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Cryotemplation for the Rapid Fabrication of Porous, Patternable Photopolymerized Hydrogels

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Introduction

Hydrogels have increasingly been used for tissue engineering applications due to their abilities to match the mechanical properties of host tissues ^{1, 2} and be functionalized with multiple chemistries to tailor their chemical presentation ^{3–5}. However, low vascularization ^{6, 7} and limited infiltration of cells both *in vitro* ⁸ and *in vivo* ⁶ in traditional hydrogels has constrained their application in tissue engineering. To expand their potential use, micrometer-sized pores have been incorporated into hydrogels. Forming hydrogels that are highly porous has enhanced their ability to support cell infiltration ⁶ and nutrient transport ⁹, and to facilitate cell transplantation ¹⁰, and controlled drug delivery ^{6, 11, 12}.

Current approaches to create porous hydrogels can be categorized into three strategies: material templation, UV-assisted templation, or cryogelation. In material templation ^{13–15}, the hydrogel precursor solution is casted inside a mold around a porogen, the monomers are polymerized, and the porogen is removed to form the final porous structure. In UV-assisted templation, photomasks ^{16–18} or directed photons ^{19, 20} define crosslinked and uncrosslinked regions for the generation of pores in photo-crosslinkable hydrogels. In chemically-polymerized cryogelation ^{21, 22}, the hydrogel precursor solution is cast into a mold, placed in a rate-controlled freeze-thaw cycle, and leached to yield the ultimate structure. The pore size for this method is achieved by the material, crosslinker, and fabrication parameters. Material templation may not be compatible with chemically-polymerized cryogelation due to the potential for the crosslinking agent to react with both the bulk material and the porogen.

Herein, we present the fabrication of porous poly(ethylene glycol) (PEG) hydrogels using cryotemplated photopolymerization, in which the precursor solution is frozen to create ice-filled pores, and then placed under ultra-violet (UV) light to crosslink the network. Freezing temperature, polymer and photoinitiator concentration, and crosslinking time were

characterized for their impact on the formation of porous hydrogels. Additionally, we investigated the use of templating materials, photolithography and the fusion of frozen pieces to create complex porous structures. Furthermore, we explored the incorporation of both cells and peptides into porous hydrogels to enhance their biological functionality. Cryotemplated photopolymerization provide a versatile platform to create porous hydrogels with a diverse range of geometries, and further development of this technique may enable cell cryostorage within these templated environments. This technology may ultimately provide a platform for a broad range of applications including high-throughput arrays^{23–25}, cellular networks and co-cultures^{26, 27}, drug delivery^{24, 28, 29}, and regenerative medicine scaffolds^{30, 31}.

Experimental

Hydrogel Fabrication

PEG-acrylate, PEG-vinylsulfone, or PEG-sulfide (4 arm, 10000–20000 molecular weight, Laysan biomaterials) was dissolved into pH 8 PBS (5%–10% w/v) with Irgacure 2959 photoinitiator (0.25%–0.5% w/v, Ciba) and pipetted into a cylindrical mold. For hydrogels, the solution was exposed to UV light (365nm, 50mW/cm²) for 15 sec to 3 min. For porous hydrogels, the solution was first frozen for 16 hours at either –20 °C or –80 °C. The formed scaffold was then immersed in PBS or distilled water until use.

Mechanical Testing

Porous PEG hydrogels were formed as before (n = 4 per condition) and trimmed to cylinders 9–10mm in height and diameter. Cylinders were compressed using the Instron 5544 at a rate of 1 mm/min until 90% deformation was reached. Modulus, ultimate strength, and percent elongation were evaluated.

Mesh Size and Swelling Properties

PEG hydrogels were formed as before (n = 4 per condition), weighed, and placed in distilled water. After 72 hours, the hydrogels were weighed (M_s), frozen in liquid nitrogen, lyophilized for 24 hours, and weighed (M_d) again. Swelling ratio (Q_M) and mesh size (ξ) was approximated with a modified Flory-Rehner model using the following equations^{32–34}:

$$\begin{aligned} Q_M &= M_s/M_d \\ 1/\bar{M}_c &= 2/\bar{M}_n - (\bar{v}/V_1)^{35/2} / [(\nu_{2,r}) \{ (\nu_{2,s}/\nu_{2,r})^{1/3} - 0.5(\nu_{2,s}/\nu_{2,r}) \}] \\ (\bar{r}_0^2)^{1/2} &= 1/C_n^{1/2} n^{1/2} \\ n &= 2\bar{M}_c/M_r \\ \xi &= \bar{v}_2^{-1/3} (\bar{r}_0^2)^{1/2} \end{aligned}$$

where ρ_p = density of polymer = 1.08, ρ_s = density of solvent = 1.00, $\bar{M}_c$ = mol. wt. between crosslinks, $\bar{M}_n$ = uncrosslinked average mol. wt. = 20000, V_1 = molar volume of solvent = 18.0153 for water, $\nu_{2,s}$ = swollen polymer fraction volume = $1/Q_M$, $\nu_{2,r}$ = relaxed polymer fraction volume, $\bar{v}$ = polymer specific volume = ρ_p/ρ_s , χ_1 = solvent-polymer interaction parameter = 0.426 for PEG in water, $(\bar{r}_0^2)^{1/2}$ = root-mean-square end to end distance of the

polymer chain, l = average monomer bond length = 0.146 nm for PEG, C_n = arm-to-monomer ratio = 4, n = no. of bonds per crosslink, M_r = repeat unit mol. wt. = 44 for PEG.

Pore Size

Porous PEG hydrogels were formed as before and placed in distilled water. After 48 hours, the hydrogels were imaged using phase microscopy at 10x magnification. Diameters were measured from random images of 3 scaffolds using FIJI/ImageJ. At least 200 diameters were assessed per condition.

Patterning with Porogens

Preformed alginate (2% with 40mM CaCl_2), gelatin (5%) or metal shapes were placed into the mold to serve as templates. Solutions (10% PEG, 0.5% photoinitiator in PBS) were then placed into the templated molds, frozen -20°C , and formed as before.

Photolithography

Laser-printed masks were made with transparency paper (HP2300 laserjet, 600 dpi). Chrome masks were designed and obtained from CAD/Art Services (Bandon, OR, USA). Solutions (10% PEG, 0.5% photoinitiator in PBS) were frozen at -20°C , placed underneath the feature mask, and formed as before.

Hydrogel Joining

Solutions (10% PEG, 0.5% photoinitiator in PBS) were placed in molds of multiple sizes, frozen at -20°C , positioned and pressed together, and formed as before.

Cell Encapsulation

Polymer solution (10% PEG, 1% photoinitiator in PBS) was conjugated to cell-adhering, plasmin-degradable peptide sequence GCYKNRGCYKNRCGRGD³⁶ (5 mM, fabricated at the Northwestern University peptide synthesis core) by Michael-type addition at 37°C for 10 min.

For cell encapsulation, when Human embryonic kidney (HEK 293T) cells were incorporated around gelatin-rich regions: a 10% gelatin solution was pipetted into a 40°C preheated mineral oil bath (1:2 ratio), cooled to 4°C , washed with acetone, and dried to create $300\text{ }\mu\text{m}$ microspheres⁶, which were subsequently mixed with the polymer solution to reach swelling equilibrium. The microsphere-laden polymer solution was mixed 1:1 v/v with cells solution (15,000 cells in DMEM with 30% FBS, 1% penicillin/streptomycin), yielding final concentrations of 10% v/v for PEG, 15% for FBS, and 10% for DMSO, and formed as before, frozen to -80°C and exposed 30 sec to UV light. When HEK 293T cells were incorporated within gelatin-rich regions: gelatin was added to the polymer solution to yield a 5% w/v concentration and formed as before. Viability was assessed after 6 hours of culture using a live/dead stain ($2\text{ }\mu\text{M}$ calcein-AM and $1\text{ }\mu\text{M}$ ethidium homodimer in cell media for 40 minutes).

Statistical Analysis

Multiple comparisons were analyzed via a one-way ANOVA with a Bonferonni post-hoc test using the software package PRISM. Significance was defined at a level of $p < 0.05$.

Results and Discussions

Photopolymerized Hydrogels

PEG hydrogels with interconnected pores were created by adapting cryogelation to a photopolymerized material (Figure 1a). A solution of PEG and photoinitiator was pipetted into a cylindrical mold, which was then frozen below $-10\text{ }^{\circ}\text{C}$ to permit the formation of ice crystals. The frozen cylinder was then exposed to UV light (365 nm , 50 mW/cm^2) to form the final structure. Upon bringing the construct to room temperature, a cylindrical porous gel was obtained. Relative to traditional hydrogels (formed at $+20\text{ }^{\circ}\text{C}$) (Figure 1b), hydrogels formed after freezing of the polymer solution at $-20\text{ }^{\circ}\text{C}$ (Figure 1c) or $-80\text{ }^{\circ}\text{C}$ (Figure 1d) were more opaque and had swelled to larger dimensions, which is likely due to rearrangement of the PEG monomers with the formation of ice crystals.

Pore structure

Three fabrication parameters for cryotemplated photopolymerization were investigated for their ability to modulate pore structure: freezing temperature^{37–39}, gelation time^{38, 39}, and ratio of polymer to photoinitiator^{21, 39, 40} (Figure 2). Pore geometry was typically spherical and randomly oriented whether the solution was frozen at $-20\text{ }^{\circ}\text{C}$ (Supp. 1a–e) or at $-80\text{ }^{\circ}\text{C}$ (Supp. 1f–j), which likely reflects the rapid, uncontrolled freezing used in this study. The median pore size ranged from $27.1\text{ }\mu\text{m}$ to $37.4\text{ }\mu\text{m}$ in the conditions investigated, whereas mesh sizes for traditional hydrogels (formed at room temperature) ranged from 9.4 nm to 16.4 nm for matched parameters (PEG %, photoinitiator %, UV time). Increasing the duration of UV exposure, which provides for longer gelation times (Figure 2a,c), led to a decreased the median pore size within the hydrogel from $30.4\text{ }\mu\text{m}$ to $26.6\text{ }\mu\text{m}$ when frozen at $-20\text{ }^{\circ}\text{C}$ and from $36.8\text{ }\mu\text{m}$ to $33.4\text{ }\mu\text{m}$ when frozen at $-80\text{ }^{\circ}\text{C}$. The pore size decreased as the light exposure was increased up to an exposure of 2 minutes, and exposure beyond 2 minutes did not further decrease the pore size. The median pore size also increased with decreasing polymer (10% to 5% w/v) or photoinitiator (0.5% to 0.25% w/v) content (Figure 2b,d) from $27.1\text{ }\mu\text{m}$ to $33.8\text{ }\mu\text{m}$ when frozen at $-20\text{ }^{\circ}\text{C}$ and from $34.1\text{ }\mu\text{m}$ to $37.4\text{ }\mu\text{m}$ when frozen at $-80\text{ }^{\circ}\text{C}$, which is attributable to an increased size of ice crystals and slower crosslinking, respectively. Reducing the temperature during freezing from $-20\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ increased pore size $5.7 \pm 2.7\text{ }\mu\text{m}$ on average, which contrasts with previous reports^{37–39}, likely due to larger ice crystal formation before crosslinking.

Swelling

Fabrication parameters also affected the extent of swelling in porous PEG hydrogels (Supp. 1, Table 1). Porous PEG hydrogels achieved swell ratios that ranged from 23.6 ± 0.3 to 71.0 ± 1.2 , depending on the preparation parameters. Greater swelling corresponded with reduced polymer or photoinitiator content, shorter UV crosslinking time, and lower temperatures, with 5% PEG hydrogels frozen at $-80\text{ }^{\circ}\text{C}$ yielding the highest swell ratio (71.0 ± 1.2). Increased swelling in porous PEG hydrogels appears to be due to a change in the

arrangement of PEG crosslinks upon the formation of ice-crystals as pore-size changed under these conditions, but dehydrated hydrogel weight did not. Further supporting this mechanism, swelling of traditional hydrogels were not substantially affected by photoinitiator content or UV crosslinking time, yielding swell ratios ranging from 11.9 ± 0.2 to 12.5 ± 0.2 . Higher swelling in traditional hydrogels was achieved only when fabricated with reduced polymer content (18.3 ± 0.4 for 5% PEG hydrogels).

PEG parameters, namely the molecular weight, and chemical end group composition (vinyl sulfone, thiol, acrylate), were also found to influence the swelling of porous hydrogels (Supp. 2). Not surprisingly, the molecular weight of the polymer influenced swelling behavior, with the higher molecular weight PEG yielding a 42% higher swelling ratio ($p < 0.05$). The composition of end groups in PEG was also identified as influencing the swelling of the porous hydrogel. Substituting PEG-acrylate with PEG-vinyl sulfone from 0% to 100% resulted in a slight, but significant ($p < 0.05$) decrease in swelling ratio, attributable to enhanced chemical reactivity under UV light. In contrast, substituting PEG-acrylate with PEG-thiol resulted in swelling ratio increasing dramatically from 34.6 ± 2.3 to 78.0 ± 7.9 ($p < 0.05$). When fabricated only with thiol-containing PEG, a hydrogel did not form (DNF).

The effect of peptide-incorporation on swelling of porous PEG was next investigated, as these hydrogels have been extensively employed in tissue engineering strategies to present cells with bioactive peptides^{5, 35, 41}. Including a tri-thiol crosslinking peptide (1:1 ratio of cysteine to acrylate groups) in the polymer solution before freezing doubled the swelling ratio from 34.6 ± 2.3 to 52.8 ± 1.0 . Furthermore, since peptides are typically incorporated into acrylate-rich PEG hydrogels via Michael-type addition, we also investigated the effect of incorporating peptides using Michael-type reaction on the swelling of porous hydrogels. However, reaction time of Michael-type addition (0 min – 45 min) did not affect swelling using our fabrication method.

Compressibility

The mechanical properties of porous PEG hydrogels were subsequently investigated as they must be sufficient to avoid porous hydrogel collapse *in vivo*^{42, 43} and can direct cellular behavior^{44–47}. Porous PEG hydrogels fabricated under multiple crosslinking times (15s to 2 min), molecular weights (10,000 to 20,000), freezing temperatures (-20°C to -80°C), polymer content (5% to 10% w/v) and photoinitiator content (0.25% to 0.5% w/v) were compressed to 90% deformation. Percent compression at yield was similar under these conditions and averaged 34.0% deformation. Young's moduli and compressive strengths using our fabrication process were found to be comparable to porous hydrogels fabricated using cryogelation methods^{37, 49–55}. Molecular weight had the greatest impact on the mechanical properties, as the low molecular weight polymer had a modulus (16.4 ± 1.4 kPa, Figure 3a) and ultimate strength (3.42 ± 0.2 kPa, Figure 3b) that was approximately an order of magnitude greater than that for the high molecular weight polymer. Other parameters such as the freezing temperature, ratio of polymer to photoinitiator, and crosslinking time had a modest effect, with moduli ranging from 0.7 ± 0.1 kPa to 3.3 ± 0.6 kPa.

Hydrogel Patterning

The architecture of hydrogels has been reported to influence cell migration *in vitro*^{56, 57} and infiltration *in vivo*⁵⁸, and control cellular expression of pro-regenerative signals^{59, 60}. We investigated the ability to design porous hydrogels of complex architectures using material templation, photomasking, and fusion of preformed structures, which has been difficult to achieve with more conventional hydrogels¹³. These studies used conditions (20k MW, 10% PEG, 0.5% photoinitiator) that produced hydrogels with mechanical properties that were in the middle of the range observed with this process (Figure 3). With an adjustment of crosslinking conditions, the formation of cryotemplated hydrogels could be extended to hydrogels other than PEG or to create hydrogels with a broader range of properties.

Material Templation

Templates have been extensively used in tissue engineering to create unique geometries in hydrogels^{6, 61}, and we investigated their compatibility with our fabrication method (Figure 4). A solution consisting of PEG and photoinitiator was pipetted inside a mold containing gelatin or alginate gels, or metal structures which served as templating materials and then frozen and UV crosslinked to form porous hydrogels. Pore formation in PEG hydrogels was unaffected by the presence of these materials. Gelatin, alginate and metal were easily separated from the porous hydrogels by heat, calcium chelation, and physical removal, respectively.

Photolithography

We next investigated the use of photomasks to enable the rapid arraying of porous hydrogels over 1 mm in height (Figure 5). Frozen solutions of PEG and photoinitiator were covered with masks fabricated on laserjet-printed transparencies (600 dpi) and exposed to UV light as before. Transparent regions were crosslinked after exposure, while opaque regions remained unpolymerized, which allowed for the creation of porous hydrogels with wide-ranging geometries including dots, square rings, and lattices. Porous structures were visualized throughout the thickness of the hydrogel, as assessed using phase microscopy (denoted with ') and light microscopy (denoted with " or "). Features of 250 μm in resolution were achieved using laserjet-based masks, which is the resolution limit for these masks^{62, 63}. Use of chrome masks enhanced feature resolution to less than 150 μm (Supp. 3). Our ability to pattern porous hydrogels with light supports the use of other mask-less photolithography approaches such as laser scanning confocal^{64, 65} and 2-photon²⁰ microscopies to pattern our hydrogels.

Hydrogel Joining

A unique feature of our fabrication process is the ability to photo-crosslink adjacent frozen solutions, and thereby, create complex three-dimensional structures over 5 mm in height (Figure 6). Solutions frozen into cylinders of multiple diameters were placed next to each other in multiple orientations. After exposure to UV, the cylinders formed into hydrogels that were joined at their points of contact. We note that the pore structure is formed during the freezing step, which is unaffected by the crosslinking reaction, and thus anticipate that this process can create complex architectures with pores distributed throughout, including

the interface of the conjoined pieces. Integration of attachment sites when rotated onto their sides (denoted with ") revealed intactness of fused hydrogels. Attachment was also assessed using frozen solutions with spherical shapes (Supp. 4). The orientation of conjoined pieces was not a limitation with the sizes and distances investigated, as seen with top (denoted with ') and side (denoted with " or ''') views.

Cell Encapsulation

We investigated the ability to encapsulate cells inside porous PEG hydrogels using temperatures and solutions that supported cell freezing (Figure 7). The solutions of PEG and photoinitiator were mixed with 10% dimethyl sulfoxide (DMSO), which is a common cryoprotectant used in cell freezing. Human embryonic kidney (HEK 293T) cells were patterned inside the PEG hydrogel using gelatin as a templating material. Gelatin has been used previously to create complex, three-dimensional architectures in hydrogels⁶, but also to incorporate cells^{66–68}, drugs⁶⁹, proteins^{70–72}, and transgenes^{73, 74}. Cells were cryopreserved in solutions, crosslinked, and then thawed in media to produce porous hydrogels with encapsulated cells (Figure 7b, 69.7 ± 8.6 %). The gels readily formed under these conditions with macroscopic features similar to hydrogels without cells. Many cells survived the freeze-thaw process, whether encapsulated around (Figure 7c, 63.3 ± 7.4 %) or within (Figure 7d, 71.8 ± 9.4 %) gelatin-rich regions, as indicated by green calcein-AM staining, but some had damaged membranes, indicated with the red ethidium homodimer. The extent of cell survival was relatively low compared to literature reports with cell encapsulation into photocrosslinked hydrogels (averaging 69.6 ± 4.4 % amongst conditions), yet was similar to cells that went through the same steps without freezing (Figure 7a, 73.4 ± 4.5 %). These results demonstrate the potential to encapsulate cells within the cryotemplated hydrogels, and the specific application of this system to a specific cell type will likely require tuning parameters such as freezing rate, photoinitiator concentration, PEG content, UV intensity, cryoprotectant, and gelation time to maximally support cell viability and function, while also creating a suitable structure.

Due to our use of gelatin and DMSO for cell encapsulation within porous PEG hydrogels, we analyzed their impact on pore structure and swelling (Supp. 5). Gelatin (1 to 3%) and/or DMSO (2 to 5%) was mixed into a warmed (~ 37 °C) PEG solution and formed as before. Increased gelatin content in porous PEG hydrogels corresponded to reduced pore size. In the presence of DMSO, PEG hydrogels crosslinked after being frozen at -20 °C did not have the pore structure typically seen using our fabrication process. DMSO has been noted to depress the freezing temperature of PEG solution⁶², which may have resulted in phase separation instead of cryotemplation under these conditions. At -80 °C, cryotemplation occurred in the presence of DMSO; however, gelatin also reduced the pore size. The composition of the solution clearly influences the hydrogel properties following cryotemplation; however, templating materials investigated in this report can be used to increase pore-size in these hydrogels.

Conclusions

These studies present a novel fabrication method termed cryotemplated photopolymerization for the formation of macroporous hydrogel scaffolds. This approach provides a versatile method for creating porous hydrogels in complex architectures. Pores can be created within the hydrogel by freezing of the water, a porogen, or spatially patterned crosslinking, with each technique providing distinct opportunities for controlling the pore properties. Porous hydrogels could also be functionalized with peptides, or could be used with cell encapsulation, though encapsulation is dependent upon having conditions in which cells can survive the freeze-thaw process. This fabrication process has been used with templation and photolithography, and appears compatible with computer-aided fabrication techniques, such as inkjet/bio-printing^{75, 76}, solid free-form/rapid prototyping^{77–79}, stereolithography^{65, 80}, and 2-photon microscopy patterning^{80, 81}, which can enhance the complexity of future designs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Schematic of the photopolymerization of frozen solutions of PEG and photoinitiator into porous hydrogels. Light microscopy image of (b) a traditional hydrogel prepared at +20 °C and porous PEG hydrogels prepared (c) at -20 °C and (d) at -80 °C using 10% polymer and 0.5% photoinitiator and crosslinked for 2 min.

Figure 2.

Influence of freezing temperature, (a,c) gelation time, and (b,d) ratio of polymer to initiator on PEG pore size ($n = 4$). Note that the pore sizes did not have a normal distribution according to the Shapiro-Wilk test and the boxes represent the interquartile range and the whiskers represent outliers (Tukey) in the plot. Porous hydrogels were prepared at (a,b) -20°C or (c,d) at -80°C . Conditions designated with different letters are statistically different ($p < 0.05$) using ANOVA with a Bonferonni post-hoc.

Figure 3.

Influence of parameters on the compressibility of porous PEG hydrogels ($n = 4$). (a) Moduli and percent compression of hydrogels. (b) Ultimate strength of hydrogels. Asterisks and pluses indicate statistically different moduli and ultimate strengths respectively ($p < 0.05$) to 10% PEG (P), 0.5% photoinitiator (I), -20 °C, 20k MW hydrogels using ANOVA with a Bonferonni post-hoc.

Figure 4.

(a) Schematic of porous PEG hydrogel formation around non-photopolymerizable template. Patterning of 10% polymer, 0.5% photoinitiator porous hydrogels prepared at -20°C with templates prepared from (b) alginate squares (c) triangular gelatin hydrogels, or (d) circular metal templates, and crosslinked for 2 min. (b', c', d') Porous hydrogels after removal of the templating (b') alginate, (c') gelatin, or (d') metal shapes.

Figure 5.

(a) Schematic of photomask-based templation of porous PEG hydrogels. Laserjet masks for (b) dots, (c) square rings, and (e) lattices. (') Light and (") phase images of 10% polymer, 0.5% photoinitiator hydrogels at -20°C and crosslinked for 1–2 min. Circular inserts reveal polymerized area in ('). Square insert indicates region for (").

Figure 6.

Porous PEG hydrogel templation by joining frozen solutions of 2 different shapes stacked (a) vertically and (b,c) horizontally into multi-tier formations. Solutions were placed (a,b) above and (c) adjacent to each other. (') denotes aerial views, (') denotes side views. Hydrogel solutions consisted of 10% polymer and 0.5% photoinitiator prepared at -20°C and crosslinked for 2 min.

Figure 7.

Viability, as assessed by a live/dead assay, of human embryonic kidney (HEK 293T) cells encapsulated into the porous PEG hydrogel ($n = 4$). Cells were patterned inside the PEG hydrogel using gelatin. Visualization of cells encapsulated (a) around or (b) within gelatin-rich regions (denoted with *G*, outlined with dashed lines) and exposed to 30 sec of UV light (365nm, 50mW/cm²). Cooling gelatin to room temperature for cell-patterning in (b) resulted in hydrogel drying and reduction in cell viability at the interface. Green fluorescence indicates viable cells that could transport GFP-fused calcein-Am. Red indicates membrane-permeable, dead or dying cells that permit the leakage of ethidium homodimer into the nucleus. Blue reveals nuclei presence for both live and dead cells