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Ploidy manipulation of zebrafish embryos with Heat Shock II 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 II (HSII). 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.
Author Comments:	We have revised the manuscript according to the comments. Please note some references had brackets in the manuscript (e.g. [2]) and I was not sure whether I had to correct that or not (I left them unchanged).
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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Madison, March 23, 2016

Dear Mr. Castaldo,

We respectfully submit a revision of our manuscript, entitled "*Ploidy manipulation of zebrafish embryos with Heat Shock II treatment"* by Baars et al, for consideration for publication in *Jove*. In this resubmission, we have tried to address all editorial and scientific concerns to the best of our abilities, resulting in what we think is an improved manuscript.

Thank you for your consideration of our manuscript in *Jove*. Sincerely,

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TITLE:
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SHORT ABSTRACT:
A modified protocol for ploidy manipulation uses a heat shock to induce a one-cycle stall in cytokinesis in the early embryo. This protocol is demonstrated in the zebrafish but may be applicable to other species.

LONG 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 II (HSII). 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.

INTRODUCTION:
This protocol allows the manipulation of ploidy in zebrafish embryos, such as in the generation of homozygous gynogenetic diploids from gynogenetic haploids (Fig. 1) or the production of tetraploids. This is achieved by inducing a delay in cytokinesis corresponding to precisely one cell cycle (Fig. 2A,B). 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 II (HSII - the term “II” reflects that

the heat pulse occurs at a later time point than the standard HS method, and that the cell cycle delay caused by HSII 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. HSII 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 HSII-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 HSII 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 HSII 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 hours 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.

1.1) 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.

1.2) 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.

1.3) 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 HSII treatment become tetraploid embryos) or UV-treated, to generate haploid zygotes (which upon HSII 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.

2.1) 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 ul of the freshly made Solution 6.

2.2) Euthanize males by overexposure to tricaine as a 0.016% solution in conditioned water.

2.2.1) 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^{o}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.

2.2.2) 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).

2.2.3) 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.

2.3) 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 minutes on a petri plate surface after dissection and before placing them in the Hank's solution.

2.4) 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 1000 µl-tip micropipette, while avoiding air bubble formation. Let the testes debris settle.

2.5) Store the sperm solution in ice, where it can keep its potency for up to 2-3 hours. 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.

3.1) 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.

3.2) 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 seconds with a 115V (60Hz, 0.68A) UV-lamp at a 19 cm (7.5 in.) 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 seconds).

3.3) 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 hours 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.

4.1) 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 minutes, until fish stop gill movement.

4.2) 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.

4.3) 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.

4.4) 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 minutes.

4.5) Activate (with water exposure) and fertilize (with sperm solution addition) eggs simultaneously and within 90 seconds 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 seconds [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.

5.1) 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.

5.2) 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.

5.3) 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].

6.1) 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.

6.2) Place the tea strainer with the embryos inside a beaker in a pre-heated bath with E3 at 28.5ºC. 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.

6.3) At 22 mpf, remove the tea strainer from the 28.5ºC 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 41.4ºC.

6.4) At 24 mpf, transfer the tea strainer back to the E3 in the water bath at 28.5ºC 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

7.1) 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.

7.2) Continue observing the fertilized embryos, selected for normal cell division during the first cell cycle, and during the second cell cycle (50-65 mpf).

7.3) Sort embryos according to the following categories (Fig. 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.

7.4) 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 (Fig. 3A,C)), in contrast to reduced axis extension and increased body thickness characteristic of haploids (Fig. 3B)), and/or assess diploidization using genetic pigment markers and other assays, such as *golden* or *albino* [2,6,9] (Fig. 3 and see below).

7.5) 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 (Fig. 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 II (HSII), uses a heat shock during the period 22-24 mpf and induces cytokinesis arrest during the second embryonic cell division at 50 mpf (Fig. 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 (Fig. 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 HSII, 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 LEGENDS:

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 II (HSII) 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.

Figure 2. HSII 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) HSII 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) HSII-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.

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 is 0.5 mm. Panels modified from reference [2], reprinted with permission.

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 hours 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 seconds 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 hours 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 minute.

A critical step for the success of diploidization using HSII 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 minutes 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 minutes 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^{o}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 HSII 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. HSII 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 (Fig. 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 HSII 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 HSII, 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 HSII, 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 HSII) 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 HSII 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.

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DISCLOSURES:

The authors do not have competing financial interests.

REFERENCES:

1 Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* **291** 293-296 (1981).

2 Heier, J., Takle, K., Hasley, A. & Pelegri, F. Ploidy manipulation and induction of alternate clevage patterns through inhibition of centrosome duplication in the early zebrafish embryo. *Dev. Dyn.* **244** 1300-1312 (2015).

3 Zhang, X. & Onozato, H. Hydrostatic pressure treatment during the first mitosis does not suppress the first cleavage but the second one. *Aquaculture* **240** 101-113 (2004).

4 Zhu, X. P., You, F., Zhang, P. J., Xu, J. H. & Sun, W. Effects of hydrostatic pressure on microtubule organization and cell cycle in gynogenetically activated eggs of olive flounder (*Paralichthys olivaceus*). *Theriogenology* **68** 873-881 (2007)

5 Vertii, A., Zimmerman, W., Ivshina, M. & Doxsey, S. Centrosome-intrinsic mechanisms modulate centrosome integrity during fever. *Mol. Biol. Cell.* **26** 3451-3463 (2015).

6 Walker, C. in *The zebrafish: Genetics and genomics* Vol. 60 *Methods in Cell Biology* eds W.H. Detrich, M. Westerfield, & L.I. Zon) 43-70 (Academic Press, 1999).
7 Blanco-Vives, B. & Sánchez-Vázquez, F. J. Synchronisation to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. *Physiol. Behav.* **98** 268-275 (2009).
8 Dekens, M. P. S. *et al.* Light regulates the cell cycle in zebrafish. *Curr. Biol.* **13** 2051-2057 (2003).
9 Pelegri, F. & Schulte-Merker, S. in *The Zebrafish: Genetics and Genomics* Vol. 60 *Methods in Cell Biology* eds W. Detrich, L.I. Zon, & M. Westerfield) 1-20 (Academic Press, 1999).
10 Hart, N. H., Becker, K. A. & Wolenski, J. S. The sperm entry site during fertilization of the zebrafish egg: localization of actin. *Mol. Reprod. Dev.* **32** 217-228 (1992).
11 Tsaadon, A., Eliyahu, E., Shtraizent, N. & Shalgi, R. When a sperm meets an egg: block to polyspermy. *Mol. Cell. Endocrinol.* **252** 107-114 (2006).
12 Wong, J. L. & Wessel, G. M. Defending the zygote: search for the ancestral animal block to polyspermy. *Curr. Top. Dev. Biol.* **72** 1-151 (2006).
13 Rappaport, R. & Rappaport, B. N. Establishment of cleavage furrows by the mitotic spindle. *J. Exp. Zool.* **189** 189-196 (1974).
14 Wühr, M., Tan, E. S., Parker, S. K., Detrich, H. W. I. & Mitchinson, T. J. A model for cleavage plane determination in early amphibian and fish embryos. *Curr. Biol.* **20** 2040-2045 (2010).
15 Yabe, T., Ge, X. & Pelegri, F. The zebrafish maternal-effect gene *cellular atoll* encodes the centriolar component Sas-6 and defects in its paternal function promote whole genome duplication. *Dev. Biol.* **312** 44-60 (2007).
16 Poss, K.D., Nechiporuk, A., Stringer, K.F., Lee, C. & Keating, M.T. Germ cell aneuploidy in zebrafish with mutations in the mitotic checkpoint gene *mps1. Genes Dev.* **18** 1527-1532 (2004).
17 Sakai, N., Burgess, S. & Hopkins, N. Delayed in vitro fertilization of zebrafish eggs in Hank's saline containing bovine serum albumin. *Mol. Mar. Biol. Biotechnol.* **6** 84-87 (1997)
18 Roco, A. S., Olmstead, A. W., Degitz, S. J., Amano, T., Zimmerman, L. B., Bullejos, M. Coexistence of Y, W, and Z sex chromosomes in *Xenopus tropicalis. Proc. Natl. Acad. Sci. USA* **112** E4752-E4761 (2015).
19 Streisinger, G., Singer, F., Walker, C., Knauber, D. & Dower, N. Segregation analyses and gene-centromere distances in zebrafish. *Genetics* **112** 311-319 (1986).
20 Dekens, M. P. S., Pelegri, F. J., Maischein, H.-M. & Nüsslein-Volhard, C. The maternal-effect gene *futile cycle* is essential for pronuclear congression and mitotic spindle assembly in the zebrafish zygote. *Development* **130** 3907-3916 (2003).
21 Pelegri, F. & Mullins, M. in *Met. Cell Biol.* Vol. 104 eds H.W. III Detrich, M. Westerfield, & L.I. Zon) 83-120 (2011).

Table 1. Preparation of Hank's solutions.

Solution	**Components**	**Storage conditions**
Hanks' Solution 1	8.0 g NaCl, and 0.4 g KCl in 100 ml ddH_2O	Store at 4ºC
Hanks' Solution 2	0.358 g Anhydrous Na_2HPO_4, and 0.6 g KH_2PO_4 in 100 ml ddH_2O	Store at 4ºC
Hanks' Solution 4	0.72 g $CaCl_2$ in 50 ml ddH_2O	Store at 4ºC
Hanks' Solution 5	1.23 g $MgSO_4 \cdot 7H_2O$ in 50 ml ddH_2O	Store at 4ºC
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 4ºC
Hanks' Solution 6	0.33 g $NaHCO_3$ in 10 ml ddH_2O	Prepare fresh the morning of the IVF procedure

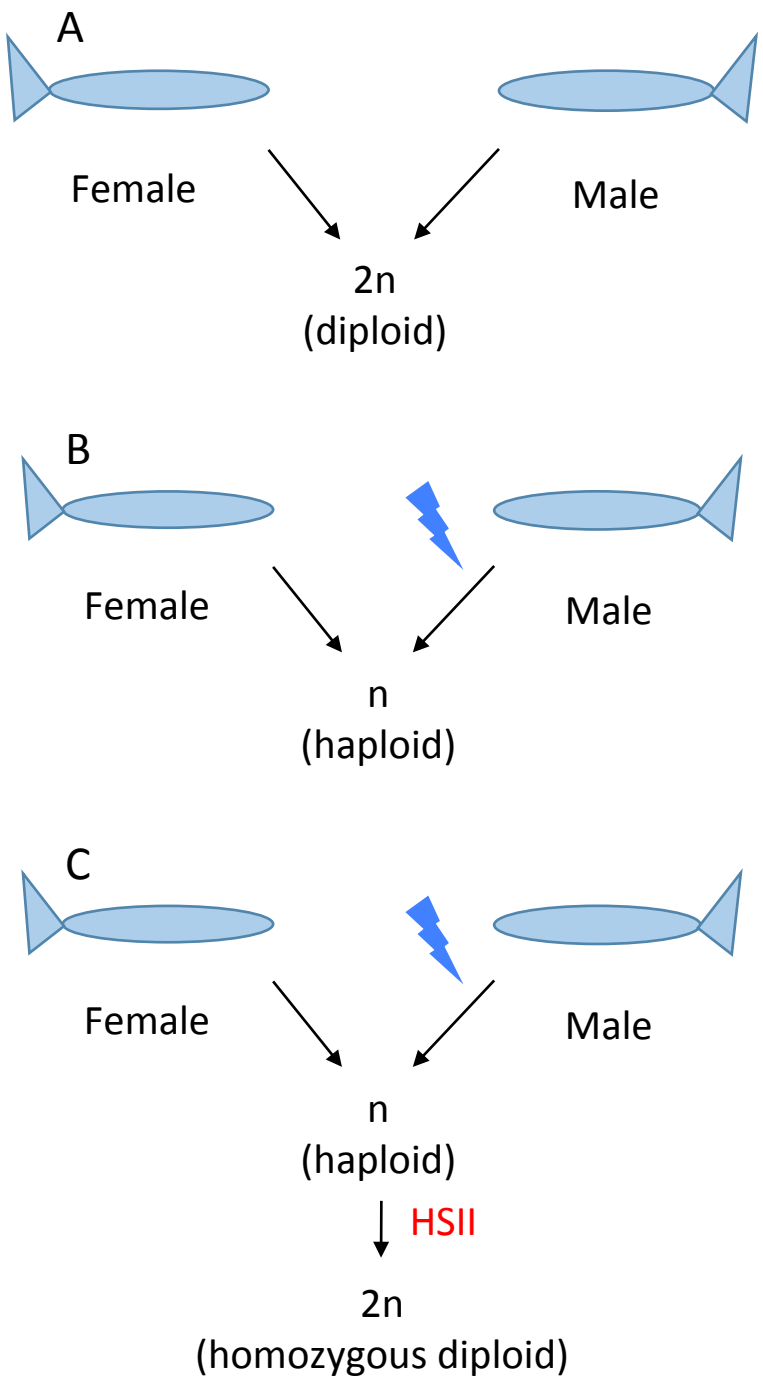


Figure 2

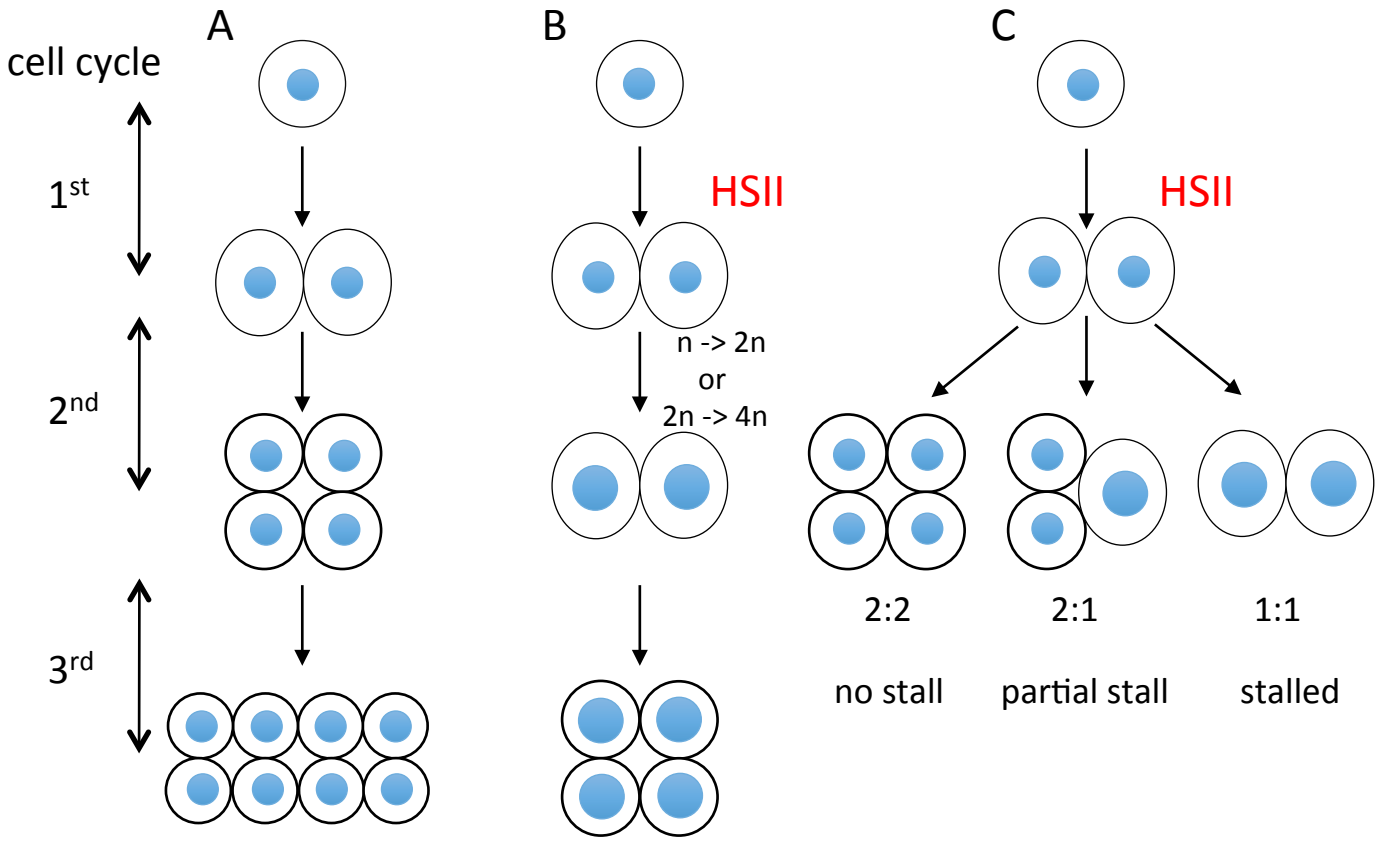
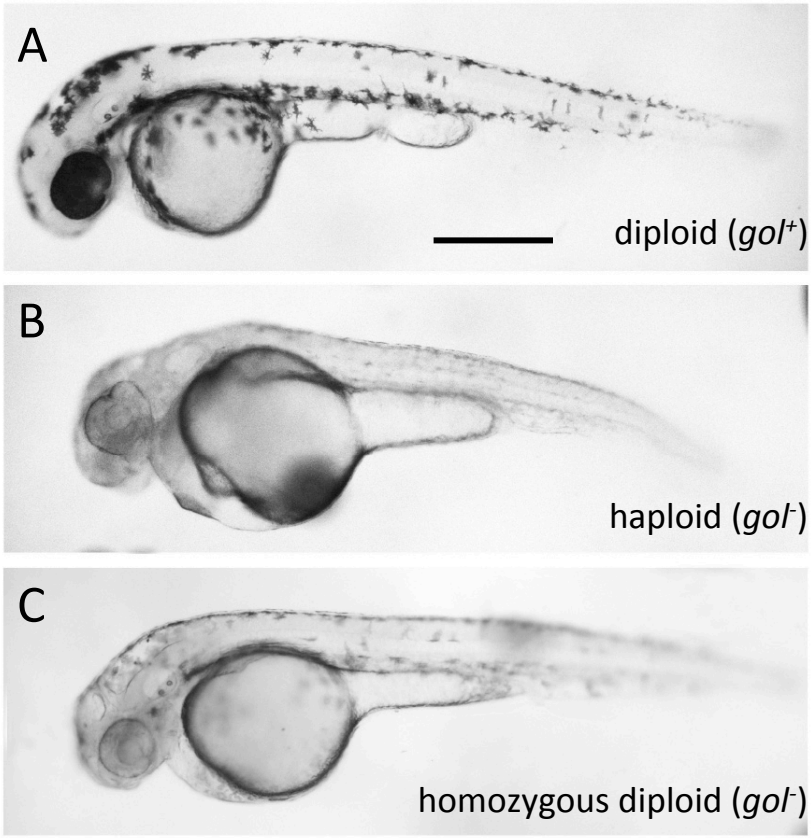


Figure 3



Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Zebrafish mating boxes	Aqua Schwarz	SpawningBox1	
NaCl	Sigma	S5886	
KCl	Sigma	P5405	
Na2HPO4	Sigma	S3264	
KH2PO4	Sigma	P9791	
CaCl2	Sigma	C7902	
MgSO4-7H2O	Sigma	63138	
NaHCO3	Sigma	S5761	
Tricaine	Western Chemical	Tricaine-D (MS 222)	FDA approved (ANADA 200-226)
Tris base	Sigma	77-86-1	to prepare 1 M Tris pH 9.0
HCl	Sigma	920-1	to prepare 1 M Tris pH 9.0
Fish net (fine mesh) (4-5 in)	PennPlax	(ThatFishThatPlace # 212370)	available in ThatFishThatPlace
Plastic spoon			available in most standard stores
Dissecting scissors	Fine Science Tools	14091-09	
Dissecting forceps	Dumont	SS	available from Fine Science Tools
Dissecting stereoscope (with transmitted light source)	Nikon	SMZ645	or equivalent
Reflective light source (LED arms)	Fostec	KL1600 LED	or equivalent
Petri plates 10 cm diameter			any maker
Eppendorf tubes 1.5 ml			any maker
Ice bucket			any maker
Pipetteman P-1000			any maker
Pipette tips 1000 µl			any maker
Narrow spatula	Fisher	14-374	
Depression glass plate	Corning Inc	722085 (Fisher cat. No 13-748B)	available from Fisher Scientific

UV lamp	UVP	Model XX-15 (cat No. UVP18006201)	available from Fisher Scientific. Although not observed by us with th
UV glasses			any maker
Paper towels			any maker
Kimwipes	Kimberly-Clark	06-666-11	available from Fisher Scientific
Timer stop watch			any maker
Wash bottle	Thermo Scientific	24020500	available from Fisher Scientific
Tea strainer			available in kitchen stores
beakers, 250 ml (2)	Corning Inc.	1000250	available from Fisher Scientific
water bath (2)			any maker, with accurary to 0.1 C (e.g. Shel Lab H2O Bath Series)
Hanks' Solution 1	see above	see above	8.0g NaCl, 0.4g KCl in 100ml ddH_2O. Store at 4oC.
Hanks' Solution 2	see above	see above	0.358g Anhydrous Na2HPO4, 0.6g KH2PO4 in 100ml ddH2O. Store at
Hanks' Solution 4	see above	see above	0.72g CaCl2 in 50ml ddH2O. Store at 4oC.
Hanks' Solution 5	see above	see above	1.23g MgSO4 · 7H2O in 50ml ddH2O. Store at 4oC.
Hank's Premix	see above	see above	add, in the following order: 10.0 ml Solution 1; 1.0 ml Solution 2; 1.
Hanks' Solution 6	see above	see above	0.33g NaHCO3 in 10ml ddH2O. Prepare fresh the morning of the IVF
Hank's Solution (final solution)	see above	see above	Combine 990ul of Hank's Premix and 10ul of freshly made Solution 6

is model, some UV sources have been observed to experience a decrease of intensity over time (if this is the case, see Modifications

t 4oC.

.0 ml Solution 4; 86.0 ml ddH2O; 1.0 ml Solution 5. Store at 4oC

procedure.

5 (NaHCO3 solution)

and Troubleshooting)

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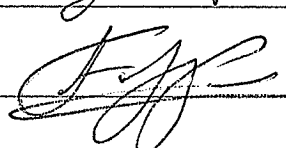
Signature:  Date: 1.13.16

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JoVE54492R1 (Ploidy manipulation of zebrafish embryos with Heat Shock II treatment" (Baars et al.)

Response to Editorial comments (unrequested changes in **bold**)

-At the start of the Protocol, an animal care/use statement should be included (this can be moved from the Acknowledgements section).

The animal care/use statement has been moved from the acknowledgement section to the beginning of the protocol (Introduction section)

-Please use complete sentences in 2.1.1 and 2.1.2. Alternatively, we suggest removing the recipes from the actual protocol, and instead include the components/amounts in a Table at the end of the manuscript.
This information has been deleted here and added to a table (Table 1) at the end of the manuscript.

-2.1.3-Please change the tense to the imperative.
Tense have been changed (please note entire section 2.1 has been condensed (after transferring information to Table 1) so there are no longer subsections

-Please check that spaces are included between numbers and their units.
The text has been revised for this

-Please remove the subheadings from the "Critical Steps" portion of the Discussion, and reformat this text into ~2 cohesive paragraphs. As written, this section resembles a bulleted list.
The subheadings have been removed and the text has been reformatted into paragraphs

-Section 6 Note: This note is long (>5 sentences), and includes a great deal of background information that may be better-suited for the Introduction section. Consider moving this text.
A portion of Note 6 has been moved to the Introduction in order to shorten that Note.

•Length - As written, the highlighted portion of the protocol exceeds the 2.75 page limit. It is recommended to remove the highlighting from Step 2.4, and possibly Steps 2.1.1 and 2.1.2.
Highlighting from Step 2.4 has been removed. Steps 2.1.1 and 2.1.2 have already been restructured and shortened, through moving of information to Table 1 (see above).

•Visualization - As 2.4 will be difficult to visualize, it is recommended to remove the highlighting from this step. However, to improve visualization, we recommended highlighting "make a longitudinal cut along the abdomen with dissecting scissors" in 2.6.
Highlighting in Step 2.4 has been removed. The statement "make a longitudinal cut along the abdomen with dissecting scissors" has been highlighted.

•Additional detail is required:

-Please include a superscript reference for the information in the first Step 1 note (dealing with egg extrusion).
A reference has been added.

-1.1-What strain/line of zebrafish is used? How does this relate to golden, mentioned in the Figure 3 legend?
We have added in Step 1.1 that the technique can be applied to zebrafish of any strain. We have also added this information to the Discussion (under "Troubleshooting and modifications"), with some specific strains as examples. There, we also make a connection to the pigment mutations that can be introduced into the strain to act as an independent marker for diploidization.

-2.3-2.5 should be substeps of 2.2. In addition, for 2.2, how is euthanasia confirmed?
Steps 2.3-2.5 have been reorganized as substeps 2.2.1-2.2.3, and other steps in section 2 have been relabeled. A statement on assessing euthanasia has been added to substep 2.2 (this statement has been left unhighlighted, but it can be highlighted if appropriate).

-2.6-How are testes identified (by what morphological characteristics)?
A statement on how to identify the testes has been added (this statement has also been left unhighlighted, but it can be highlighted if appropriate).

-7.4-If possible, please add a note detailing what specific morphological characteristics are assessed at the 24 hpf stage. In this note, also include a reference for pigment markers/other assays that can be performed.
A brief description of morphological characteristics has been added (unhighlighted). Two examples of pigment markers and references have been added.

•Branding should be removed:

-2.7, 3.3, Discussion-Pipetteman
changed to "micropipette"

-2.8, 3.3-Eppendorf
changed to "microfuge"

-4.3-labwipes
changed to "lab wipes" (this was what seemed to be the best generic term from an internet search)

Additional change:

- An additional funding source (RO1 GM065303) has been added to the acknowledgements.

•Results

-Please include an image in the Results section of actual zebrafish embryos demonstrating the different types/cell distributions mentioned in Step 7.3. This would greatly help supplement the current Figure 2.
A diagram representing the various embryo cleavage types has been added to Figure 2.

Additional references to Figures have been added earlier in the text in order to maintain the figure presentation order

-Please include a scale bar in Figure 3.
We have added a scale bar to the figure.

Response to Reviewer's comments (unrequested changes in **bold**)

We thank both Reviewers for their time and helpful critiques. We have tried to address these as best as possible, resulting in what we think is an improved manuscript.

Reviewer 1
None

Minor comments: The term "gynogenetic diploid" is used throughout the manuscript. I'm under the impression this term can be confused with embryos produced by suppression of 2nd polar body formation, which may not be completely homozygous (see ZFIN methods). Other terms, such as 'double haploid' or 'homozygous diploid' may be preferable for the embryos produced by the method described here.

We have revised the manuscript to avoid confusion between terms.

We have added the following sentence in the introduction (we have also simplified the term referring to gynogenetic haploids):

"For conciseness, we refer to gynogenetic haploid embryos as "haploids", and homozygous, gynogenetic diploid embryos as "homozygous diploids"."

The substituted these terms accordingly.

In 'Representative Results', it would be useful to provide some kind of yield numbers with which to gauge successful experiments. With good quality parents, what fraction of offspring can be recovered from haploid to diploid?

We have added the following statement to the "Representative Results" section "Using an unselected mixed AB/Tübingen background genetic strain, yields of homozygous diploid induction by HSII, as assayed by morphology at 24 hpf, vary from 10% to 50% [2]. Selection of lines by propagation through HSII is expected to increase the effective yield [1]."

Additional Comments to Authors:

One phenomenon that has been observed in other lower vertebrate species (e.g. Xenopus) is spontaneous polar body failure. Haploid Xenopus embryos sometimes spontaneously recover diploidy, and can complicate analysis of production of double haploids by pressure suppression of first cleavage (Roco et al 2015 doi: 10.1073/pnas.1505291112). Perhaps the authors can comment on this phenomenon (has been observed in Danio?) given the potential application of the method to other species.

Polar body failure has not been observed in wild-type zebrafish. We have, however, added a reference to this phenomenon in the "Limitations and troubleshooting" section. We specifically state how the suggested genetic marking system can detect spontaneous polar body failure.

Some UV sources (used for inactivation of sperm) degrade fairly quickly; the authors may wish to comment.

We have no experienced UV source decreases while using two different UV source set ups. However, we have added a note to the Excel table cautioning users of this potential problem.

Reviewer 2

Some molecular or cytogenetic evidence should be provided to validate their claims of what is accomplished through these manipulations and to provide a way for investigators using their method to confirm that they have accomplished their goals.

References to assess successful diploidization (viability of gynogenotes, chromosome counts, quantitation of DNA diameter) have been added to the Representative Results section. With regards to the HSII method, evidence of successful diploidization has already been published, in the article on which this JoVE article is based on (Heier et al 2015).
Additional evidence has been added to direct the reader towards molecular methods to assess ploidy (Poss et al 2004).

Some of these methods have been published before, and are incompletely cited.

This JoVE article is based on a recently article published in Developmental Dynamics (Heier et al, 2015). These studies were in turn based on earlier work by Stressinger and co-workers (1981). The purpose of this manuscript is to present the already published methodology in technical and visual detail, to facilitate its implementation.

We have tried to appropriately cite all relevant references, in particular the seminal contribution by Streisinger and colleagues to this methodology. We have added a sentence to more clearly spell out the contribution for two additional studies (Zhang and Onozato, 2004; Zhu et al 2007), which showed the stalling effect on the cell cycle and chromosome duplication. Findings in Heier et al (2015) were obtained independently of these two later studies, but also acknowledge their contribution. Our work in Heier et al. (2015) further developed the original Streissinger method and additional findings to assess its genetic usefulness with regard to the generation of viable diploidized embryos and genetic segregation of alleles present in the mother.

Second, given the known frequency of just coding mutations alone in humans (on the order of 10,000 per person), it is not really easy to believe that a doubled haploid can be viable to adulthood; this affects some of the comments made. The implications of arrested development and lethality in haploid and doubled haploid embryos should be addressed for genetic screens.

Streisinger et al (1981) showed that these methods, including HS (the HSII precursor) led to viable adults. Such adults could be maintained for multiple generations through gynogenetic methods. Yields were low in the initial experiments, but increased through selection using these methods. This increase in yield suggests a "purging" of deleterious alleles through selection.

We have previously grown HS-derived fish to adulthood and established lethal-free lines with these fish (Pelegri and Schulte-merker, 1999). We have indeed used gynogenetic methods to carry out genetic screens, but those screens were based on the EP method, which has some heterozygosity after inhibition of meiosis II. The current improvements on HSII yield now allow inhibition of mitosis as the basis for improved genetic screens, which we are currently undertaken. For this purpose, our laboratory has re-established lethal-free lines (using HSII) which are currently in their third generation. Results from HSII-based screens will require several years, but we believe there is ample evidence that shows that HS- and HSII-derived fish can be viable and can be selected to become robust lines, and that the HS-method can constitute the basis for a genetic screen. The information will be useful to scientists in other model system, each with its own peculiarities with regards to the application of gynogenesis. It is the hope that publication of this JoVE article will promote the use of this technique in a variety of model systems, in addition to zebrafish research.

Third, the arguments would be more easily understood if Figure 2 showed nuclei and their ploidy.

Nuclei and ploidy number have been added to Figure 2. The diploidization event is indicated. A Statement in the legend has been added to direct the reader to Heier et al. 2015, which has diagrams representing centrioles (we preferred not to present centrioles in this manuscript in order to emphasize the methods and not their underlying cause)

Minor Concerns:

1) Grammatical problem in the second sentence of the abstract.

this has been corrected

2) Why the name HSII?

The term "II" differentiates this abbreviation from that for the standard HS method, and reflects that fact that the cell cycle delay caused by this method occurs during the second cell cycle, not the first as in the standard HS. We have expanded the introductory paragraph to better convey the history of the method and included this information in it.

A reference to a recent article on heat-induced centrosome degradation has been added to the introduction (Vertii et al)

3) "Every single locus" (not loci, which is plural)

This has been corrected

4) Lines 499-500 "of each other" is not needed.

These words have been deleted

Copyright clearance for Fig. 3