Video Article

Ploidy Manipulation of Zebrafish Embryos with Heat Shock 2 Treatment

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Abstract

Manipulation of ploidy allows for useful transformations, such as diploids to tetraploids, or haploids to diploids. In the zebrafish *Danio rerio*, specifically the generation of homozygous gynogenetic diploids is useful in genetic analysis because it allows the direct production of homozygotes from a single heterozygous mother. This article describes a modified protocol for ploidy duplication based on a heat pulse during the first cell cycle, Heat Shock 2 (HS2). Through inhibition of centriole duplication, this method results in a precise cell division stall during the second cell cycle. The precise one-cycle division stall, coupled to unaffected DNA duplication, results in whole genome duplication. Protocols associated with this method include egg and sperm collection, UV treatment of sperm, *in vitro* fertilization and heat pulse to cause a one-cell cycle division delay and ploidy duplication. A modified version of this protocol could be applied to induce ploidy changes in other animal species.

Video Link

The video component of this article can be found at https://www.jove.com/video/54492/

Introduction

This protocol allows the manipulation of ploidy in zebrafish embryos, such as in the generation of homozygous gynogenetic diploids from gynogenetic haploids (**Figure 1**) or the production of tetraploids. This is achieved by inducing a delay in cytokinesis corresponding to precisely one cell cycle (**Figure 2A**, **2B**). The key one-cycle delay in cytokinesis is achieved by treatment with heat shock. The standard protocol of Heat Shock (HS) as originally described by Streisinger and colleagues involved a temperature pulse during the period 13-15 mpf, resulting in a one-cycle cell division stall during the first cell cycle [1]. The efficiency of this protocol has been recently improved by scanning the early cell cycles with a sliding temporal window of heat shock treatment. This scan identified a later time point for a heat shock, still within the first cell cycle (22-24 mpf), that results in a higher rate of embryos with a one-cycle cell division stall, which in this case affects the second cell cycle [2]. The observation that experimental manipulations during the first cell cycle interfere with cell division during the second cell cycle and cause DNA content duplication has also been reported in other fish species [3,4]. We refer to this modified protocol as Heat Shock 2 (HS2 – the term "2" reflects that the heat pulse occurs at a later time point than the standard HS method, and that the cell cycle delay caused by HS2 occurs during the second cell cycle). These studies showed that the basis for cytokinesis arrest after heat shock is the inhibition of centriole duplication during the heat pulse, which affects spindle formation and furrow induction in the following cell cycle. HS2 results in yields of cell cycle arrest nearing 100%, and rates of ploidy duplication up to 4 times higher than standard HS [2].

Embryos treated with a heat shock during blastomere cell cycling exhibit many deleterious effects, suggesting that heat shock affects multiple processes required for cell division [2]. On the other hand, if the heat shock is applied prior to the initiation of cell cycling (time period 0-30 mpf), it appears to have effects consistent with specific interference with centriole duplication and does not seem to affect other essential cell processes [2]. These studies showed that the time prior to the initiation of blastomere division appears to be a developmental period amenable to using Heat Shock as a tool to specifically manipulate ploidy through centriole inhibition. The underlying cause of the apparent selectivity for heat shock on centriole duplication is unknown, but may be related to a selective degradation of centrosome substructures observed under heat stress in certain cell types, such as leukocytes [5].

Temporal synchronization of embryonic development is achieved by in vitro fertilization (IVF). Use of untreated sperm during fertilization results in diploid embryos that upon HS2-induced one-cycle cytokinesis stall become tetraploid. Use of UV-treated sperm, which carries crosslinks that inactivate its DNA, results in gynogenetic haploid embryos [6], which upon HSII-induced one-cycle cytokinesis stall become gynogenetic diploids [2]. Because of the resulting whole genome duplication, the latter gynogenetic diploids are homozygous at every single locus across the genome. For conciseness, we refer to gynogenetic haploid embryos as "haploids", and homozygous gynogenetic diploid embryos as "homozygous diploids". If viable and fertile, homozygous diploids can be used to initiate sterile and lethal-free lines. Direct homozygosity induced by HS2 should also be readily incorporated into genetic analysis or genetic screens, since homozygous diploids from females that are heterozygous carriers of mutations exhibit rates of homozygosity at high and fixed (50%) ratios [2].

The following protocol describes steps to perform HS2 and induce ploidy duplication with full homozygosity. For tetraploid production, sperm solution should be untreated. For homozygous diploid production, sperm should be inactivated by UV treatment. In addition, as described in the Discussion, visible pigment markers can also be used to facilitate identification of homozygous diploids. Zebrafish mate primarily during the first 3 hr of the initiation of their light cycle [7], and both adults and eggs are sensitive to circadian rhythms [8], so for best results the IVF procedure should occur within this time period.

Protocol

All animal experiments were conducted according to University of Wisconsin – Madison and Institutional Animal Care and Use Committee (IACUC) guidelines (University of Wisconsin – Madison Assurance number A3368-01).

1. Selecting Females for Egg Collection via Interrupted Mating

NOTE: IVF-based protocols rely on the extrusion of mature eggs from females through manual pressure [9]. Previous protocols have used females directly from tanks or in pair matings without the females undergoing egg release behavior, but only a small fraction of these females (about 20% or less, depending on the zebrafish line) yield extruded and competent eggs upon manual pressure. In an improved procedure, females are pre-sorted for egg laying by direct visual observation, followed by immediate interruption of mating. This procedure is very effective, as nearly all females pre-selected through this interrupted mating step yield extruded and competent eggs upon manual pressure.

1. The afternoon before the experiment, set up mating pairs of the desired zebrafish strain in standard zebrafish mating boxes. Keep males in the same chamber as females yet physically separated from them, either through a mating box division or by placing the male under an egg-laying insert and the female inside the insert.
2. The morning of the experiment, remove the physical partition, placing both the male and female within the same egg-laying insert, so that mating begins.
3. Visually inspect tanks containing mating pairs to detect extrusion of eggs during natural mating. At the first signs of egg extrusions, separate male and female to interrupt breeding. After separation from males, keep the pre-selected females either separately or pooled in the same tank. Use multiple females depending on the number of embryos desired (typically 50-150 eggs/female).

2. Preparing a Sperm Solution

NOTE: IVF relies on exposure of mature eggs to a sperm solution. This solution can be untreated, to generate diploid zygotes (which upon HS2 treatment become tetraploid embryos) or UV-treated, to generate haploid zygotes (which upon HS2 treatment become homozygous diploid embryos). Previous sperm preparation protocols suggested the use of capillary tubes to collect milt from the anal region of live males, but this was an ineffective process as only a small fraction of males yielded milt [9]. The protocol presented below relies instead on sperm preparation from sheared dissected testes, which yields more reliable results.

1. Prepare Hank's solution ahead of time as a Hank's premix solution, comprising of all components (Solutions 1, 2, 4 and 5) except the sodium bicarbonate solution (Solution 6). Prepare Solution 6 fresh and add to the premix the morning of the experiment (**Table 1**). To make Hanks' solution (final solution, prepare the morning of the IVF procedure), combine 990 ul of Hank's Premix and 10 µl of the freshly made Solution 6.
2. Euthanize males by overexposure to tricaine as a 0.016% solution in conditioned water.
    1. Prepare tricaine as a 0.2% stock solution in water (buffer to pH 7.0 with 1M Tris pH 9.0) and keep at 4 °C. Add 8 ml of tricaine stock solution per 100 ml of water in a beaker, and use a net to transfer the males to the tricaine solution.
    2. Use the equivalent of testes for one male per clutch needed to be fertilized in a volume of Hank's solution corresponding to 100 µl per male (*e.g.* testes from 10 males collected in 1 ml Hank's solution, to fertilize 10 clutches with 100 µl/clutch).
    3. Confirm euthanasia by cessation of gill movements for 15 minutes. After euthanasia, remove the males from the beaker with a spoon. Rinse the males briefly with conditioned water and dry them lightly by placing them briefly on several locations of a paper towel.
3. To remove the testes, first decapitate euthanized males using dissecting scissors or a razor blade, and make a longitudinal cut along the abdomen with dissecting scissors.Under a dissecting microscope with a reflected light source, remove internal organs with dissecting forceps. Pull out each of the testes masses with forceps and place them inside the microcentrifuge tube containing Hank's solution.
NOTE: Testes can be identified as each of two elongated structures of translucent appearance found alongside the body walls and which converge near the cloaca. Testes can stay 2-3 min on a petri plate surface after dissection and before placing them in the Hank's solution.
4. Release the sperm into the solution by shearing the testes with a narrow spatula and/or gently pipetting up and down 5-6 times the testes in solution with a 1,000 µl-tip micropipette, while avoiding air bubble formation. Let the testes debris settle.
5. Store the sperm solution in ice, where it can keep its potency for up to 2-3 hr. If proceeding to UV-treatment of sperm solution, transfer the solution into a clean microcentrifuge tube leaving the pieces of testes behind.

3. UV Treatment

NOTE: UV treatment is used to crosslink sperm DNA in order to render it inactive in the embryo. This step is only used when producing gynogenetic haploid or homozygous gynogenetic diploid embryos. Sperm solution for UV treatment should be separated from pieces of testes (step 2.4), as large pieces may shield sperm from the UV treatment.

1. Transfer the sperm solution to a clean, dry well of a depression glass plate sitting on an ice bed (*e.g.* ice inside a petri plate). Use up to 1 ml of sperm solution per glass plate well.

2. Expose the sperm solution to UV by placing the depression glass plate on the ice bed under a UV lamp. Treat the sperm solution for 90 sec with a 115 V (60 Hz, 0.68A) UV-lamp at a 19 cm (7.5 inches) distance. With the end of a pipette tip, gently mix the solution every 30 seconds during UV treatment (use a clean pipette tip every 30 sec).
3. Using a clean pipet tip and micropipette, transfer the treated sperm solution to a new microcentrifuge tube. Store on ice until needed for IVF (no longer than 2-3 hr from extraction).

4. Manual Extrusion of Mature Eggs

NOTE: Females obtained by interruption of natural matings will readily yield eggs under anesthesia and manual pressure. During this procedure, tricaine treatment should be carefully controlled to avoid overexposure that may prevent recovery of the females.

1. Anesthetize females by light exposure to tricaine solution: transfer females with a net to tricaine solution in conditioned water (made by adding 8 ml of Tricaine 0.2% stock solution to 100 ml of conditioned fish water) for about 2-5 min, until fish stop gill movement.
2. As soon as a female stops gill movement, use a spoon to collect it and briefly rinse it in conditioned water. Still using the spoon to move the fish, dry it lightly by placing it briefly and repeatedly on several locations of a paper towel, then transfer it to a clean, dry 10 cm diameter petri plate. Approach the fish with the spoon in the anterior to posterior direction, to avoid potentially damaging the gill operculum.
3. Use lab wipes or soft tissue to gently further dry the anal fin area, to prevent any released eggs from prematurely being activated by water. Dampening the fingers lightly with water (to avoid them sticking to fish scales), place one finger of one hand on the female's back as support, and with a finger of the other hand apply slight pressure along the female's abdomen until eggs become extruded.
4. Use a narrow spatula to move the eggs away from the female's body. Place the female back into a tank with conditioned water for recovery. NOTE: Once eggs are extruded while the female is lying on one side of her body, she can be flipped over and the process repeated on her other side to obtain additional eggs. To insure full recovery of the female, apply only gentle manual pressure on the abdomen, and carry out the procedure from gill movement stop to return to water in less than 2 min.
5. Activate (with water exposure) and fertilize (with sperm solution addition) eggs simultaneously and within 90 sec after extrusion (see section 5).

5. *In Vitro* Fertilization

NOTE: Zebrafish fertilization in natural crosses is external, dependent on the simultaneous release and activation by water of eggs and sperm during mating. In vitro fertilization mimics this process by exposing the eggs to sperm solution in the presence of water. Water volume is originally small (1 ml) in order to increase the effective sperm concentration. Binding by sperm occurs within 15-20 sec [10], and water volume can then be increased. Chorion lifting further contributes to the close synchronization of the clutch by limiting the window for competence for sperm binding [11,12]. The resulting embryos therefore exhibit largely simultaneous cell division cycles during the early cleavage stages.

1. Add 100 µl of sperm solution (corresponding to the equivalent of testes from one male – see Part 2) to the clutch of extruded eggs on a petri plate. Gently swirl the pipette tip used to add the sperm solution among the eggs to mix the sperm and eggs together, being sure to not lift the tip from the surface of the petri plate to avoid egg damage.
2. Immediately activate the eggs by adding 1 ml of embryonic medium (E3) solution (conditioned water is also acceptable as a substitute for E3 in Parts 5 through 7) and again gently mix eggs and sperm by gently swirling with the pipette tip. Start a timer to initiate timing relative to fertilization.
3. At 1 mpf in the 1 ml volume, flood the plate with E3. Before continuing, leave undisturbed until 10-12 mpf to allow egg activation, including full chorion expansion.

6. Heat Shock Treatment

NOTE: A heat shock applied in the early embryo inhibits centriole duplication, resulting in an incomplete complement of centrioles to drive spindle formation during the subsequent cell cycle [2]. The absence of spindle in turn results in the lack of furrow formation [13].

1. After expansion of the chorions (10-12 mpf), pour the embryos from the petri plate into a tea strainer. Rinse the petri plate using a wash bottle with E3 in order to collect any remaining embryos in the tea strainer.
2. Place the tea strainer with the embryos inside a beaker in a pre-heated bath with E3 at 28.5 °C. Pre-equilibrate the beaker and E3 to the water bath temperature, and make sure there is enough E3 so that all embryos in the tea strainer are exposed to the medium.
3. At 22 mpf, remove the tea strainer from the 28.5 °C water bath, briefly blot it onto a paper towel to remove excess moisture, and place it inside a similarly preheated E3 beaker in a heat bath at 41.4 °C.
4. At 24 mpf, transfer the tea strainer back to the E3 in the water bath at 28.5 °C after brief blotting. At 29 mpf, use a wash bottle with E3 to transfer the embryos from the tea strainer to a 10 cm Petri plate.

7. Selection for Embryos with a One-cycle Cytokinesis Stall

1. During the time period 35-45 mpf, under a dissecting microscope with a transmitted light source, select those embryos that are undergoing symmetrical cleavage into the 2-cell stage, and which are therefore fertilized. Remove embryos that are not undergoing cell cleavage.
2. Continue observing the fertilized embryos, selected for normal cell division during the first cell cycle, and during the second cell cycle (50-65 mpf).
3. Sort embryos according to the following categories (**Figure 2C**): 4 cells ("no stall", in the 2:2 arrangement standard for a 4-cell embryo); 3 cells ("partial stall", in an aberrant 2 smaller cells:1 large cell arrangement); and 2 cells ("stalled", embryos exhibiting a one-cell cycle delay in a 1:1 arrangement identical to that of a 2-cell embryo). Sort the stalled embryos into a fresh petri plate. NOTE: At this stage, embryos in the 2:2 arrangement correspond to those in which during the second cell cycle neither blastomere underwent a cell division stall; embryos in an aberrant 2 smaller cells:1 large cell arrangement correspond to those where heat shock caused

a cell cycle stall during the second cell cycle in one blastomere but not the other; and embryos "stalled" in a 1:1 arrangement correspond to those where during the second cell cycle both blastomeres underwent the desired cell division stall. Stalled embryos should resume cell cleavage during the following cell cycle period (65-80 mpf). The arrangement of blastomeres may be variable, due to the incomplete cues from the previous cell cycle to stabilize the spindle [2,14], but most embryos will form a relatively normal blastula that can undergo normal development.

4. Allow embryos to develop in the Petri plate, with a limit of 80 embryos per 10 cm plate. At 24 hpf, observe the embryos to determine whether they have a normal morphology characteristic of diploid or homozygous diploid embryos [6] (normal extent of axis extension (**Figure 3A**, **3C**)), in contrast to reduced axis extension and increased body thickness characteristic of haploids (**Figure 3B**)), and/or assess diploidization using genetic pigment markers at 36 mpf, such as *golden* or *albino* and other such assays (**Figure 3** and see below) [2,6,9].
5. Remove any embryos that appear to have a haploid morphology, or that have lysed or exhibit other grossly abnormal defects.
NOTE: If desired, allow embryos to develop until 5 days post fertilization, while continuing to remove lysed or grossly abnormal embryos on a daily basis and adding fresh E3 to refresh the medium. NOTE: Surviving embryos can also be grown after swimbladder inflation on day 5 by transferring to a hatchery system and feeding under standard conditions.

Representative Results

In spite of the one-cell cycle cytokinesis stall, DNA replication occurs normally in such embryos, resulting in the duplication of the DNA content of the embryo (**Figure 1**). The Streisinger Heat Shock protocol (standard HS) involves a heat pulse during the period 13-15 minutes post fertilization (mpf) and induces primarily cytokinesis arrest during the first embryonic cell division at 35 mpf [1,2], whereas the derived method described here, referred to as Heat Shock 2 (HS2), uses a heat shock during the period 22-24 mpf and induces cytokinesis arrest during the second embryonic cell division at 50 mpf (**Figure 2**; [2,3,4]). At 24 hpf, observation of the embryos allow determining whether they have a normal morphology characteristic of diploid or homozygous diploid embryos, or the shorter and wider body axis morphology characteristic of haploid embryos (**Figure 3**) [6]. If properly selected for a one-cell cycle delay, all embryos should exhibit a normal diploid morphology. In addition, in the absence of deleterious mutations homozygous diploids are viable, whereas haploid embryos invariably exhibit lethality after 2-3 days of development. Using an unselected mixed AB/Tübingen background genetic strain, yields of homozygous diploid induction by HS2, as assayed by morphology at 24 hpf, vary from 10% to 50% [2]. Selection of lines by propagation through gynogenetic methods has been shown increase the yield of homozygous diploid production [1]. Confirmation of the precise whole genome duplication expected from the inhibition of one mitotic cycle can be obtained by chromosome counts [2,3,4,15] or quantitation of nuclear diameter [3,4]. Normal development in zebrafish as other animals is highly sensitive to chromosome number abnormalities [16], and the observation that homozygous diploids become viable and fertile adults [1,2,9] provides additional evidence for successful diploidization. Ploidy in zebrafish embryo can also be assessed using molecular methods such as fluorescent in situ hybridization (FISH) of nuclear gene count and fluorescent-activated cell sorting (FACS) to quantify DNA content [16].

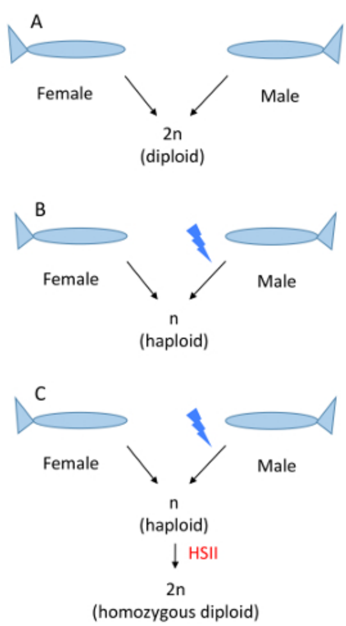

Figure 1: Fertilization types. (**A**) Natural mating. Male and female gametes are untreated, resulting in a natural diploid. (**B**) The sperm is treated with UV, resulting in the destruction of the paternal DNA and the production of haploid zygotes. If untreated, such haploids do not survive past day 2-3 of development. (**C**) Treatment of haploid zygotes with Heat Shock 2 (HS2) inhibits one cycle of cytokinesis of mitosis in the early embryo. This cell cycle stall, coupled to unaffected DNA replication, generates homozygous diploids that can become viable and fertile adults. Please click here to view a larger version of this figure.

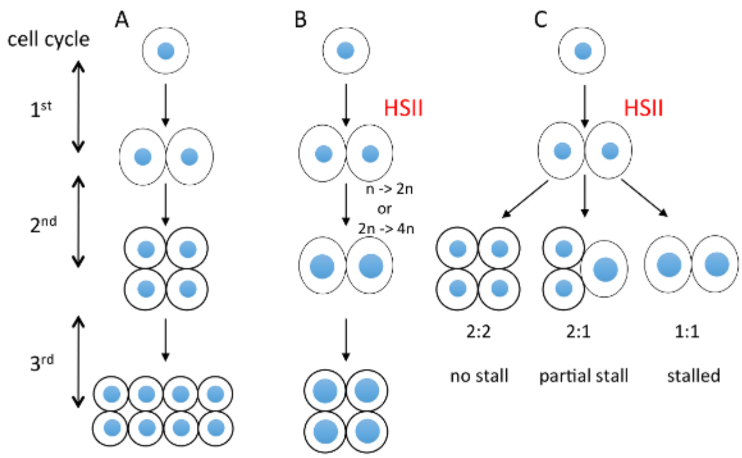

Figure 2: HS2 promotes whole genome duplication by stalling the second cell cycle. (**A**) Cell cleavage pattern of an untreated embryo during the first three cell cycles, corresponding to 2-, 4-, and 8-cell embryos. (**B**) HS2 treatment results in a one-cycle stall of cell division during the second cell cycle. During the stall, ongoing DNA synthesis results in embryos undergoing diploidization, from either haploid to diploid (n -> 2n) or diploid to tetraploid (2n -> 4n) (see text). After this stall, the embryo resumes cell division, with the newly acquired ploidy. (**C**) HS2-treated embryos can exhibit a variety of blastomere arrangements depending whether blastomeres exhibit a cell division delay during the second cell cycle: i) "no stall": neither blastomere exhibits a delay, resulting in a 2:2 arrangement characteristic of 4-cell stage embryos, ii) "partial stall": only one of two blastomeres exhibits a delay, resulting in an aberrant 2:1 arrangement, and iii) "stalled": both blastomeres exhibit a delay, resulting in a 1:1 arrangement characteristic of 2-cell stage embryos. In (**A**-**C**), nuclei are represented as blue circles, with the diploidization event represented by an expansion of the nuclear size. See reference [2] for a diagram depicting centriolar behavior as the proposed basis for the cell division stall. Please click here to view a larger version of this figure.

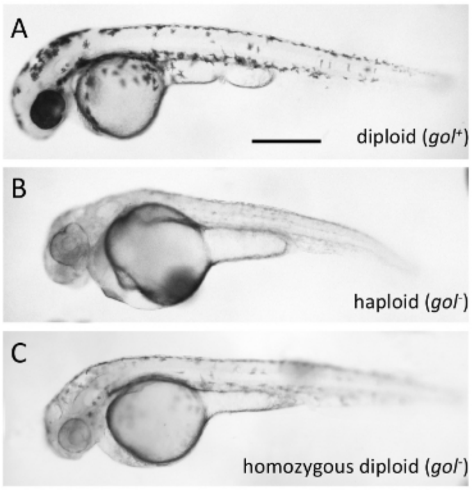

Figure 3: Morphology of natural diploids, haploids and gynogenotes. (**A**) Normal embryonic morphology of a diploid obtained through natural crosses. (**B**) Haploid embryos exhibit a shorter and wider body axis. (**C**) Homozygous diploids have normal body morphology. Embryos additionally carry a recessive mutation in the pigmentation gene *golden* to facilitate tracking parental DNA inheritance: mothers are homozygous carriers for a mutation in *golden,* and fathers wild-type for this gene. Thus, natural diploids, heterozygous for *golden*, exhibit wild-type pigmentation, whereas both haploids (hemizygous for *golden*) and homozygous diploids (homozygous for *golden*) exhibit mutant pigmentation. Scale bar = 0.5 mm. Panels modified from reference [2], reprinted with permission. Please click here to view a larger version of this figure.

Solution	Components	Storage conditions
Hanks' Solution 1	8.0 g NaCl, and 0.4 g KCl in 100 ml ddH_2O	Store at 4°C
Hanks' Solution 2	0.358 g Anhydrous Na_2HPO_4, and 0.6 g KH_2PO_4 in 100 ml ddH_2O	Store at 4°C
Hanks' Solution 4	0.72 g $CaCl_2$ in 50 ml ddH_2O	Store at 4°C
Hanks' Solution 5	1.23 g $MgSO_4 \cdot 7H_2O$ in 50 ml ddH_2O	Store at 4°C
Hank's Premix	Add, in the following order: 10.0 ml Solution 1, 1.0 ml Solution 2, 1.0 ml Solution 4, 86.0 ml ddH2O, and 1.0 ml Solution 5	Store at 4°C
Hanks' Solution 6	0.33 g $NaHCO_3$ in 10 ml ddH_2O	Prepare fresh the morning of the IVF procedure

Table 1. Preparation of Hank's solutions.

Discussion

Critical steps

It is critical to work under conditions of effective in vitro fertilization. To insure a good supply of mature eggs (step 1), females set up for mating should not have been set up in mating crosses for at least 5 days and should appear gravid. During interruption of breeding, an observer can monitor 15-30 tanks adequately for the first appearance of natural egg extrusion. Interruption of mating should occur as soon as possible when the first eggs are released through natural matings, in order to allow most eggs to remain inside the females for the IVF procedure. These females, which are now ready for manual extrusion of mature eggs, can be kept separated for up to 2 hr without an apparent effect on subsequent egg release. To prepare an effective sperm solution (step 2), males should appear robust and ideally with a reddish tint, which is characteristic of breeding zebrafish males. A clean dissection typically involves removing the intestinal track by pulling it towards to the posterior of the body, and the swim bladder by pulling it anteriorly. After removal of central internal organs, the testes remain as translucent elongated masses alongside each side of the internal body wall. Avoid including unwanted organs (*e.g.* intestines) into the sperm solution; if needed separate these from testes by working on a dry petri plate surface before being placed into the Hank's solution. To achieve high fertilization rates during IVF (step 4), it is essential that extruded eggs are maintained competent until sperm addition. Eggs competent for fertilization exhibit a slightly yellow tone and are translucent. Avoid eggs having any contact with water prior to sperm addition, since water will trigger egg activation and premature activation will preclude fertilization. Water activation and fertilization should occur within 90 sec of extrusion from the female in order to avoid dehydration of eggs, which will lead to their degradation. If needed, extruded eggs can be kept for longer periods (up to 1.5 hr or more) in 100-200 µl of Hank's solution supplemented with 0.5% Bovine Serum Albumin (BSA) [17] (when preparing the Hank's solution for this specific purpose, prepare the premix to correct for water content added during BSA supplementation). If eggs have been kept competent with the help of Hank's BSA solution, remove excess Hank's BSA from the eggs using a micropipette and fertilize within 1 min.

A critical step for the success of diploidization using HS2 is the sorting of stalled embryos (step 7). Initial sorting of symmetrically cleaving 2-cell embryos selects for embryos that become fertilized and begin cell division (occasional dispermy during IVF results in embryos that transition directly from 1- to 4-cells at 35-50 mpf – such embryos should be discarded). Cell cycling occurs every 15 min during these early embryonic stages (35-50 mpf for the first cell cycle, 50-65 mpf for the second cell cycle, and so on), so sorting of cleaving embryos during the first 10 min of each cell cycle insures an absence of overlap between cycles and accurate identification of a cell division stall.

Modifications and troubleshooting

The strength of the UV treatment in step 3 (*e.g.* exposure time, lamp power, distance to lamp) can be adjusted if needed by testing initial fertilization rates as well as the frequency of haploids in the absence of subsequent HSII treatment: the correct amount of UV exposure does not have a noticeable effect on fertilization rates while resulting in 100% of haploid embryos (see **Representative Results** to distinguish haploid from diploid embryos). Too much UV exposure causes reduced fertilization rates presumably due to deleterious effects on sperm function, while insufficient UV exposure produces diploid embryos due to incomplete inactivation of sperm DNA.

If embryos exhibit developmental defects or lethality after resumption of cell cleavage (step 7), the heat shock temperature in step 6 can be reduced slightly (*e.g.* to 41.0 °C) to increase embryo survival; conditions that result in approximately equal fractions of aberrant 3-cell and stalled 2-cell embryos during the second cell cycle (and are therefore near threshold for an effect on centriolar duplication) result in minimal subsequent developmental defects and increased survival after resumption of cell cycling.

The HS2 method incorporates a convenient and intrinsic mechanism to assess cell division stall (and therefore whole genome duplication), since direct observation of embryos as they develop allows tracking the various cell cycles. Manual selection of embryos that have undergone a one-cell cycle division stall allows generating a uniform population of diploidized embryos. HS2 can be carried out in any zebrafish genetic strain (*e.g.* AB, Tübingen, WIK), as the one-cell division stall acts as an intrinsic mechanism to confirm DNA duplication. Additionally, visible genetic markers introduced into the strain can be used to facilitate the identification of diploidized embryos (**Figure 3**). For example, the use of eggs from females that are homozygous for recessive mutations in pigmentation genes, such as *golden* or *albino,* can be combined with sperm derived from wild-type strains carrying normal alleles for the same pigmentation gene. In this situation, because UV-treated sperm cannot contribute wild-type pigment alleles, haploid and homozygous diploid embryos exhibit the recessive pigment mutation. In addition, homozygous diploids exhibit wild-type morphology at 24 hpf, in sharp contrast to the less extended haploid morphology. The combination of the appearance of the maternal recessive trait and overall embryo morphology confirms successful ploidy duplication. Further confirmation can be obtained through chromosome counts of treated and untreated embryos.

The above-described genetic marking system, based on recessive visible genetic markers, also allows confirming the absence of sperm-derived DNA in the progeny, since the resulting embryos exhibit wild-type pigmentation only if sperm has not been fully inactivated by the UV treatment. In the absence of a genetic marking system, a sample of embryos can be allowed to develop in the absence of heat shock to confirm that all embryos exhibit the haploid morphology at 24 hpf, and thus that sperm DNA inactivation was complete. In conjunction with fully inactivated sperm, this same genetic marking system also allows detecting potential spontaneous polar body failure. Such an event would result in the appearance of recessively marked embryos with diploid morphology without a diploidization treatment. Spontaneous polar body failure has not been observed in zebrafish but is reported in *Xenopus tropicalis* [18]. If present in a given system, this spontaneous event would have to be reduced or controlled for in order to optimally implement ploidy manipulation.

Limitations of the technique

The ability to produce viable gynogenotes relies on the absence of background gene variants which when homozygous are deleterious. Thus, success of the procedure will vary depending on the genetic strain used. Selection of genetic strains through multiple generations of gynogenesis has been shown to improve viability [1].

The HS2 method, when used in conjunction with untreated sperm to produce diploid embryos, also allows production of tetraploid embryos at a high frequency. However, in this case, due to the diploid to tetraploid transformation, genetic markers do not readily allow for the confirmation of whole genome duplication. In this case, observation of the one cell cycle stall in synchronized embryos and selection of stalled embryos should insure tetraploid selection, and chromosome counts can be used to further confirm ploidy.

Significance with respect to existing/alternate methods

Although described by Streisinger over 40 years ago [1], the standard HS method has not been widely used due to poor yield. Instead, ploidy manipulation has almost exclusively relied on the alternative and relatively more efficient method of Early Pressure, which results in ploidy duplication through the inhibition of the second meiotic division. However, Early Pressure results in variable proportions of gene homozygosity [9,19], which lessens its value as a genetic tool. Recent studies show that HS2, a modification of the standard HS protocol, namely the application of the heat pulse at a different time during the first cell cycle (the 22-24 mpf period in HS2, compared to 12-14 mpf in standard HS) results in up to 4 times increased yield of homozygous diploid productions.

The action of the heat pulse can be inferred to occur by virtue of the inhibition of centriole duplication during the time of heat shock (22-24 mpf, during the first cell cycle), which consequently lacks the proper complement to generate a spindle and mediate cell division during the following cell cycle (50-65 mpf, corresponding to the second cell cycle). Centriole duplication can resume in the absence of heat shock during subsequent cell cycles, allowing development to proceed after a precise one-cell cycle stall. Because centriole duplication and DNA replication cycles are interdependent [20], DNA replication proceeds normally even during the stalled division in the second cell cycle. This results in precise whole genome duplication and complete homozygosis.

Future applications

Ploidy manipulation is a convenient method to facilitate genetic analysis. In particular, the coupling of haploid embryo production (through in vitro fertilization with UV-treated sperm) and ploidy duplication (through HS2) allows the direct (in one generation) homozygosis in a high and fixed proportion (50%) of mutations present in a heterozygous female. This in turn helps bypass additional generations that would be required to reach homozygosis using natural crosses, reducing the amount and space needed for such genetic schemes. Such method can facilitate genetic schemes such as genetic screens, in particular those that would require multiple generations involving adult and/or parental-effect genes, or those involving multiple alleles, such as in suppressor/enhancer screens [21].

Approaches similar to HS2 can be applied to other species with external fertilization, even those not currently being used as genetic systems. This method in particular takes advantage of a period of time from fertilization through the initiation of blastomere cycling where a heat pulse can affect centriole duplication and generate a precise cell division stall and whole genome duplication without producing deleterious developmental effects. Thus, this early time period can be explored for heat shock sensitivity to develop ploidy manipulation-based genetic methods in other animal systems.

Disclosures

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