximize cryoprotection and minimize toxicity, lower concentrations of cryoprotectants, shorter exposure times and lower temperatures are beneficial [34], [117]. Sometimes, mixtures of cryoprotectants are employed to reduce toxicity. For articular chondrocytes, five cryoprotectants were investigated (DMSO, ethylene glycol,

propylene glycol, glycerol and formamide) and it was found that combinations of three cryoprotectants at a 3 M concentration reduced cryoprotectant toxicity compared to individual cryoprotectants at the same concentration [61].

Reducing the toxicity of DMSO is important to cryopreserve many cells. A common strategy is to combine HES with a lower concentration of DMSO [172]. Cryopreservation of peripheral blood progenitor cells was performed using DMSO and HES where it was concluded that at least 5% DMSO and 6% HES is necessary [165]. A combination of 5% DMSO and 6% HES has been used to cryopreserve many cells, including: *i)* umbilical cord blood cells [15], [46]; *ii)* bone marrow [168], [169], [170], [187]; *iii)* peripheral blood stem cells, [6], [11], [16], [40], [54], [63], [65], [66], [92], [148]; *iv)* granulocytes [81], [82], [199]; *v)* monocytes [51]; *vi)* canine femoral artery and vein endothelial cells [49]; *vii)* canine bone marrow $CD34^+$ cells [52]; *viii)* canine pancreatic islet cells [93]; and *ix)* rat molars [67] . Cryopreservation of hematopoietic stem cells showed that using 5% DMSO and 6% HES resulted in quicker recovery of patients white blood cell count compared to cryopreservation using 10% DMSO alone [154]. Other studies have shown that the results for cryopreservation using 5% DMSO and 6% HES did not differ from the results for cryopreservation using 10% DMSO for bone marrow [89] and peripheral blood stem cells [105], [174].

Combinations of DMSO and HES other than 5% DMSO and 6% HES have been studied. For mesenchymal stem cells, 5% DMSO and 5% HES was recommended, although 8% DMSO and 2% HES showed the highest viability [130]. Umbilical cord mesenchymal cells were successfully cryopreserved using a 10% DMSO and 20% HES solution [80]. Cryopreservation of rat granulocytes was optimal using 10% DMSO and 5% HES [7]. Chinese hamster fibroblasts were cryopreserved using 5% DMSO and 5% HES, where 5% DMSO resulted in higher viabilities after two-step freezing [106]. Cryopreservation of human pancreatic islets was performed using 5% DMSO and 4% HES resulting in a viability of 71.2% [68]. Cryopreservation of peripheral blood stem cells showed that using 5% DMSO and 3% HES resulted in higher viabilities than using 10% DMSO [23].

Combining DMSO and HES does not always result in the highest recovery. Platelet recovery was lower after cryopreservation using 5% DMSO and 6% HES compared to DMSO alone [4]. Pluripotent stem cells had a higher viability using a combination of 5% DMSO, 6% HES, and 5% ethylene glycol compared to 5% DMSO and 6% HES [53]. Although combining

HES with DMSO is a common strategy to reduce DMSO toxicity, other methods are available to optimize cryopreservation procedures.

1.4 HUVEC Cryopreservation Studies

HUVEC cryopreservation has been studied using: the intact umbilical vein [9], the HUVEC cell line (ECV304) [135], [193], and HUVEC suspensions [78], [77], [140], [147].

In cryopreserving ECV304 in 10% DMSO, slower cooling rates were better for monolayers [135]; however, in cryopreserving cell suspensions, the optimum viability was observed over a wide cooling range between 0.3 °C/min and 10 °C/min. Two factors may have contributed to a wide optimal cooling range: *i)* not controlling the temperature of ice formation and *ii)* centrifugation of cells after thawing for DMSO removal. After thawing, centrifugation and DMSO dilution could be damaging [198].

Using an intact umbilical vein, HUVEC cryopreservation was investigated throughout the HUVEC extraction process: *i)* cryopreserving the intact umbilical vein, *ii)* cryopreserving HUVEC suspensions after extraction from the umbilical vein and, *iii)* cryopreserving HUVEC suspensions after culturing [9]. Cryopreservation was done by cooling at 0.5 °C/min in a 10% DMSO solution to −80 °C and holding for 12 hours before liquid nitrogen storage for 8 weeks. No recovery of HUVEC was possible after thawing the umbilical vein due to a fracture which prevented endothelial cell harvesting. Also, cryopreservation directly after extraction did not result in recovery of HUVEC after thawing. It was thought that the HUVEC extraction process was stressful and that additional stress from cryopreservation resulted in no recovery of viable cells. Recovery of HUVECs was possible if cells were cryopreserved after they had undergone a sub-culture prior to cryopreservation. It was concluded that HUVECs must first be extracted from the umbilical vein and cultured prior to cryopreservation [9].

Cryopreservation of HUVEC suspensions resulted in a wide range of cell recovery [77]. Cooling was performed at 1 °C/min in a 10% DMSO solution in CPTes followed by storage for 7 to 36 days in the liquid nitrogen vapour phase. Upon thawing, a retrieval of 66% ± 5% was obtained (mean ± S.D., n = 31) ranging from 32% to 88% measured using trypan blue. It was concluded that the wide response range was due to variations in the umbilical cord material and differences in investigator performance.

In a subsequent report [78], a high viability of HUVECs was obtained after cryopreservation using M199 with 20% foetal calf serum (FCS) and 10% DMSO. Lehle *et al.* investigated cryopreservation of HUVECs in defined media CPTes and 10% DMSO, or media M199 with 10% DMSO and 20% foetal calf serum. A wide range of viabilities were reported, but it is difficult to compare their results directly with this work because it is unclear from the methods whether viabilities were measured before or after centrifugation and how their reported "retrieval" and "viability" should be combined. Also their report that cryopreservation outcome was dependent on storage time in liquid nitrogen (1 to 12 months) is unexpected; making it difficult to compare this work with other cryopreservation studies.

A study by Polchow *et al.* [140] reported the development of good manufacturing practice in the processing of HUVECs including cryopreservation. HUVECs were cryopreserved in 10% DMSO and 18% human serum albumin, cooled nominally at 1 °C/min to –80 °C and stored in the liquid nitrogen vapour phase. Reported viabilities of HUVECs after cryopreservation were 66.3% ± 4.4% (7 days in liquid nitrogen vapour) and 69.2% ± 2.1% (1 year in liquid nitrogen vapour). Ranges in cooling rates or other variables important in cryopreservation procedures were not investigated. Also, the procedure using good manufacturing practices [140] is a routine cryopreservation procedure which has not been optimized for HUVECs.

1.4.1 Interrupted Cooling Protocols

Interrupted cooling protocols provide much more detail to optimize cryopreservation procedures. Interrupted cooling protocols allow many variables to be compared by measuring: *i)* viability after cooling to and thawing from intermediate sub-zero temperatures (direct thaw); and *ii)* viability after cooling to and thawing from liquid nitrogen (plunge-thaw). Interrupted cooling has improved understanding of cell response to freeze-thaw conditions with computer simulations to provide a detailed characterization of the cryobiological response of TF-1 cells [150], [151], [152]. Interrupted cooling protocols have been applied to many cells, including: fibroblasts [36], [39], [106], [107], [108], [109], [113], [178], [195]; lymphocytes [36], [111], [113], [186]; granulocytes [5], [113], [196]; chick skin [176]; bull spermatozoa [88], [90], [141], human whole blood [144]; human articular cartilage [128]; human corneal endothelial cell monolayers [29]; human foetal pancreas tissues [160]; human granulocytes [5], [196]; human

umbilical vein endothelial cells [147]; porcine articular cartilage [195]; sheep articular cartilage [126]; malarial parasites [188]; Madin-Darby Canine Kidney cells [3], porcine split-thickness skin [197]; mouse oocytes [64], [94], [100], [102], [119], [158]; mouse lymphocytes [14]; bovine embryos [69], [94], [181]; rumen ciliate protozoa [70], [132]; parasitic helminths [185]; rat embryos [83], rat Islets of Langerhans [8]; naplius larvae [133]; bromegrass [55]; microalgae [25]; and semen of the African catfish *Clarias gariepinus* [184].

The two interrupted cooling protocols of interest are two-step freezing and graded freezing. HUVECs have been studied in the absence of cryoprotectants using two-step freezing and graded freezing [147]. Two-step freezing is an interrupted rapid cooling procedure [151] and graded freezing is an interrupted slow cooling procedure [152]. To study HUVECs, two-step freezing was used to examine the effect of rapid cooling to intermediate sub-zero temperatures (hold temperatures) and hold times at those temperatures. Depending on the hold temperature, an optimal hold time was in the range of 3 minutes for plunge-thaw from −9 °C or 10 minutes for plunge-thaw from −12 °C with viabilities of 11% and 15% respectively. Graded freezing was used to examine controlled cooling to intermediate sub-zero temperatures (experimental temperatures). The optimal cooling rate was 0.1 °C/min as observed before plunge-thaw from −20 °C with a viability of 48% [147]. The interrupted cooling protocols demonstrated for HUVECs that slow cooling results in higher viabilities than rapid cooling in the absence of cryoprotectant [147].

Interrupted cooling protocols provide a means to determine a range of optimal cooling conditions; however the responses are specific to the types of cells. Hydraulic conductivity characterizes a cells' water permeability, an important characteristic to predict whether a particular cooling protocol will maintain optimal cell viability throughout cryopreservation procedures [103]. HUVECs have the lowest hydraulic conductivity of the cells shown in Table 1-1 and the lowest hydraulic conductivity for a cell examined using interrupted cooling. Some publications examined a range of conditions to cryopreserve cells and optimal cryopreservation procedures could be determined which are presented in Table 1-1. Based on hydraulic conductivity, it is hypothesized that HUVECs have a higher survival after slow cooling. HUVECs in the absence of cryoprotectant have also been shown to respond better to slow cooling than rapid cooling [147].

Table 1-1. Hydraulic conductivities reported for various types of cells

Type of cell [reference]	Temperature during measurement	Hydraulic conductivity, $\mu m^3/\mu m^2/min/atm$	Optimal Cryopreservation [reference]
HUVECs [149]	20 °C	0.148	1.0 °C/min in 10% DMSO [140]
Canine pancreatic islets [189]	22 °C	0.193	0.24 °C/min to −43 °C in 2.57 M ethylene glycol before plunge-thaw [190]
Human granulocytes [196]	Room temperature	0.195	1 °C/min to −15 °C before plunge-thaw without CPA [196]
Porcine articular cartilage chondrocytes [191]	26 °C	0.236	Not available
Bovine spermatozoa [19]	22 °C	0.28	30 °C/min from −5 °C to −50 °C before plunge-thaw without CPA [74]
Human bone marrow progenitor cells [31]	20 °C	0.283	1 °C/min, 5% DMSO and 6% HES [187]
Bovine articular cartilage chondrocytes [110]	24 °C	0.305	Predict 10 to 30 °C/min without CPA [110]
TF-1 [149]	20 °C	0.339	Rapid cooling to −12 °C, 3 minute hold before plunge-thaw without CPA [151]
Human corneal epithelial cells [27]	22 °C	0.39	Predict 7 °C/min, 1 M DMSO [30]

Type of cell [reference]	Temperature during measurement	Hydraulic conductivity, $\mu m^3/\mu m^2/min/atm$	Optimal Cryopreservation [reference]
Mouse oocytes [10]	22 °C	0.40	Rapid cooling to −25 °C, 30 minute hold before plunge-thaw without CPA [102]
Human lymphocytes [47]	25 °C	0.46	2 °C/min, 5% DMSO [37]
Rat embryos [83]	25 °C	0.54	1 °C/min to −20 °C in 1.0 M adonitol before plunge-thaw [182]
Human corneal keratocytes [27]	22 °C	0.55	Predict 7 °C/min, 1 M DMSO [30]
Human corneal endothelial cells [28]	22 °C	0.62	1 °C/min to −40 °C before plunge-thaw, 1 M DMSO [29]
Bovine embryos [60]	25 °C	1.2	0.3 °C/min to −30 °C in 1.5 M ethylene glycol and 0.2 M trehalose [181]
Human pancreatic islets [189]	22 °C	1.56	1 °C/min, 5% DMSO and 6% HES [68]
Fibroblast [31]	22 °C	1.82	1 °C/min, 5% DMSO, plunge-thaw from −30 °C or −40 °C [107]
Human spermatozoa [42]	22 °C	1.84	10 °C/min to −80 °C in 0.85 M glycerol before plunge-thaw [48]
Red blood cells [201]	19.8 °C	18.11	1000 °C/min in 10% DMSO or 30% PVP [125]

1.5 Thesis Scope

It is hypothesized that interrupted cooling protocols can be used compare the effects of: *i)* flow cytometry versus fluorescence microscopy membrane integrity measurements, *ii)* cooling protocols, *iii)* cooling rates, *iv)* cryoprotectant addition procedures, *v)* cryoprotectant compositions and *vi)* plunge temperature.

In general, cells with low hydraulic conductivity survive slow cooling and cells with high hydraulic conductivity survive rapid cooling; however as can be seen in Table 1-1, there are exceptions. For example, fibroblasts have a very high hydraulic conductivity; however they survive better using slow cooling [107]. Based on the low hydraulic conductivity of HUVECs and previous research findings [78], [147], it is hypothesized that slow cooling rates will be beneficial for HUVEC survival. Cryopreservation of HUVECs in the presence of DMSO [9], [77], [78], [135], [140], [147], [193] results in higher membrane integrities than cryopreservation in the absence of cryoprotectant [147]. Considering the routine cryopreservation procedure using 1 °C/min in the presence of 10% DMSO, it will be valuable to determine how this cryopreservation procedure can be optimized for HUVECs.

The first objective of this thesis is to compare fluorescence microscopy and flow cytometry for membrane integrity assessment of HUVECs after interrupted cooling protocols.

The second objective of this thesis is to compare the membrane integrities of HUVECs in response to interrupted cooling protocols in the presence and absence of cryoprotectant using the more stringent flow cytometry membrane integrity assessment method. Variables tested using interrupted cooling protocols were: *i)* cooling rates, *ii)* cooling protocols, *iii)* cryoprotectant addition procedures, *iv)* cryoprotectant compositions, and *v)* plunge temperatures.

The third objective of this thesis is to compare the membrane integrity of HUVECs following the best cryopreservation procedure in this work to the viabilities reported in the literature and the viability of HUVECs as supplied after commercial cryopreservation.

Chapter Two: Viability Assessment

2.1 Introduction

In cryobiology, assessing cell survival can be a challenge. Loss of cell survival increases cell debris that causes an increase in background noise, making it difficult to consistently resolve cells. Fluorescence microscopy and flow cytometry have a long history of facilitating cell biology research. Delving into instrument operation for the sake of optimization is intimidating but necessary to obtain the high experiment repeatability required to determine optimal conditions for HUVEC cryopreservation. Previous application of membrane integrity assessment for HUVECs provides a basis for optimization [146], [147].

G. G. Stokes first coined the term *fluorescence* in 1852 while studying quinine, a naturally occurring compound from the bark of the cinchona tree [171]. Since then, fluorescence techniques have developed. Fluorescence microscopy provides specificity and contrast not possible through optical microscopy techniques because the specimen is the light source [95]. A fluorescence microscope uses optical filters to separate incident ultraviolet light from fluorescent emissions of the specimen [58]. Treating cells with fluorochromes allows cells to fluoresce vibrantly upon excitation by ultraviolet light. Fluorochrome excitation wavelengths are shorter than emission wavelengths, the difference being referred to as Stokes' shift, where a greater difference in wavelengths allows for better resolution of fluorescence from excitation and background light [163]. Additional complexity in separation occurs when multiple types of fluorochromes are present. As well, fluorochrome fluorescence intensity is time-sensitive from effects of fluorochromes binding nucleic acids and from photobleaching effects of UV-visible light [120]. Also, after cells are exposed to freeze-thaw stress, membrane damage may continue to manifest over time [113]. Cell culturing and preparation methods affect cell–cell contact [1], [2], modifying cell response and reducing the ability to resolve cells for accurate quantification [50], [124]. Distinguishing membrane-intact from membrane-damaged cells using fluorescence microscopy is impacted by many variables requiring consideration.

Flow cytometry is a powerful technique combining fluidics, lasers, multiple sensors, electronics and sophisticated computer software to record interaction of light with a cell in a

flowing system [159]. Flow cytometers measure the laser light scatter and fluorescence emission from thousands of cells within seconds [159]. Laser light interacts with a cell, causing light to scatter and be detected by two sensors, the forward light scatter (FS) sensor and the side light scatter (SS) sensor. Scattered light is influenced by: *i*) particle size; *ii*) surface topography; *iii*) optical density; and *iv*) internal cell structure [91]. The many different variables affecting light scatter make it challenging to interpret the information. Previous studies demonstrate the difficulty in assessing freeze-thaw damage from light scatter alone [112]. Fluorescence measurements made it possible to assess HUVEC freeze-thaw damage, as light scattering properties alone were ineffective to distinguish membrane-intact cells, membrane-damaged cells and background light scatter [146].

2.2 Materials and Methods

2.2.1 HUVEC Culturing and Preparation

2.2.1.1 HUVEC Source and Culture Initiation

HUVECs from Clonetics Umbilical Vein Endothelial Cell Systems (Lonza Group Ltd., Walkersville, MD, USA) were purchased as pooled primary cells frozen after the first sub-culture. HUVECs were supplied in a cryopreservation medium containing endothelial growth medium with 10% foetal bovine serum (FBS) and 10% DMSO. HUVECs were shipped (Cedarlane, Burlington, ON, Canada) in a polystyrene container of dry ice and immediately stored in liquid nitrogen until required.

Optically clear, sterile, tissue-culture treated polystyrene flasks with a 0.2-µm vent cap (T-flasks) (Corning Inc., NY, USA) were used for HUVEC growth with EGM-2 endothelial growth medium (EGM) (CC-3162, Lonza). Preparing for experiments, a 1-mL cryovial of HUVECs (~500,000 cells/mL) was thawed in a 37 °C water bath and the contents diluted in 5 mL of EGM using good asceptic technique. After thawing, two 75-cm^2 T-flasks containing 15 mL of pre-warmed EGM were seeded with 3 mL of diluted supplied HUVECs.

2.2.1.2 HUVEC Contamination

Inspecting the T-flask for HUVEC growth and contamination was performed with the naked eye and using the Labovert phase contrast microscope (Leitz, Los Angeles, CA, USA). Under sterile conditions, the media was transparent and red/pink, but became turbid in the presence of contaminants. Microscopically, HUVEC shape and morphology were observed using the EF 10X objective lens and checked for contamination.

When contamination was observed, cell cultures were discarded and culture lab equipment (e.g. biosafety cabinet, incubator, pipettors) was decontaminated with 70% ethanol and Accel TB wipes (a hydrogen peroxide wipe) (Accel, Oakville, ON, Canada). Lab gowns were laundered, disposable supplies involved in the lab activity were discarded (e.g. growth medium) or autoclaved (e.g. glass Pasteur pipettes) and the lab bench areas decontaminated.

2.2.1.3 HUVEC Trypsinization

Media was changed every 2 days using 25 mL of pre-warmed EGM until the cell culture reached 50% to 80% surface coverage as observed using the phase contrast microscope. Images of attached HUVECs were captured for cell quality reference using an attached Diractor camera (Pixera, Santa Clara, CA, USA). Sub-culturing was done using the Clonetics procedures which involved trypsin/EDTA detachment solution (CC-5012), HEPES Buffered Saline (CC-5024) and trypsin neutralizing solution (CC-5002). HUVECs were observed using the phase contrast microscope while detachment proceeded at room temperature for approximately 2.5 to 3 minutes until more than 90% of cells were rounded. Placing the T-flask on the lab bench and tapping it against the base of the microscope caused the rest of the cells to detach.

2.2.1.4 HUVEC Sub-cultures and Experiments

To sub-culture, a total of 750,000 cells were seeded in 150-cm^2 T-flasks (5,000 cells/cm^2) containing 50 mL of pre-warmed EGM and cultured in an incubator humidified using a tray filled with water and maintained at 37 °C with 5% CO_2 in air (VWR, Sheldon Manufacturing

Inc., Cornelius, OR, USA). As it could mask contamination from poor aseptic technique, the 0.5-mL vial of GA-1000 (30 µg/mL gentamicin and 15 ng/mL amphotericin B after addition to growth media) was not used to supplement the EGM.

HUVECs were sub-cultured after 2 days until cells reached 50% to 80% surface coverage; after which time cells were sub-cultured and grown until the desired cell density was obtained. Cells were removed from T-flasks using supplier protocols involving trypsin and counted using a Z2 Coulter particle count and size analyzer (Beckman Coulter, Inc., Mississauga, ON, Canada). HUVEC concentration was measured after diluting 100 µL of HUVEC suspension in 10 mL of Isoton II diluent (Beckman Coulter, Inc.). For fluorescence microscopy analysis, a concentration of approximately 2×10^6 to 4×10^6 cells/mL was used and for flow cytometry analysis, a concentration of approximately 1×10^6 to 2×10^6 cells/mL was used. Typically, four or five T-flasks were required to prepare 8 to 9 mL of cell suspension for experiments but to continue sub-culturing; a total of 12 to 15 mL was prepared depending on the measured cell concentration. Those cells used for experiments were placed on wet ice at 0 °C for 2 to 4 hours prior to experiments.

2.2.1.5 HUVEC Lifetime

Cells were grown to a maximum of approximately 15 population doublings, as calculated from equation (2.1) as rearranged to equation (2.2),

$$N_i \times 2^n = N_f \tag{2.1}$$

$N_i \equiv$ initial cell seeding number
$N_f \equiv$ final cell number
$n \equiv$ number of doublings

$$n = \frac{ln\left(\frac{N_f}{N_i}\right)}{ln(2)} \tag{2.2}$$

In calculating the cell doublings after the first passage, the cell concentration as supplied was used to calculate the initial cell seeding number. The initial cell seeding number was

adjusted since the contents of a 1-mL cryovial were split between two 75-cm^2 tissue culture flasks. The certificate of analysis from the supplier provided the supplier cell concentration, the lot number and other information related to cell quality and expected cell doubling time.

2.2.2 Temperature Measurement

A T-type thermocouple and OMB-DAQ-55 data acquisition module (OMEGA Engineering Inc., Stamford, Connecticut, USA) were used to measure temperature. A methanol bath (FTS Systems, Stone Ridge, NY, USA) was used to manipulate the cooling bath temperature and cooling rates in all interrupted cooling experiments. A thermocouple was placed directly into the methanol bath, another thermocouple was used to measure room temperature and another thermocouple was placed in a borosilicate glass culture tube (VWR, Edmonton, Alberta, Canada) containing 200 µL of EGM that was placed in the methanol bath to act as a proxy for HUVEC suspensions.

Thermocouple measurements were referenced for accuracy. Temperature referencing was performed by placing thermocouples in the water portion of an ice-water bath and recording the measurement of each thermocouple at 0 °C. At experimental temperatures, the thermocouple temperature was recorded in the lab book and adjusted during analysis using the temperature reference.

2.2.3 Statistics

All results were reported as mean ± standard error of the mean. Differences between means were interpreted assuming two normal population distributions with unknown and unequal variances [121]. The null hypothesis H_0 is shown in equation (2.3) which states that the population means were not different.

$$\mu_1 - \mu_2 = 0 \tag{2.3}$$

$\mu_1 \equiv$ population mean of first sample

$\mu_2 \equiv$ population mean of second sample

The test statistic T_0^*, shown in equation (2.4), was approximated and compared using the Student's *t* distribution because there was not an exact test statistic assuming unknown and unequal variances.

$$T_0^* = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\mathrm{SEM}_1^2 + \mathrm{SEM}_2^2}} \tag{2.4}$$

$T_0^* \equiv$ test statistic

$\bar{X}_1 \equiv$ sample mean of first sample

$\bar{X}_2 \equiv$ sample mean of second sample

$\mathrm{SEM}_1 \equiv$ standard error of the first sample mean

$\mathrm{SEM}_2 \equiv$ standard error of the second sample mean

The degrees of freedom were calculated using equation (2.5) and rearranged to equation (2.6).

$$v = \frac{\left(\frac{\mathrm{s}_1^2}{n_1} + \frac{\mathrm{s}_2^2}{n_2}\right)^2}{\frac{\left(\frac{\mathrm{s}_1^2}{n_1}\right)^2}{n_1 - 1} + \frac{\left(\frac{\mathrm{s}_2^2}{n_2}\right)^2}{n_2 - 1}} \tag{2.5}$$

$$v = \frac{(\mathrm{SEM}_1^2 + \mathrm{SEM}_2^2)^2}{\frac{\mathrm{SEM}_1^4}{n_1 - 1} + \frac{\mathrm{SEM}_2^4}{n_2 - 1}} \tag{2.6}$$

Using the Student's *t* distribution with *v* degrees of freedom, two-tailed p-values that were less than 0.05 were considered to have significantly different population means.

2.2.4 Membrane Integrity Measurement by Fluorescence Microscopy

HUVECs were treated with green-fluorescent SYTO 13 (Molecular Probes, Eugene, OR, USA) and red-fluorescent ethidium bromide (Sigma-Aldrich, Mississauga, ON, Canada) that are permeable and non-permeable nucleic acid dyes, respectively. The nuclei of membrane-intact cells fluoresce green after SYTO 13 diffuses through cell membranes and binds to nucleic acids and is not quenched by ethidium bromide (EB) that cannot enter. Membrane-damaged cells fluoresce red after EB diffuses through damaged cell membranes and binds to nucleic acids. In preparing and adding SYTO 13 and EB (SYTOEB) to HUVEC suspensions, SYTOEB was first diluted in 1X Dulbecco's Phosphate Buffered Saline modified without calcium chloride and magnesium chloride (DPBS) (Sigma-Aldrich). Table 2-1 shows the volume-composition of reagents used to prepare the SYTOEB solution prior to addition to HUVECs. In staining HUVECs, 40 µL of SYTOEB was added to a 400 µL HUVEC suspension. The final concentration in the cell suspension was 11.4 µM of SYTO 13 and 22.7 µM of EB. In culture tubes, the cells were incubated with SYTOEB at room temperature for 2 minutes in the dark. Treated cells were transferred to a hemocytometer, 10 µL per well, and were illuminated in the 440 to 480 nm visible light range by a UV mercury lamp through a 3-λ PLOEMOPAK fluorescence illuminator (Leitz). The 10X NPL FLUOTAR objective lens was used to magnify SYTOEB-stained cells.

Table 2-1. SYTOEB composition for fluorescence microscopy

Reagents	Volume, µL
SYTO 13, 5 mM in DMSO	20
EB, 2.6 mM in 1X DPBS	80
DPBS, 1X	700

To capture images, a Leitz Dialux 22 microscope (Leitz, Wetzlar, Germany) was used with an attached Infinity3 camera and Infinity Capture software (Lumenera Corporation, Ottawa, ON, Canada). Fluorescence images and brightfield images were captured at the centre of the hemocytometer wells and four more fluorescence images per well were captured outside the centre.

The Viability3 program (version 3.2, Great Canadian Computer Company, Spruce Grove, AB, Canada) facilitated the measurement of membrane integrity by automatically marking green and red cells for all captured images. The Viability3 program was used at default settings, as shown in Table 2-2. For each sample, 10 images were captured and membrane integrity was calculated as a percentage of green from coloured cells as shown in equation (2.7).

$$\%\mathrm{MI} = \frac{\text{Number of green cells}}{\text{Number of green cells} + \text{Number of red cells}} \times 100\% \qquad (2.7)$$

%MI ≡ percent membrane integrity

Table 2-2. Default settings for Viability3 counting program

Parameter (range)	Value
Upper threshold (0-255)	255
Lower threshold (0-255)	100
Cumulative threshold (0-99%)	85

To measure membrane integrity by fluorescence microscopy, the image capture settings are shown in Table 2-3. These settings were used for all fluorescence microscopy interrupted cooling experiments.

Table 2-3. Image capture settings optimized for fluorescence microscopy

Parameter	Value
Image Capture Resolution	1024 x 768
Exposure (ms)	33
Gain	1.65
Light Source Type	Fluorescent
Light Source Frequency (Hz)	60
Contrast	10
Brightness	0
Gamma	1.00
Hue	0
Saturation	0
Red	1.19
Green	1.00
Blue	2.50

2.2.5 Membrane Integrity Measurement by Flow Cytometry

An Epics XL-MCL flow cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser was used for flow cytometry. Forward light scatter (FS) and side light scatter (SS) sensors detected laser light scatter and the fluorescent light (FL) sensors detected light in the 200 nm to 800 nm spectral range. Laser light scatter was filtered for the FS and SS sensors by means of a 488-nm dichroic filter and laser light scatter was blocked from the FL sensors by a 488-nm laser-blocking filter. Green fluorescence emission was separated from other light using a 505-nm to 545-nm dichroic filter for the FL1 sensor. Red fluorescence emission was separated from other light using a 605-nm to 635-nm dichroic filter for the FL3 sensor.

2.2.5.1 Optimization Strategy to Quantify Membrane Integrity

Counting membrane-intact and membrane-damaged HUVECs for membrane integrity measurements was performed using red and green fluorescence measurements. The optimal separation of membrane-intact and membrane-damaged populations is illustrated schematically in Figure 2-1 showing the location of the: *i)* background light scatter (Bkgd); *ii)* the membrane-intact cell population (SYTO); and *iii)* the membrane-damaged cell population (EB). EB fluorescence detection was plotted on the y-axis and SYTO 13 fluorescence detection was plotted on the x-axis. The dashed lines represent the first decade where background light scatter was isolated. To isolate background light scatter to the first decade, HUVECs were measured for fluorescence in the absence of fluorochromes. The total gain was initially set very low to view all background light scatter and then the gain was increased until the background light scatter was isolated to the first decade. Two histograms were required to define the quadrants: *i)* the histogram of the green fluorescence from Figure 2-2a that was used to measure the separation of high green fluorescence from background green fluorescence; and *ii)* the histogram of the red fluorescence from Figure 2-2b that was used to measure the separation of high red fluorescence from background red fluorescence. The high green fluorescence was identified from the minimum between the peaks in Figure 2-2a and used to define the vertical line dividing the quadrants in Figure 2-1. The high red fluorescence was identified from the minimum between the peaks in Figure 2-2b and used to define the horizontal line dividing the quadrants in Figure 2-1. Events that had both high green and high red fluorescence could be counted in the Dbl quadrant and were assumed to be membrane-damaged. The red and green fluorescence measured from each cell provides a method to define the quadrants to measure membrane integrity.

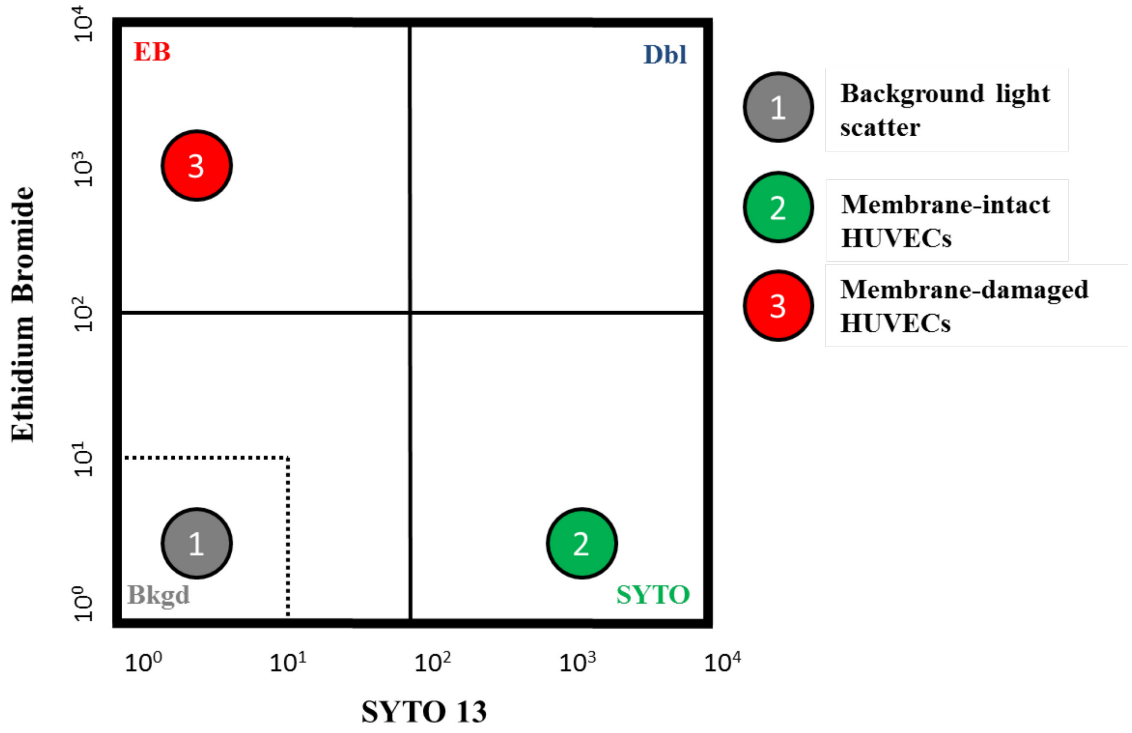


Figure 2-1. Schematic of EB and SYTO 13 fluorescence detection by flow cytometry

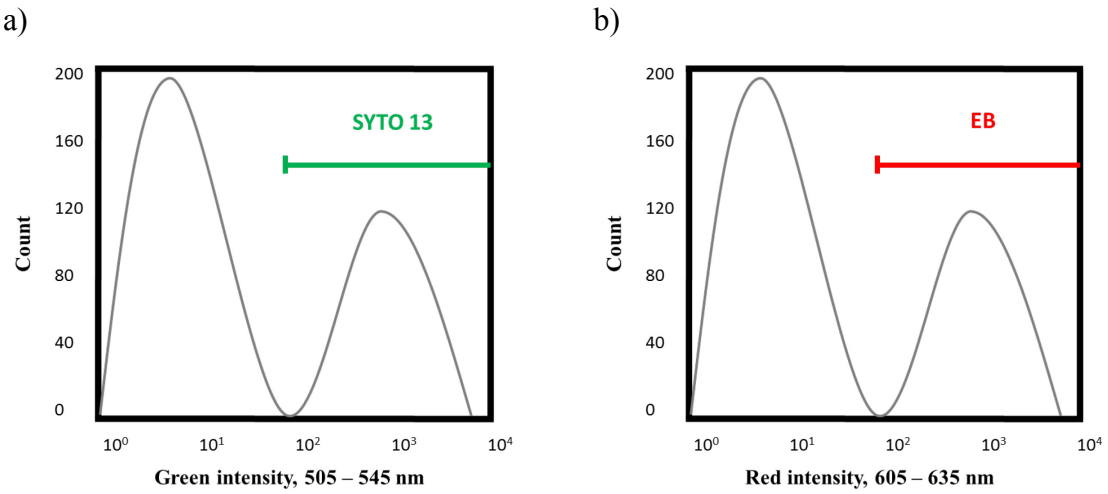


Figure 2-2. Schematic histogram of: a) SYTO 13 and b) EB detection by flow cytometry

2.2.5.2 Fluorescence Colour Compensation

SYTO 13 has a range of fluorescence emission wavelengths, some of which can be detected by the red sensor. Therefore, fluorescence colour compensation was necessary. Applying fluorescence colour compensation to SYTO 13 resulted in only the green fluorescence emissions being displayed; the red fluorescence emissions were reduced mathematically (compensated) after the fluorescence measurements were acquired. Figure 2-3 shows red and green fluorescence detected before compensation, where SYTO 13 fluorescence emissions were detected by the green and red sensor and displayed in the Dbl quadrant. Fluorescence colour compensation was adjusted using equation (2.9) until the geometric mean of the high green fluorescence population was the same as the background light scatter with respect to the sensor for red detection. This allows the best separation of membrane-intact HUVECs, membrane-damaged HUVECs and background light scatter to quantify membrane integrity.

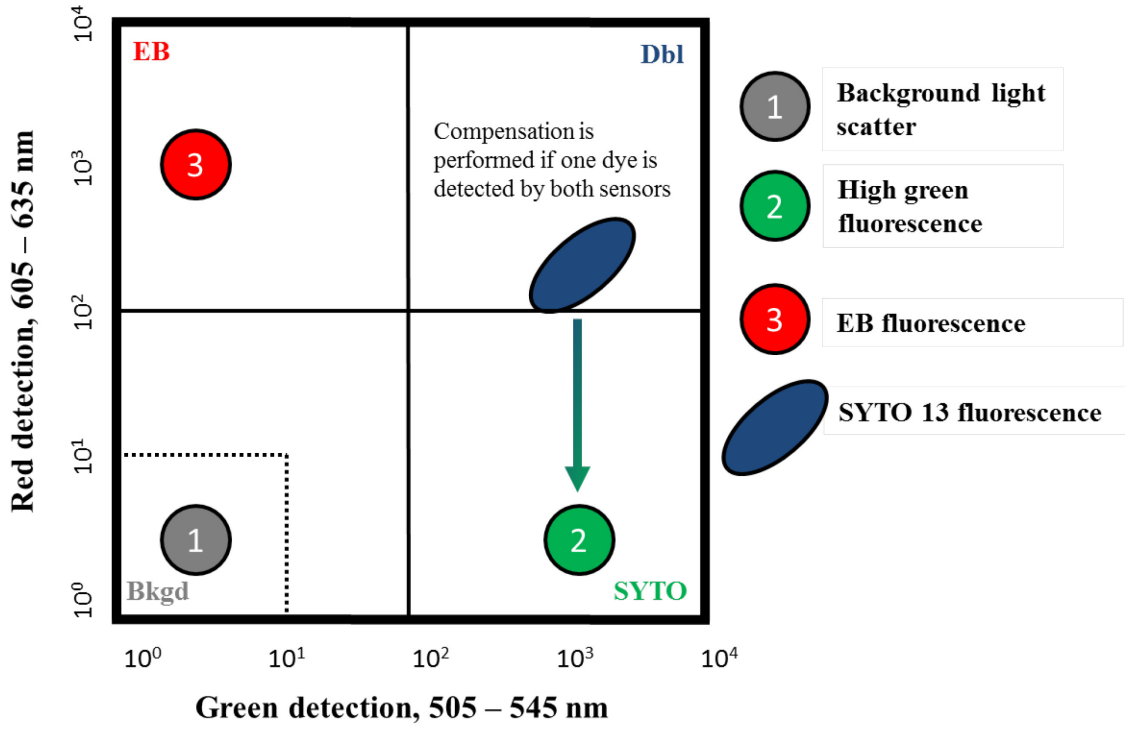


Figure 2-3. Schematic of fluorescence colour compensation for SYTO 13

2.2.5.3 Interaction of SYTO 13 and EB

To situate membrane-intact HUVECs at the third decade, the SYTO 13 fluorescence conditions were examined. Background light scatter was isolated to the first decade using HUVECs in the absence of fluorochromes. The concentration of SYTO 13 was varied; however background light scatter increased in intensity with increasing SYTO 13. The total gain was decreased with increasing concentrations of SYTO 13 to readjust the fluorescence measurements so that the background light scatter remained within the first decade. For optimization, the background light scatter must be localized within the first quadrant; however, the separation between background light scatter and membrane-intact HUVECs did not improve. Therefore, incubation time of HUVECs with SYTO 13 was varied between 2 minutes and 10 minutes. The 10 minute incubation time resulted in high green fluorescence separated from the background light scatter. However, the membrane-damaged HUVECs showed high green and high red fluorescence which required consideration to reduce the high green fluorescence. EB did not require compensation; therefore, it was hypothesized that there may be a competition between SYTO 13 and EB for nucleic acid binding sites. SYTO 13 and EB bind to DNA at similar sites by intercalation [45], [134]. After adding more EB, the green fluorescence decreased in the membrane-damaged HUVECs. Therefore, the concentration of EB was increased until the geometric mean of the membrane-damaged HUVECs was the same as the geometric mean of the background light scatter with respect to the green sensor. The interaction of SYTO 13 and EB with nucleic acids required that a higher concentration of EB and a longer incubation time be used to obtain optimal separation of membrane-intact HUVECs, membrane-damaged HUVECs and background light scatter.

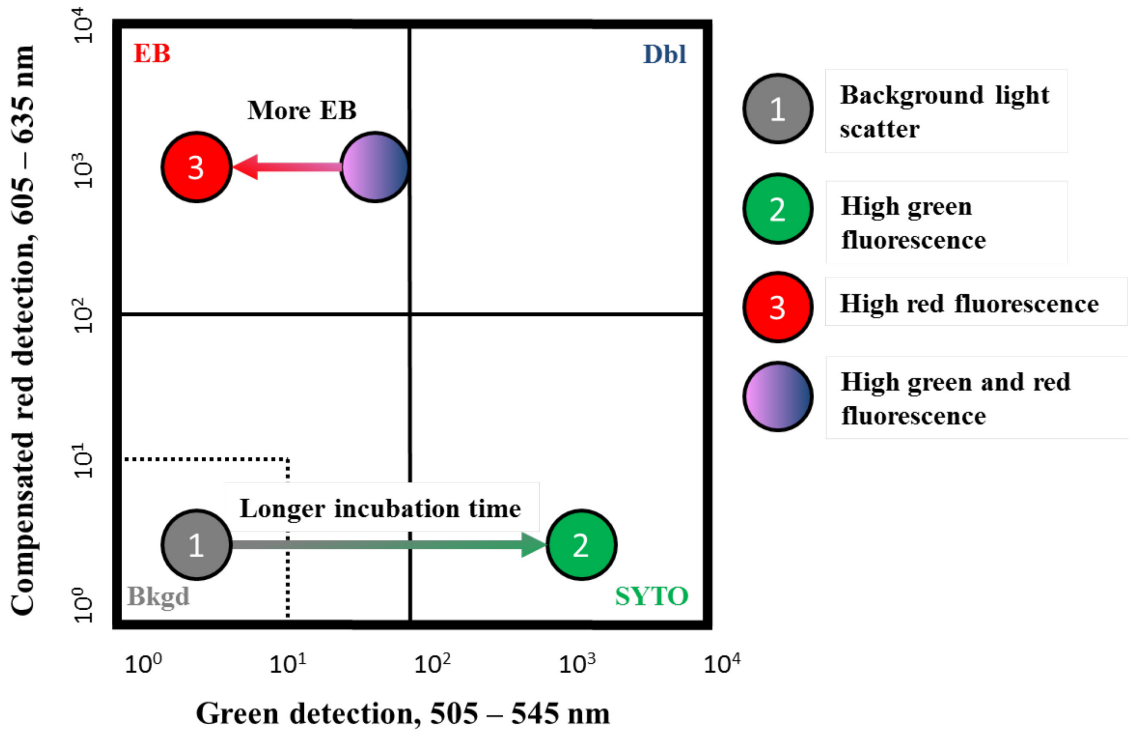


Figure 2-4. Schematic of difficulties for flow cytometry membrane integrity assessment

2.2.5.4 Optimized Flow Cytometer Settings

The optimized flow cytometer settings for membrane integrity measurements are shown in Table 2-4. The fluorescence colour compensation was adjusted to 32.0%, except in the presence of 20% DMSO where it was set to 29.0%. The total gain can be calculated based on equation (2.8) and the compensation can be applied using equation (2.9).

$$\text{Total Gain} = \text{Gain} \times [1 + (0.003 \times \text{voltage})] \tag{2.8}$$

$$\text{FL3 sensor display} = \text{FL3} - c \times \text{FL1} \tag{2.9}$$

Table 2-4. Optimized flow cytometer settings for SYTOEB membrane integrity measurements

Sensor	Voltage	Linear amplification (gain)
FS	150	2
SS	550	10
FL1	300	7.5
FL3	315	7.5

2.2.5.5 Membrane Integrity Assay

To prepare SYTOEB, Table 2-5 shows the required volumes of SYTO 13, EB and water. The total volume of SYTOEB was 524 µL, of which 20 µL was added to 400 µL of HUVECs before incubating in the dark for 10 minutes at room temperature. The final concentration of SYTOEB was 11.4 µM SYTO 13 and 92.2 µM EB in the HUVEC suspension.

Table 2-5. SYTOEB solution for flow cytometry membrane integrity assessment

Reagents	Volume, µL
SYTO 13, 5 mM in DMSO	25
EB, 26 mM in water	40
Water	459

2.2.5.6 Optimized Flow Cytometry with SYTOEB

A mixture of membrane-intact and membrane-damaged HUVECs incubated with SYTOEB was measured by flow cytometry to show the separation of: membrane-intact HUVECs, membrane-damaged HUVECs, and background light scatter. Membrane integrity was measured using the optimized flow cytometry methods described previously in the sections "Optimized Flow Cytometer Settings" and "Membrane Integrity Assay". Figure 2-5 shows the separation of membrane-intact HUVECs, membrane-damaged HUVECs and background light scatter. Membrane-intact HUVECs were prepared from fresh HUVECs and membrane-damaged

HUVECs were prepared from thawing HUVECs previously plunged without controlled cooling into liquid nitrogen. The gridlines used to separate Figure 2-5c into quadrants were defined using histograms shown in Figure 2-5a and Figure 2-5b. The grid centre was determined from: *i)* the minimum between the background and High green domain in Figure 2-5a, and *ii)* the minimum between the background and High red domain in Figure 2-5b. Fluorescence measurements in the SYTO quadrant were coloured green (membrane-intact cells), fluorescence measurements in the EB quadrant were coloured red (membrane-damaged cells) and fluorescence measurements in the Dbl quadrant were coloured blue (included in membrane-damaged cell counts). The colour of fluorescence measurements from Figure 2-5c was conserved for Figure 2-5d, showing that membrane-intact cells have a high forward scatter and membrane-damaged cells have a low forward scatter as has been previously observed [146]. This optimized sensor display shows a good separation between membrane-intact HUVECs, membrane-damaged HUVECs and background debris. To calculate membrane integrity, equation (2.10) was used, where membrane-intact cells were counted from the SYTO quadrant and membrane-damaged cells were counted from the Dbl and EB quadrants.

$$\%\mathrm{MI} = \frac{\text{Syto events}}{\text{Syto events} + \text{ Dbl events} + \text{EB events}} \times 100\% \qquad (2.10)$$

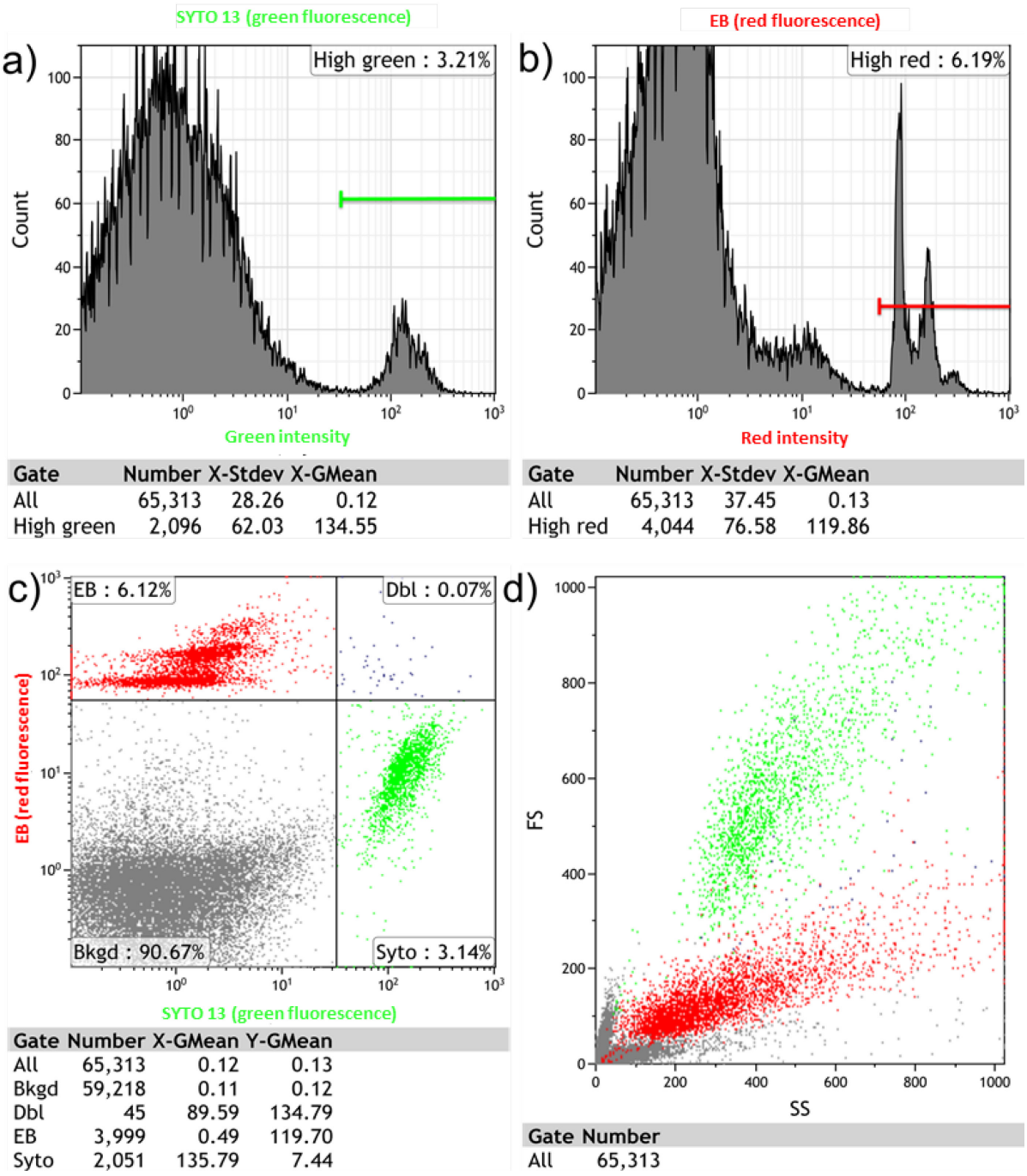


Figure 2-5. Flow cytometry measurement of membrane integrity using SYTOEB: a) histogram for high green detection; b) histogram for high red detection; c) fluorescence measurements after compensation; d) FS and SS of events, coloured using fluorescence quadrants.

2.2.5.7 Propidium Iodide as an Alternative to EB

The hazards inherent to using EB made it prudent to consider alternative stains. According to the material safety data sheet, EB has acute toxicity through inhalation (Category 3) and germ cell mutagenicity (Category 2) based on the Globally Harmonized System of Classification and Labelling of Chemicals. Propidium iodide (PI) in combination with SYTO 13 (SYTOPI) is a commonly used method to measure membrane integrity [194]. PI has fewer health and environmental effects, although it is still hazardous. It has a health hazard of 1 based on the Hazardous Materials Identification System and has specific mutagenic effects based on animal experiments that showed mutagenic and teratogenic effects.

To determine the composition of SYTOPI to measure membrane integrity, the concentration of PI was varied while maintaining the same addition and incubation conditions as the SYTOEB membrane integrity assessment by flow cytometry. This made it easier to determine the appropriate concentration of PI, as SYTO 13 fluorescence intensity would remain unchanged. Initially, low concentrations of PI were tested, however membrane-damaged HUVECs showed green fluorescence. Therefore, the concentration of PI was increased until the geometric mean of the membrane-damaged HUVECs was the same as the geometric mean of the background with respect to the green fluorescence sensor. Table 2-6 shows the required volumes of SYTOPI to measure membrane integrity using flow cytometry. The total volume of SYTOPI was 524 µL, of which 20 µL was added to 400 µL of HUVEC suspension before incubating in the dark for 10 minutes at room temperature. The final concentration of SYTOPI in the HUVEC suspension was 11.4 µM SYTO 13 and 67.8 µM PI.

Table 2-6. SYTOPI solution for flow cytometry membrane integrity assessment

Reagents	Volume, µL
SYTO 13, 5 mM in DMSO	25
PI, 1.5 mM in water	499

A mixture of membrane-intact and membrane-damaged HUVECs incubated with SYTOPI was measured by flow cytometry to show the separation of membrane-intact HUVECs, membrane-damaged HUVECs, and background debris. Membrane integrity was measured using

the optimized flow cytometry methods described previously in the sections “Optimized Flow Cytometer Settings” and “Membrane Integrity Assay”. Figure 2-6 shows the separation of membrane-intact HUVECs, membrane-damaged HUVECs and background debris. Membrane-intact HUVECs were prepared from fresh HUVECs and membrane-damaged HUVECs were prepared from thawing HUVECs previously plunged without controlled cooling into liquid nitrogen. The gridlines used to separate Figure 2-6c into quadrants were defined using histograms shown in Figure 2-6a and Figure 2-6b. The grid centre was determined from: *i)* the minimum between the background and High green domain in Figure 2-6a, and *ii)* the minimum between the background and High red domain in Figure 2-6b. Events detected in the SYTO quadrant were coloured green (membrane-intact cells), events detected in the PI quadrant were coloured red (membrane-damaged cells) and events detected in the Dbl quadrant were coloured blue (included in membrane-damaged cell counts). The colour of each fluorescence measurement from Figure 2-6c was conserved for Figure 2-6d, showing that membrane-intact cells have a high forward scatter and membrane-damaged cells have a low forward scatter. This optimized sensor display shows a good separation between membrane-intact HUVECs, membrane-damaged HUVECs and background debris. To calculate membrane integrity, equation (2.10) was used, where membrane-intact cells were counted from the SYTO quadrant and membrane-damaged cells were counted from the Dbl and PI quadrants.

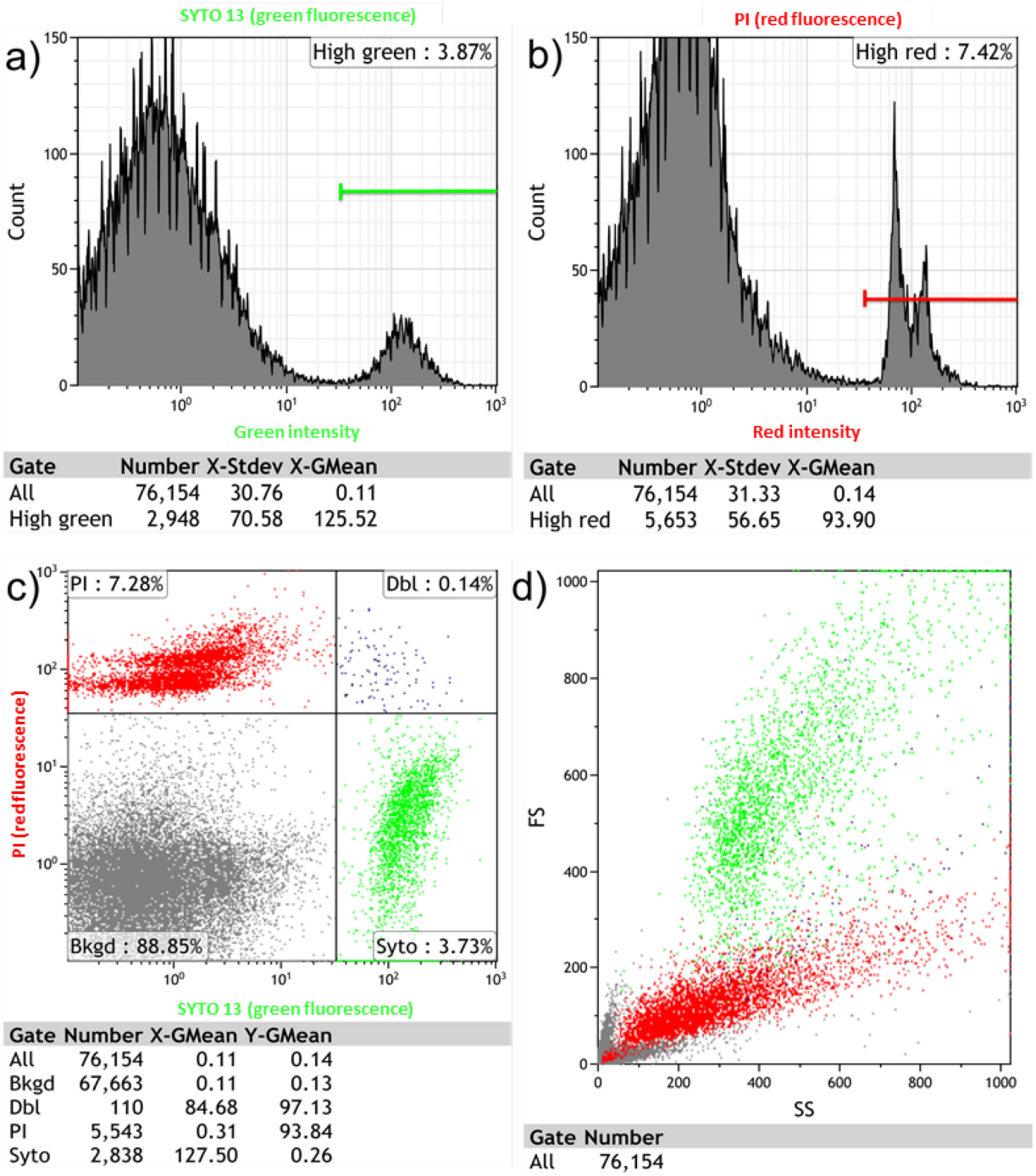


Figure 2-6. Flow cytometry measurement of membrane integrity using SYTOPI: a) histogram for high green detection; b) histogram for high red detection; c) fluorescence measurements after compensation; d) FS and SS of events, coloured using fluorescence quadrants.

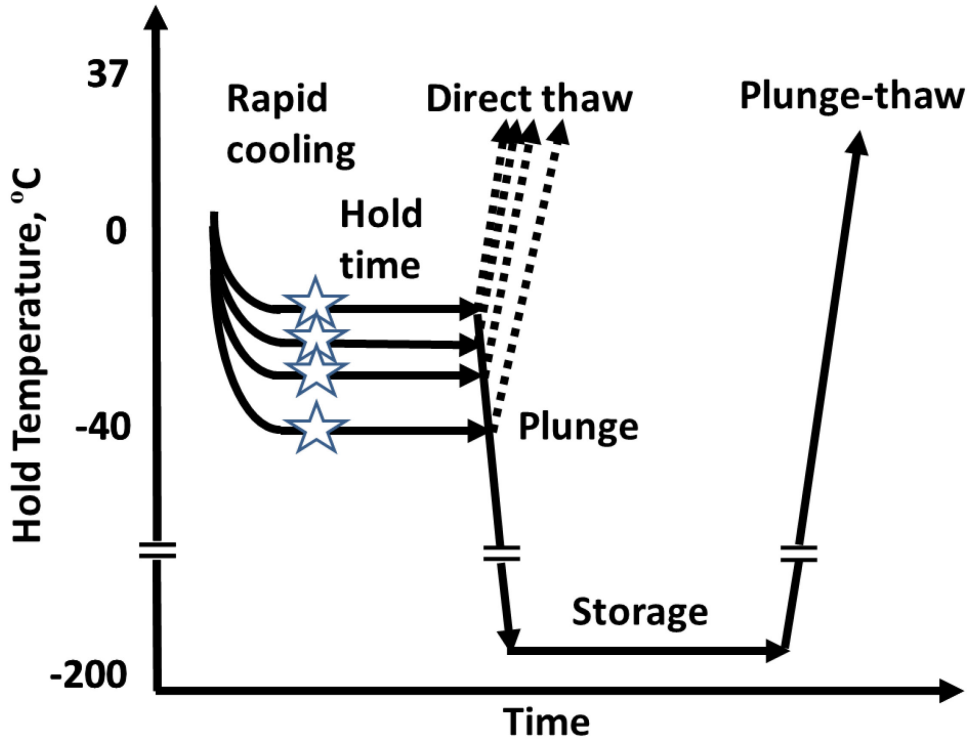
2.2.5.8 Summary

An optimization method was developed to improve the separation of membrane-intact HUVECs from background events. However, the interaction between SYTO 13, EB and nucleic acids made optimization more complicated. To define an optimization endpoint, separation of membrane-intact HUVECs, membrane-damaged HUVECs and background events was considered optimized when quadrants could be defined from green and red fluorescence measurements. There were several key variables that required optimization: *i)* flow cytometry total gain; *ii)* flow cytometry compensation; *iii)* SYTOEB incubation time; and *iv)* SYTOEB concentration. Learning the flow cytometer operation was complicated; however, developing an optimization strategy resulted in excellent separation of membrane-intact and membrane-damaged HUVECs to measure membrane integrity. The optimization strategy developed for SYTOEB facilitated optimization of SYTOPI to measure membrane integrity. The quadrants allowed some flexibility in interpreting membrane integrity, therefore a strict method was chosen for counting membrane-intact HUVECs.

2.2.6 Two-Step Freezing

Two-step freezing was used to investigate the effects of rapid cooling on HUVECs. Figure 2-7 illustrates schematically the details of two-step freezing. Two-step freezing involved: *i)* rapid cooling to intermediate sub-zero temperatures (hold temperatures), *ii)* induced ice formation, *iii)* hold times at intermediate sub-zero temperatures *iv)* rapid plunge into liquid nitrogen, *v)* storage in liquid nitrogen and *vi)* rapid thawing. Steps *i)*, *ii)*, *iii)* and *vi)* were performed for direct thaw and all steps were performed for plunge-thaw. HUVEC suspensions were kept on ice for 2 to 4 hours before rapid cooling. Culture tubes were rapidly cooled to an intermediate sub-zero hold temperature, which was between −3 °C to −40 °C in the absence of cryoprotectants. The intermediate sub-zero hold temperature range was −5 °C to −40 °C when using 10% DMSO (Fisher Scientific, Edmonton, Canada) and −10 °C to −45 °C when using 10% DMSO and HES (hydroxyethyl starch from Bristol-Myers Squibb, Dublin, Ireland) or when using 20% DMSO. After a two minute thermal equilibration time, ice nucleation was induced using liquid nitrogen-cooled forceps and a three minute hold time was allowed for cell dehydration. HUVEC

suspensions were either thawed directly from intermediate sub-zero hold temperatures (direct thaw) or plunged from an intermediate sub-zero hold temperature into liquid nitrogen and then thawed (plunge-thaw). Storage was for at least an hour in liquid nitrogen. All thawing steps were performed using a 37 °C water bath until the last sliver of ice had melted. After thawing, cells were left at room temperature for immediate membrane integrity assessment. A detailed procedure is available in the Appendix in the section "Interrupted Cooling Protocols".



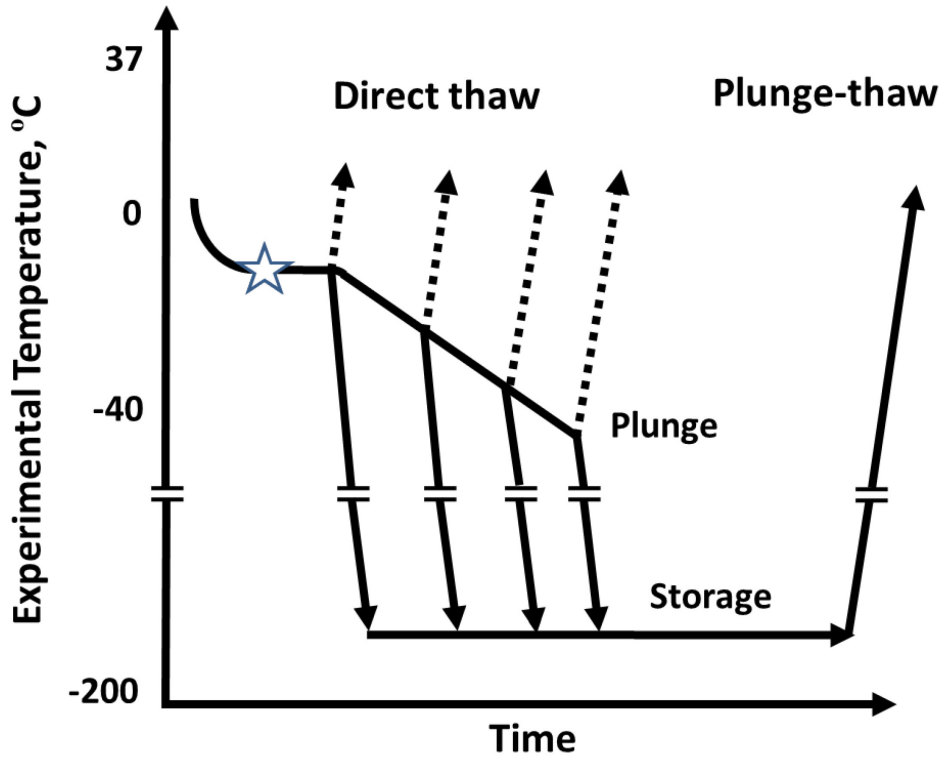
☆Ice nucleation was induced using liquid nitrogen-cooled forceps

Figure 2-7. Schematic details of two-step freezing

2.2.7 Graded Freezing

Graded freezing was used to investigate the effects of slow cooling on HUVECs. Figure 2-8 illustrates schematically the details of graded freezing. Graded freezing involved: *i)* induced ice formation, *ii)* controlled slow cooling to intermediate sub-zero temperatures (experimental temperatures), *iii)* rapid plunge into liquid nitrogen, *iv)* storage in liquid nitrogen and *v)* rapid thawing. Steps *i)*, *ii)* and *v)* were performed for direct thaw and all steps were performed for

plunge-thaw. HUVEC suspensions were kept on ice for 2 to 4 hours before cooling. Culture tubes were cooled from 0 °C to the first experimental temperature. The experimental temperature range was −3 °C to −40 °C in the absence of cryoprotectant, −5 °C to −40 °C when using 10% DMSO and −10 °C to −45 °C when using 10% DMSO and HES or when using 20% DMSO. After a two minute thermal equilibration time at the first experimental temperature, ice nucleation was induced using liquid nitrogen-cooled forceps and three minutes was allowed for latent heat removal and cell dehydration prior to beginning the slow cooling. HUVEC suspensions were either thawed directly from experimental temperatures (direct thaw) or plunged from experimental temperatures into liquid nitrogen and then thawed (plunge-thaw). Storage was for at least one hour in liquid nitrogen. All thawing steps were performed using a 37 °C water bath until the last crystal of ice had melted. After thawing, cells were left at room temperature for immediate membrane integrity assessment. A detailed procedure is available in the Appendix in the section “Interrupted Cooling Protocols”.



☆Ice nucleation was induced using liquid nitrogen-cooled forceps

Figure 2-8. Schematic details of graded freezing

2.3 Results and Discussion

2.3.1 HUVEC Culturing, Preparation and Viability as Received

HUVECs from the supplier were measured for viability using the SYTOEB or SYTOPI membrane integrity assessment methods for flow cytometry. Thawing of HUVECs from the supplier was performed and membrane integrities were measured. Table 2-7 shows the membrane integrity assessment results, the supplier lot number and the membrane integrity of HUVECs after diluting 1 mL of HUVECs in 5 mL of EGM without DMSO removal and without centrifugation. Table 2-8 shows the membrane integrity assessment results, the supplier lot number and the membrane integrity of HUVECs immediately after thawing, still in the presence of 10% DMSO. The membrane integrity of HUVECs in the presence of DMSO without dilution was 64.8% ± 2.2% (n = 6) and the membrane integrity of HUVECs after DMSO dilution was 49.6% ± 1.5% (n = 6). The difference in membrane integrity may be the result of diluting the cryovial contents with EGM.

Table 2-7. Membrane integrity of supplied HUVEC after dilution

Assay	%MI	Lot number
SYTOEB	52.5	0000311953
SYTOEB	54.8	0000311953
SYTOEB	46.7	0000339730
SYTOEB	51.1	0000339730
SYTOEB	47.4	0000342222
SYTOPI	45.3	0000342222

Table 2-8. Membrane integrity of supplied HUVEC without dilution

Assay	%MI	Lot number
SYTOPI	58.2	0000342222
SYTOPI	67.2	0000342222
SYTOPI	67.4	0000342222
SYTOPI	57.9	0000394986
SYTOPI	68.1	0000394986
SYTOPI	69.8	0000394986

There were difficulties to thaw HUVECs to supplier specifications. Transferring HUVEC cryovials from the liquid nitrogen storage tank and subsequent thawing was time-sensitive. The instructions recommended that thawing must be completed within 2 minutes, otherwise cell recovery could decrease. Thawing was done in a 37 °C water bath until the last sliver of ice had melted. The use of Cryo-Gloves (Tempshield Cryo-Protection, Burlington, ON, Canada) in handling ultra-cold cryovials was required to protect people from frostbite; however this introduced some complications. The cold temperature and the affixed label prevented view of the frozen contents making it difficult to thaw HUVEC cryovials within 2 minutes. The Cryo-Gloves were removed so the label could be removed. The difficulty in removing the label from the frozen vials resulted in a lower warming rate.

Of particular consideration was the procedure to remove 10% DMSO. HUVECs from the supplier were delivered as a frozen cell suspension in 10% DMSO, but as described by the supplier and by Yang *et al.* [198], cryoprotectant removal by centrifugation could introduce mechanical stress and damage the fragile cells. Instead, the supplier states that HUVECs should be diluted in 5 mL of EGM, seeded in a T-flask, and then the EGM should be removed after incubation for 24 hours.

To demonstrate HUVEC culturing technique, phase contrast images were taken of HUVECs after growth in the absence of antibiotics. All images from Figure 2-9 were taken using an EF 10X objective lens. Figure 2-9a shows HUVECs in their attached, growing state in a contaminated T-flask; Figure 2-9b shows pure HUVECs in their attached, growing state in a T-flask; Figure 2-9c shows pure HUVECs on a hemocytometer after keeping cells on ice as a cell suspension; and Figure 2-9d shows pure HUVECs as seen in their attached state approaching

100% surface coverage. The contamination, as shown in Figure 2-9a, was only observed from one HUVEC culture. The contamination was the result of poor asceptic technique which could have been from the pipette coming in contact with the outside of the flask before media replacement. Since there were no antibiotics present in the growth medium, contamination was easily identified using the phase contrast microscope. After contamination was identified, the decontamination procedure was effective at keeping HUVEC cultures free from contamination. In Figure 2-9b, the uncontaminated HUVECs were attached in the T-flask, where dark circles from Weibel-Palade bodies were visible within the cells and the brighter cells were HUVECs undergoing mitosis. In Figure 2-9c, HUVECs were small and round as a cell suspension after being kept on wet ice at 0 °C for 2 – 4 hours and there were cell bodies visible as small, dark objects. In Figure 2-9d, there were spaces between the cells; however for interrupted cooling experiments, HUVECs were never grown beyond this stage. The phase contrast microscopy images of HUVECs in various states demonstrate the range of growth conditions from following the HUVEC culturing and preparation methods.

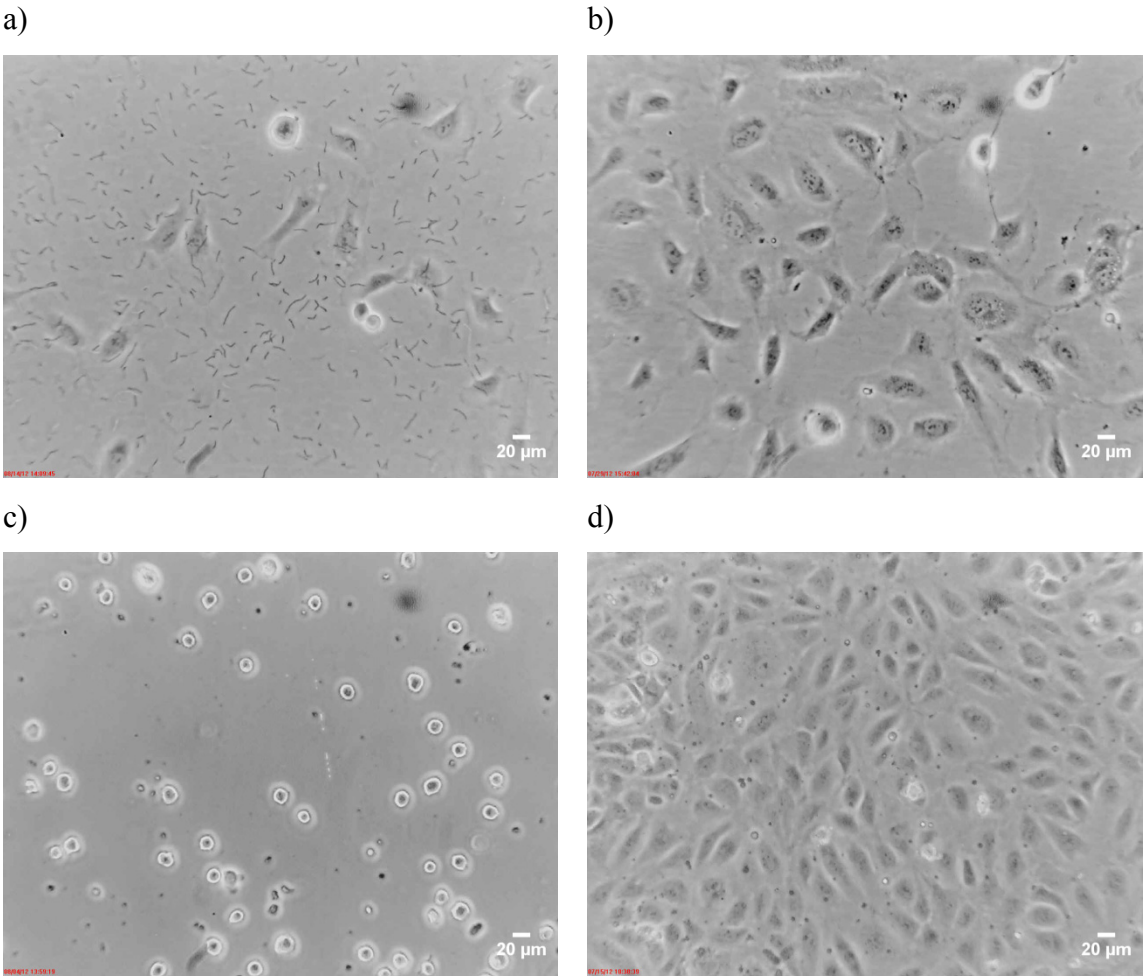


Figure 2-9. Phase contrast images of HUVECs either: a) attached in a contaminated T-flask; b) attached in a contaminant-free T-flask; c) on a hemocytometer as a cell suspension; and d) attached and approaching 100% culture flask surface coverage

2.3.1.1 Summary

Thawing supplier-provided HUVECs for optimal cell recovery was a challenge due to the cold temperature from storage of HUVECs in liquid nitrogen. The supplier label on the cryovial prevented viewing of the frozen contents and removing the label interrupted the thawing process. Supplier-provided HUVECs diluted using EGM had a lower membrane integrity than non-diluted HUVECs. This may require further investigation for DMSO removal strategies after cryopreservation. Although HUVEC culturing and preparation procedures were provided by the supplier, modifications were made. The supplier recommended the use of antibiotics and did not

specify maintenance of HUVEC suspensions; therefore two important changes made in this work were the avoidance of antibiotics in cell cultures and maintenance of HUVEC suspensions at 0 °C for 2 – 4 hours after subculture prior to experiments. Antibiotics were not used so that issues with aseptic techniques could be easily identified and low-level contamination could be avoided. HUVECs were held at 0 °C prior to experiments to reduce cell clumping and to maintain viability until the experiment setup was ready. As demonstrated later in the results and discussions for fluorescence microscopy, the modifications to supplier-provided instructions contributed to highly repeatable experiments and high membrane integrities. Simultaneous learning from fluorescence microscopy and HUVEC culturing and preparation methods provided excellent HUVEC source material for flow cytometry applications.

2.3.2 Membrane Integrity Measurement by Fluorescence Microscopy

2.3.2.1 HUVEC Controls

To demonstrate the impact of HUVEC culturing and preparation methods, fluorescence images are presented in Figure 2-10. Figure 2-10a shows membrane-intact HUVEC controls from fresh HUVEC suspensions and Figure 2-10b shows membrane-damaged HUVEC controls from HUVEC suspensions that were plunged directly into liquid nitrogen and thawed using a 37 °C water bath. HUVEC suspensions were grown for 2 days in the T-flask before attaining 60% coverage after their 11^{th} population doubling. HUVECs had been kept on ice for 2 – 4 hours prior to measuring membrane integrity. On six separate days, ten images per sample were captured each day and a membrane integrity of 92.7% ± 0.9% (n = 6) and 0.03% ± 0.02% (n = 6) was observed for the membrane-intact and membrane-damaged controls, respectively. The HUVEC culturing and preparation methods allowed the control of population doubling, culture flask coverage and handling temperature. As a result, the membrane integrity was quantified in a repeatable manner using the Viability3 cell counting program.

a) b)

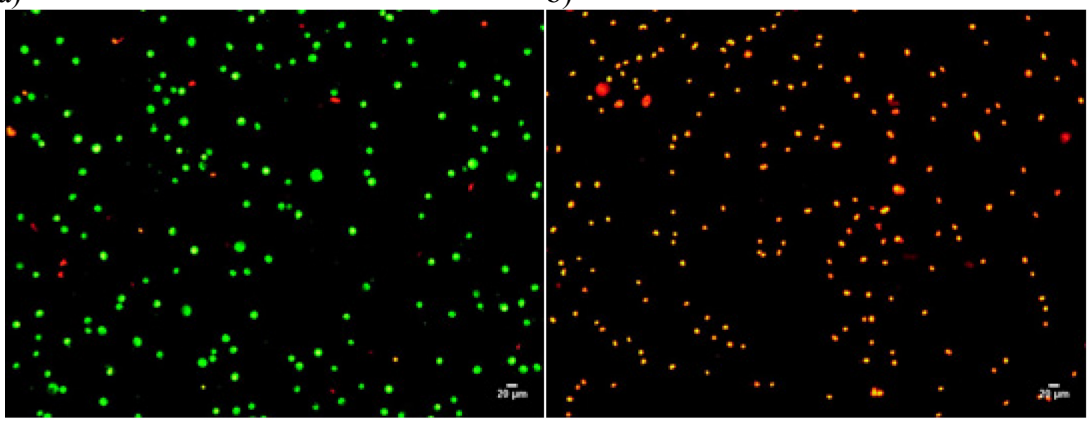


Figure 2-10. Fluorescence microscopy images of HUVEC: a) membrane-intact HUVEC controls; and b) membrane-damaged HUVEC controls

2.3.2.2 Optimization Strategy to Quantify Membrane Integrity

To optimize the exposure time, differences in cell counts were minimized between the Viability3 cell counting program and manual counts. It was required to use brightfield microscopy and high gain settings for fluorescence microscopy to identify the cells. Once cells were identified, exposure time and gain were adjusted for accurate marking of green and red cells. The optimal exposure time was chosen based on minimizing differences in percent membrane integrity between Viability3 program counts and manual counts. Equation (2.11) and equation (2.12) show how differences were calculated, where $\Delta\%\mathrm{MI}$ and $\Delta\mathrm{Count}$ were both desired to be as close to zero as possible.

$$\Delta\%\mathrm{MI} = |\text{manual }\%\mathrm{MI} - \text{Viability3 }\%\mathrm{MI}| \tag{2.11}$$

$$\Delta\mathrm{Count} = |\text{manual cell counts} - \text{Viability3 cell counts}| \tag{2.12}$$

2.3.2.3 Reproducibility of Membrane Integrity Measurements

Exposure time and gain settings for fluorescence microscopy were optimized and graded freezing experiments were repeated using a 0.2 °C/min cooling rate. Graded freezing experiments were performed in triplicate and each replicate is shown in Figure 2-10. Membrane integrity measurements after direct thaw are shown by dashed lines and membrane integrity measurements after plunge-thaw are shown by solid lines. The post-thaw times were controlled, in that culture tubes were left at room temperature immediately after thawing for immediate membrane integrity measurements. Table 2-3 in the previous section, "Membrane Integrity Measurement by Fluorescence Microscopy" describes the optimized fluorescence microscopy settings to measure membrane integrity. For the three runs, the maximum membrane integrity after plunge-thaw from −20 °C was 21.7%, 24.6% and 20.9%. The pattern of membrane integrity response was the same, showing that −20 °C was the optimal temperature for plunge-thaw. The membrane integrity measurements were repeatable after optimizing the microscope cameras exposure time and gain. The optimization of image capture settings using the Viability3 program as a reference and HUVEC culturing and preparation methods resulted in consistent membrane integrity results.

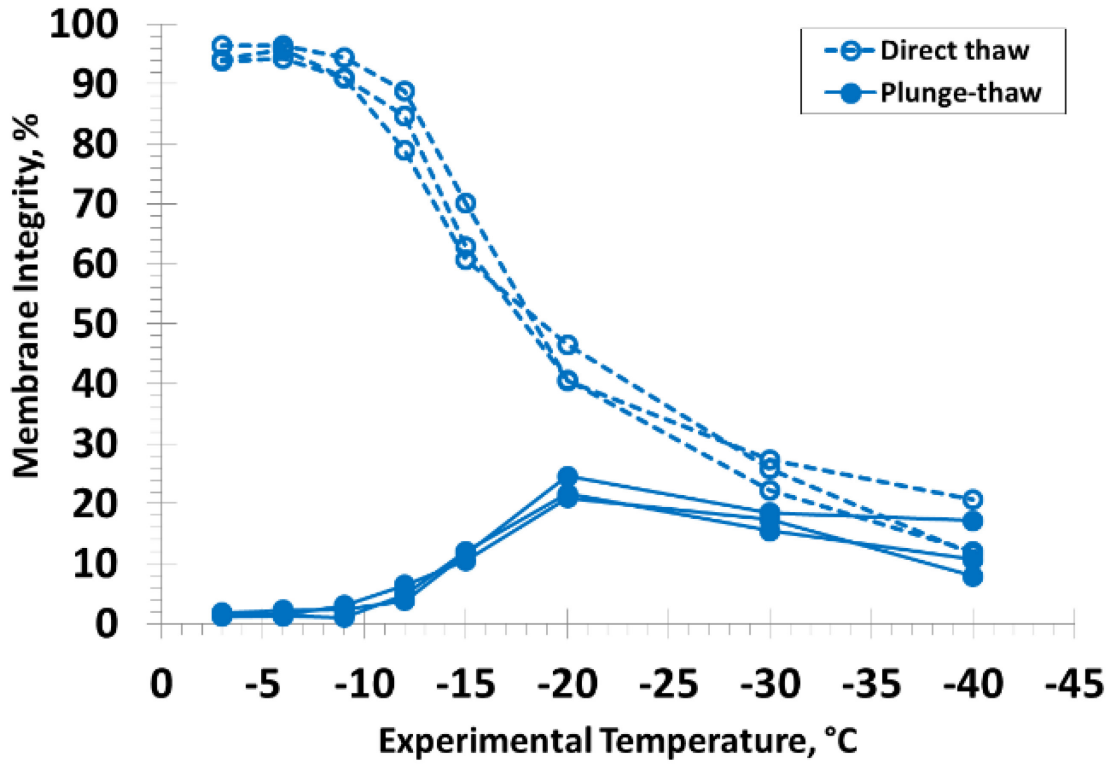


Figure 2-11. Graded freezing at a 0.2 °C/min cooling rate after optimizing key variables for experiment repeatability

2.3.2.4 Summary

The image capture area was reduced to maintain focus within the field of view, and exposure time and gain were optimized using the Viability3 cell counting program for reference. Also, it was discovered that to eliminate cell clumping and ensure high repeatability of membrane integrity measurements, four variables must be controlled: *i*) population doublings; *ii*) culture flask surface coverage; *iii*) cell handling temperature; and *iv)* thawing conditions.

2.3.3 Membrane Integrity Measurement by Flow Cytometry versus Fluorescence Microscopy

Flow cytometry and fluorescence microscopy were optimized in the previous chapter which required different SYTOEB incubation conditions with HUVECs. SYTOEB incubation conditions are longer and require more EB, making the incubation conditions potentially more damaging to HUVECs. Membrane integrity can be calculated in more than one manner, making it important to compare flow cytometry and fluorescence microscopy methods for measuring membrane integrity. To compare flow cytometry and fluorescence microscopy, the two-step freezing and graded freezing protocols were used with SYTOEB in the absence of cryoprotectants. As demonstrated previously by Ross-Rodriguez *et al.* using TF-1 cells, the damaging effects of supercooling were observed from two-step freezing [151] and a combination of supercooling effects and osmolality effects were observed from graded freezing [152]. Supercooling can result in membrane damage that can be measured immediately after thaw and osmolality effects can result in membrane damage that can manifest over time [113]. Therefore, measuring membrane integrity by flow cytometry and fluorescence microscopy could result in different interpretations of membrane integrity, where flow cytometry involves: *i)* a longer incubation time, *ii)* a higher concentration of ethidium bromide and, *iii)* a more stringent method for selecting membrane-intact cells. For fluorescence microscopy (FM), membrane integrity was calculated as previously described in equation (2.7). For flow cytometry, fluorescence events detected in the Dbl quadrant were interpreted as membrane-damaged cells that could have been interpreted as membrane-intact cells. Therefore, two methods of calculating membrane integrity were possible for flow cytometry, the one as described using equation (2.10) (FC) and the alternate method that counts events in the Dbl quadrant as membrane-intact cells (FC Dbl), as shown in equation (2.13).

$$\%\mathrm{MI} = \frac{\text{Syto events} + \text{Dbl events}}{\text{Syto events} + \text{Dbl events} + \text{EB events}} \times 100\% \qquad (2.13)$$

2.3.3.1 Two-Step Freezing

Two-step freezing was performed in the absence of cryoprotectant to compare fluorescence microscopy and flow cytometry methods of measuring membrane integrity. Membrane integrity can be calculated in two ways using flow cytometry; therefore Figure 2-12 shows three methods to measure membrane integrity: *i)* FC (represented by green circles); *ii)* FC Dbl (represented by blue diamonds); and *iii)* FM (represented by black triangles). Only at −20 °C was a significant difference observed, where flow cytometry measured lower membrane integrities than fluorescence microscopy. However at −20 °C, membrane integrities were less than 5%, making it difficult to conclude which method was more stringent. Comparing membrane integrities after plunge-thaw, the following differences were observed: *i)* at −20 °C, membrane integrities were lower for FC and FC Dbl than FM; *ii)* at −9 °C, membrane integrity was lower for FC than FM; and *iii)* at −15 °C, −20 °C and −40 °C, membrane integrity was lower for FC than FC Dbl. After plunge-thaw, membrane integrities were less than 2%, making it difficult to conclude whether any method was more stringent. Therefore, using two-step freezing, differences between methods were only observed when membrane integrity was very low, making it difficult to conclude whether any method was more stringent.

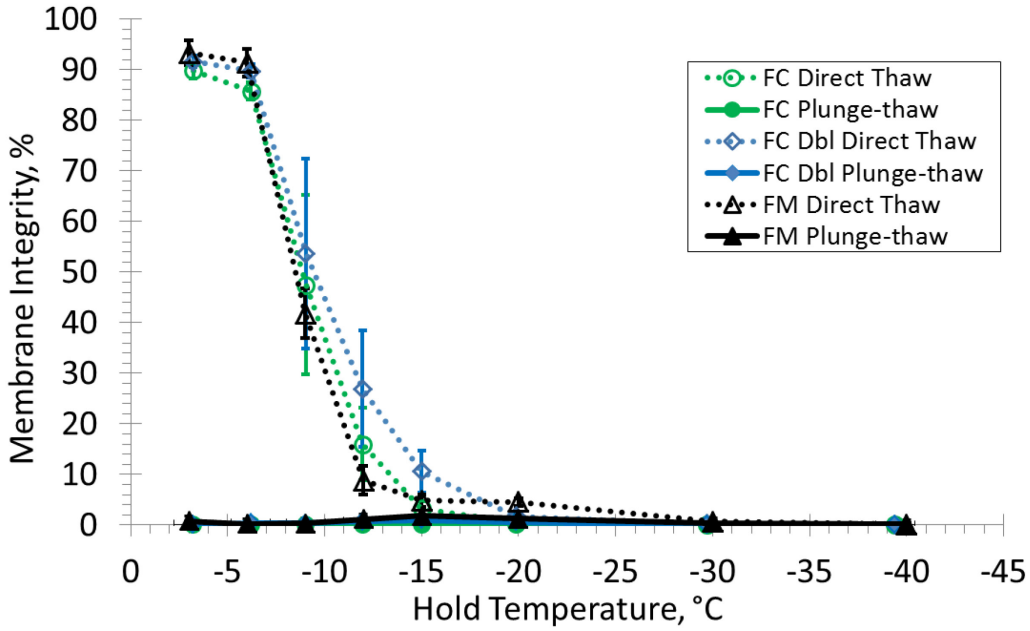


Figure 2-12. Flow cytometry and fluorescence microscopy comparison of two-step freezing in the absence of cryoprotectant

2.3.3.2 Graded Freezing

Graded freezing was performed in the absence of cryoprotectant to compare fluorescence microscopy and flow cytometry methods of measuring membrane integrity. Figure 2-13 shows three methods to measure membrane integrity: *i)* FC (represented by green circles); *ii)* FC Dbl represented by blue diamonds); and *iii)* FM (represented by black triangles). After direct thaw, all membrane integrities measured by flow cytometry were significantly lower than membrane integrities measured by fluorescence microscopy. Membrane integrities measured by FC Dbl were significantly lower than those measured by FM after direct thaw from −3 °C, −6 °C, −15 °C, −20 °C and −30 °C. Membrane integrities measured by FC were significantly lower than those measured by FC Dbl after direct thaw from −9 °C, −12 °C, −15 °C, −20 °C, −30 °C and −40 °C. After plunge-thaw, membrane integrities measured by FC and FC Dbl were significantly lower than those measured by FM after plunge-thaw from −3 °C, −6 °C, −12 °C, −15 °C, −20 °C and −30 °C. Membrane integrities measured by FC were significantly lower than those measured by FC Dbl after plunge-thaw from −6 °C, −20 °C, −30 °C and −40 °C. Therefore, graded freezing showed significant differences between methods after direct thaw and plunge-thaw. Measuring membrane integrity using FC as described in equation (2.10) was more stringent than the other methods.

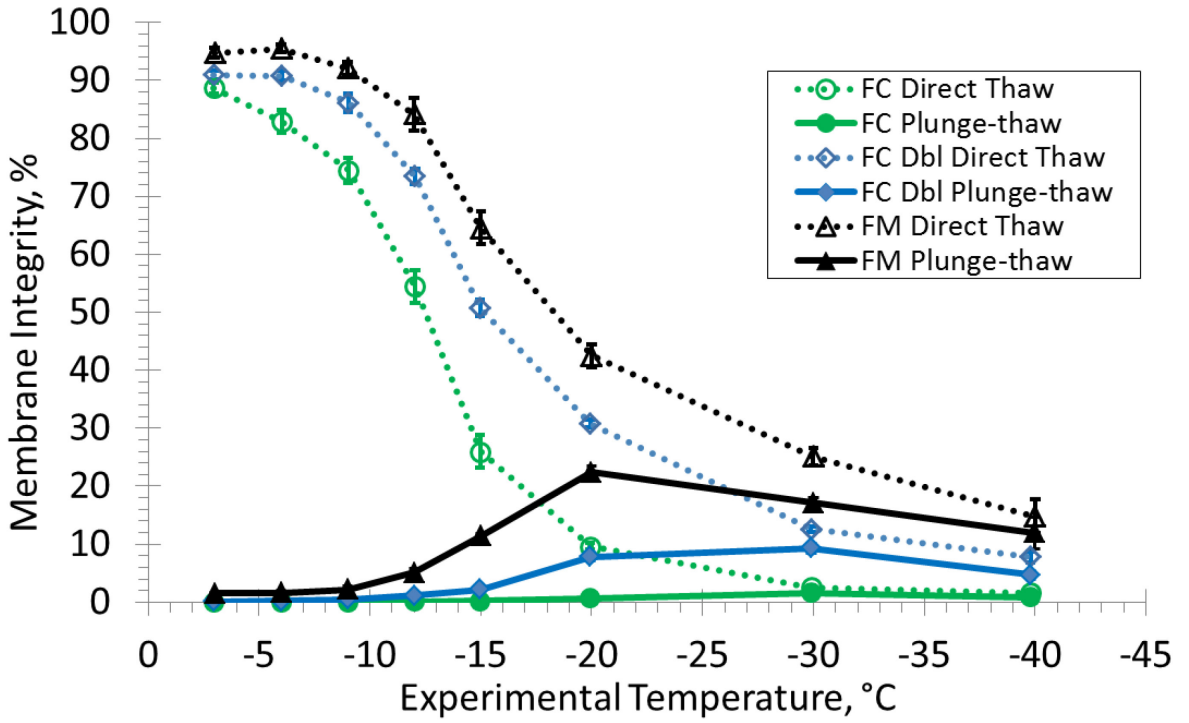


Figure 2-13. Flow cytometry and fluorescence microscopy comparison of graded freezing at 0.2 °C/min in the absence of cryoprotectant

Graded freezing was performed in the presence of 10% DMSO to compare fluorescence microscopy and flow cytometry methods of measuring membrane integrity. Figure 2-13 shows three methods to measure membrane integrity: *i)* FC (represented by green circles); *ii)* FC Dbl (represented by blue diamonds); and *iii)* FM (represented by black triangles). To add DMSO, a 20% w/w DMSO solution was added to HUVECs to a final concentration of 10% w/w DMSO with HUVECs followed by a 15 minute exposure at 0 °C. For FM graded freezing experiments, prior to controlled cooling, a 5 minute equilibration time at −3 °C was followed by induced ice formation and a 3 minute hold time. For FC and FC Dbl graded freezing experiments, prior to controlled cooling, a 2 minute equilibration at −5 °C was followed by induced ice formation and a 3 minute hold time. Membrane integrity was measured for FM with SYTOEB and all measurements were repeated three times except for direct thaw and plunge-thaw from −40 °C which were repeated two times. Membrane integrity was measured for FC and FC Dbl with SYTOEB, except for one replicate with SYTOPI for the graded freezing at a 1.0 °C/min cooling rate in the absence of cryoprotectant. In the presence of 10% DMSO, the membrane integrity of

HUVECs measured by FM did not change significantly after direct thaw, ranging from 95.3% ± 0.9% to 96.8% ± 0.7%. Also measured by FM, the optimal temperature for plunge-thaw was −30 °C resulting in a membrane integrity of 88.4% ± 1.7%. The membrane integrity measured by FC did not change significantly after direct thaw, ranging from 92.5% ± 0.3% to 90.7% ± 0.7%. Also measured by FC, the maximum membrane integrity after plunge-thaw was 67.4% ± 1.8% from −35 °C. Membrane integrities measured by FC Dbl were statistically similar to membrane integrities measured by FC at all experimental temperatures after direct thaw; however membrane integrities measured by FC Dbl were significantly higher at all experimental temperatures after direct thaw. Measuring membrane integrity by FC Dbl was statistically similar to membrane integrities measured by FM at all experimental temperatures after direct thaw and after plunge-thaw from −9 °C to −25 °C; however membrane integrities were significantly lower measured by FC Dbl after plunge-thaw from −5 °C, −30 °C, −35 °C and −40 °C. In the presence of 10% DMSO, higher membrane integrities are observed using FM compared to FC; however membrane integrities are similar at several experimental temperatures using FM and FC Dbl.

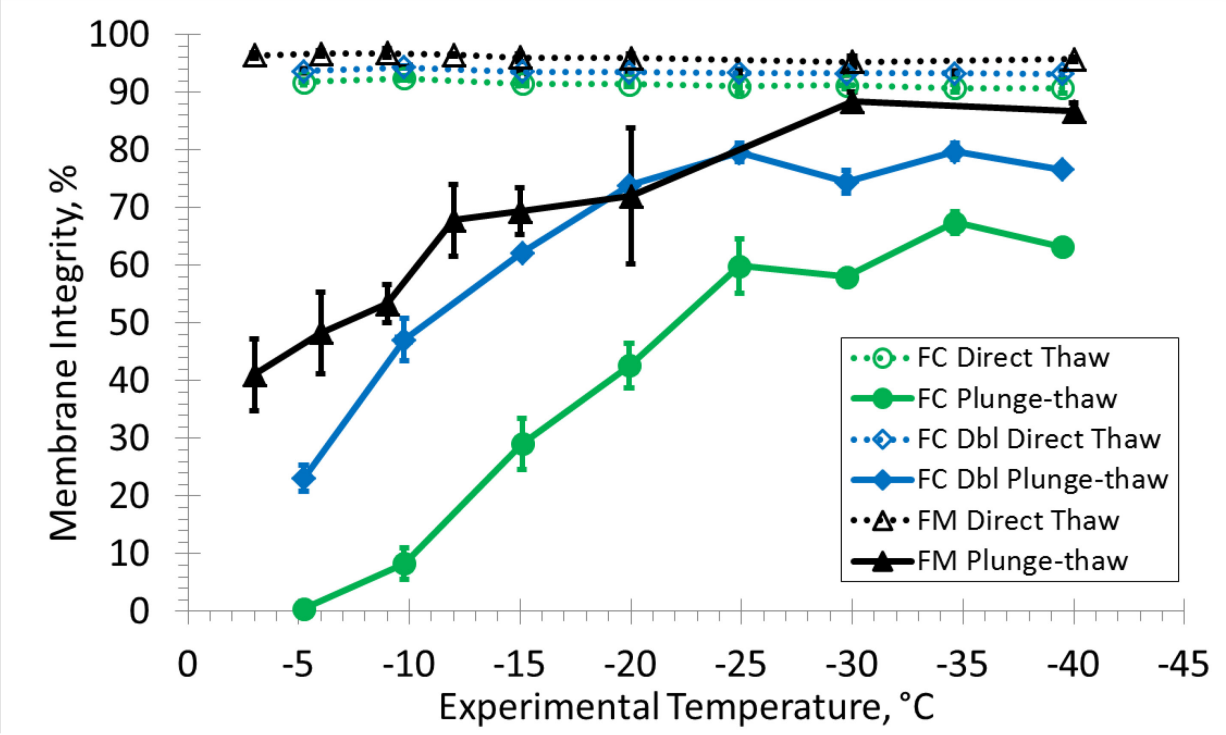


Figure 2-14. Flow cytometry and fluorescence microscopy comparison of graded freezing at 1 °C/min in the presence of 10% DMSO

2.3.3.3 Summary

In the absence of cryoprotectant, membrane integrity was measured and calculated in three ways. For fluorescence microscopy, membrane integrity was calculated using the Viability3 program. For flow cytometry, membrane integrity was calculated in two different ways, one being more stringent for selecting membrane-intact cells than the other. The flow cytometry incubation conditions were more toxic to HUVECs, involving: *i)* a longer incubation time, *ii)* a higher concentration of ethidium bromide and, *iii)* a more stringent method for selecting membrane-intact cells. Two-step freezing was applied to HUVECs; however it was difficult to conclude whether any method was more stringent for selecting membrane-intact HUVECs. The results for graded freezing, however, demonstrated significant differences measuring membrane integrities after direct thaw from all experimental temperatures and after plunge-thaw from most experimental temperatures. The flow cytometry method of measuring membrane integrity used in this thesis as described in equation (2.10) was the more stringent method for selecting membrane-intact HUVECs. Since there were differences between measurement methods from graded freezing, but not from two-step freezing, this demonstrates that membrane damage from supercooling effects can be observed quickly and osmolality effects can manifest as membrane damage over time.

Chapter Three: Interrupted Cooling Protocols

3.1 Introduction

In the past, protocols developed to optimize cryopreservation of HUVEC suspensions have met with difficulties due to large variation in the measured viability response [78]. The variables manipulated include: *i)* cell culture and preparation [9], *ii)* cooling rates [78], [135] *iii)* cryopreservation media [78], *iv)* cryoprotectant addition procedure [135] and *iv)* storage time [78]. Using interrupted cooling protocols for HUVECs, other variables were identified which include: *i)* experimental or hold temperature, *ii)* hold time, and *iii)* viability assessment method [147]. To identify key variables to optimize cryopreservation of HUVEC suspensions, interrupted cooling protocols work well [147]. In this chapter, interrupted cooling protocols were used to identify key variables to optimize HUVEC cryopreservation in the presence and absence of cryoprotectant.

3.2 Materials and Methods

All the methods are described in Chapter Two: Viability Assessment, namely: HUVEC culturing and preparation methods, temperature measurement methods, statistics, methods to measure membrane integrity by fluorescence microscopy, methods to measure membrane integrity by flow cytometry, two-step freezing methods and graded freezing methods. Also, detailed procedural steps for two-step freezing and graded freezing are available in the Appendix within the "Interrupted Cooling Protocols" section. All experiments were performed in triplicate unless otherwise stated and differences between means were stated as being significant if the level of significance, or p-value, was less than 0.05. Variations in temperature and membrane integrity are reported as standard errors of the means.

3.3 Fluorescence Microscopy – Interrupted Cooling Experiments

In the absence of cryoprotectant, the impact of two-step freezing and graded freezing on HUVECs was measured. Figure 3-1 shows membrane integrities after two-step freezing and graded freezing at a 0.2 °C/min cooling rate. For two-step freezing, the maximum membrane integrity after plunge-thaw was 1.9% ± 0.6% from −15 °C. The membrane integrity after direct thaw dropped rapidly at hold temperatures lower than −6 °C. For graded freezing, the maximum membrane integrity after plunge-thaw was 22.4% ± 1.1% when plunging from −20 °C. For samples after direct thaw, the membrane integrity decreased with decreasing experimental temperature. The membrane integrity was similar after direct thaw or plunge-thaw from −40 °C. In the absence of cryoprotectant, membrane integrity was higher after graded freezing at a 0.2 °C/min cooling rate than after two-step freezing. The optimal temperature for plunge-thaw was identified for interrupted slow cooling using graded freezing and interrupted rapid cooling using two-step freezing.

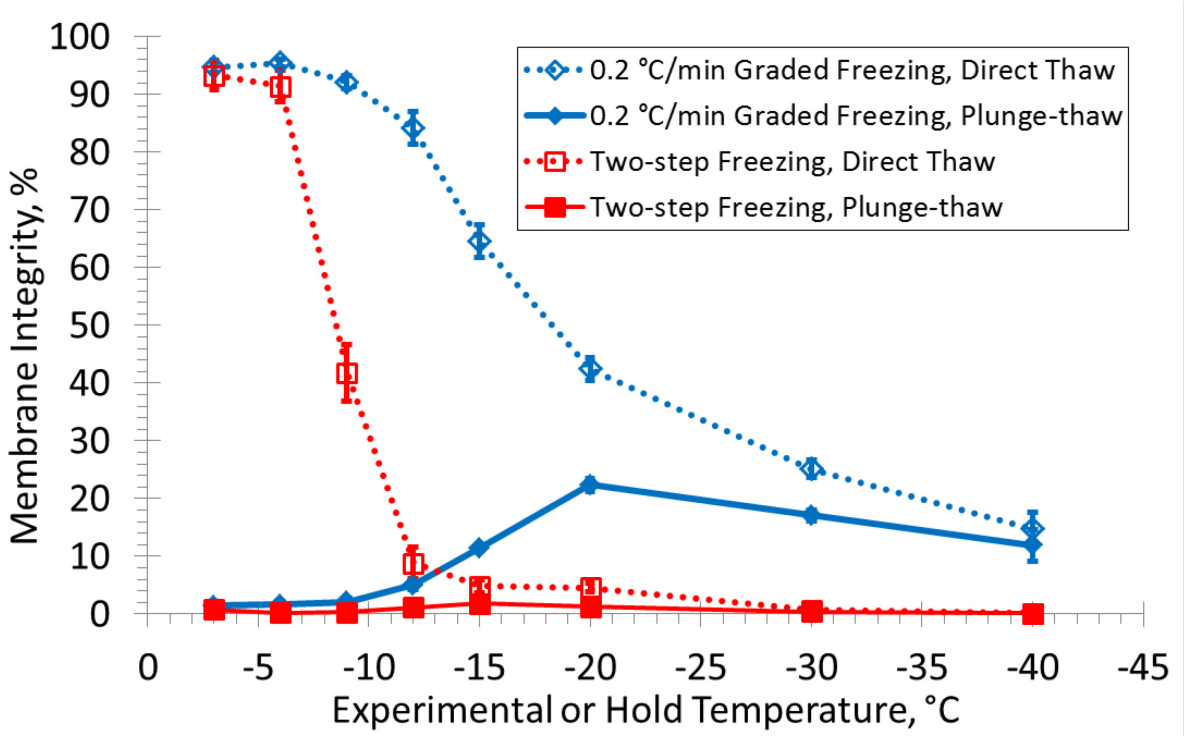


Figure 3-1. Interrupted cooling of HUVECs measured by fluorescence microscopy in the absence of cryoprotectant

Two-step freezing was used to observe the effect of DMSO on HUVEC membrane integrity. Figure 3-2 shows membrane integrities after two-step freezing in the presence of 4.2% DMSO by mass. One 8.4% DMSO solution was prepared and used for all three experiments and experiments were performed every other day, where 4 mL of DMSO solution was added to 4 mL of HUVEC suspension; 200 µL was transferred to 36 culture tubes and incubated at room temperature for 15 minutes followed by incubation at 0 °C for 5 minutes. For the first hold temperatures, ice formation was induced at −3 °C. Four red squares were used to group statistically similar membrane integrities. The membrane integrity of fresh HUVECs was 95.5% ± 1.2%. Membrane integrity after direct thaw did not significantly decrease compared to fresh HUVECs until −30 °C and −40 °C. The membrane integrity after plunge-thaw was optimal after hold temperatures between −6 °C and −20 °C, with a maximum of 61.8% ± 7.2% after plunge-thaw from −15 °C. HUVECs were not membrane-damaged after direct thaw from hold temperatures in the range of −3 °C to −20 °C; however HUVECs were membrane-damaged after plunge-thaw from hold temperatures in the range of −3 °C to −20 °C. After direct thaw from −30 °C or −40 °C, HUVECs were membrane-damaged; however membrane integrities were similar after direct thaw and plunge-thaw from −30 °C or −40 °C. Adding 4.2% DMSO resulted in higher HUVEC membrane integrities; therefore cryoprotectant is important to optimize HUVEC cryopreservation. An optimal temperature for plunge-thaw was identified. Considering the manner of cryoprotectant addition, there may have been an effect on determining the optimal plunge-thaw temperature. DMSO exposure time at 0 °C was not controlled, as DMSO was added to HUVECs at 0 °C and two-step freezing was performed in sequence from −3 °C to −40 °C. For future experiments it will be valuable to control for cryoprotectant exposure time. Comparisons between membrane integrities after direct thaw and plunge-thaw suggest that if membrane integrity can be maintained down to −30 °C, then HUVECs will survive plunge-thaw with minimal additional membrane damage.

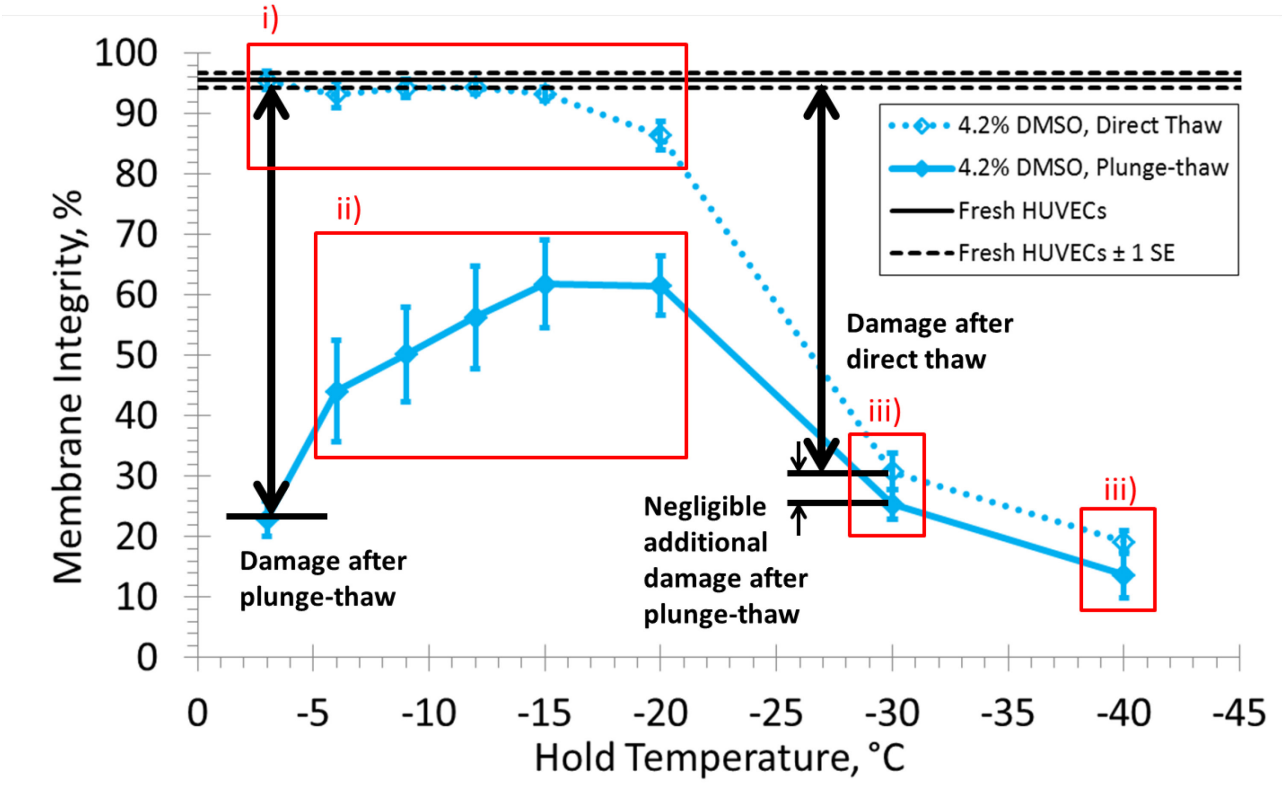


Figure 3-2. Two-step freezing of HUVECs measured by fluorescence microscopy with 4.2% DMSO. Red squares indicate: i) membrane integrities not significantly different from fresh HUVECs, ii) membrane integrities not significantly different from HUVECs after plunge-thaw from −15 °C, iii) membrane integrities after direct thaw not significantly different from plunge-thaw membrane integrities

3.3.1 Summary

In the absence of cryoprotectant, membrane integrity was higher after graded freezing than after two-step freezing; however, HUVEC membrane integrity was very low after plunge-thaw. Interrupted cooling protocols in the absence of cryoprotectant could be used to identify an optimal cooling profile which was cooling at 0.2 °C/min to −20 °C before plunge-thaw. Also, an optimal cooling profile was identified using two-step freezing in the presence of 4.2% DMSO to be rapid cooling to −20 °C followed by a 3 minute hold before plunge-thaw. The key variables affecting HUVEC membrane integrity were: *i)* plunge temperature, *ii)* the type of interrupted cooling protocol, and *iii)* the presence or absence of cryoprotectant. Comparing membrane

integrities after direct thaw and plunge-thaw suggested that cooling to −30 °C or lower without membrane damage would result in negligible additional damage after plunge-thaw.

3.4 Flow Cytometry – Interrupted Cooling Experiments

3.4.1 Description of Key Variables to Optimize Cryopreservation

Key variables to optimize cryopreservation of HUVEC suspensions were identified using fluorescence microscopy and interrupted cooling experiments. To study the impact of these variables, Figure 3-3 shows the experiment design. As cryoprotectants can impose an osmotic stress resulting in excessive cell shrinkage during addition and cell expansion during removal [136], [192], graded freezing at a 1.0 °C/min cooling rate was used to compare three cryoprotectant addition procedures: *i)* adding a 20% DMSO solution in EGM to the HUVEC suspension to a final concentration of 10% DMSO with 15 minute exposure at 0 °C; *ii)* adding a 20% DMSO solution in EGM to the HUVEC suspension to a final concentration of 10% DMSO with 30 minute exposure at 0 °C; and *iii)* adding a 20% DMSO solution in EGM to the HUVEC suspension to an initial concentration of 3% DMSO, followed by a 10 minute exposure at 0 °C and then adding more 20% DMSO to a final concentration of 10% DMSO, followed by a 20 minute exposure at 0 °C. This third procedure is the procedure proposed by Pegg [135], except that there is no centrifugation step used in this work.

To compare interrupted cooling protocols in the presence of cryoprotectant, the cryoprotectant addition procedure that resulted in the highest membrane integrity after graded freezing at a 1.0 °C/min cooling rate was used as a basis. In the presence and absence of 10% DMSO, three interrupted cooling procedures were compared: *i)* two-step freezing; *ii)* graded freezing at a 0.2 °C/min cooling rate; and *iii)* graded freezing at a 1.0 °C/min cooling rate.

To compare cryoprotectant solutions, the cryoprotectant addition procedure and the interrupted cooling protocol that resulted in the highest membrane integrity were used as a basis. Three cryoprotectant solutions were compared: *i)* 20% DMSO; *ii)* 10% DMSO plus 5% HES; and *iii)* 10% DMSO plus 8% HES. The highest HUVEC membrane integrity in this work was compared to the HUVEC viabilities after cryopreservation as reported in the literature [78], [140], and to the viability of supplier-provided HUVEC suspension (as determined in this thesis,

see Chapter Two: Viability Assessment in the "Results and Discussion" section). SYTOEB or SYTOPI was used for membrane integrity assessment by flow cytometry. The membrane integrity assessment method is specified during the discussion of each experiment.

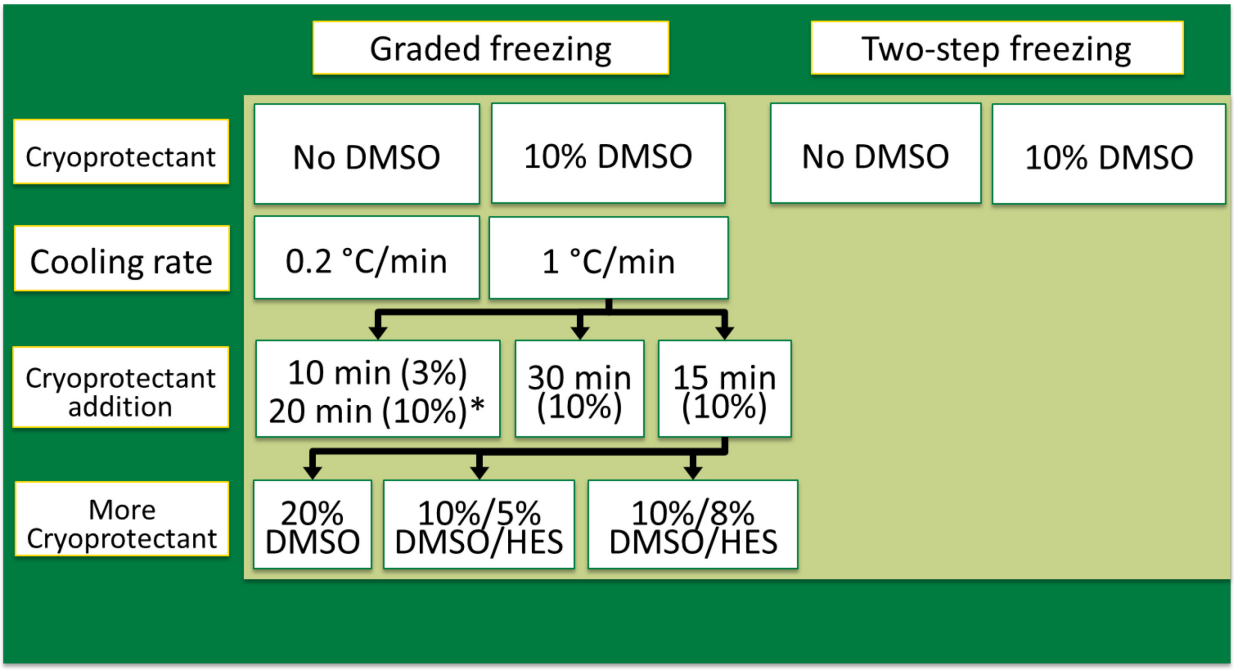


Figure 3-3. Key variables to optimize cryopreservation of HUVEC suspensions

3.4.2 Interrupted Cooling Protocols in the Absence of Cryoprotectant

To determine the best interrupted cooling procedure for HUVEC in the absence of cryoprotectant, graded freezing and two-step freezing were compared for membrane integrity. Figure 3-4 shows membrane integrity results in the absence of cryoprotectant for: *i)* two-step freezing using a 3 minute hold time (represented by purple jewels); *ii)* graded freezing at a 0.2 °C/min cooling rate (represented by red squares); and *iii)* graded freezing at a 1.0 °C/min cooling rate (represented by blue diamonds). Membrane integrity was measured using flow cytometry with SYTOEB, except for one replicate using flow cytometry with SYTOPI for graded freezing at a 1.0 °C/min cooling rate. Membrane integrities were similar comparing all three interrupted cooling procedures at direct thaw from −3 °C, −6 °C and −9 °C. The pattern of response was different, where membrane integrity declined more steeply with decreasing temperature and graded freezing at 1 °C/min declined least steeply. Direct thaw from −12 °C and −15 °C showed

that graded freezing at a 1.0 °C/min cooling rate resulted in higher membrane integrities than graded freezing at a 0.2 °C/min cooling rate. Direct thaw from −12 °C and −15 °C showed that two-step freezing resulted in the lowest membrane integrities. There was no measure of membrane integrity larger than 2% after plunge-thaw for the interrupted cooling procedures. It is difficult to make a conclusion as to which interrupted cooling procedure is best for HUVECs in the absence of cryoprotectant. If comparisons from direct thaw are made, then graded freezing at a 1.0 °C/min cooling rate was best for HUVEC survival.

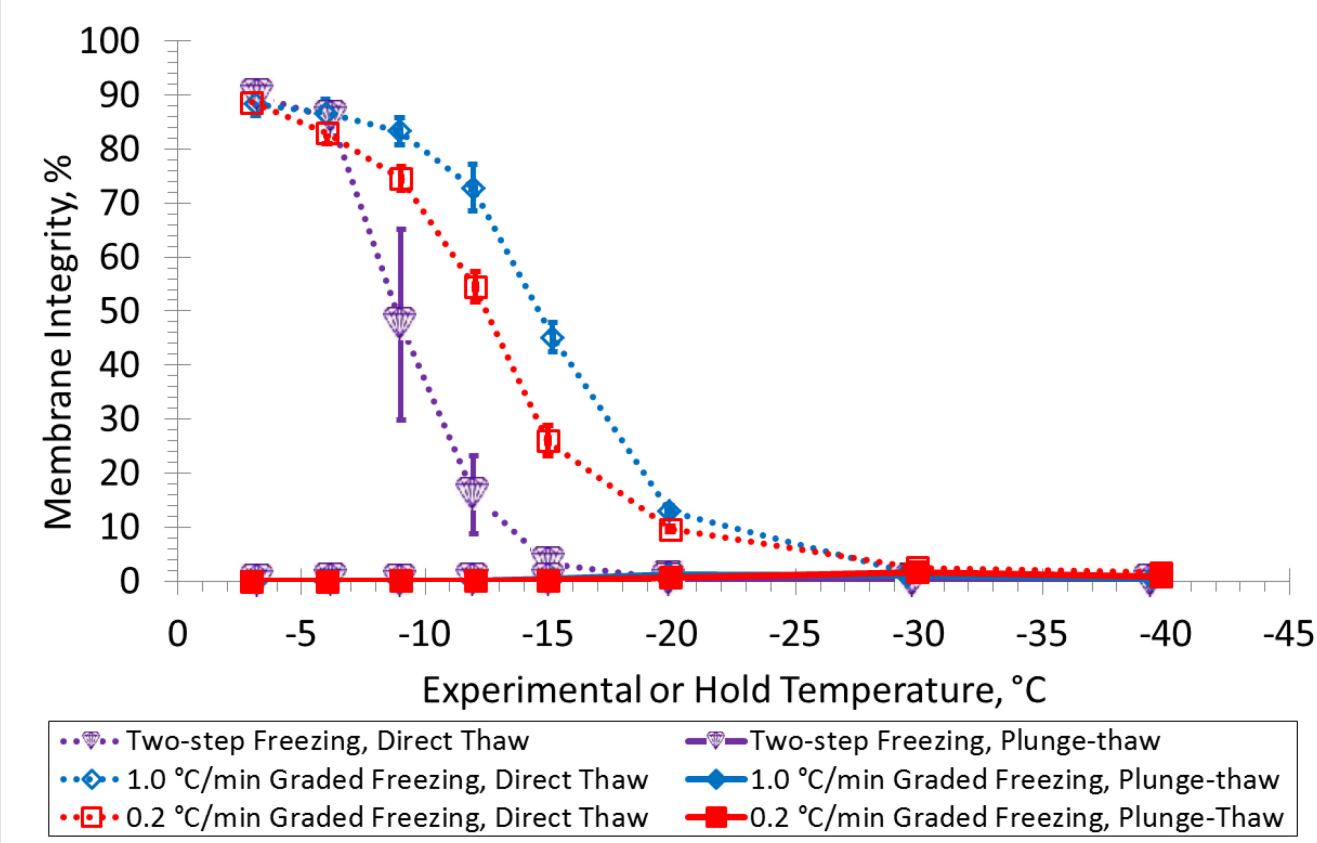


Figure 3-4. Interrupted cooling of HUVECs in the absence of cryoprotectant

3.4.3 Cryoprotectant Addition Procedures

To determine the best cryoprotectant addition procedure for HUVECs, graded freezing at a 1.0 °C/min cooling rate was used with 10% DMSO. Figure 3-5 shows membrane integrity results for three different cryoprotectant addition procedures at 0 °C: *i)* HUVECs exposed to 10% DMSO for 15 minutes (represented by green circles); *ii)* HUVECs exposed to 10% DMSO for

30 minutes (represented by blue triangles) and *iii)* HUVECs exposed to 3% DMSO for 10 minutes and then exposed to 10% DMSO for 20 minutes (represented by yellow squares) [135]. To add the DMSO, a 20% w/w solution was prepared and 5 mL of 20% DMSO solution was added to 5 mL of HUVEC suspension to achieve a 10% w/w DMSO with HUVEC solution. Membrane integrity was measured using flow cytometry with SYTOEB. There are no large differences in membrane integrities; however comparing 15 minute exposure to the 30 minute exposure, membrane integrity was significantly higher using a 15 minute exposure after direct thaw from −5 °C, −10 °C and −20 °C. Also, comparing the 15 minute exposure to the 30 minute exposure, membrane integrity was significantly higher using a 15 minute exposure after plunge-thaw from −30 °C (p-value of 0.041). There was only one significant difference observed between the 15 minute exposure and the 10/20 minute exposure where membrane integrity was significantly higher using a 10/20 minute exposure after plunge-thaw from −5 °C. Therefore adding 10% DMSO using a 15 minute exposure at 0 °C was used for all subsequent cryoprotectant addition procedures.

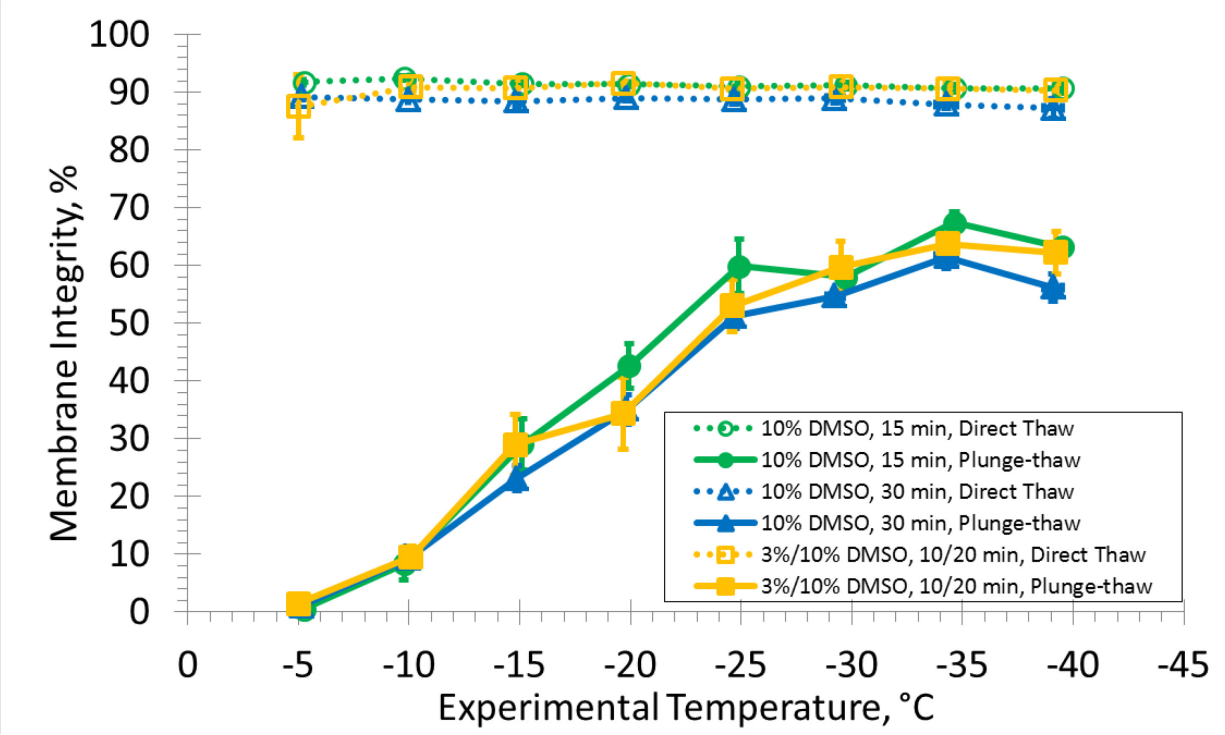


Figure 3-5. Graded freezing at 1.0 °C/min comparing DMSO addition procedures

3.4.4 Interrupted Cooling Protocols in the Presence and Absence of 10% DMSO

To observe the impact of 10% DMSO, HUVEC membrane integrity was measured after graded freezing at a 1.0 °C/min cooling rate in the presence and absence of 10% DMSO. Figure 3-6 shows membrane integrity results of: *i)* graded freezing at a 1.0 °C/min cooling rate in the absence of cryoprotectant (represented by blue diamonds); and *ii)* graded freezing at a 1.0 °C/min cooling rate in the presence of 10% DMSO (represented by green circles). Membrane integrity was measured by flow cytometry with SYTOEB, except for one replicate with SYTOPI for the graded freezing at a 1.0 °C/min cooling rate in the absence of cryoprotectant. In the presence of 10% DMSO, the membrane integrity of HUVECs did not change significantly after direct thaw, ranging from 92.5% ± 0.3% to 90.7% ± 0.7%. Also in the presence of 10% DMSO, the maximum membrane integrity after plunge-thaw was 67.4% ± 1.8% from −35 °C. In the absence of cryoprotectant after direct thaw, membrane integrity decreased gradually with decreasing temperature and was significantly lower than fresh HUVECs after direct thaw from temperatures of −12 °C and lower. Also in the absence of cryoprotectant, the membrane integrity was not more than 2% after plunge-thaw, preventing any observation of an optimal temperature for plunge-thaw. Using graded freezing at a 1.0 °C/min cooling rate in the presence of 10% DMSO, plunge-thaw was optimal from −25 °C, −35 °C or −40 °C.

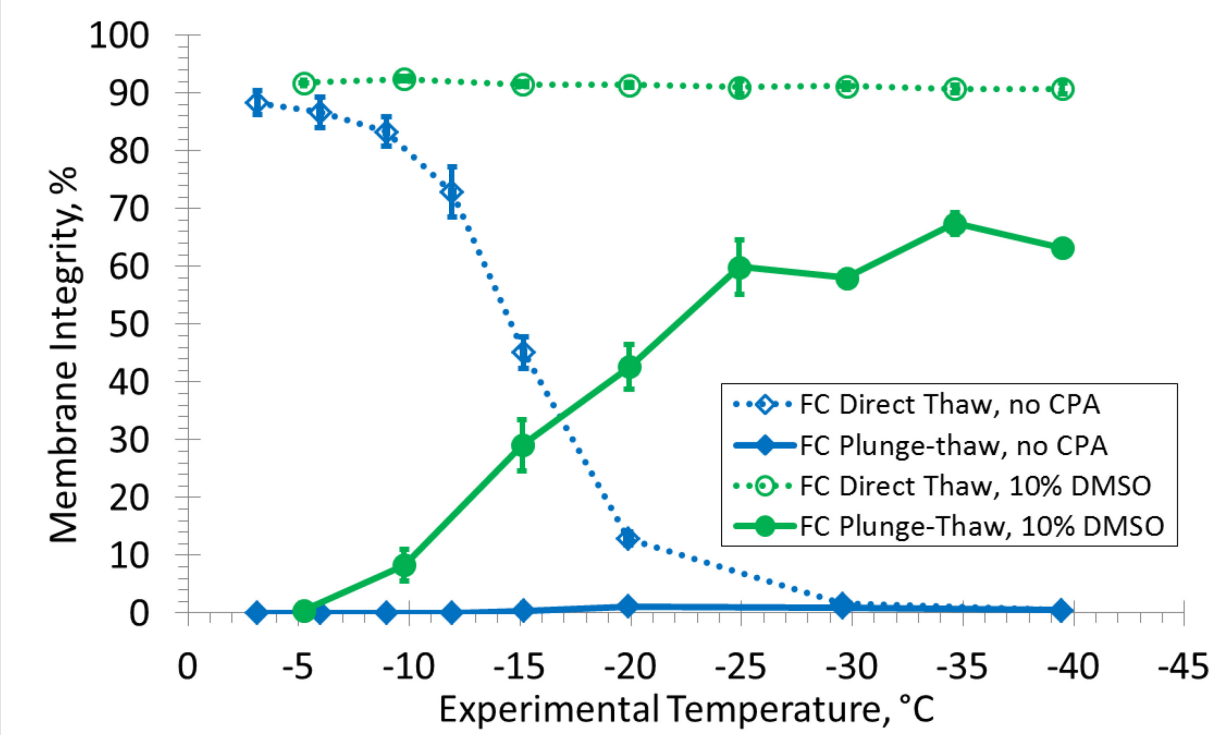


Figure 3-6. Graded freezing at 1.0 °C/min in the presence and absence of 10% DMSO

To observe the impact of 10% DMSO, HUVEC membrane integrity was measured after graded freezing at a 0.2 °C/min cooling rate in the presence and absence of 10% DMSO. Figure 3-7 shows membrane integrity results of: *i)* graded freezing at a 0.2 °C/min cooling rate without cryoprotectant (represented by red squares); and *ii)* graded freezing at a 0.2 °C/min cooling rate with 10% DMSO (represented by yellow triangles). Membrane integrity was measured using flow cytometry with SYTOEB, except for two replicates using flow cytometry with SYTOPI from graded freezing at a 0.2 °C/min cooling rate with 10% DMSO. Compared to fresh HUVECs (92.4% ± 0.4%), the membrane integrity in 10% DMSO decreased significantly after direct thaw from −25 °C and −40 °C, with membrane integrities of 90.7% ± 0.4% to 88.1% ± 0.9%, respectively. In the absence of cryoprotectant, membrane integrity decreased gradually after direct thaw with decreasing temperature and was significantly lower than fresh HUVEC after direct thaw from −9 °C and lower. In the absence of cryoprotectant, membrane integrity was not more than 2% after plunge-thaw, preventing any observation of an optimal temperature for plunge-thaw. In the presence of 10% DMSO, membrane integrity was as high as 57.6% ±

1.7% after plunge-thaw from −40 °C. Using graded freezing at a cooling rate of 0.2 °C/min, plunge-thaw was optimal with 10% DMSO from −30 °C, −35 °C or −40 °C.

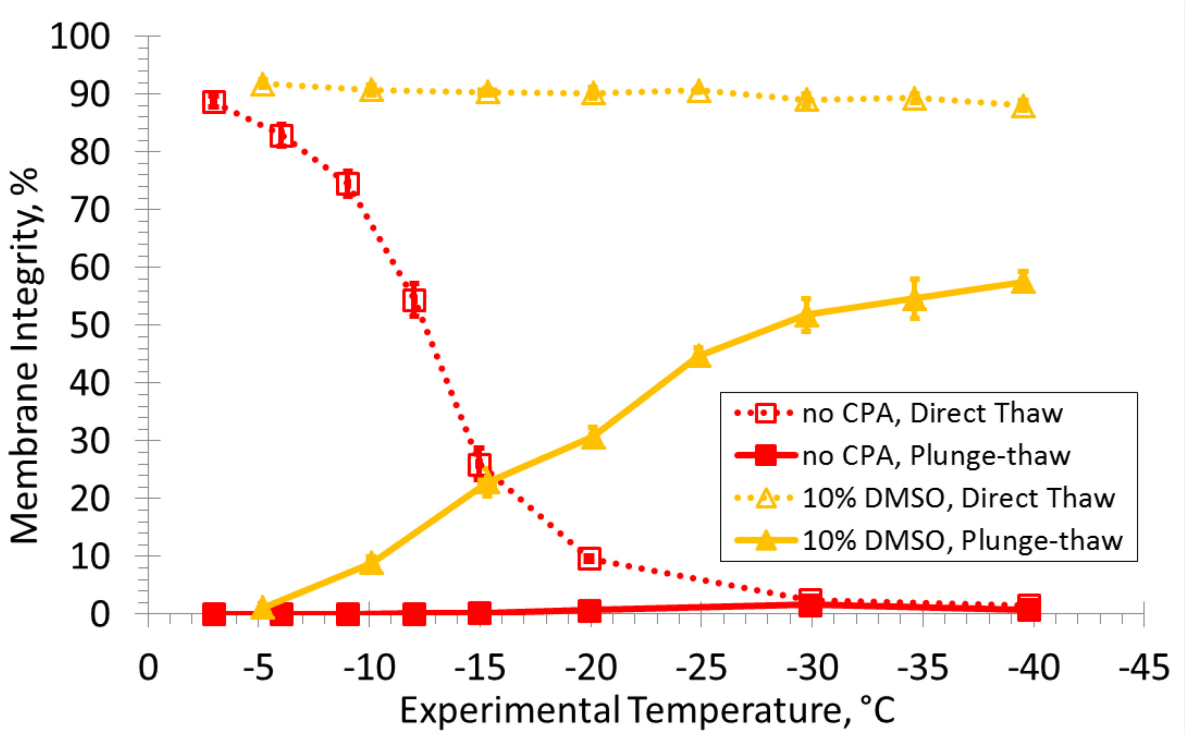


Figure 3-7. Graded freezing at 0.2 °C/min in the presence and absence of 10% DMSO

To observe the impact of DMSO, HUVEC membrane integrity was measured after two-step freezing in the presence and absence of 10% DMSO. Figure 3-8 shows membrane integrity results of: *i)* two-step freezing using a 3 minute hold time in the absence of cryoprotectant, (represented by purple jewels); and *ii)* two-step freezing using a 3 minute hold time in the presence of 10% DMSO, (represented by black stars). Membrane integrity was measured using flow cytometry with SYTOEB, except for two replicates using flow cytometry with SYTOPI for two-step freezing in the presence of 10% DMSO. In the absence of cryoprotectants, there is more damage at hold temperatures preventing an optimal hold temperature from being observed. In 10% DMSO, the membrane integrity decreased in a constant manner after direct thaw from −20 °C to −40 °C. In 10% DMSO, the optimal hold temperature for plunge-thaw was from −20 °C to −30 °C.

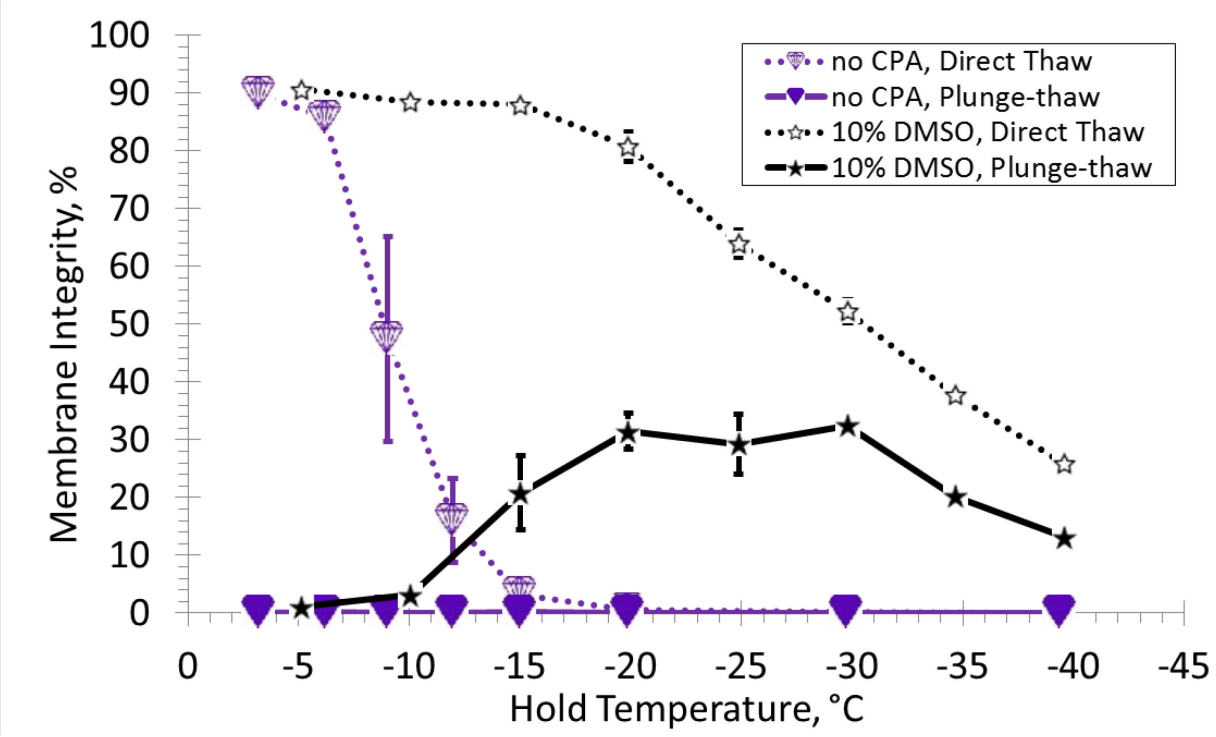


Figure 3-8. Two-step freezing in the presence and absence of 10% DMSO

3.4.5 Interrupted Cooling Protocols in the Presence of 10% DMSO

To determine the best interrupted cooling protocol in the presence of 10% DMSO, graded freezing and two-step freezing were compared. Figure 3-9 shows membrane integrity results in the presence of 10% DMSO for: *i)* graded freezing at a 1.0 °C/min cooling rate (represented by green circles); *ii)* graded freezing at a 0.2 °C/min cooling rate (represented by yellow triangles); and *iii)* two-step freezing using a 3 minute hold time (represented by black stars). Membrane integrity was measured using flow cytometry with SYTOEB, except for two replicates for two-step freezing using SYTOPI and two replicates for graded freezing at a 0.2 °C/min cooling rate using SYTOPI. Membrane integrities after direct thaw were higher for graded freezing compared to two-step freezing for experimental temperatures in the range of −25 °C to −40 °C. Membrane integrities after plunge-thaw were higher after graded freezing than two-step freezing when plunging from −25 °C or lower. Membrane integrities were higher after plunge-thaw from graded freezing using a 1.0 °C/min cooling rate only after plunge-thaw from −35 °C compared to

graded freezing at a 0.2 °C/min cooling rate. The highest membrane integrity after plunge-thaw was 67.4% ± 1.8% cooling at a rate of 1.0 °C/min to −35 °C.

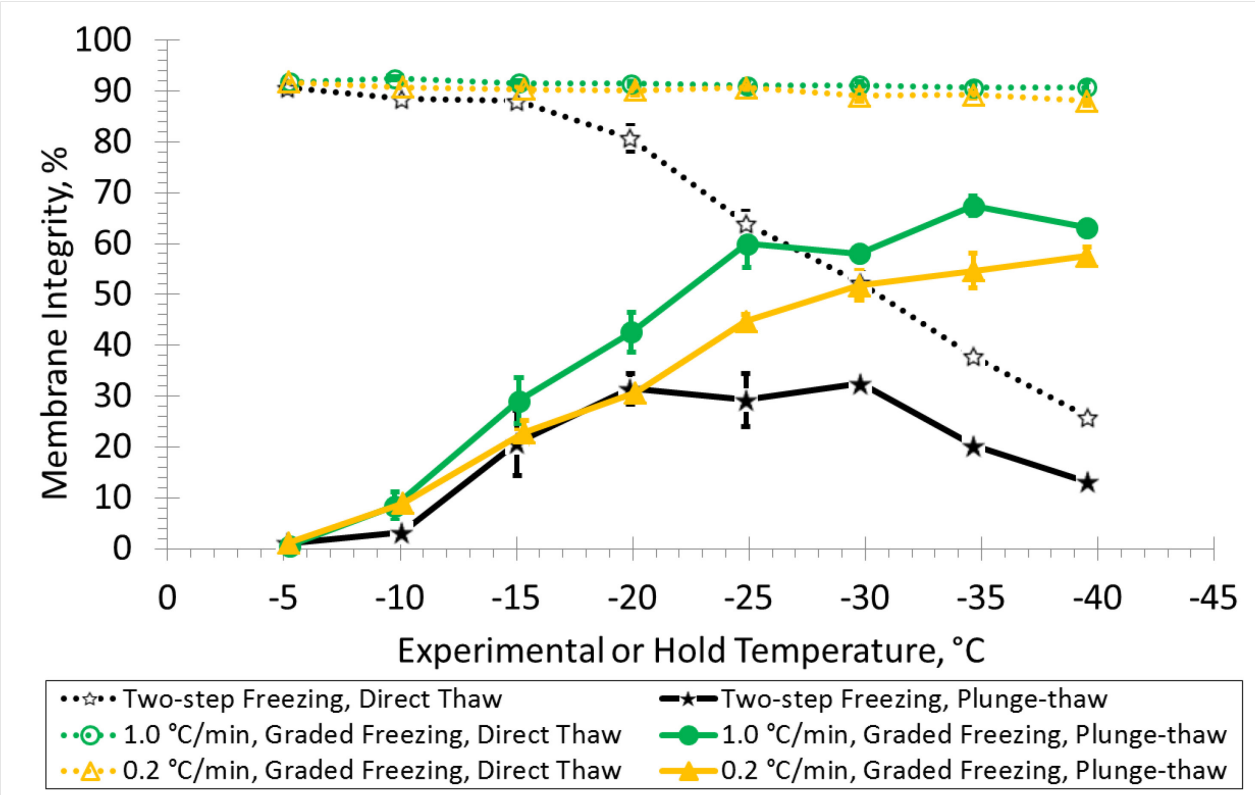


Figure 3-9. Interrupted cooling of HUVECs in the presence of 10% DMSO

3.4.6 Interrupted Cooling Protocols with More Cryoprotectant

To determine the best cryoprotectant concentration and cryoprotectant mixture, the best interrupted cooling experiment was used with the 15 minute exposure to cryoprotectant addition procedure. Figure 3-10 shows membrane integrity results for graded freezing at a 1.0 °C/min cooling rate with HUVECs in: *i)* 10% DMSO (represented by green circles); *ii)* 10% DMSO plus 5% HES (represented by yellow squares); *iii)* 10% DMSO plus 8% HES (represented by blue diamonds); and *iv)* 20% DMSO (represented by red circles). Membrane integrity was measured using flow cytometry with SYTOEB only for the 10% DMSO experiment. The other experiments (20% DMSO, 10% DMSO plus 5% HES, and 10% DMSO plus 8% HES) were measured for membrane integrity using SYTOPI. The membrane integrities were not

significantly different comparing all four graded freezing experiments after direct thaw. The membrane integrity was higher for 10% DMSO compared to 20% DMSO after plunge-thaw from −35 °C; however the detection of background light scatter was much higher in the presence of 20% DMSO, and as well the compensation was reduced from 32.0% to 29.0% in the presence of 20% DMSO due to a decrease in fluorescence from membrane-intact cells. The effect of 20% DMSO on background light scatter was also observed by comparing the number of events detected in the Bkgd quadrant for EGM alone and 20% DMSO, where 128 events were detected from EGM and more than 25,000 events were detected from 20% DMSO. The impact of more backgound light scatter was to cause the flow cytometer to exceed its measuring capabilities, such that cells may not have been counted due to an excessive number of events being detected. The membrane integrities were higher for 10% DMSO plus 5% HES after plunge-thaw from −10 °C, −30 °C, −35 °C and −40 °C compared to 10% DMSO. The membrane integrities were higher for 10% DMSO plus 8% HES after plunge-thaw from −10 °C, −15 °C, −20 °C, −30 °C, −35 °C and −40 °C compared to 10% DMSO. Using 10% DMSO plus 8% HES, there was no clear optimum temperature for plunge-thaw as membrane integrity increased with decreasing experimental temperature. The highest membrane integrity after plunge-thaw was from −30 °C to −45 °C using 10% DMSO plus 8% HES. The highest membrane integrity after plunge-thaw was 83.6% ± 1.6% from −45 °C, which was significantly less than the fresh HUVECs (91.7% ± 0.7%) and fresh HUVECs in the presence of 10% DMSO (93.2% ± 0.7%). The highest membrane integrity after plunge-thaw was achieved by cooling at 1.0 °C/min in the presence of 10% DMSO plus 8% HES to −45 °C before plunge-thaw.

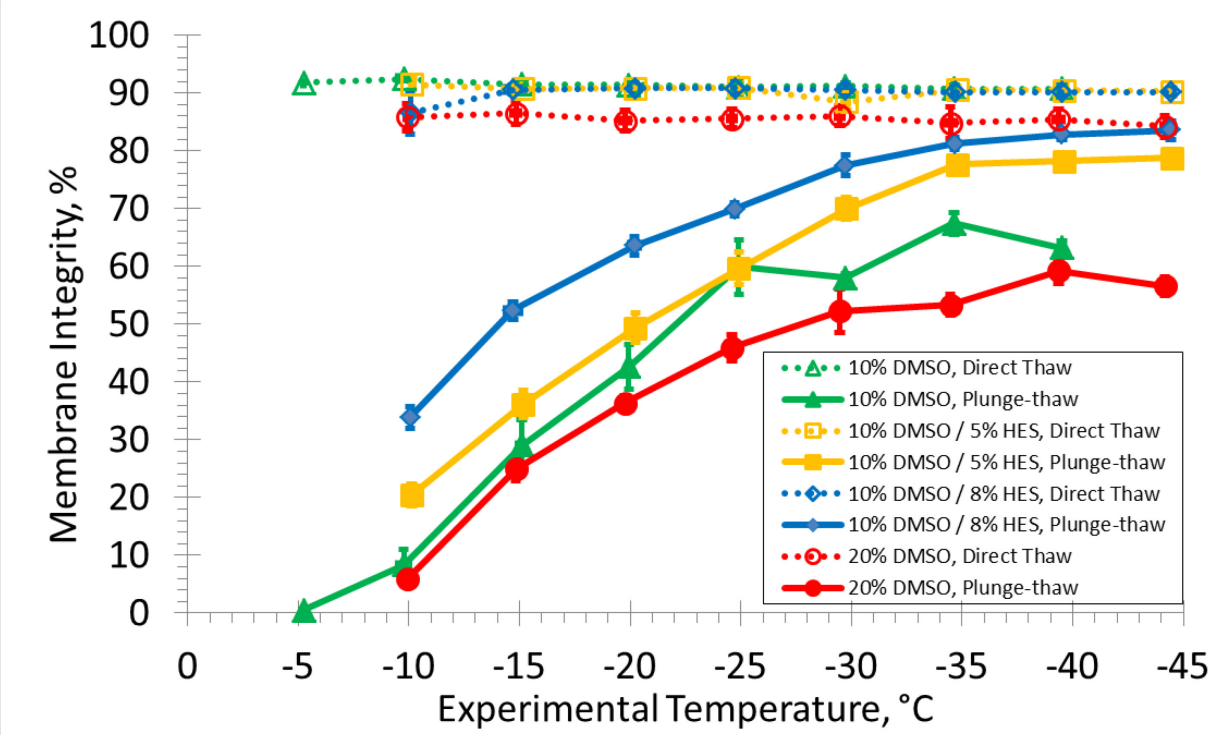


Figure 3-10. Graded freezing at 1.0 °C/min comparing 10% DMSO in the presence or absence of HES

Chapter Four: Conclusions

The key variables to optimize HUVEC cryopreservation procedures are: *i)* cooling protocols, *ii)* cooling rates, *iii)* cryoprotectant compositions, and *iv)* plunge temperatures. Only the cryoprotectant addition procedure did not require optimization. A previously defined cryoprotectant addition procedure [135] did not provide additional benefits compared to the direct addition of 10% DMSO.

The high reproducibility of experiments and the use of interrupted cooling protocols facilitated the identification of key variables to optimize HUVEC cryopreservation procedures. The high reproducibility was the result of careful consideration of HUVEC culturing and preparation as well as membrane integrity measurements. Phase contrast microscopy provided a learning opportunity to culture HUVECs aseptically in the absence of antibiotics. Fluorescence microscopy provided a learning opportunity to prepare HUVECs as cell suspensions.

The different mechanisms of cryoinjury were observed from measurements of membrane integrity by flow cytometry and fluorescence microscopy. Graded freezing demonstrated that flow cytometry is more stringent than fluorescence microscopy to measure membrane integrity; however membrane integrity measurements after two-step freezing showed no significant differences between the membrane integrity measurements. Slow cooling damage from graded freezing was likely the reason for the different membrane integrity measurements as membrane damage from supercooling effects and solute effects [152] can manifest as membrane damage over time [113]. Rapid cooling damage from two-step freezing was likely the reason for the similar membrane integrity measurements as supercooling effects [151] result in high cell damage. Membrane damage from supercooling effects can be observed quickly after thawing and membrane damage from osmolality effects can be observed gradually after thawing.

As hypothesized based on the low hydraulic conductivity and on previous research [78], [147], HUVECs were able to survive interrupted slow cooling better than interrupted rapid cooling. In the literature, TF-1 cells have a higher hydraulic conductivity than HUVECs and survive rapid cooling [151], [152]; however fibroblasts have a very high hydraulic conductivity and survive slow cooling [106], [107]. To improve predictions of optimal cooling for other types of cells, other factors can be considered, including solute permeability [30], [32], [56], [57], [192] and intracellular solution osmotic virial coefficients [153].

Graded freezing using a 1.0 °C/min cooling rate was used to determine an optimal concentration of DMSO. Also, adding a non-permeating cryoprotectant increased HUVEC survival when used with DMSO during slow cooling. These results are consistent with many other studies illustrating that DMSO toxicity is reduced by combining DMSO and HES; however in previous research using two-step freezing for fibroblasts, DMSO plus HES was less beneficial than DMSO alone [106]. The lower concentration of DMSO and the use of two-step freezing for fibroblasts could be the reason for the different response from fibroblasts.

The highest membrane integrity using the best cryopreservation procedure herein is higher than the viability reported in the literature (69.2% ± 2.3%) for the standard good manufacturing practices protocol (cooling at 1 °C/min in the presence of M199 with 18% serum and 10% DMSO) [140]and higher than the membrane integrity of 64.8% ± 2.2% measured in this thesis for the supplier-provided HUVECs. Our procedure is nearly serum-free (2% FCS), takes less time than cooling to −80 °C and the procedural detail provided in this work is appropriate to ensure reproducibility of results. We also note that the flow cytometry method to measure membrane integrity is more stringent than the fluorescence microscopy method, and we have identified the effects of the key variables important to improve the cryopreservation of HUVECs. Interrupted cooling protocols are useful to improve the HUVEC cryopreservation procedure, a protocol design which can also be used to improve cryopreservation of other types of cells.

4.1 Future Studies

In the presence of cryoprotectants, membrane integrity was measured without cryoprotectant removal or dilution. Considering the lower membrane integrity measurement from the supplier and the literature [136], a cryoprotectant dilution procedure might be beneficial for future routine cryopreservation of HUVECs and other types of cells in the presence of permeating cryoprotectants.

4.1.1 GelRed as an EB and PI Replacement

The SYTOEB membrane integrity assay uses SYTO 13 and EB and the SYTOPI membrane integrity assay uses SYTO 13 and PI, which are potentially dangerous to personal health and the environment. According to the Material Safety Data Sheet (MSDS), EB has acute toxicity through inhalation (Category 3) and germ cell mutagenicity (Category 2) based on the Globally Harmonized System of Classification and Labelling of Chemicals (E1510, Sigma-Aldrich). PI (P3566, Invitrogen Corporation) has a health hazard of 1 based on the Hazardous Materials Identification System and has specific mutagenic effects as animal experiments showed mutagenic and teratogenic effects. EB was ordered in an already dissolved form (10.0 mg/mL in water) which reduces the likelihood of inhalation. PI was purchased as a 1.0 mg/mL solution in water. However due to health and environmental effects, EB and PI can be replaced.

GelRed Nucleic Acid Stain (41003, Biotium, Inc.) has a health hazard of 0 based on the Hazardous Materials Identification System and is purchased as a 10,000 × solution in water. GelRed was evaluated using flow cytometry for appropriate concentrations based on the optimized flow cytometer settings. The addition procedure for the stain was kept constant, 20 µL of premix solution to 400 µL of HUVEC suspension and incubating at room temperature for 10 minutes. Since the concentration of SYTO 13 was kept constant and incubation time remained the same as for the SYTOEB and SYTOPI assays, fluorescence colour compensation remained the same. To determine an appropriate amount of GelRed, the concentration was gradually increased until the membrane-damaged population was located in the GelRed quadrant. Figure 4-1 shows a mixture of membrane-intact and membrane-damaged HUVECs incubated with 36.4X GelRed and 11.4 µM SYTO 13. Table 4-1 shows the composition of SYTO 13 and GelRed (SYTOGelRed) prepared and kept on ice, of which 20 µL was added to 400 µL HUVEC suspension. To calculate membrane integrity, equation (2.10) was used, where membrane-intact cells were counted from the SYTO quadrant and membrane-damaged cells were counted from the Dbl and GelRed quadrants. Based on the safety and flow cytometry, SYTOGelRed is a good alternative membrane integrity assay to SYTOEB and STYOPI.

Table 4-1. Premix solution of SYTOGelRed

SYTOGelRed	Volume, µL
SYTO 13, 5 mM in DMSO	25
GelRed, 10,000 X in water	40
Water	459

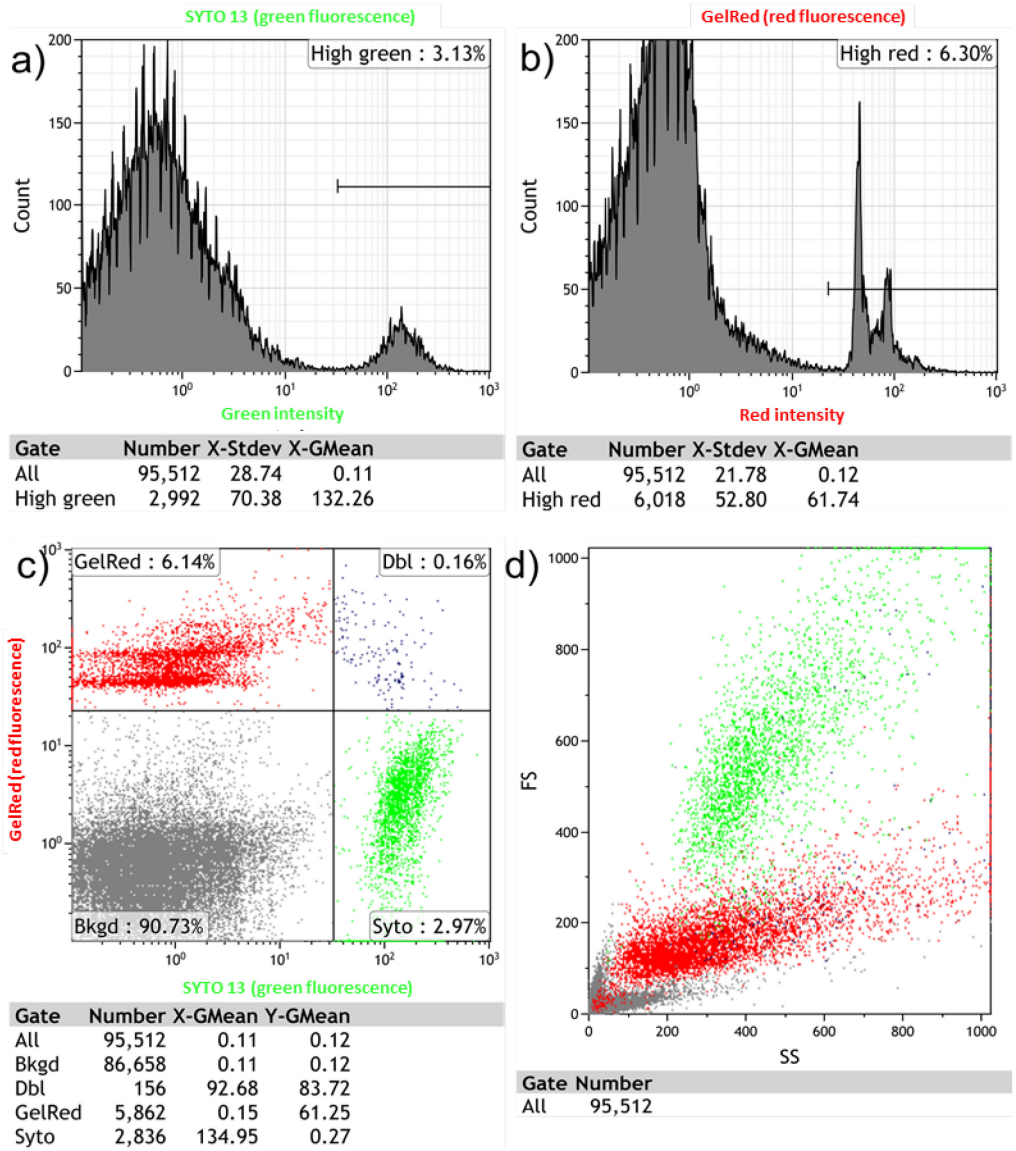


Figure 4-1. Flow cytometry measurement of membrane integrity using SYTOGelRed: a) histogram for high green detection; b) histogram for high red detection; c) fluorescence measurements after compensation; d) FS and SS of events, coloured using fluorescence quadrants.

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Appendix

Permissions

I, Michal Zielinski, give permission for Ahmad Billal Sultani to use the following figure, HUVEC growth after seeding from subculture, in his thesis.

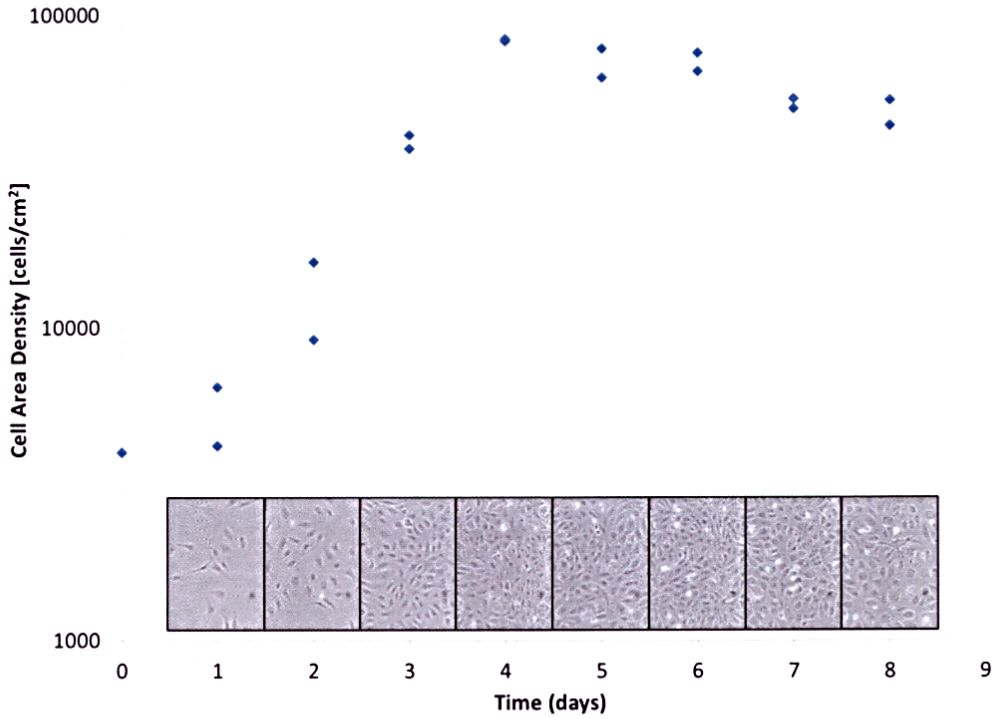

Figure A01. HUVEC growth after seeding from subculture prepared by Michal Zielinski

Michal Zielinski

2015-06-16

Fluorescence Microscopy Data

Table A1. Measured cooling rates and cryoprotectant conditions for fluorescence microscopy

Date of experiment	Cooling rate setpoint, °C/min	Cooling rate measured, °C/min	% CPA	CPA	Tn
2012-07-15	0.2	0.196	0	none	−3
2012-07-18	0.2	0.194	0	none	−3
2012-07-20	0.2	0.195	0	none	−3
2012-07-31	0.2	0.1946	0	none	−3
2012-08-02	0.2	0.194	0	none	−3
2012-08-04	0.2	0.192	0	none	−3
2012-08-28	Two-step	na	0	none	−3
2012-08-30	Two-step	na	0	none	−3
2012-09-01	Two-step	na	0	none	−3
2012-09-11	Two-step	na	4.2	DMSO	−3
2012-09-13	Two-step	na	4.2	DMSO	−3
2012-09-15	Two-step	na	4.2	DMSO	−3
2012-09-26	1.0	not recorded	9.8	DMSO	−3
2012-09-28	1.0	not recorded	14.3	DMSO	−3
2012-09-30	1.0	0.969	11.7	DMSO	−3

na – not applicable

CPA – cryoprotectant

Tn – induced nucleation temperature

Table A2. Measured cell conditions for fluorescence microscopy

Date of experiment	[Cell], 10^6 cells/ml	Cell doublings	Lot #	MI assay
2012-07-15	3.3	7.5	204434	SYTOEB FM
2012-07-18	2.0	10.8	204434	SYTOEB FM
2012-07-20	1.3	13.5	204434	SYTOEB FM
2012-07-31	1.5	6.9	6F4437	SYTOEB FM
2012-08-02	2.4	9.1	6F4437	SYTOEB FM
2012-08-04	2.1	11.4	6F4437	SYTOEB FM
2012-08-28	1.7	na	6F4437	SYTOEB FM
2012-08-30	2.9	na	6F4437	SYTOEB FM
2012-09-01	2.9	na	6F4437	SYTOEB FM
2012-09-11	2.7	6.3	197807	SYTOEB FM
2012-09-13	3.4	8.5	197807	SYTOEB FM
2012-09-15	3.8	10.3	197807	SYTOEB FM
2012-09-26	1.3	5.8	78524	SYTOEB FM
2012-09-28	2.5	8.1	78524	SYTOEB FM
2012-09-30	2.6	10.3	78524	SYTOEB FM

Table A3. Membrane integrity without cryoprotectant for graded freezing at 0.2 °C/min

	Direct thaw		Plunge-thaw	
T, °C	%MI	SE (%MI)	%MI	SE (%MI)
0	91.7	0.35	0.1	0.03
−3	94.7	0.84	1.5	0.19
−6	95.5	0.67	1.6	0.32
−9	92.1	1.12	2.1	0.58
−12	84.2	2.84	5.0	0.76
−15	64.6	2.84	11.4	0.46
−20	42.5	2.00	22.4	1.13
−30	25.1	1.53	17.1	0.89
−40	14.8	2.93	11.9	2.76

Table A4. Membrane integrity without cryoprotectant for two-step freezing

	Direct thaw		Plunge-thaw	
T, °C	%MI	SE (%MI)	%MI	SE (%MI)
0	93.6	1.77	0.0	0.00
−3	93.3	2.53	0.8	0.60
−6	91.4	2.75	0.3	0.17
−9	41.8	4.89	0.3	0.07
−12	8.8	2.78	1.2	0.64
−15	4.8	1.17	1.9	0.64
−20	4.6	0.75	1.2	0.14
−30	0.7	0.26	0.5	0.47
−40	0.2	0.13	0.1	0.07

Table A5. Membrane integrity after two-step freezing with 4.2% DMSO

	Direct thaw		Plunge-thaw	
T, °C	%MI	SE (%MI)	%MI	SE (%MI)
0	95.5	1.23	3.0	0.47
−3	95.5	1.55	23.0	2.99
−6	93.2	2.11	44.1	8.43
−9	94.3	1.53	50.2	7.80
−12	94.3	1.17	56.4	8.47
−15	93.3	1.11	61.8	7.24
−20	86.4	2.38	61.6	4.92
−30	30.8	2.95	25.4	2.44
−40	19.2	1.87	13.8	3.95

Flow Cytometry Data

$$\%\text{Recov} = \frac{\text{SYTO count after treatment}}{\text{SYTO count before treatment}} \times 100\% \qquad \textit{Equation (A1)}$$

Table A6. Measured cooling rates and cryoprotectant conditions for flow cytometry

Date of experiment	Cooling rate setpoint, °C/min	Cooling rate measured, °C/min	% CPA	CPA	Tn
2013-09-27	0.2	0.156	0	none	−3
2013-09-29	0.2	0.156	0	none	−3
2013-10-03	0.2	0.157	0	none	−3
2013-10-20	Two-step	na	0	none	−3
2013-11-29	Two-step	na	0	none	−3
2013-12-10	Two-step	na	0	none	−3
2014-01-17	1.0	0.892	0	none	−3
2014-02-11	1.0	0.79495	0	none	−3
2014-04-04	1.0	0.892	0	none	−3
2013-10-28	1.0	0.841	10.0	DMSO	−5
2013-12-14	1.0	0.820	10.0	DMSO	−5
2014-01-19	1.0	not recorded	10.1	DMSO	−5
2014-02-17	1.0	0.824	3.0/10.0	DMSO	−5
2014-03-03	1.0	not recorded	3.2/10.4	DMSO	−5
2014-03-05	1.0	not recorded	3.1/10.1	DMSO	−5
2013-10-30	1.0	0.816	10.2	DMSO	−5
2014-03-07	1.0	0.870	10.1	DMSO	−5
2014-03-09	1.0	0.861	10.0	DMSO	−5
2014-01-21	0.2	0.156	10.1	DMSO	−5
2014-03-29	0.2	0.157	10.1	DMSO	−5
2014-03-31	0.2	0.157	9.9	DMSO	−5
2014-01-23	Two-step	na	10.0	DMSO	−5

Date of experiment	Cooling rate setpoint, °C/min	Cooling rate measured, °C/min	% CPA	CPA	Tn
2014-04-02	Two-step	na	10.0	DMSO	−5
2014-04-06	Two-step	na	10.0	DMSO	−5
2014-04-16	1.0	not recorded	20.7	DMSO	−10
2014-04-18	1.0	not recorded	20.5	DMSO	−10
2014-04-20	1.0	not recorded	20.4	DMSO	−10
2014-04-22	1.0	not recorded	10.4/5.3	DMSO/HES	−5
2014-04-24	1.0	not recorded	10.3/5.3	DMSO/HES	−5
2014-04-26	1.0	not recorded	10.6/5.4	DMSO/HES	−5
2014-07-23	1.0	0.792	10.2/8.2	DMSO/HES	−10
2014-07-25	1.0	0.823	10.1/8.2	DMSO/HES	−10
2014-07-27	1.0	0.822	10.0/7.9	DMSO/HES	−10

Table A7. Measured HUVEC conditions for flow cytometry

Date of experiment	[Cells], 10^6 cells/ml	Cell doublings	Lot #	MI assay
2013-09-27	0.9	7.3	311953	SYTOEB FC
2013-09-29	1.2	9.5	311953	SYTOEB FC
2013-10-03	1.1	14.2	311953	SYTOEB FC
2013-10-20	1.5	8.0	311953	SYTOEB FC
2013-11-29	0.9	12.2	339730	SYTOEB FC
2013-12-10	0.8	7.5	339730	SYTOEB FC
2014-01-17	1.1	8.2	339730	SYTOEB FC
2014-02-11	1.2	7.3	339730	SYTOEB FC
2014-04-04	0.4	9.3	342222	SYTOPI
2013-10-28	1.1	17.0	311953	SYTOEB FC
2013-12-14	1.5	11.9	339730	SYTOEB FC
2014-01-19	1.2	10.3	339730	SYTOEB FC
2014-02-17	1.4	14.1	339730	SYTOEB FC
2014-03-03	1.0	6.2	342222	SYTOEB FC

Date of experiment	[Cells], 10^6 cells/ml	Cell doublings	Lot #	MI assay
2014-03-05	1.6	8.8	342222	SYTOEB FC
2013-10-30	1.0	19.0	311953	SYTOEB FC
2014-03-07	1.0	11.0	342222	SYTOEB FC
2014-03-09	1.0	13.1	342222	SYTOEB FC
2014-01-21	1.1	12.2	339730	SYTOEB FC
2014-03-29	0.6	5.0	342222	SYTOPI
2014-03-31	0.4	6.2	342222	SYTOPI
2014-01-23	1.3	14.2	339730	SYTOEB FC
2014-04-02	0.5	7.8	342222	SYTOPI
2014-04-06	0.6	10.6	342222	SYTOPI
2014-04-16	0.6	4.7	342222	SYTOPI
2014-04-18	0.8	6.9	342222	SYTOPI
2014-04-20	0.8	9.1	342222	SYTOPI
2014-04-22	0.8	11.4	342222	SYTOPI
2014-04-24	0.6	13.4	342222	SYTOPI
2014-04-26	0.8	15.7	342222	SYTOPI
2014-07-23	0.7	5.8	342222	SYTOPI
2014-07-25	0.8	8.1	342222	SYTOPI
2014-07-27	0.7	10.3	342222	SYTOPI

Table A8. HUVEC response without cryoprotectant to graded freezing at 0.2 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			91.2	0.35	100.0	0.0
Fresh_Post			91.5	0.87	122.7	11.5
Dead			0.3	0.05	0.4	0.1
Direct Thaw						
−3	−3.0	0.03	88.7	0.91	109.3	8.6
−6	−6.0	0.03	82.9	1.91	99.4	5.3
−9	−9.0	0.03	74.5	2.19	94.2	7.8
−12	−12.0	0.03	54.5	2.83	73.2	14.4
−15	−15.0	0.03	26.0	2.78	32.7	5.8
−20	−19.9	0.07	9.6	0.50	13.5	1.9
−30	−29.9	0.10	2.5	0.30	3.2	0.2
−40	−39.8	0.20	1.6	0.31	2.1	0.4
Plunge-thaw						
−3	−3.0	0.03	0.0	0.01	0.0	0.0
−6	−6.0	0.03	0.0	0.01	0.0	0.0
−9	−9.0	0.03	0.0	0.02	0.0	0.0
−12	−12.0	0.03	0.1	0.05	0.2	0.1
−15	−15.0	0.03	0.2	0.02	0.3	0.0
−20	−19.9	0.07	0.7	0.04	0.9	0.0
−30	−29.9	0.10	1.6	0.14	2.2	0.1
−40	−39.8	0.20	0.8	0.10	1.1	0.1

Table A9. HUVEC response without DMSO to two-step freezing

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			93.6	1.45	100.0	0.0
Fresh_Post			93.2	1.89	90.3	8.6
Dead			0.2	0.07	0.2	0.0
Direct Thaw						
−3	−3.2	0.03	89.8	1.62	95.0	8.1
−6	−6.2	0.00	85.8	1.64	85.6	5.9
−9	−9.0	0.09	47.5	17.71	53.7	22.7
−12	−12.0	0.03	16.0	7.22	20.7	11.4
−15	−15.0	0.10	3.2	1.39	3.8	1.9
−20	−19.9	0.07	0.5	0.23	0.6	0.2
−30	−29.7	0.03	0.2	0.09	0.1	0.1
−40	−39.4	0.00	0.0	0.00	0.0	0.0
Plunge-thaw						
−3	−3.2	0.03	0.0	0.00	0.0	0.0
−6	−6.2	0.00	0.1	0.10	0.1	0.1
−9	−9.0	0.09	0.0	0.00	0.0	0.0
−12	−12.0	0.03	0.1	0.03	0.1	0.1
−15	−15.0	0.10	0.1	0.00	0.1	0.0
−20	−19.9	0.07	0.0	0.03	0.0	0.0
−30	−29.7	0.03	0.0	0.00	0.0	0.0
−40	−39.4	0.00	0.0	0.00	0.0	0.0

Table A10. HUVEC response without DMSO to graded freezing at 1.0 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			92.9	0.68	100.0	0.0
Fresh_Post			90.6	1.16	83.2	9.4
Dead			0.1	0.03	0.1	0.0
Direct Thaw						
−3	−3.1	0.03	88.3	2.09	82.8	13.3
−6	−6.0	0.07	86.7	2.63	71.6	5.2
−9	−9.0	0.07	83.3	2.50	87.6	10.9
−12	−11.9	0.09	72.8	4.34	54.1	3.3
−15	−15.2	0.03	45.1	2.71	38.0	5.6
−20	−19.9	0.12	12.9	1.06	13.0	2.8
−30	−29.5	0.07	1.6	0.17	1.6	0.3
−40	−39.4	0.09	0.5	0.07	0.6	0.1
Plunge-thaw						
−3	−3.1	0.03	0.0	0.00	0.0	0.0
−6	−6.0	0.07	0.0	0.00	0.0	0.0
−9	−9.0	0.07	0.0	0.00	0.0	0.0
−12	−11.9	0.09	0.0	0.00	0.0	0.0
−15	−15.2	0.03	0.4	0.28	0.3	0.2
−20	−19.9	0.12	1.2	0.18	1.1	0.2
−30	−29.5	0.07	1.0	0.09	0.9	0.2
−40	−39.4	0.09	0.5	0.06	0.4	0.1

Table A11. 15 minute exposure of 10% DMSO to graded freezing at 1.0 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			92.8	0.25	100.0	0.00
CPA_pre			93.3	0.66	132.5	33.40
Fresh_Post			92.4	0.44	107.3	6.66
CPA_post			91.7	1.48	116.7	22.61
Dead			0.6	0.38	0.6	0.25
CPA_dead			0.7	0.38	0.8	0.47
Direct Thaw						
−5	−5.2	0.03	91.7	0.01	106.8	6.77
−10	−9.8	0.28	92.5	0.32	125.4	15.99
−15	−15.1	0.20	91.5	0.42	105.9	10.46
−20	−19.9	0.03	91.4	0.38	112.2	16.97
−25	−24.9	0.06	91.1	1.12	127.6	15.73
−30	−29.7	0.03	91.2	0.53	114.7	7.49
−35	−34.6	0.03	90.7	0.68	121.4	14.28
−40	−39.5	0.00	90.8	0.98	118.7	11.91
Plunge-thaw						
−5	−5.2	0.03	0.5	0.05	0.6	0.13
−10	−9.8	0.28	8.3	2.77	8.1	2.83
−15	−15.1	0.20	29.0	4.50	30.3	4.97
−20	−19.9	0.03	42.6	3.91	43.2	7.27
−25	−24.9	0.06	59.9	4.71	78.7	22.22
−30	−29.7	0.03	58.0	0.74	60.9	9.93
−35	−34.6	0.03	67.4	1.85	85.7	3.29
−40	−39.5	0.00	63.2	1.22	70.9	6.93

Table A12. 10 / 20 minute exposure of 3% / 10% DMSO to graded freezing at 1.0 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			93.1	0.05	100.0	0.00
CPA_pre			93.0	0.54	107.7	19.38
Fresh_Post			91.4	0.61	86.9	9.56
CPA_post			91.0	2.23	109.9	17.29
Dead			0.6	0.21	0.6	0.17
CPA_dead			4.6	0.25	4.8	0.72
Direct Thaw						
−5	−5.0	0.00	87.6	5.54	107.9	23.26
−10	−10.1	0.10	91.0	1.23	111.3	12.89
−15	−14.8	0.00	90.8	1.41	111.3	21.25
−20	−19.7	0.07	91.6	0.44	128.3	4.01
−25	−24.6	0.00	90.6	0.25	106.6	5.37
−30	−29.5	0.00	91.0	1.20	121.3	21.58
−35	−34.3	0.03	90.7	0.92	111.7	9.79
−40	−39.2	0.03	90.4	1.11	116.1	17.91
Plunge-thaw						
−5	−5.0	0.00	1.4	0.13	1.6	0.11
−10	−10.1	0.10	9.6	1.75	7.5	4.12
−15	−14.8	0.00	29.0	5.24	25.9	10.75
−20	−19.7	0.07	34.4	6.24	39.0	9.92
−25	−24.6	0.00	53.0	4.54	53.7	15.71
−30	−29.5	0.00	59.8	4.52	68.3	19.02
−35	−34.3	0.03	63.7	1.45	68.5	4.66
−40	−39.2	0.03	62.2	3.62	76.0	16.79

Table A13. 30 minute exposure of 10% DMSO to graded freezing at 1.0 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			91.2	1.28	100.0	0.00
CPA_pre			91.4	1.33	120.0	15.58
Fresh_Post			90.3	0.61	87.8	12.03
CPA_post			90.5	0.93	104.1	7.15
Dead			0.1	0.02	0.2	0.03
CPA_dead			2.1	0.67	2.4	0.81
Direct Thaw						
−5	−5.1	0.03	89.2	0.45	101.7	14.17
−10	−10.0	0.03	88.7	0.12	106.2	17.55
−15	−14.9	0.07	88.5	1.11	98.8	4.56
−20	−19.8	0.15	89.0	0.44	104.0	6.89
−25	−24.7	0.10	88.8	0.78	102.6	6.89
−30	−29.2	0.37	88.9	0.87	111.2	8.14
−35	−34.3	0.47	87.9	0.71	112.8	4.01
−40	−39.1	0.42	87.3	1.71	105.6	7.19
Plunge-thaw						
−5	−5.1	0.03	1.1	0.33	1.2	0.36
−10	−10.0	0.03	9.3	2.03	9.8	1.00
−15	−14.9	0.07	23.2	2.20	25.1	1.21
−20	−19.8	0.15	35.1	2.54	37.7	0.53
−25	−24.7	0.10	51.2	2.15	63.7	1.86
−30	−29.2	0.37	54.7	0.59	60.0	4.15
−35	−34.3	0.47	61.5	2.04	71.3	1.55
−40	−39.1	0.42	56.2	2.29	61.1	6.23

Table A14. 15 minute exposure of 10% DMSO to graded freezing at 0.2 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			92.9	0.33	100.0	0.00
CPA_pre			92.4	0.38	99.3	8.35
Fresh_Post			90.7	0.57	71.9	1.53
CPA_post			91.0	1.05	95.4	7.13
Dead			0.3	0.18	0.3	0.22
CPA_dead			2.3	0.88	2.4	1.05
Direct Thaw						
−5	−5.2	0.06	91.8	0.61	117.4	4.95
−10	−10.1	0.06	90.7	0.91	113.5	3.80
−15	−15.3	0.33	90.4	0.59	112.4	11.01
−20	−20.1	0.10	90.2	0.91	91.1	3.08
−25	−24.9	0.03	90.7	0.37	98.8	13.45
−30	−29.7	0.03	89.1	1.05	97.6	5.49
−35	−34.6	0.03	89.3	0.80	91.3	8.98
−40	−39.5	0.07	88.1	0.88	100.6	9.21
Plunge-thaw						
−5	−5.2	0.06	1.2	0.29	1.3	0.38
−10	−10.1	0.06	8.9	0.89	9.3	0.61
−15	−15.3	0.33	22.8	2.30	21.5	2.11
−20	−20.1	0.10	30.7	1.62	33.0	2.32
−25	−24.9	0.03	44.7	1.38	57.2	4.38
−30	−29.7	0.03	51.8	2.88	64.6	5.01
−35	−34.6	0.03	54.6	3.45	63.6	2.06
−40	−39.5	0.07	57.6	1.71	72.4	6.04

Table A15. HUVEC response with 15 minute exposure of 10% DMSO to two-step freezing

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			91.1	0.84	100.0	0.00
CPA_pre			90.6	1.21	105.8	13.85
Fresh_Post			90.9	0.21	103.8	21.32
CPA_post			90.5	0.46	124.1	19.73
Dead			0.5	0.14	0.6	0.14
CPA_dead			1.0	0.47	1.2	0.55
Direct Thaw						
−5	−5.1	0.07	90.7	0.21	154.3	15.55
−10	−10.1	0.07	88.5	0.42	134.8	25.33
−15	−15.0	0.09	88.1	0.42	108.8	8.52
−20	−19.9	0.00	80.7	2.50	123.4	22.20
−25	−24.9	0.03	64.0	2.35	87.8	8.82
−30	−29.8	0.00	52.3	2.00	70.3	7.41
−35	−34.7	0.07	37.8	0.98	47.6	5.74
−40	−39.6	0.03	25.8	0.77	31.2	3.54
Plunge-thaw						
−5	−5.1	0.07	1.0	0.16	1.2	0.26
−10	−10.1	0.07	3.2	0.56	3.6	0.33
−15	−15.0	0.09	20.8	6.41	22.9	7.81
−20	−19.9	0.00	31.5	3.03	35.4	5.59
−25	−24.9	0.03	29.2	5.25	37.4	9.18
−30	−29.8	0.00	32.5	0.81	42.2	2.31
−35	−34.7	0.07	20.3	1.31	25.3	0.77
−40	−39.6	0.03	13.1	1.18	16.0	1.63

Table A16. HUVEC response with 15 minute exposure of 20% DMSO to graded freezing at 1.0 °C/min

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			92.0	1.59	100.0	0.00
CPA_pre			84.6	0.44	64.8	18.20
Fresh_Post			91.1	1.16	82.1	16.70
CPA_post			77.1	2.41	30.7	15.05
Dead			0.2	0.12	0.5	0.38
CPA_dead			2.0	0.15	1.2	0.26
Direct Thaw						
−10	−9.9	0.12	85.8	2.36	57.4	7.61
−15	−14.8	0.17	86.5	1.92	67.9	7.97
−20	−19.8	0.19	85.2	1.74	76.9	5.05
−25	−24.6	0.15	85.6	1.65	75.8	7.07
−30	−29.5	0.15	86.0	1.67	77.3	7.30
−35	−34.5	0.23	84.8	2.76	68.7	4.64
−40	−39.4	0.19	85.4	1.75	72.9	3.89
−45	−44.2	0.15	84.2	1.86	72.0	5.09
Plunge-thaw						
−10	−9.9	0.12	6.0	1.09	5.0	1.16
−15	−14.8	0.17	25.0	1.99	17.5	2.29
−20	−19.8	0.19	36.4	1.32	24.5	4.89
−25	−24.6	0.15	45.9	2.28	33.4	5.71
−30	−29.5	0.15	52.3	3.74	33.5	6.79
−35	−34.5	0.23	53.3	1.75	32.8	2.63
−40	−39.4	0.19	59.2	2.21	41.5	2.79
−45	−44.2	0.15	56.6	1.64	36.8	3.79

Table A17. HUVEC response with 15 minute exposure of 10% DMSO plus 5% HES to graded freezing at a 1.0 °C/min cooling rate

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			94.2	0.84	100.0	0.00
CPA_pre			93.1	0.47	82.4	12.52
Fresh_Post			93.4	0.38	92.3	14.23
CPA_post			91.3	0.42	80.8	13.83
Dead			0.3	0.03	0.3	0.06
CPA_dead			1.5	0.38	1.1	0.40
Direct Thaw						
−10	−10.1	0.07	91.5	0.95	95.2	9.46
−15	−15.2	0.07	90.7	0.77	94.3	11.67
−20	−20.2	0.23	90.8	0.49	100.9	8.02
−25	−24.9	0.06	90.9	0.92	99.1	11.70
−30	−29.8	0.09	88.5	1.55	94.1	14.55
−35	−34.8	0.09	90.6	0.57	99.7	11.38
−40	−39.6	0.00	90.5	0.85	98.6	13.52
−45	−44.5	0.06	90.3	0.74	101.0	12.46
Plunge-thaw						
−10	−10.1	0.07	20.5	1.97	17.9	3.24
−15	−15.2	0.07	36.1	2.36	31.0	5.36
−20	−20.2	0.23	49.4	2.58	44.2	9.59
−25	−24.9	0.06	59.6	2.79	59.0	9.62
−30	−29.8	0.09	70.0	1.82	67.1	8.66
−35	−34.8	0.09	77.6	0.60	74.7	10.43
−40	−39.6	0.00	78.2	0.28	79.3	11.45
−45	−44.5	0.06	78.8	0.88	78.8	11.98

Table A18. HUVEC response with 15 minute exposure of 10% DMSO plus 8% HES to graded freezing at a 1.0 °C/min cooling rate

	T, °C	SE (T)	%MI	SE (%MI)	%Recov	SE (%Recov)
Fresh_Pre			91.7	0.69	100.0	0.00
CPA_pre			93.2	0.72	88.8	5.67
Fresh_Post			90.9	1.04	98.2	10.57
CPA_post			91.7	1.07	90.8	6.87
Dead			0.5	0.29	0.5	0.26
CPA_dead			2.3	0.34	2.0	0.33
Direct thaw						
−10	−10.1	0.12	86.6	3.75	86.6	5.29
−15	−14.7	0.36	90.6	0.80	92.6	2.64
−20	−20.2	0.23	90.9	0.97	99.0	7.77
−25	−24.7	0.09	90.9	1.10	105.6	10.41
−30	−29.7	0.12	90.6	1.18	100.0	7.67
−35	−34.7	0.09	90.1	1.23	100.7	10.08
−40	−39.5	0.10	90.2	1.13	100.6	4.92
−45	−44.4	0.12	90.2	0.57	101.2	4.60
Plunge-thaw						
−10	−10.1	0.12	33.9	1.97	33.2	3.64
−15	−14.7	0.36	52.3	1.50	53.7	2.90
−20	−20.2	0.23	63.6	1.60	64.3	5.81
−25	−24.7	0.09	69.9	1.17	74.9	6.34
−30	−29.7	0.12	77.5	1.77	82.5	6.61
−35	−34.7	0.09	81.2	1.07	86.4	5.87
−40	−39.5	0.10	82.9	0.85	83.5	3.80
−45	−44.4	0.12	83.6	1.58	88.7	6.02

HUVEC Culturing Procedure

Thawing of Cryopreserved HUVECs from LONZA

1. Clean biosafety cabinets using Accel TB wipes, ethanol may also be used. Use (2) wipes and make sure to wipe the entire working surface including the grate and outer edge.
2. Get ready sterile pipettes, pipettor and sterilized Pasteur pipettes.
3. Materials required include:
    a. (3) 5-mL serological pipettes (Fisherbrand, 1367811D)
    b. (2) 25-mL serological pipettes (Fisherbrand, 1367811)
    c. Autopipettor
    d. EGM-2 (Clonetics, CC-3162)
    e. (2) 50-mL centrifuge tubes (Fisherbrand, 0644319)
    f. (2) 75-cm^2 tissue culture flasks (T-flask) (Corning, 1012637)
    g. Individually packaged 1000 µL pipette tips (Eppendorf, 0540382)
    h. 100 – 1000 µL pipette tips
    i. Accel TB wipes
    j. Solution of 70% histoprep ethanol
    k. Phase contrast microscope with image capture software
4. Warm EGM-2 to at least room temperature in a 37 °C water bath
5. Clean EGM-2 bottle using paper towels soaked in 70% ethanol before transferring to the biosafety cabinet
6. Add 15 mL EGM-2 to each 75-cm^2 T-flask
7. Place in 37 °C incubator until HUVEC (Clonetics, c2519a) are thawed and ready
8. Transfer 5 mL or more EGM-2 to a 50-mL centrifuge tube
9. Thaw the 1 mL cryovial in a 37 °C water bath

At this point, membrane integrity of *undiluted* HUVEC can be measured. Refer to the following section, “Membrane Integrity Analysis of Supplied HUVEC”.

10. Transfer to a 50-mL centrifuge tube
11. Add dropwise 5 mL EGM-2 to the 1 mL HUVEC. This can be done using a 1000 µL pipette with an individually wrapped sterile tip for each milliliter of media.

At this point, membrane integrity of *diluted* HUVEC can be tested. Refer to the following section, "Membrane Integrity Analysis of Supplied HUVEC".

12. Transfer 3 mL of EGM+HUVEC to each 75-cm^2 T-flask
13. Rock T-flasks to mix well
14. Label T-flasks using a black sharpie and place in incubator at 37 °C and 5% CO_2 overnight
15. Clean biosafety cabinet as described in Step 1

Within 24 hours,

16. Clean the biosafety cabinet as described in Step 1
17. Get ready sterile pipettes, pipettor and sterilized Pasteur pipettes. Materials required include:
    a. EGM-2, at least 30 mL
    b. (2) 25mL serological pipette
    c. Autopipettor
18. Let reagents warm to at least room temperature in the 37 °C water bath
19. Using the Labovert phase contrast microscope, capture images of HUVEC adhered within the T-flask
20. If there is contamination, STOP experiments until contamination issue has been resolved.
21. Transfer all reagents to the biosafety cabinet using proper aseptic technique which requires that reagent bottles be wiped down with paper towels soaked in 70% ethanol solution
22. Turn on vacuum and aspirate media from the T-flasks using Pasteur pipettes and collect in the vacuum flask with bleach for disposal
23. Add 15 mL EGM-2 to each 75-cm^2 T-flask using the autopipettor

24. Tighten lids, return T-flask to 37 °C incubator at 5% CO_2, and return reagents to the fridge. Make sure all reagents are labeled with your initials and the date it was opened.
25. Empty vacuum flask when necessary, as vacuum flasks which contain too much liquid can be aspirated into the vacuum filter thereby plugging it.
26. Clean biosafety cabinets as above in step 1
27. Make sure microscopes are turned off, water bath is covered and to discard gloves in biosafety waste boxes before leaving the lab area
28. Change media every 2 days until criteria for passaging HUVEC are met; refer to the section, "Criteria for Passaging HUVEC".

Membrane Integrity Analysis of Supplied HUVECs

To analyze *undiluted* HUVEC for membrane integrity, take 400 µL after step 9 and perform membrane integrity assessment. Use the remaining 600 µL for cell culture.

To analyze *diluted* HUVEC for membrane integrity, the Materials required include:

a. EGM-2, at least 30 mL
b. (2) 25 mL serological pipette
c. Autopipettor

1. This procedure is to be followed after step 11 for *diluted* HUVEC from the section above, "Thawing of Cryopreserved HUVECs from LONZA".
2. Take 1 mL from the 6 mL EGM+HUVEC sample
3. Measure membrane integrity for (2) 400 µL samples using flow cytometry

Criteria for Passaging HUVECs

To have confidence in the consistency of cells from experiment to experiment, refer to Figure A1, the HUVEC growth curve prepared by Michal Zielinski. It provides concentrations of cell suspensions as measured using the Coulter counter and phase contrast images of HUVEC in culture flasks for comparison. HUVEC are passaged after 2 days.

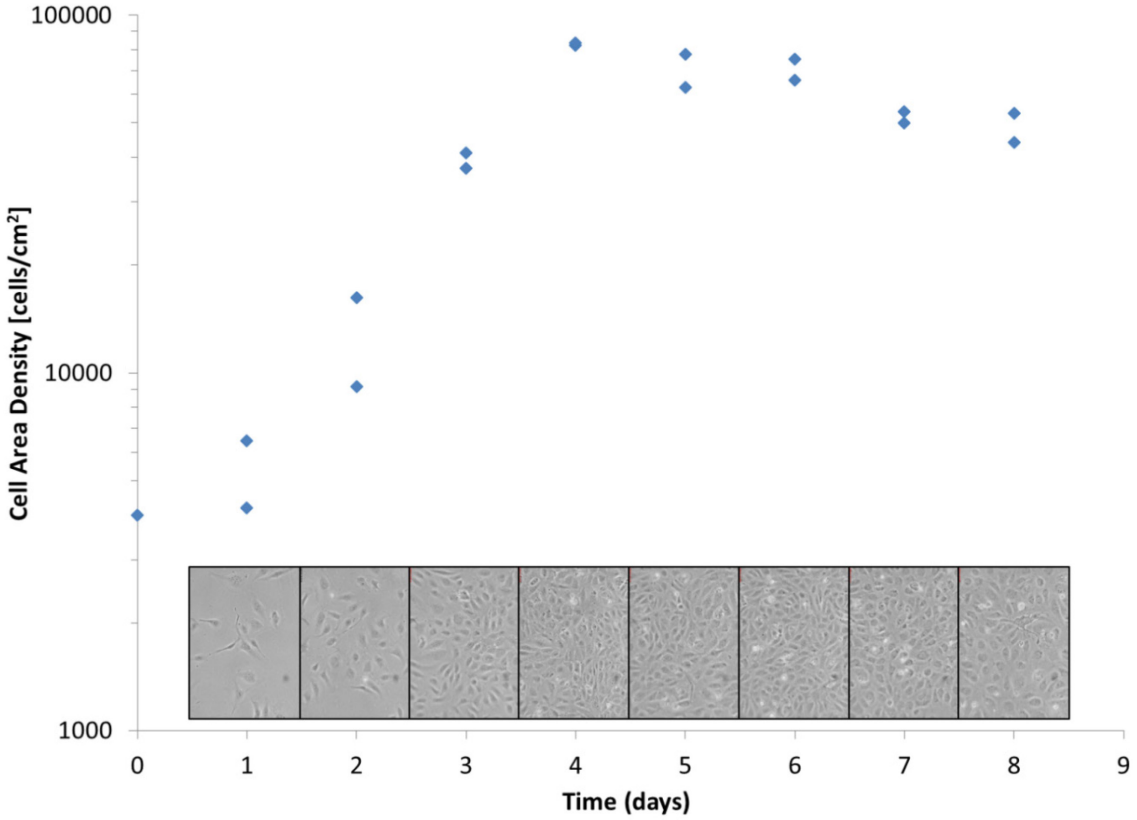

Figure A1. HUVEC growth after seeding from sub-culture prepared by Michal Zielinski

Changing Endothelial Growth Medium (EGM-2)

If HUVEC cultures are to be grown within the same tissue culture flask (T-flask) for more than two days, then the EGM-2 must be changed. To feed adherent HUVEC:

1. Change EGM-2 every 2 days unless passaging HUVEC within 2 days of seeding
2. Make sure the vacuum flask has sufficient volume for experiments and contains 100 – 200 mL bleach
3. Clean the biosafety cabinets using Accel TB wipes. Use (2) wipes and make sure to wipe the entire working surface including the grate and outer edge
4. Get ready sterile pipettes, pipettor and sterilized Pasteur pipettes. Refer to Table A1 for necessary volumes to select pipettes. For example, (1) 150-cm^2 T-flask planning for >45% coverage requires the following materials:

a. EGM-2, at least 60 mL
b. (1) 50mL serological pipette (maximum volume is 60 mL)
c. Autopipettor
d. (1) Pasteur pipette

5. Let reagents warm to at least room temperature in the 37 °C water bath
6. Using the Labovert phase contrast microscope, capture images of HUVEC adhered within the T-flask
7. If there is contamination, STOP experiments until contamination issue has been resolved.
8. Transfer all reagents to the biosafety cabinet using proper asceptic technique which requires that reagent bottles be wiped down with 70% ethanol solution
9. Turn on vacuum and aspirate media from the T-flasks using Pasteur pipettes and collect in the vacuum flask for discard
10. Add fresh EGM-2 to cells using the autopipettor
11. Tighten lids, return T-flask to 37 °C incubator at 5% CO_2, and return reagents to the fridge. Make sure all reagents are labeled with your initials and the date it was opened.
12. Empty vacuum flask if necessary.
13. Clean biosafety cabinets as above in step 4
14. Make sure microscopes are turned off, water bath is covered and to discard gloves in biosafety waste boxes before leaving the lab area

Table A19. Endothelial growth medium (EGM-2) volumes required depending on flask size and cell coverage at passaging

Tissue culture flask	Coverage <25%	Coverage 25-45%	Coverage >45%
75 cm^2	15 mL	22.5 mL	30 mL
150 cm^2	30 mL	45 mL	60 mL

Note that EGM-2 must be changed every 2 days at least. Whenever the flasks are removed from the incubator, pictures of cells must be taken to ensure proper growth without contamination or improper seeding.

Passaging and Seeding HUVECs

Passaging HUVECs is basically the removal of the endothelial cells from the growing surface of the tissue culture flask (T-flask) either for experiments or to continue growth in another T-flask. Seeding is the act of adding a certain number of cells to a T-flask so as to grow to a larger population. Table A20 shows the volumes of reagents required for the passaging of HUVEC and the procedural steps are provided below.

Table A20. Reagent volumes used in passaging of HUVEC for (2) different T-flask sizes

Step	Reagent	Reagent volume	
		75-cm^2 T-flask	150-cm^2 T-flask
1	HEPES buffered saline	15 mL	30 mL
2	Trypsin	6 mL	12 mL
3	Trypsin neutralizing solution	12 mL	24 mL
4	HEPES buffered saline	6 mL	12 mL
5	Endothelial growth media (EGM-2)	1 mL	2 mL

1. Make sure the vacuum flask has a sufficient volume available to aspirate all T-flasks and reagents throughout the passaging procedure. Volumes of solution include the EGM-2 previously in the T-flask, HEPES buffered saline from Table A20, Step 1 and after centrifugation, the contents from Table A20, Step 2 to Step 4.
2. Ensure vacuum flask contains approximately 200 mL bleach
3. Clean biosafety cabinets using Accel TB wipes. Use (2) wipes and make sure to wipe the entire working surface including the grate and outer edge
4. Get ready sterile pipettes, pipettor and sterilized Pasteur pipettes. Refer to Table A19 and Table A20 for necessary volumes to select pipettes. For example, (1) 150-cm^2 T-flask for passaging and seeding requires the following materials:
    a. Passaging
        i. HEPES buffered saline, 42 mL
        ii. Trypsin, 12mL
        iii. Trypsin neutralizing solution, 24 mL

iv. EGM-2, 2 mL
v. Autopipettor
vi. (2) 25 mL serological pipettes
vii. (2) 10 mL serological pipettes
viii. (2) 50 mL centrifuge tubes
ix. Centrifuge tube rack or holder
x. Vacuum flask with inline vacuum filter and connecting tubing
xi. 100 – 1000 µL pipette
xii. 100 – 1000 µL individually wrapped pipette tips
xiii. Timer
xiv. Labovert phase contrast microscope for visualization
xv. PC to capture images of HUVEC for quality analysis
xvi. Incubator, 37 °C, 5% CO2

b. Seeding
i. Tissue culture flasks (T-flask)
ii. EGM-2, refer to Table A19 for volume required for T-flask
iii. (1) 50 mL centrifuge tube
iv. Particle Counter
v. 10-100 µL pipette and individually wrapped tips
vi. 100-1000 µL pipette and individually wrapped tips
vii. Calculator

5. Let reagents thaw in the 37 °C water bath
6. Using the Labovert phase contrast microscope, capture images of HUVEC adhered within the T-flask
7. If there is contamination, STOP experiments until contamination issue has been resolved.
8. Transfer all reagents to the biosafety cabinet using proper asceptic technique which requires that reagent bottles be wiped down with 70% ethanol solution
9. At this time, if seeding will take place, add EGM-2 to T-flasks (to be seeded with HUVEC) and place in the incubator at 37 °C and 5% CO_2 until the seeding volume has been calculated

10. Turn on vacuum and aspirate media from the T-flasks using Pasteur pipettes and collect in the vacuum flask for discard
11. Add HEPES buffered saline to T-flask as referred to in Table A20, Step 1. Rock T-flasks to mix well and aspirate the HEPES buffered saline from the T-flask
12. This is a time-sensitive step. Note the timings may vary between 1:30 to 7:00 minutes depending on the temperature and activity of trypsin
13. Add trypsin as referred to in Table A20, Step 2; Rock T-flasks to mix well.
14. Watch HUVEC rounding up under the Labovert phase contrast microscope:
    a. At about 2:00 minutes tap the T-flask against the counter to dislodge loose HUVEC
    b. By about 2:30 minutes >90% of HUVEC should be detached – return to biosafety cabinet
15. At 3:00 minutes, add trypsin neutralizing solution as referred to in Table A20, Step 3. Use the autopipettor to pipette up and down vigorously to mix well and to stop HUVEC from clumping.
16. If HUVEC are not mostly detached within 3:00 minutes, this could be a result of using low activity trypsin or mistakenly skipping the wash step with HEPES buffered saline. Wait for >90% HUVEC to detach or wait until 7:00 minutes. If HUVEC does not detach within 7:00 minutes, discard the T-flask or add trypsin neutralizing solution, transfer the contents to a centrifuge tube and try the trypsin and trypsin neutralizing solution step again.
17. Transfer HUVEC suspension from the T-flask to the 50-mL centrifuge tube. At this point, this solution contains trypsin and trypsin neutralizing solution.
18. Wash flask with HEPES buffered saline as referred to in Table A20, Step 4 to recover remaining HUVEC
19. Transfer HEPES buffered saline from the T-flask to the 50-mL centrifuge tube. At this point, the 50-mL centrifuge tube contains trypsin, trypsin neutralizing solution and HEPES buffered saline.
20. Before using the centrifuge, refer to the centrifuge safe operating procedure.
21. Centrifuge the 50-mL centrifuge tube using the Beckman TJ-6 centrifuge located in Dr. Acker's lab.

22. Centrifuge for 5 minutes at 1000 rpm, which corresponds to the pre-set program 3.
23. Meanwhile check the empty T-flasks using the Labovert phase contrast microscope for any remaining cells
24. Return centrifuge tubes to the biosafety cabinet, noting the position of the cell pellet in the bottom of the centrifuge tube
25. Carefully aspirate off the supernatant leaving approximately 100 µL of liquid above the undisturbed pellet. Aspirate by holding the centrifuge tube at an angle to minimize disturbing the cell pellet.
26. Resuspend the pellet in EGM-2 as referred to in Table A20, Step 5; using a 100-1000 µL pipet and pipetting up and down until no clumps are visible. Avoid creating foam with too vigorous pipetting
27. Prepare the Coulter counter to determine the cell density by running the 'flush aperture' function using a newly obtained 10 mL isoton solution. Ensure there are no bubbles in the line which can affect the accuracy of the 10 mL isoton solution and increase variability when calculating cell concentration.
28. Mix well the HUVEC suspension in the 50-mL centrifuge tube before adding 100 µL HUVEC suspension in 10 mL ISOTON
29. Using the Coulter counter, perform two sequential counts
    a. Note cell distribution (graphical display). Record the cell counts for events between 10 µm and 20 µm.
    b. To calculate the concentration of HUVECs, multiply the number provided by the Coulter counter (which has units of cells/mL) by the dilution factor. The dilution factor is 10.1 mL/ 0.1 mL which is 101.
    c. To calculate the total number of HUVEC, multiply the concentration by the volume of HUVEC suspension.
    d. Debris and cell aggregates will give erroneous cell titers – it is good to count a 10 µL HUVEC suspension with a hemocytometer (grid volume = 0.1 µL) for comparison.
30. Take T-flasks out of incubator and place them in the biosafety cabinet for seeding.
31. Seed T-flasks for 5000 cells/cm^2

32. For experiments, extra EGM-2 should be kept in a 50-mL centrifuge tube; perhaps 10 mL or more to be used for temperature measurements and preparation of cryoprotectant solutions
33. Tighten lids, return T-flask to 37 °C incubator at 5% CO_2, and return reagents to the fridge. Make sure all reagents are labeled with your initials and the date it was opened.
34. Empty vacuum flask when necessary.
35. Clean biosafety cabinets as above in step 3
36. Make sure microscopes are turned off, the water bath is covered and discard gloves in biosafety waste boxes before leaving the lab area

Interrupted Cooling Protocols

After passaging, keep cells on ice in preparation for experiments. Placing HUVECs on ice reduces HUVEC clumping.

Freezing Point

Take special note that with the addition of cryoprotectants, the freezing point may affect the −3 °C direct thaw and plunge-thaw sample. If the freezing point is lower, then it may be necessary to calculate the freezing point to choose an appropriate temperature for the first direct thaw and plunge-thaw sample. To calculate the freezing point, use equation 2 [143]:

$$T_{FP}^{0} - T_{FP} = \frac{\left[W_1/(\overline{s_1^{0L}} - \overline{s_1^{0S}})\right] R T_{FP}^{0} \pi}{1 + \left\{\left[W_1/(\overline{s_1^{0L}} - \overline{s_1^{0S}})\right] R\pi\right\}} \qquad \textit{Equation (A2)}$$

T_{FP}^{0} ≡ Freezing point of pure water (273.15 K)

T_{FP}≡ Freezing point of solution (K)

W_1 ≡ Molecular weight of water, 0.01802kg/mol

$\overline{s_1^{0L}}$ ≡ Entropy per mole of pure liquid water, J/mol×K

$\overline{s_1^{0S}}$ ≡ Entropy per mole of pure water in the solid phase, J/mol×K

$\overline{s_1^{0L}} - \overline{s_1^{0S}}$ = 22.00 J/mol×K

R ≡ Universal gas constant, 8.314 J/mol×K

π ≡ Osmolality

To calculate the osmolality of a DMSO solution, equation 3 can be used:

$$\pi = m_1 + B_1 m_1^2 \qquad \textit{Equation (A3)}$$

m_1 ≡ molality, mol/kg

B_1 ≡ First osmotic virial coefficient for DMSO, 0.108 kg/mol [143]

Methanol Bath

As with all lab equipment, instructions on safe operation can be found in the binder, "Equipment Use and Experimental Procedures". There are also manufacturer's instructions which can be found in the filing cabinet in the lab. Some important things to do are to read the MSDS about methanol, which can be found in one of the (5) binders of MSDS.

To use the methanol bath, there are three buttons on the methanol bath, the 'Power' button, a '△' button and a '▽' button. The manufacturer's instructions provide more information.

1. Press the 'Power' button to turn on the methanol bath
    a. The display now reads the current temperature of methanol in the bath
2. Press the '△' button to access the setpoint temperature (displayed as SP)
    a. Press the '△' or '▽' button to change the setpoint temperature
    b. The temperature setpoint is fixed when the display has stopped blinking (approximately 3 seconds)
3. Press and hold the '△' and '▽' buttons at the same time, for about 2 seconds, to access the PID controller
    a. Press the 'Power' button to select the PID menu
    b. Ramp rate (rr) is displayed, so use the '△' or '▽' button to set the cooling rate to 0.2°C/min
    c. Press the 'Power' button to cycle through the PID menu (rr, HPb, It, dt)
    d. Once you reach the current temperature display, the cooling rate is set. If you hit the 'Power' button too many times, you may accidentally turn off the methanol bath but turning the methanol bath back on is an easy fix. Make sure the methanol bath is mixing when you turn it back on.
4. Set stirrer speed by pressing the '▽' button
    a. Pressing once toggles the stirrer on or off
    b. To adjust stirrer speed, use the '△' or '▽' to adjust the stirrer speed (at least 70)
    c. Stirrer speed is set once display returns to the current methanol bath temperature
5. To set the temperature display between °C, °F and Kelvin, press both the '△' and '▽' button and hold for 2 seconds
    a. Use the '△' button to display 'Etc'

b. Use the ‘Power’ button to select ‘Etc’
c. Use the ‘Power’ button to select ‘deG’
d. Use the ‘△’ or ‘▽’ button to choose
    i. 0 ≡ °C
    ii. 1 ≡ °F
    iii. 2 ≡ K
e. Press the ‘Power’ button (3) times to return to the methanol bath temperature display

Two-Step Freezing Procedure

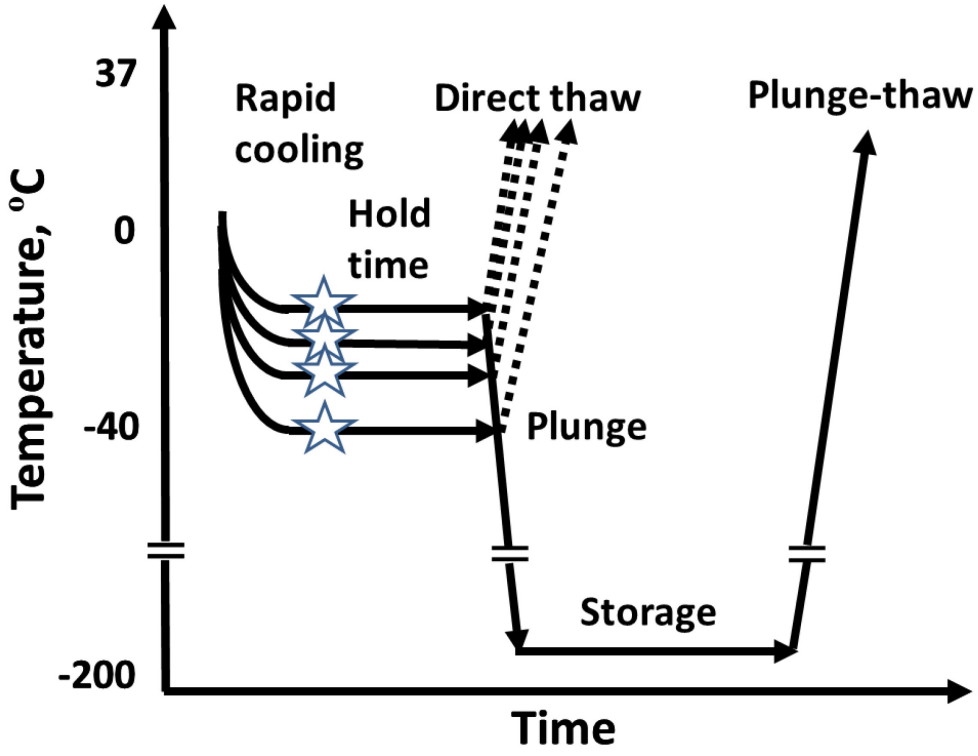

Figure A2. Temperature versus time for the two-step freezing procedure

Table A21 shows all the HUVEC samples prepared for both two-step and graded freezing without cryoprotectant. Two culture tubes containing 200 µL HUVEC suspensions were prepared for each sample. Thermocouples were referenced using a 0 °C ice-water bath and temperature was recorded for each sample. HUVECs were either thawed directly from hold temperatures or plunged into liquid nitrogen from hold temperatures and stored for at least an hour prior to rapid thawing. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL. Hold temperatures were between −3 °C and −40 °C.

Table A21. HUVEC samples for two-step and graded freezing experiments

Sample description	Comments
Live cells (pre-experiment)	Fresh HUVECs on ice after cell culture and handling
Live cells (post-experiment)	HUVECs on ice throughout the experiment
Dead cells	HUVECs plunged into liquid nitrogen from 0 °C
−3 °C direct thaw	
−3 °C plunge-thaw	
−6 °C direct thaw	
−6 °C plunge-thaw	
−9 °C direct thaw	
−9 °C plunge-thaw	
−12 °C direct thaw	
−12 °C plunge-thaw	
−15 °C direct thaw	
−15 °C plunge-thaw	
−20 °C direct thaw	
−20 °C plunge-thaw	
−30 °C direct thaw	
−30 °C plunge-thaw	
−40 °C direct thaw	
−40 °C plunge-thaw	

Figure A2 shows the two-step freezing procedure and Table A21 above describes the samples which are discussed below.

1. Put on appropriate personal protective equipment (PPE) (e.g. gloves, safety glasses, lab gown)
2. Set the programmable methanol cooling bath to −3 °C with mixing speed of at least 70

3. Ensure methanol bath is mixing and insert a polystyrene float to allow samples to be placed without falling into the methanol bath
4. Turn on flow cytometer and fluorescence microscope if necessary
5. Prepare SYTOPI and keep it on ice protected from light
6. Begin temperature data acquisition program to record the temperature throughout the experiment
7. Reference thermocouples using an ice-water bath (0 °C at equilibrium).
8. Place (2) culture tubes with 200 µL EGM-2 into the methanol bath with type-T thermocouples
9. Nucleate the (2) culture tubes using liquid nitrogen cooled forceps
10. Place (1) thermocouple directly into the methanol bath
11. Leave (1) thermocouple to record room temperature
12. Leave (1) thermocouple in the ice-water bath
13. Place O-rings on (38) culture tubes
14. Add 100 µL HUVECs to (8) culture tubes
15. Add 100 µL EGM-2 to these same (8) culture tubes
16. Start timer and incubate for 15 minutes on ice
17. During the incubation time, place corks on the (8) culture tubes
18. Leave (2) culture tubes of HUVECs on ice until experiments for direct thaw are complete
19. Plunge (2) culture tubes of HUVECs into liquid nitrogen for 'Dead cells'
20. Analyze (2) culture tubes of fresh HUVECs without any dye to observe light scatter
21. Analyze the 'Live cells (pre-experiment)' using the SYTOPI membrane integrity assay
22. Place forceps into liquid nitrogen
23. Add 100 µL HUVECs to each of (4) culture tubes
24. Add 100 µL EGM-2 to each of these same (4) culture tubes (two culture tubes for direct thaw and two for plunge-thaw)
25. Cork the tubes and incubate 15 minutes on ice
26. Record the hold temperature to the nearest decimal place using the (2) thermocouples in culture tubes in the methanol bath
27. Place the (4) culture tubes in the methanol bath
28. Start timer

29. Allow culture tubes to equilibrate for 2 minutes at the −3 °C hold temperature
30. Use liquid nitrogen cooled forceps to induce ice nucleation in the (4) samples
31. Incubate at the −3 °C hold temperature for the 3 minute hold time
32. Transfer the −3 °C plunge-thaw samples to liquid nitrogen
33. Transfer the −3 °C direct thaw samples to the 37 °C water bath only until thawing is complete.
34. After thawing is complete, perform SYTOPI membrane integrity analysis
35. Set the methanol bath to the next hold temperature
36. While waiting for the methanol bath to equilibrate at its setpoint temperature, analyze the direct thaw samples
37. Repeat steps 21 through 35 until all direct thaw and plunge-thaw samples are complete
38. Turn off the methanol bath
39. Analyze the ‘Live cells (post-experiment)’
40. Analyze the plunge-thaw samples
41. Analyze the ‘Dead cells’
42. Turn off the microscope and allow 15 minutes cool down before replacing the cover
43. Save the temperature data using the data acquisition system
44. If using the fluorescence microscope, backup the saved images
45. If using the flow cytometer, begin the cleanup panel then backup the data and turn off the flow cytometer
46. Dispose of SYTOPI-contaminated samples in an empty bleach bottle
47. Use 70% ethanol to clean thermocouples
48. Leave excess liquid nitrogen to evaporate
49. Save O-rings and corks for future experiments
50. Discard gloves in biohazard waste boxes
51. Clean up and wash hands before leaving the lab area

Two-Step Freezing Procedure with Cryoprotectant

Addition of cryoprotectant required that the freezing point be calculated prior to experimentation and the methanol bath was set to −3 °C or at least 3 °C less than the freezing point. Note that cryoprotectant was not removed prior to membrane integrity assessment.

Table A22 shows all the HUVEC samples prepared for both two-step and graded freezing with cryoprotectant. Two culture tubes containing 200 μL HUVEC suspensions in the presence of cryoprotectant were prepared for each sample. Thermocouples were referenced using a 0 °C ice-water bath and temperature was recorded for each sample. HUVECs were either thawed directly from hold temperatures or plunged into liquid nitrogen from hold temperatures and stored for at least one hour prior to rapid thawing. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL. Hold temperatures were between −5 °C and −40 °C.

Table A22. HUVEC samples for two-step and graded freezing experiments with cryoprotectant

Sample	Description	Comments
1	Live cells (pre-experiment)	Fresh HUVECs on ice after cell culture and handling
2	Live cells (post-experiment)	HUVECs on ice throughout the experiment
3	Live cells with cryoprotectant (pre-experiment)	Fresh HUVECs on ice after cell culture and handling in the presence of cryoprotectant
4	Live cells with cryoprotectant (post-experiment)	HUVECs on ice throughout the experiment in the presence of cryoprotectant
5	Dead cells	HUVECs plunged into liquid nitrogen from 0 °C
6	Dead cells with cryoprotectant	HUVECs plunged into liquid nitrogen from 0 °C in the presence of cryoprotectant
7	−5 °C direct thaw*	These temperatures depend on the freezing point of HUVECs in cryoprotectant solution
8	−5 °C plunge-thaw*	
9	−10 °C direct thaw	
10	−10 °C plunge-thaw	
11	−15 °C direct thaw	

Sample	Description	Comments
12	−15 °C plunge-thaw	
13	−20 °C direct thaw	
14	−20 °C plunge-thaw	
15	−25 °C direct thaw	
16	−25 °C plunge-thaw	
17	−30 °C direct thaw	
18	−30 °C plunge-thaw	
19	−35 °C direct thaw	
20	−35 °C plunge-thaw	
21	−40 °C direct thaw	
22	−40 °C plunge-thaw	

* These temperatures may be different depending on the freezing point of the solution

For two-step freezing, cryoprotectant was added to each sample in the culture tubes (as opposed to preparing a bulk solution of HUVECs with cryoprotectant for graded freezing). The following steps can be followed assuming preparation of 10% DMSO with HUVECs. Note that the stock cryoprotectant solution was prepared before lunch to avoid the risk of contamination. Calculating the percentage of cryoprotectant was different using a powdered cryoprotectant because a volume-percent calculation was not possible.

1. Stock cryoprotectant solution (20% DMSO)
    - Label (1) 50-mL centrifuge tube as ‘DMSO’ with initials and date
    - Record the mass of the centrifuge tube
    - Add 5.0g EGM-2 (approximately 5.0 mL, assuming 1.0 g/mL EGM-2 density)
    - Record the mass and volume of EGM-2 added
    - Add 1.25g DMSO (approximately 1110 µL, assuming 1.1 g/mL DMSO density)
    - Record the mass and volume of DMSO added
    - Keep on ice
    - Calculate the % mass of DMSO (it should be close to 20%)

2. Addition of stock cryoprotectant solution to HUVECs in EGM-2
    - HUVECs in EGM-2 is kept at 0 °C
    - Add 100 µL HUVECs in EGM-2 to each of (4) culture tubes (two culture tubes for direct thaw and two for plunge-thaw)
    - Add 100 µL of 20% DMSO in each of the same (4) culture tubes
    - Mix the 200 µL culture tubes by pipetting up and down at least three times
    - Allow 15 minute equilibration time at 0 °C
    - Calculate the % mass of DMSO (it should be close to 10% and assumes that the density of HUVECs in EGM-2 is 1.0 g/mL)

Figure A2 shows the two-step freezing procedure and Table A22 describes all the samples which are discussed below. This procedure assumed that −5 °C hold temperature was the first desired setpoint temperature. Since cryoprotectant was used, the freezing point was calculated and the first setpoint temperature was adjusted to −5 °C or at least 3 °C below the freezing point. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL:

1. Put on appropriate personal protective equipment (PPE) (e.g. gloves, safety glasses, lab gown)
2. Set the programmable methanol cooling bath to −5 °C or 3 °C less than the freezing point with mixing speed of at least 70
3. Ensure methanol bath is mixing and insert a polystyrene float to allow samples to be placed without falling into the methanol bath
4. Turn on the flow cytometer and fluorescence microscope if necessary
5. Prepare SYTOPI and keep it on ice protected from light
6. Begin temperature data acquisition program to record the temperature throughout the experiment
7. Record thermocouple temperature using an ice-water bath (0 °C at equilibrium).
8. Place (3) culture tubes with 100 µL EGM-2 and 100 µL stock cryoprotectant solution into the methanol bath with type-T thermocouples

9. Nucleate the (2) culture tubes using liquid nitrogen cooled forceps
10. Place (1) thermocouple directly into the methanol bath
11. Leave (1) thermocouple to record room temperature
12. Leave (1) thermocouple in the ice-water bath
13. Place O-rings on (44) culture tubes
14. Add 100 µL HUVECs to (12) culture tubes
15. Add 100 µL EGM-2 to (6) of the (12) culture tubes and 100 µL stock cryoprotectant solution to the other (6) of the (12) culture tubes from the previous step
16. Start timer and incubate for 15 minutes at 0 °C
17. During the incubation time, place corks on the (12) culture tubes
18. Leave (2) culture tubes of HUVECs and (2) culture tubes of HUVECs in cryoprotectant on ice until experiments for direct thaw are complete
19. Plunge (2) culture tubes of fresh HUVECs into liquid nitrogen for 'Dead cells' and (2) culture tubes of fresh HUVECs with cryoprotectants for 'Dead cells with cryoprotectant' into liquid nitrogen
20. Analyze (2) culture tubes of fresh HUVECs without any dye and (2) culture tubes of fresh HUVECs in cryoprotectant without any dye to observe light scatter
21. Analyze the 'Live cells (pre-experiment)' and the 'Live cells with cryoprotectant (pre-experiment)' using the SYTOPI membrane integrity assay
22. Prepare liquid nitrogen cooled forceps
23. Add 100 µL HUVECs to each of (4) culture tubes
24. Add 100 µL of stock cryoprotectant solution to each of these same (4) culture tubes (two culture tubes for direct thaw and two for plunge-thaw)
25. Cork the tubes and incubate for 15 minutes at 0 °C
26. Record the hold temperature to the nearest decimal place using the (2) thermocouples in culture tubes in the methanol bath
27. Place the (4) culture tubes in the methanol bath
28. Start timer
29. Allow culture tubes to equilibrate for 2 minutes at the hold temperature
30. Use liquid nitrogen cooled forceps to induce ice nucleation in the (4) samples
31. Incubate at the hold temperature for the 3 minute hold time

32. Transfer the plunge-thaw samples to liquid nitrogen
33. Transfer the direct thaw samples to the 37 °C water bath only until thawing is complete.
34. After thawing is complete, perform SYTOPI membrane integrity analysis
35. Set the methanol bath to the next hold temperature
36. While waiting for the methanol bath to equilibrate at its setpoint temperature, analyze the direct thaw sample
37. Repeat steps 21 through 35 until all direct thaw and plunge-thaw samples are prepared
38. Turn off the methanol bath
39. Analyze the 'Live cells (post-experiment)' and 'Live cells with cryoprotectant (post-experiment)'
40. Analyze the plunge-thaw samples
41. Analyze the 'Dead cells' and the 'Dead cells with cryoprotectant'
42. Turn off the microscope and allow 15 minutes cool down before replacing the cover
43. Save the temperature data using the data acquisition system
44. If using the fluorescence microscope, backup the saved images
45. If using the flow cytometer, begin the cleanup panel then backup the data and turn off the flow cytometer
46. Dispose of SYTOPI-contaminated samples in an empty bleach bottle
47. Use 70% ethanol to rinse thermocouples
48. Leave excess liquid nitrogen to evaporate
49. Save O-rings and corks for future experiments
50. Discard gloves in biohazard waste boxes
51. Clean up and wash hands before leaving the lab area

Graded Freezing Procedure

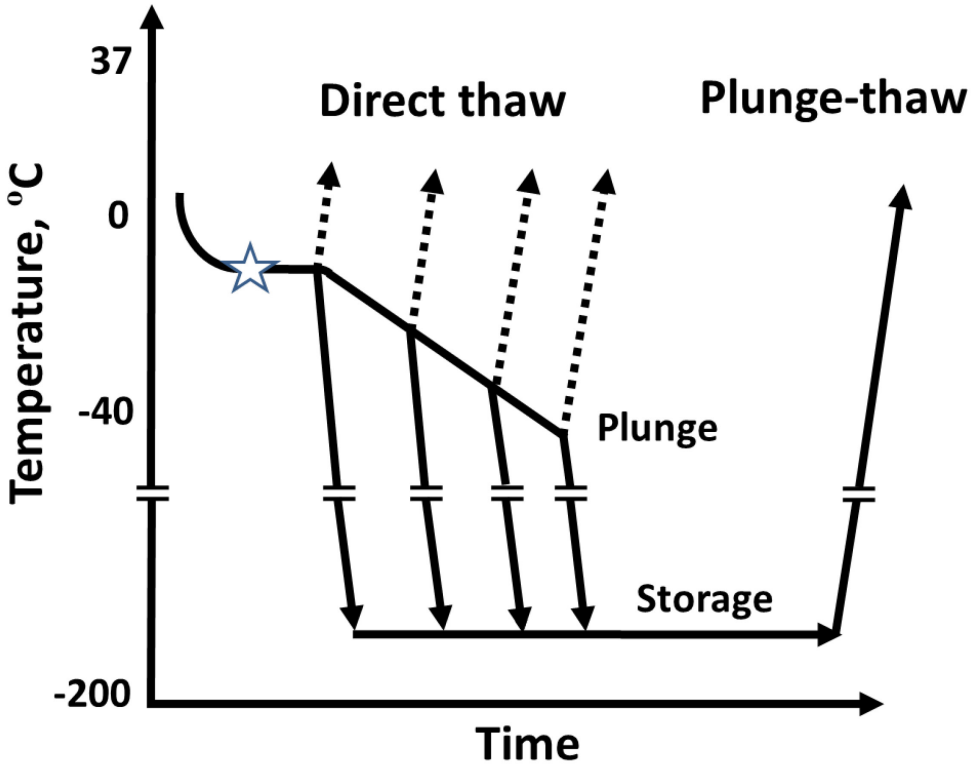

Figure A3. Temperature versus time for the graded freezing procedure

Two culture tubes containing 200 µL HUVEC suspensions were prepared for each sample. Thermocouples were referenced using a 0 °C ice-water bath and temperature was recorded for each sample. HUVECs were either thawed directly from experimental temperatures or plunged into liquid nitrogen from experimental temperatures and stored for at least one hour prior to rapid thawing. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL. Experimental temperatures were between −3 °C and −40 °C.

1. Put on appropriate personal protective equipment (PPE) (e.g. gloves, safety glasses, lab gown)
2. Set the programmable methanol cooling bath to −3 °C with a mixing speed of at least 70
3. Ensure the methanol bath is mixing and insert a polystyrene float to allow samples to be placed without falling into the methanol bath
4. Set the cooling rate to 0.2 °C/min
5. Turn on the flow cytometer and fluorescence microscope if necessary
6. Prepare SYTOPI dye for membrane integrity assay and keep on ice protected from light
7. Begin temperature data acquisition program to record the temperature throughout the course of the entire experiment
8. Reference thermocouples using an ice-water bath (0 °C at equilibrium)
9. Place (2) culture tubes with 200 µL EGM-2 into the methanol bath with T-type thermocouples
10. Nucleate the (2) culture tubes using liquid nitrogen cooled forceps
11. Place (1) thermocouple directly into the methanol bath
12. Leave (1) thermocouple to record 0 °C
13. Place O-rings on (38) culture tubes
14. Add 5 mL EGM-2 to 5 mL HUVECs
15. Mix well by pipetting up and down
16. Start timer and incubate for 15 minutes at 0 °C
17. During the 15 minute incubation, add 200 µL HUVECs in EGM-2 to (38) culture tubes and place corks on all (38) culture tubes
18. Leave (2) culture tubes of HUVECs on ice until experiments for direct thaw are complete
19. Plunge (2) culture tubes of HUVECs into liquid nitrogen for ‘Dead cells’
20. Analyze (2) culture tubes of fresh HUVECs without any dye to observe light scatter
21. Analyze the ‘Live cells (pre-experiment)’ using the SYTOPI membrane integrity assay
22. Record the experimental temperature to the nearest decimal place using the (2) thermocouples in culture tubes in the methanol bath
23. Place (2) forceps into liquid nitrogen
24. Place (32) culture tube samples in the methanol bath
25. Start timer

26. Allow culture tubes to equilibrate for 2 minutes at the −3 °C experimental temperature
27. Use liquid nitrogen cooled forceps to induce ice nucleation in all (32) samples
28. Incubate at the −3 °C experimental temperature for 3 minutes
29. Set the methanol bath to −45 °C or 5 °C below the final setpoint temperature, whichever is lower
30. Transfer the −3 °C plunge-thaw samples into liquid nitrogen
31. Transfer the −3 °C direct thaw samples to the 37 °C water bath only until thawing is complete.
32. After thawing is complete, perform SYTOPI membrane integrity analysis
33. Repeat steps 29 to 31 until all direct thaw samples are analyzed
34. Turn off the methanol bath
35. Analyze the ‘Live cells (post-experiment)’
36. Analyze the plunge-thaw samples
37. Analyze the ‘Dead cells’
38. Turn off the microscope and allow 15 minutes cool down before replacing the cover
39. Save the temperature data using the data acquisition system
40. If using the fluorescence microscope, backup the saved images
41. If using the flow cytometer, begin the cleanup panel, backup saved files and turn off the flow cytometer
42. Dispose of SYTOPI-contaminated samples in an empty bleach bottle
43. Use 70% ethanol to rinse thermocouples
44. Leave excess liquid nitrogen to evaporate
45. Save O-rings and corks for future experiments
46. Discard gloves in biohazard waste boxes
47. Clean up and wash hands before leaving the lab area

Graded Freezing Procedure with Cryoprotectant

Addition of cryoprotectant required that the freezing point be calculated prior to experimentation and the methanol bath was set to −3 °C or at least 3 °C less than the freezing point. Note that cryoprotectant was not removed prior to membrane integrity assessment.

Two culture tubes containing 200 µL HUVEC suspensions in the presence of cryoprotectant were prepared for each sample. Thermocouples were referenced using a 0 °C ice-water bath and temperature was recorded for each sample. HUVECs were either thawed directly from experimental temperatures or plunged into liquid nitrogen from experimental temperatures and stored for at least one hour prior to rapid thawing. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL. Experimental temperatures were between −3 °C and −40 °C.

1. Stock cryoprotectant solution (20% DMSO)
    - Label (1) 50 mL centrifuge tube as 'DMSO' and (1) 50 mL centrifuge tube as 'HUVEC + DMSO'
    - Record the mass of the centrifuge tube labeled 'DMSO'
    - Add 5.0g EGM-2 (approximately 5.0 mL, assuming 1.0 g/mL EGM-2 density)
    - Record the mass and volume of EGM-2 added
    - Add 1.25g DMSO (approximately 1100µL, assuming 1.1 g/mL DMSO density)
    - Record the mass and volume of DMSO added
    - Calculate the % mass of DMSO (it should be close to 20%)
    - Use the osmometer to measure the osmolality of the stock cryoprotectant solution

2. Addition of cryoprotectant solution to HUVECs in EGM-2
    - Record the mass of the centrifuge tube labeled 'HUVEC + DMSO'
    - Add 5.0 mL HUVECs
    - Record the mass and volume of HUVECs in EGM-2 added
    - Add 5.0 mL of 20% DMSO
    - Calculate the % mass of DMSO (it should be close to 10%)

Figure A3 above shows the graded freezing procedure, which is explained below. This procedure assumed that −3 °C was the first desired setpoint temperature, but cryoprotectant was used so the freezing point was calculated and the first setpoint temperature was set to −3 °C or at least 3 °C below the freezing point. Table A22 describes the samples which are also discussed below. The total volume of HUVECs required was 6 mL at a concentration of $2 – 4 \times 10^6$ cells/mL:

1. Put on appropriate personal protective equipment (PPE) (e.g. gloves, safety glasses, lab gown)
2. Set the programmable methanol cooling bath to −3 °C or 3 °C below the freezing point with mixing speed of at least 70
3. Ensure the methanol bath is mixing and insert a polystyrene float to allow samples to be placed without falling into the methanol bath
4. Set the cooling rate to 0.2 °C/min
5. Turn on the flow cytometer and fluorescence microscope if necessary
6. Prepare SYTOPI and keep on ice protected from light
7. Begin the temperature data acquisition program to record the temperature throughout the course of the entire experiment
8. Reference thermocouples using an ice-water bath (0 °C at equilibrium).
9. Place (2) culture tubes each with 100 µL EGM-2 and 100 µL stock cryoprotectant solution into the methanol bath with T-type thermocouples
10. Nucleate the (2) culture tubes using liquid nitrogen cooled forceps
11. Place (1) thermocouple directly into the methanol bath
12. Leave (1) thermocouple to record room temperature
13. Leave (1) thermocouple in the ice-water bath
14. Place O-rings on (44) culture tubes
15. Add 5 mL stock cryoprotectant solution to 5 mL HUVECs
16. Mix well by pipetting up and down
17. Start timer and incubate for 15 minutes at 0 °C
18. During the 15 minute incubation, add 200 µL HUVECs in cryoprotectant to (44) culture tubes and place corks on all (44) culture tubes

19. Leave (2) culture tubes of HUVECs and (2) culture tubes of HUVECs in cryoprotectant on ice until experiments for direct thaw are complete
20. Plunge (2) culture tubes of HUVECs and (2) culture tubes of HUVECs with cryoprotectant into liquid nitrogen for ‘Dead cells’ and ‘Dead cells with cryoprotectant’
21. Analyze the ‘Live cells (pre-experiment)’ and the ‘Live cells with cryoprotectant (pre-experiment)’ using the SYTOPI membrane integrity assay
22. Record the experimental temperature to the nearest decimal place using the (2) thermocouples in culture tubes in the methanol bath
23. Place (2) forceps into liquid nitrogen
24. Place (32) culture tube samples in the methanol bath
25. Start timer
26. Allow culture tubes to equilibrate for 2 minutes at the −3 °C experimental temperature
27. Use liquid nitrogen cooled forceps to induce ice nucleation in all (32) samples
28. Incubate at the −3 °C experimental temperature for 3 minutes
29. Set the methanol bath to −45 °C or 5 °C below the final setpoint temperature, whichever is lower
30. Transfer the −3 °C plunge-thaw samples into liquid nitrogen
31. Transfer the −3 °C direct thaw samples to the 37°C water bath only until thawing is complete.
32. After thawing is complete, perform SYTOPI membrane integrity analysis
33. Repeat steps 29 to 31 until all direct thaw and plunge-thaw samples are analyzed
34. Turn off the methanol bath
35. Analyze the ‘Live cells (post-experiment)’ and ‘Live cells with cryoprotectant (post-experiment)’
36. Analyze the plunge-thaw samples
37. Analyze the ‘Dead cells’ and the ‘Dead cells with cryoprotectant’
38. Turn off the microscope and allow 15 minutes cool down before replacing the cover
39. Save the temperature data using the data acquisition system
40. If using the fluorescence microscope, backup the saved images
41. If using the flow cytometer, begin the cleanup panel, backup saved files and turn off the flow cytometer

42. Dispose of SYTOPI-contaminated samples in an empty bleach bottle
43. Use 70% ethanol to rinse thermocouples
44. Leave excess liquid nitrogen to evaporate
45. Save O-rings and corks for future experiments
46. Discard gloves in biohazard waste boxes
47. Clean up and wash hands before leaving the lab area