STATE OF ACTIN CYTOSKELETON AND DEVELOPMENT OF SLOW-FROZEN AND VITRIFIED RABBIT PRONUCLEAR ZYGOTES

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Abstract

This study was focused on the effect of cryopreservation on the state of actin cytoskeleton and development of rabbit pronuclear zygotes. Zygotes were collected from superovulated females and immediately used for 1) slow-freezing in a solution containing 1.5M 1,2-propanediol and 0.2M sucrose, or 2) vitrification in a solution containing 42.0% (v/v) of ethylene glycol, 18.0% (w/v) of dextran and 0.3M sucrose as cryoprotectants. After thawing or warming, respectively, zygotes were evaluated for 1) actin distribution, 2) *in vitro* or 3) *in vivo* development to blastocyst. Comparing actin filaments distribution, a significantly higher number of vitrified zygotes with actin distributed in cell border was observed (55 ± 7.7 vs. 74

± 6.1 % for slow-frozen vs. vitrified, respectively). After 24 and 72h of *in vitro* development, significant differences in the cleavage and morula rate among the groups were observed (9 ± 2.4 and 3 ± 1.3 vs. 44 ± 3.0 and 28 ± 2.7 % for slow-frozen vs. vitrified, respectively). None of the slow-frozen zygotes reached the blastocyst stage, in contrast to the vitrified counterparts (11 ± 1.9 %). Under *in vivo* culture conditions, a significant difference in blastocyst rate was observed between vitrified and fresh embryos (6 ± 1.5 vs. 35 ± 4.4 % respectively). Our results showed that alterations in actin cytoskeleton and deteriorated development are more evident in slow-frozen than vitrified pronuclear zygotes. Vitrification method seems to be a more effective option for rabbit zygotes cryopreservation, although pronuclear zygotes manipulation *per se* resulted in a notable decrease in embryo development.

Keywords: Pronuclear; Slow-freezing; Vitrification; Actin filaments; Development; Rabbit

Introduction

Successful freezing of mammalian embryos, including rabbits, has been the subject of intensive research over many years [35]. It maintains the advantage of full genetic complement of sire and dam conservation, protecting species and population integrity as well as heterozygosity [43]. Moreover, it is widely used in assisted reproductive technologies (ARTs) in both laboratory and domestic animals [33]. Banks of genetic resources are a valuable tool in livestock improvement schemes, where population control is necessary to measure the current rate of genetic gain or to preserve the present selected lines [32]. It has been shown that long-term storage of embryos in cryobank entails the advantage of maintaining similar pregnancy rate, fertility and survival at birth for at least 15 years [45,32].

At present, conventional slow-freezing and vitrification are the two major methods of embryo cryopreservation [57,50], although there are still concerns regarding whether one

technique is better than the other [1]. It has been suggested that vitrification might be the cryopreserving procedure of choice for rabbit embryos, as non-cellular investments (*zona pellucida* and mucin coat) are frequently damaged during conventional freezing/thawing and with the vitrification approach it may be possible to reduce the damage [29,51]. Moreover, there is evidence that vitrification provides better implantation and birth rates for rabbit embryos than slow-freezing [48].

As with other species, survival of cryopreserved rabbit embryos depends on the cryoprotective agent (CPA) and the embryonic stage of development [22,36]. In rabbits, morula stage embryos in particular are commonly cryopreserved with generation of live offspring (ranging between 25 % and 65 %) using either slow-freezing [59,60,41,47,48] or vitrification [29,61,37,41,39,48]. However, both rabbit oocytes and pronuclear zygotes are completely different scenarios and there are only a few publications reporting live offspring after rabbit oocyte (3.3 – 13.2%) [4,26,27] and pronuclear zygote (3.7 – 36 %) [22,23,36] cryopreservation. The difficulties throughout the studies on oocyte and zygote cryopreservation in rabbits might be due to singularities (low surface/volume ratio, not activated genome, sensitivity of microtubules and microfilaments to high CPA concentration) which make the early stages highly sensitive to the cryopreservation process [42,40,5,26,27]. Nevertheless, there are reasons which make the cryostorage of freshly fertilized rabbit ova interesting. Zygotes are important in transgenic animal production, as the pronuclear microinjection of exogenous DNA is the most conventional and reliable method for transgenesis [21]. Transgenic rabbits are suitable tools for protein production, such as human interleukin-2 [7], insulin-like growth factor-1 [63] or human clotting factor VIII [9]. However, the rabbit is a unique mammal in that its embryos have thick mucin coat deposited during oviductal passage. Therefore, rabbit embryos cultured from the 1-cell stage *in vitro* to the

morula or blastocyst stage have no mucin coat and after transfer the lack of mucin coat significantly increases pregnancy failure rates [28].

The aim of this study was to compare the effect of slow-freezing and vitrification procedure on the actin cytoskeleton status and *in vitro* or *in vivo* development competence of rabbit pronuclear zygotes.

Materials and methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma–Aldrich Química S.A (Madrid, Spain).

Animals

Five-month-old rabbit does belonging to the New Zealand White breed from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV) were used as donors and recipients. All experimental procedures involving animals were approved by the Research Ethics Committee of the UPV and licensed by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

Pronuclear stage zygote recovery

Eighteen New Zealand White females were superovulated by administration of 5 doses (7 IU each 12 h) of porcine follicle-stimulating hormone (pFSH, FOLLTROPINTM, BionicheTeoranta,Galway,Ireland) and 0.7UI of recombinant human luteinizing hormone (rhLH, Luveris®, Serono, MW, London, UK). The does were artificially inseminated with pooled semen at a density of 40×10^6 spermatozoa/mL in Tris-citric-glucose extender [62] (0.5 mL per doe)and induced to ovulate by intramuscular injection of 1 µg of Buserelin acetate 12 h after the last superovulation dose. Presumptive pronuclear stage zygotes were

recovered from the oviducts 19 h after AI by flushing of each oviduct with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2 % of bovine serum albumin (BSA), 0.132 g/L of calcium chloride ($CaCl_2$) and antibiotics (sodium penicillin G300.000 UI, procaine G,penicillin 700.000 UI and dihydrostreptomycin sulphate 1250 mg, Penivet1, Divasa Farmavic, Barcelona, Spain).

Cryopreservation procedures

The slow-freezing procedure was adapted from previously described methods [52]. After recovery, zygotes were incubated for 10 min in a solution containing 1.5 M 1,2-propanediol (PROH) in a base medium (BM: DPBS + 20 % foetal bovine serum, FBS). Zygotes were then placed into the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in BM for 5 min and then mounted between two air bubbles in 0.25 ml sterile French mini straws (IMV Technologies. L´Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20°C to -7°C at a rate of -2°C/min. Manual seeding was performed at -7°C. Temperature was then lowered to -30°C at a rate of -0.3°C/min. Finally, the straws were plunged directly into liquid nitrogen (LN_2) and stored until later use. For thawing, the straws were held at ambient temperature for 10-15 s and plunged into a water bath (20°C). Zygotes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in BM) for 5 min before being equilibrated for 10 min in TCM-199 containing 10 % FBS. Afterwards, the zygotes were cultured in TCM-199 medium supplemented with 10 % of FBS at 38.5°C and 5% of CO_2 in humidified atmosphere.

The vitrification procedure was performed following the method of minimum essential volume (MEV), using Cryotop® as a device [31]. After recovery, zygotes were placed into equilibration solution containing 20% (v/v) of ethylene glycol (EG) in DPBS at 20-25°C for 3

min, and then transferred into the vitrification solution composed of 42.5 % EG (v/v), 18.0 % (w/v) dextran and 0.3M sucrose in DPBS. Three to five zygotes were placed in a cryotop in 2 µl of vitrification solution and plunged directly into LN_2 within one minute. After storage in LN_2, the zygotes were warmed by immersing the cryotop into the warming solution composed of 0.5 M sucrose in DPBS. After 3 min, zygotes were washed three times in a solution composed of 0.25 M sucrose in DPBS.Finally, zygotes were washed in DPBS and equilibrated for 10 min in TCM-199 containing 10 % FBS. After warming, the zygotes were cultured as stated above.

Actin cytoskeleton staining

Evaluation of cytoskeletal actin filaments was performed in the three experimental groups: slow-frozen,vitrified and fresh zygotes. Zygotes were washed in DPBS and then fixed in 4% (w/v) paraformaldehyde in DPBS (pH 7.2-7.4) for 45 min at 38.5°C. After washing in DPBS, permeabilization was performed by 50 min incubation of zygotes in 0.5% Triton X-100 in DPBS. Afterwards, the zygotes were washed three times in DPBS-PVP solution (DPBS with 4mg/ml polyvinylpyrrolidone; Sigma-Aldrich Chemie, Steinheim, Germany) and were then placed in the solution of phallodine-TRITC conjugate (Chemicon International; stock solution was diluted in DPBS at 1:500) for labelling of actin filaments, for 45 min. Thereafter the zygotes were transferred onto a microscopic slide and covered with 5µl of Vectashield anti-fade mounting medium, containing nuclear DAPI stain (Vector Laboratories, Burlingame, CA, USA). The coverslip was attached to the microslide using nail polish. All treatments were performed at ambient temperature.

Stained zygotes were evaluated using a laser scanning microscope (LSM 700; ZEISS) equipped with an Axio Imager Z2 scanning unit. Phallodine-TRITC and DAPI fluorescence signals were excited using 546 and 405 nm laser, respectively.The images were acquired and

processed using ZEN software. The actin cytoskeleton was classified as belonging to the grades according to [38] on the basis of appearance of actin filaments in rabbit morula stage embryos. We adapted the methodology for pronuclear stage zygotes, and classified Grade I as best (sharply stained actin filaments with continuous cell border) and fair (slightly non-continuous actin filaments stained in cell border) quality zygotes and Grade II as poor (large areas lacking actin staining in cell border or visible actin largely aggregated into intracytoplasmic clumps) quality zygotes(Figure 1).

***In vitro* embryo development**

After thawing/warming, only zygotes with intact *zona pellucida*were considered suitable for culture. Slow-frozen, vitrified and freshpronuclear stage zygotes were cultured in TCM-199 supplemented with 10 % of FBS at 38.5 °C and 5% of CO_2 in humidified atmosphere. Cleavage rate of the zygotes and development to morula or blastocyst stage was examined after 24, 72 and 120 h of culture, respectively.

***In vivo* embryo development**

Fifteen recipient females were induced to ovulate 20 h prior to embryo transfer. The intraoviductal transfer procedure was adapted from a previously described technique used in rabbit [6]. The equipment used was a Hopkins® Laparoscope, which is a 0°-mm straight viewing laparoscope, 30 cm in length, with a 5 mm working channel (Karl StorzEndoscopiaIbérica S.A. Madrid). Recipients were sedated by intramuscular injection of 5 mg/kg ofxylazine (Rompun, Bayer AG, Leverkusen, Germany) and anaesthesia was induced by an intravenous injection of 6 mg/Kg ketamine hydrochloride (Imalgene, Merial SA, Lyon, France). To evaluate *in vivo* development, vitrified and fresh zygotes, both types classified as normal (homogeneous cytoplasm, no vacuoles or granulations and an intact *zona*

pellucida), were transferred into both oviducts of 15 recipient does (20 to 40 embryos per recipient, depending on the number of zygotes available in each session) by laparoscopy. To evaluate the late blastocyst developmental rate, recipient does were euthanized 120 h after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 20 mL of the same solution as used for zygote recovery. The developmental rate was estimated as the number of late blastocysts recovered per uterine horn divided by the number of embryos transferred into the oviduct.

Statistical analysis

The general linear model was used to evaluate the state of cytoskeletal actin filaments and to compare*in vitro* and *in vivo* development using the type of embryo (slow-frozen, vitrified and fresh) as a fixed factor and session, females and the cryopreservation procedure by session interaction as random factors. The session, female and interaction were non-significant, so were removed from the model. The error was designated as having a binomial distribution using the probity link function. Binomial data were assigned a value of 1if it had achieved the desired stage or 0 if it had not. A value of $P \leq 0.05$ was considered significant. Data are shown as least squares means ± standard error of the mean of combined data from all the replicate experiments. All analyses were performed with SPSS 16.0software package (SPSS Inc., Chicago, IL, USA, 2002).

Experimental design

The experimental design followed in this study is shown in Figure 2. Briefly, to assess the actin cytoskeleton alteration, fluorescence staining was performed to compare actin filaments status of slow-frozen, vitrified or fresh pronuclear zygotes. To evaluate *in vitro* development competence of slow-frozen, vitrified or fresh pronuclear zygotes, the cleavage

rate, morula and blastocyst rates after 24, 72 and 120 h of *in vitro* culture, respectively were compared. To evaluate *in vivo* development ability of vitrified and fresh zygotes, late blastocyst ratio 120 h after embryo transfer was evaluated.

Results

Actin filament status in the pronuclear zygotes

Significant differences in numbers of grade I embryos among the slow-frozen, vitrified and fresh group were noted (Table 1). A higher number ($P \leq 0.05$) of grade I zygotes was found in vitrified compared to slow-frozen group (74±6.1% vs. 55±7.7%), but it was less than in the fresh control (97±3.4%).

In vitro development

A total of 514 rabbit pronuclear zygotes were cryopreserved either by slow-freezing or vitrification. After the thawing or warming procedure, only zygotes with intact *zona pellucida* were considered as suitable for culture (60.1 % of slow-frozen and 100.0 % of vitrified zygotes; Table 2). After 24and 72 h of *in vitro* development, significant differences in the cleavage and morula rates among the groups (9 ± 2.4 % and 3 ± 1.3 % vs. 44 ± 3.0 % and 28 ± 2.7 % vs.97.0 ± 8.0 % and 74.0 ± 2.3 %of slow-frozen, vitrified and fresh zygotes, respectively) were observed. None of the slow-frozen zygotes reached the blastocyst stage, in contrast to the vitrified and fresh zygotes (11±1.9% and 49 ± 2.6 %, $P \leq 0.05$, respectively).

*In vivo*development

A total of 123 fresh and 305 vitrified zygotes were transferred to 5 and 10 recipient does, respectively. Significant difference was observed between vitrified (6±1.5%) and fresh (35±4.4%) zygotes that reached late blastocyst stage 120 h after transfer to recipients.

DISCUSSION

Pronuclear zygotes are important in production of transgenic animals and transgenic rabbits are suitable tools for recombinant protein production [7,63,9]. Therefore, due to the limited literature regarding pronuclear zygotes cryopreservation in rabbits, we aimed to analysethe effect of either slow-freezing or vitrification procedure on the actin cytoskeleton status and early developmental competence of rabbit pronuclear zygotes. To the best of our knowledge, no literature describing actin cytoskeleton state in cryopreserved rabbit pronuclear zygotes is available. Nevertheless, this assessment method has been used in oocytes, zygotes and embryos of various species [24,54,14,12] including rabbit oocytes [49] and morulas[38]. Classification of the slow-frozen and vitrified pronuclear zygotes into the actin grades indicated that both cryopreservation procedures induced a high rate of detectable damage to actin cytoskeleton. A similar homogenous distribution of actin filaments with continuous cell borders in intact fresh zygotes was markedly affected upon cryopreservation, as a higher degree of cytoskeletal disorganization and actin clustering was found in cryopreserved zygotes. However, according to the staining patterns determined, we observed that slow-frozen zygotes suffered significantly from greater damage to actin cytoskeleton compared to their vitrified counterparts. It has already been proven that the cytoskeleton of mammalian embryos changes in response to cooling or cryopreservation [15] and the deleterious effect of slow-freezing and vitrification on distribution of actin filaments have already been described [14,2,38,12,13]. Actin filaments play an important role in fertilization and early embryonic development events, including compaction, cell differentiation, hatching and elongation in blastocyst [19]. Therefore, irreversible disruption of the cytoskeleton elements may compromise the survival of cryopreserved embryos [54,53,13]. In our study, the higher presence of pronuclear zygotes with damaged cytoskeleton in the slow-frozen group is consistent with the results of *in vitro* culture, as none of the slow-frozen zygotes reached the

252 blastocyst stage. This might be due to differences in susceptibility of rabbit pronuclear
253 zygotes to various cryopreservation protocols.

254 Moreover, after thawing, a high degree of *zona pellucida* fractures was found in the
255 slow-frozen zygotes (39.9 %), whilst no fracture of *zona pellucida* was detected after
256 vitrification. Although vitrification procedure might also negatively affect embryo viability,
257 its influence was not apparent in the morphology of warmed zygotes, as all the recovered
258 zygotes appeared normal. It is known that *zona pellucida* and mucin coat are essential for
259 rabbit embryo development and implantation [28] and damage to *zona pellucida* is common
260 phenomenon when embryos are cryopreserved in normal straws [44,30,58,20]. On the other
261 hand, cryopreservation by ultra-rapid vitrification utilizing the MEV method and accelerated
262 cooling/warming rates might avoid such damage [8]. Likewise, high recovery rates of the
263 *zona pellucida*-intact pronuclear zygotes might also be attributed to the composition of
264 vitrification media. We used dextran as a macromolecule additive and it was already
265 demonstrated that the addition of dextran into vitrification media elevates the viscosity of the
266 solution, reducing its tendency to crack, hence the *zona pellucida* and mucin coat might be
267 better preserved against cryoinjury [51].

268 Despite this, and based on the bibliography, rabbit pronuclear zygotes are particularly
269 difficult to cryopreserve. In the present study, under *in vitro* conditions, cleavage efficiency
270 was 9 % and 44% for slow-frozen and vitrified pronuclear zygotes respectively, and the
271 efficiency of development to the blastocyst stage was 11% only for vitrified pronuclear
272 zygotes, which was comparable to previous reports [3,17,18,36]. Based on the size of a
273 pronuclear zygote, as well as a surface/volume ratio which is similar to that of an oocyte, we
274 decided to use the slow-freezing methodology that has recently been applied successfully in
275 rabbit oocytes cryopreservation with the result of liveborn rabbits [25,26]. There are only two
276 works dealing with slow-freezing of rabbit pronuclear zygotes with very different results (10

% vs 52 % blastocyst after *in vitro* culture) [3,22]. Only Hochi et al. [22,23] report a higher development rate to blastocyst stage for both cryopreservation procedures (52 % and 51 % for slow-frozen and vitrified pronuclear zygotes, respectively). Although the information available is very limited, rates of offspring for slow-frozen and microinjected pronuclear zygotes are 4.5% [22] and rates for vitrified pronuclear zygotes are between 3.7% and 36% [23,36]. Nevertheless, direct comparison among these studies must be applied carefully owing to their inherent experimental differences (donor genotypes, cryopreservation procedures and CPA used, number of recipients used, among others). It has already been shown that different genotypes might be taken into account when working with different lines [60,39].

In addition, both cell size and lipid content of zygotes are factors thought to influence their sensitivity to CPAs and to cryopreservation [42,40]. Even though rabbit zygotes contain small amount of lipid droplets, the diameter of rabbit zygotes is large (130-150 µm) and is close to zygotes of large domestic species like cattle and sheep [22]. As embryo development proceeds, the surface/volume ratio increases, which alters permeability properties of the embryo. This ratio has a direct influence on transport of water and CPAs through the membranes [36], so the dehydration process is more complicated for the single cell of the oocytes and pronuclear zygotes than for later stage embryos [16,10]. Likewise, the chilling sensitivity of an embryo is dependent on the embryonic stage of development [55,56] and has mainly been attributed to the destruction of plasma membrane following exposure to low temperature without freezing [5]. Interestingly, in cryopreserved oocytes it has been reported that the rabbit species is highly sensitive to low temperatures and high levels of cryoprotectants, and cryopreservation causes damage to the organization of the microtubules and meiotic spindle, inducing exocytosis, disorder of cortical granules and chromosome aberration [26]. Consequently, live birth was achieved only four times, once in the 80s and three times recently [2,25,26,27]. Therefore, we could hypothesize that pronuclear zygote has

302 a similar sensitivity to low temperatures as rabbit oocytes. It was concluded that rabbit
303 embryos at or beyond the 8-cell stage acquired higher cryotolerance [36]. The findings of
304 Leandri et al. [34] demonstrated that the prominent increase in survival and blastocyst rates
305 might be also associated with embryonic genome activation, which in the rabbit occurs
306 around the 8- to 16-cell stage and which might cause intrinsic changes in the membrane
307 properties of embryos as the genes associated with membrane structure might have turned on
308 [36].

309 In the present study, due to the low development rates of slow-frozen pronuclear
310 zygotes under *in vitro* culture, only vitrified pronuclear zygotes were evaluated under *in vivo*
311 conditions. Consistently with *in vitro* embryo development, vitrified-warmed and transferred
312 pronuclear zygotes cultured until late blastocyst (preimplantation embryo) exhibit similar
313 rates. Interestingly, we found that the efficiency of fresh pronuclear zygotes after transfer was
314 affected (35%), indicating that the pronuclear zygote handling or the oviduct manipulation
315 during transfer technique is critical to successful development. Although the information
316 available is very limited, the rates of offspring for fresh transfer pronuclear zygotes are
317 between 28% and 53% [23,36]. Embryo transfer technique is regarded as safe and is not
318 considered a manipulation with adverse outcomes in normal gene expression. However, some
319 studies have found that embryo transfer itself results in the misexpression of several imprinted
320 genes [46], although this hypothesis needs to be tested.

321 Based on our results, we conclude that damage to the *zona pellucid* and alterations in
322 actin cytoskeleton are more evident in slow-frozen than in vitrified rabbit pronuclear zygotes.
323 The slow-frozen zygotes also showed significantly lower developmental competence after *in*
324 *vitro* culture. Vitrification method seems to be a more effective option for rabbit pronuclear
325 zygotes cryopreservation, although pronuclear zygotes manipulation *per se* resulted in a

notable decrease in embryo development rate. Nevertheless, further studies should be undertaken to improve pronuclear zygote cryopreservation in rabbit.

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509 **Table 1** Distribution of actin cytoskeleton in slow-frozen, vitrifiedand fresh rabbit zygotes.

Type	N	Grade I (%)	Grade II (%)
Slow-frozen	42	55 ±7.7^{c}	45 ± 7.7^{c}
Vitrified	53	74 ± 6.1^{b}	26 ± 6.1^{b}
Fresh	29	97 ± 3.4^{a}	3 ± 3.4^{a}

510 N = number of zygotes examined. Grade I = best (sharply stained actin filaments with continuous cell border)
511 and fair (slightly non-continuous actin filaments stained in cell border) quality zygotes. Grade II = poor (large
512 areas lacking actin staining in cell border or visible actin largely aggregated into intracytoplasmic clumps)
513 quality zygotes. Data are shown as least square means ± standard error of the mean. Different superscripts per
514 column are statistically different ($P \leq 0.05$).

515

516 **Table 2.** *In vitro* developmental rates of slow-frozen, vitrified and fresh rabbit pronuclear

517 zygotes.

Type	N	n	Cleavage (%)	Morula (%)	Blastocyst (%)
Slow-frozen	248	149	9 ± 2.4^{c}	3 ± 1.3^{c}	-
Vitrified	266	266	44 ± 3.0^{b}	28 ± 2.7^{b}	11 ± 1.9^{b}
Fresh	373	373	97 ± 8.0^{a}	74 ± 2.3^{a}	49 ± 2.6^{a}

518 N = number of pronuclear zygotes cryopreserved. n=number of pronuclear zygotes cultured. Data are shown as

519 least square means ± standard error of the mean. Different superscripts per column are statistically different

520 ($P \leq 0.05$).

521

Fig. 1 Grades of actin cytoskeleton observed under a confocal laser scanning microscope using 40x objective. Grade I = best (A,D; sharply stained actin filaments with continuous cell border) and fair (B,E; slightly non-continuous actin filaments stained in cell border) quality zygotes; Grade II = poor (C,F; large areas lacking actin staining in cell border or visible actin largely aggregated into intracytoplasmic clumps) quality zygotes, 3D reconstruction (upper panel) and 2D optical section (lower panel) of the zygotes stained with phallodine-TRITC for actin (red), and with DAPI for pronuclei (blue). Scale bar represents 30µm.

Fig. 2 Experimental design.