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# Multi-color FISH facilitates analysis of cell-type diversification and developmental gene regulation in the *Parasteatoda* spider embryo

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## Materials and methods

*Probe synthesis*

Four types of labeled antisense RNA probes, digoxigenin (DIG)-, fluorescein (FLU)-, biotin-, and 2,4-dinitrophenyl (DNP)-labeled probes, were prepared by in vitro transcription. The former three were synthesized using 10× RNA labeling mixes, each of which contained a modified UTP, DIG-11-UTP, FLU-12-UTP, or biotin-16-UTP (Roche 1277073, 1685619, and 1685597). DNP-labeled probes were synthesized using DNP-11-UTP (PerkinElmer NEL555001EA) at a concentration of 0.14 mM in the transcription reaction solution, which was 40% of the concentration of other modified UTPs, because a higher concentration of DNP-11-UTP tends to result in a lower probe yield. T7 and SP6 RNA polymerase-plus (Ambion AM2716 and AM2701) were used in the transcription reaction. The cDNA templates that were used for probe synthesis were as follows: *At-fork head* (*fkh*), *At-orthodenticle* (*otd*), (Akiyama-Oda and Oda 2003), *At-twist* (*twi*) (Yamazaki *et al.* 2005), *At-short gastrulation* (*sog*) (Akiyama-Oda & Oda 2006), *At-Delta* (*Delta*), *At-hedgehog* (*hh*) (Oda *et al.* 2007), *At-labial* (*lab*), *022_P10*, *012_A08* (Akiyama-Oda & Oda, 2010), *At-odd paired* (*opa*) (Kanayama *et al.* 2011), and *sox2* (FY220555|At_eW_012_P20).

*Embryo fixation*

Spider embryos were fixed as previously described (Akiyama-Oda & Oda 2003) with some modifications. One modification entailed the piercing of vitelline membranes with a glass capillary to make small holes. This piercing was performed in Halocarbon oil 700 (Sigma H8898) after the embryos were dechorionated with 100% commercial bleach, washed with water and covered with oil, and this step led to the prompt permeation of the fixative. Piercing was followed by thoroughly washing the oil with

heptane and transferring the embryos into a two-phase fixative (heptane and 5.5% formaldehyde in PEMS (100 mM piperazine-1,2-bis(2-ethanesulfonic acid) (PIPES), 1 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM $MgSO_4$, pH 6.9)). The second modification entailed the adjustment of the fixation time to 45-50 minutes, as a longer fixation time leads to greater background fluorescence in the yolk. The third modification was the use of PEMS + 0.1% Polyoxyethylene (20) sorbitan monolaurate (Tween-20) to wash the fixative, and in this solution the vitelline membranes were removed with forceps before gradual replacement with methanol, followed by ethanol. The embryos were then stored in ethanol at -20°C until used for hybridization.

*In situ hybridization and detection*

The pretreatment, hybridization, and washing of excessive probes were performed in a similar manner as for *Drosophila* embryos (Lehmann & Tautz 1994), with the exception of the following two points. First, in the pretreatment steps, $H_2O_2$ treatment (20 minutes of incubation with 2% $H_2O_2$ in methanol) was added to inactivate endogenous peroxidase before rehydration with phosphate-buffered saline + 0.1% Tween-20 (PBT). Second, in prehybridization and hybridization solutions, dextran sulfate sodium salt (Sigma-Aldrich D8906) was added at a concentration of 50 μg/mL. These two changes were performed according to Lauter *et al.* (2011a). The contents of the prehybridization and hybridization solutions were as follows: 50% formamide; 5× standard sodium citrate (SSC); 0.1% Tween-20; 50 μg/mL heparin; 100 μg/mL salmon sperm DNA (Invitrogen 15632-011); and 50 μg/mL dextran sulfate sodium salt. In the hybridization solution, a maximum of four differently labeled RNA probes were included.

After overnight hybridization, excess probes were washed with a wash solution (50% formamide, 5× SSC, 0.1% Tween-20, and 50 μg/mL heparin), followed by gradual replacement with PBT and then with Tris-NaCl-Tween (TNT) buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween-20). Embryos were then blocked with Tris-NaCl-Blocking (TNB) buffer (0.5% blocking reagent (PerkinElmer FP1020) in 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl) for 30 minutes. This step was followed by incubation with an antibody or with streptavidin diluted with TNB buffer for 1.5 hours at room temperature (approximately 25°C). The antibodies that were used were as follows: anti-DIG-POD (Roche 1633716) at a dilution of 1:2000; anti-Fluorescein-HRP

(PerkinElmer NEF710001EA) at a dilution of 1:400; and anti-DNP-HRP (PerkinElmer FP1129) at a dilution of 1:200. Streptavidin-HRP (PerkinElmer NEF750001EA) was used at a dilution of 1:2000 to detect biotin probes. After the incubation step, the antibody or streptavidin was washed with TNT buffer, followed by peroxidase reaction with tyramide signal amplification (TSA) systems. The TSA reaction was performed according to the method outlined by Lauter *et al.* (2011b); in brief, embryos were rinsed twice with borate buffer (100 mM borate (pH 8.5), 0.1% Tween-20) and were then incubated for 15 minutes (or for a variety of minutes in the experiment shown in Fig. 4) in a TSA reaction solution (a tyramide reagent, 0.003% $H_2O_2$, 2% dextran sulfate sodium salt, and 0.3 mg/mL 4-iodophenol (Sigma I10201) in borate buffer). A commercial tyramide reagent, coumarin-tyramide, which was supplied in the TSA Coumarin system (PerkinElmer NEL703001KT), was used at a dilution of 1:100. Other tyramide reagents were synthesized in the laboratory (see below) and used at a dilution of 1:200 or 1:400. Following the TSA reaction, embryos were washed several times with PBT and incubated in 6% $H_2O_2$ in PBT for 10-30 minutes to inactivate peroxidase. When multiple RNA probes were hybridized, the individual probes were detected in succession; i.e., several rounds of blocking, antibody/streptavidin incubation, peroxidase reaction, and peroxidase inactivation steps were repeatedly performed. Some samples were counterstained with 4′,6-diamidino- 2-phenylindole (DAPI; Sigma-Aldrich).

*Fluorescent tyramide synthesis*

The following succinimidyl esters were used for tyramide reagent synthesis: 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes C-1311); DyLight488 *N*-hydroxysuccinimide (NHS) ester (Thermo Scientific 46402); AlexaFluor568 NHS ester (Molecular Probes A20003); DyLight594 NHS ester (Thermo Scientific 46412); and DyLight680 NHS ester (Thermo Scientific 46418). The tyramide reagents were synthesized according to the method of Lauter *et al.* (2011b). In brief, each succinimidyl ester was dissolved in dimethylformamide (Sigma-Aldrich D4551) at a concentration of 10 mg/mL. Tyramine hydrochloride (Sigma-Aldrich T2879) solution was prepared at a concentration of 10 mg/mL in dimethylformamide. This tyramine solution also contained triethylamine (Sigma-Aldrich T0886) at a concentration of 10 μL/mL. For the fluorescent tyramide reagent synthesis reaction, the

tyramine hydrochloride and succinimidyl ester solutions were mixed at a molar ratio of 1:1.1. The synthesis reaction proceeded overnight in the dark at room temperature (approximately 25°C), after which the products were diluted to a concentration of 1 mg/mL with 100% ethanol.