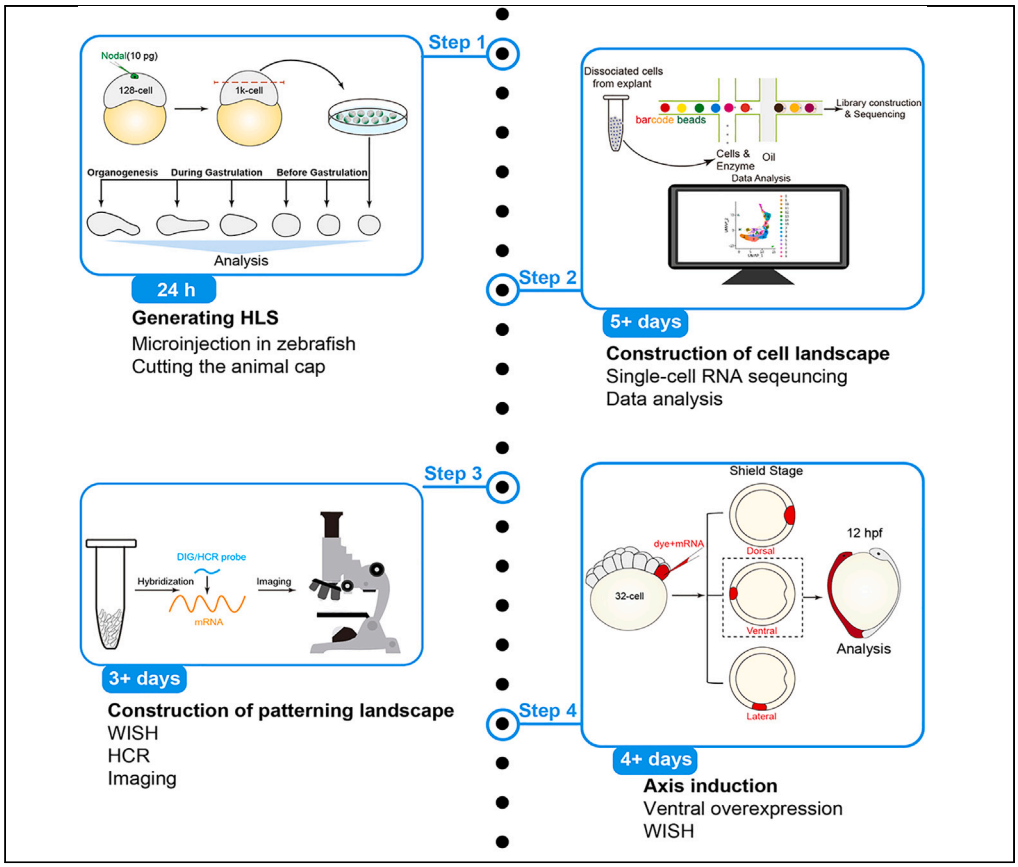
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for generation and assessment of head-like structure in zebrafish



In vitro embryonic analogue models, such as gastruloids, trunk-like structures and embryoids, have been developed to understand principles of early development and morphogenesis. However, models that can fully mimic head formation are still missing. Here, we present a protocol for generating the head-like structure (HLS) in zebrafish embryonic explants. We describe steps for dissection and constructing cell and patterning landscapes. We then detail assessment of this structure through axis induction.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Tao Cheng, Yan-Yi Xing, Yang Dong, Peng-Fei Xu

pengfei_xu@zju.edu.cn

Highlights

Steps to generate zebrafish explants with a single morphogen gradient

Investigation of cell types in the explants by single-cell RNA-seq

Exploration of cell patterns in the explants by *in situ* hybridization

Evaluation of axis induction ability of a single gene by ventral overexpression assay

Cheng et al., STAR Protocols 4, 102553
December 15, 2023
https://doi.org/10.1016/j.xpro.2023.102553

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for generation and assessment of head-like structure in zebrafish

Tao Cheng,[1,3,4] Yan-Yi Xing,[1,2,3] Yang Dong,[1,3] and Peng-Fei Xu[1,5,*]

[1]Women's Hospital, and Institute of Genetics, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

[2]Zhejiang Provincial Key Laboratory of Genetic & Developmental Disorders, Hangzhou, Zhejiang, China

[3]These authors contributed equally

[4]Technical contact: chengtao2ldo@zju.edu.cn

[5]Lead contact

*Correspondence: pengfei_xu@zju.edu.cn
https://doi.org/10.1016/j.xpro.2023.102553

SUMMARY

***In vitro* embryonic analogue models, such as gastruloids, trunk-like structures and embryoids, have been developed to understand principles of early development and morphogenesis. However, models that can fully mimic head formation are still missing. Here, we present a protocol for generating the head-like structure (HLS) in zebrafish embryonic explants. We describe steps for dissection and constructing cell and patterning landscapes. We then detail assessment of this structure through axis induction.**

For complete details on the use and execution of this protocol, please refer to Cheng et al. (2023).[1]

BEFORE YOU BEGIN

This protocol describes the main steps to generate and assess the head-like structure in zebrafish. The protocol is defined by four general steps: (1) generation of zebrafish embryonic explants with a Nodal signaling gradient in different developmental stages, (2) single-cell RNA-seq for the explants, 3) WISH/HCR for the explants, and (4) axis induction by ventral overexpression assay. Prior to beginning this protocol, it is necessary to breed the wild-type zebrafish line (AB), synthesize the *ndr2* mRNA and prepare the culture medium for zebrafish explants.

Institutional permissions

Zebrafish strains were used as per standard procedures from the Institutional Review Board of Zhejiang University. The animal protocol was reviewed and approved by The Animal Ethics Committee of the School of Medicine, Zhejiang University (ZJU20220375). Adult zebrafish and embryos were raised and maintained in the zebrafish core facility of Zhejiang University School of Medicine at 28°C on 14 h light/10 h dark rhythmical cycles.

Construction of pCS2+-ndr2 plasmid

Timing: 2 days

1. Extract total RNA from zebrafish embryos by phenol/chloroform-based method[2] using RNA isolater Total RNA Extraction Reagent (Vazyme).

Note: The embryos during gastrulation stage (6–10 hpf) are selected for RNA extraction, because *ndr2* is highly expressed during that stage.

2. cDNA template synthesis.
   a. Reverse transcribe the extracted RNA into cDNA using Takara's PrimeScript RT Master Mix, according to the following protocol.

Reagent	Amount
5× Master Mix	4 μL
Extracted RNA	1 μg
ddH_2O	Add to 20 μL

   b. Incubate at 37°C for 30 min, and then heat at 85°C for 5 s.
   c. Store cDNA in a -20°C freezer for later use (The cDNA can be stored at -20°C over 6 months).
3. Use the homologous recombination primers to amplify the gene coding (*ndr2*) sequence (https://www.ncbi.nlm.nih.gov/nuccore/NM_139133.1).

PCR Reaction system:	
Reagent	Amount
KOD one PCR Master Mix	12.5 μL
ndr2-F primer	0.5 μL
ndr2-R primer	0.5 μL
cDNA template	1 μL
ddH_2O	Add to 25 μL

PCR reaction: Denaturation at 98°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 2 min, 35 cycles. Run on a 1% gel for 15 min. The expected DNA band size is about 1.2k bp.

ndr2-F primer: cttgttctttttgcaggatccATGCACGCGCTCGGAGTC.

ndr2-R primer: gaattcgaatcgatgggatccTCACAGGCATCCGCACTCC.

4. Use PCR products for homologous recombination (Insert the fragments into PCS2+ vector). ClonExpress II One Step Cloning Kit (Vazyme) is used in this experiment.

Reaction system:	
Reagent	Amount
PCR products (purified)	1 μL
5× CE II Buffer	2 μL
Exnase II	1 μL
PCS2+ vector (linearized)	1 μL
ddH_2O	5 μL
Incubate the mixture at 37°C for 30 min.	

Note: Use the *XbaI* enzyme to linearize PCS2+ vector.

5. Transformation.
   a. Add 10 μL ligation system into 50 μL competent cells.
   b. Keep in ice block at 4°C for 30 min.
   c. Heat shock at 42°C for 30 s.
   d. Return to ice block at 4°C for 2 min.

e. Add in 300 μL LB medium.
f. Incubate in shaking incubator at 37°C and 220 rpm shaking speed for 45 min.
g. Plate cells on LB plate with 1‰ ampicillin (working concentration: 50 μg/mL).

6. Perform colony PCR to detect insert fragments.

PCR Reaction system:	
Reagent	Amount
2× Taq Master Mix	10 μL
ndr2-F primer	1 μL
ndr2-R primer	1 μL
ddH_2O	7 μL
Colony DNA (Template)	1 μL
PCR reaction: Denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min, 35 cycles. Run on a 1% gel for 15 min and send the positive colonies for sequencing.	

Note: Pick a bacterial colony and inoculate it into an Eppendorf tube (EP tube) containing 500 μL of LB medium (with 50 μg/mL ampicillin). After fully mixing, incubate the mixture in shaking incubator at 37°C and 220 rpm for 4–5 h and then pipette 1 μL for use as the PCR template.

ndr2 mRNA *in vitro* transcription

Timing: 20–24 h

7. Perform a *NotI* cleavage using a 50 μL restriction enzyme digest reaction.

Reaction system:	
Reagent	Amount
10× *NotI* Fast Digest Buffer	5 μL
NotI Enzyme	2 μL
pCS2+-ndr2 plasmid	23 μL (10 μg)
ddH_2O	20 μL
Incubate the mixture at 37°C for 2 h.	

8. *ndr2* mRNA *in vitro* synthesis using mMESSAGE mMACHINETM SP6 transcription kit.

Reaction system:	
Reagent	Amount
10× Reaction Buffer	2 μL
2× NTP/CAP	10 μL
Enzyme Mix	2 μL
Linearized pCS2+-ndr2	1 μg
ddH_2O	Add to 20 μL

a. Incubate at 37°C for 4 h (3–5 h).
b. Add in 1.5 μL TURBO DNase to degrade DNA.
c. Incubate at 37°C for 20 min (15–30 min).
d. Add in 30 μL RNase-free H_2O & 30 μL LiCl.

e. Put the mixture in -20°C freezer for 12 h–16 h to precipitate RNA.

9. Purify the mRNA.
   a. Centrifuge the precipitated reaction product at 15,000 g, 4°C, for 15 min. Discard the supernatant.
   b. Add 500 μL of pre-cooled 75% ethanol to wash 1–2 times, and then centrifuge at 15,000 g, 4°C, for 5 min. Discard the supernatant.
   c. Place the product in a super-clean workbench to air dry naturally.
   d. Add 30–50 μL of ddH_2O to dissolve the product.
   e. Use agarose gel electrophoresis to evaluate the purity. The purified mRNA is stored in a -80°C freezer for later use.

Prepare for culture medium

Timing: 1 h

Non-Serum medium (500 mL)	
Reagent	Amount
DMEM/F-12	474.5 mL
HEPES	5 mL
MEM NEAA	5 mL
Sodium Pyruvate	5 mL
antibiotic-antimycotic	5 mL
2-mercaptoethanol	5 mL (10 mM)
Gentamycin	500 μL

Note: Store in a 4°C freezer for later use. MEM: Minimum Essential Medium, NEAA: non-essential amino acids. Non-Serum medium is best to be used within one month.

Culture medium (zebrafish cell)	
Reagent	Amount
Non-Serum medium	44.65 mL
KnockOut Serum Replacement	5 mL
$CaCl_2$ (1M)	350 μL

Note: Store in a 4°C freezer for later use, and it is best to use it within one month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-fluorescein-AP, Fab fragments (1:2000)	Sigma-Aldrich	11426338910
Anti-digoxigenin-AP, Fab fragments (1:10000)	Sigma-Aldrich	11093274910
Bacterial and virus strains		
DH5α	TransGen Biotech	Cat#CD201-02
Chemicals, peptides, and recombinant proteins		
Cycloheximide	MCE	CAS No. 66-81-9
HCR probe	Molecular Technologies	N/A
HCR amplifiers	Molecular Technologies	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Probe hybridization buffer	Molecular Technologies	N/A
Probe wash buffer	Molecular Technologies	N/A
Amplification buffer	Molecular Technologies	N/A
PBS powder	Servicebio	G0002
Paraformaldehyde (PFA)	Sigma-Aldrich	158127
Citric acid trisodium salt	Sigma-Aldrich	C3674
tRNA from wheat germ	Sigma-Aldrich	R7876
Citric acid	Sigma-Aldrich	C2404
Bovine Serum Albumin (BSA)	Sigma-Aldrich	B2064
Sodium chloride (NaCl)	Sigma-Aldrich	S5886
$MgCl_2 \cdot 6H_2O$	Sigma-Aldrich	M2393
BCIP	Sigma-Aldrich	11383221001
Calcium chloride ($CaCl_2$)	Sigma-Aldrich	C5670
2-Mercaptoethanol	Sigma-Aldrich	M3148
Hepes	Sigma-Aldrich	H3375
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma-Aldrich	M2773
Potassium chloride (KCl)	Sigma-Aldrich	P5405
Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$)	Sigma-Aldrich	C1396
Proteinase K	Sangon Biotech	B600169
Formamide deionized	Sangon Biotech	A600211
Heparin sodium	Sangon Biotech	A603251
Tween 20	Sangon Biotech	A600560
NBT	Sangon Biotech	A610379
Gentamycin sulfate	Sangon Biotech	A506614
DIG RNA labeling mix	Sigma-Aldrich	11277073910
LB Broth	Generay Biotech	GL7002
Ampicillin	Generay Biotech	GA0339
RNA isolater total RNA extraction reagent	Vazyme	R401-01
Agarose	Tsingke Biotech	TSJ001
Normal sheep serum	Jackson ImmunoResearch	013-000-121
KOD one PCR master mix	Toyobo	KMM-201
2× Taq master mix	Vazyme	P111
FastDigest *XbaI*	Thermo Fisher	FD0685
FastDigest *NotI*	Thermo Fisher	FD0595
Antibiotic-Antimycotic	Thermo Fisher	15240062
DMEM/F12	Thermo Fisher	11320033
MEM NEAA	Thermo Fisher	10370021
Sodium Pyruvate	Thermo Fisher	11360070
Pronase	Roche	63163721
Dextran rhodamine B	Invitrogen	Cat#D1824
Dextran fluorescein	Invitrogen	Cat#D1821
Trypan blue solution	Sigma-Aldrich	T8154
TrypLE Select	Thermo Fisher	12563011
Propidium iodide	MedChemExpress	Cat#25535-16-4
Critical commercial assays		
Single Cell 3′Library & gel Bead kit v3.1	10× Genomics	Cat# PN-1000121
mMESSAGE mMACHINE™ SP6 transcription kit	Thermo Fisher	AM1340
T7 RNA Polymerase	Promega	Cat# P2075
T3 RNA Polymerase	Promega	Cat# P2083
pEASY Blunt Zero Cloning vector	TransGen Biotech	CB501
PrimeScript RT master mix	Takara	RR036A
ClonExpress II one step Cloning kit	Vazyme	C112

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Bulk and single-cell RNA-seq of Nodal explants	This study	GEO: GSE165654
Single-cell RNA-seq data of wild-type zebrafish explant	This study	GEO: GSE221220
Experimental models: Organisms/strains		
Zebrafish: *Danio rerio*, wild type AB strain, embryos, sex not determined at this stage	Dr. Sean Megason's lab (Department of systems Biology Harvard Medical School)	N/A
Oligonucleotides		
fzd8b primer for ISH probe Forward: 5′-CCGAGGATGGACTCGCCT-3′		N/A
fzd8b primer for ISH probe Reverse: 5′- GCTGCAGTTGCTCAGTTTG-3′		N/A
tbxta primer for ISH probe Forward: 5′-TCAAGCTTTAT TTGATCGGAAATATG-3′		N/A
tbxta primer for ISH probe Reverse: 5′-GTATGCCTCGGT ATGATACAGATGTG-3′		N/A
Recombinant DNA		
pCS2+-ndr2	This study	N/A
Software and algorithms		
ImageJ	Open Source/National Institutes of Health	https://imagej.nih.gov/ij/
Imaris 9.5	Oxford Instruments	https://imaris.oxinst.com/
R	The R Project	https://www.r-project.org/
R Studio		https://rstudio.com
Python	The Python Project	https://www.python.org
Seurat v2.3.4	Butler et al.[3]	https://satijalab.org/seurat/
Seurat v3.1.0	Stuart et al.[4]	https://satijalab.org/seurat/
Cell Ranger v3.0.2	10 X Genomics	https://github.com/10XGenomics/cellranger/
DEseq2	Love et al.[5]	https://bioconductor.org/packages/DESeq2/
STAR v2.7.1a	Dobin et al.[6]	https://github.com/alexdobin/STAR
SAMtools v1.8	Danecek et al.[7]	https://github.com/samtools/samtools
featureCounts v1.6.0	Liao et al.[8]	https://subread.sourceforge.net
clusterProfiler	Yu et al.[9]	https://bioconductor.org/packages/clusterProfiler/
ComplexHeatmap	Gu et al.[10]	https://github.com/jokergoo/ComplexHeatmap
ggplot2	Wickham[11]	https://ggplot2.tidyverse.org
Pheatmap	Kolde[12]	https://CRAN.R-project.org/package = pheatmap
Other		
Zebrafish embryonic single-cell dataset	Wagner et al.[13]	GEO: GSE112294
Zebrafish embryonic single-cell dataset	Farrell et al.[14]	GEO: GSE106587
Pi-injector	Warner	PLI-90A
Greenough stereo microscope	Leica	S9D
Fluorescence stereo microscope	Leica	M205 FCA
Microscope camera	Leica	DFC7000 T
Laser scanning microscope	Olympus	FV12-IXCOV
Tower workstation	Dell	Precision 7920

MATERIALS AND EQUIPMENT

1 M $CaCl_2$		
Reagents	Final concentration	Amount
$CaCl_2$	1 M	11.1 g
ddH_2O	N/A	Up to 100 mL
Total	N/A	100 mL
1 M $CaCl_2$ can be stored at 20°C–25°C for one year.		

1 M citric acid		
Reagents	Final concentration	Amount
Citric acid	1 M	19.21 g
ddH_2O	N/A	Up to 100 mL
Total	N/A	100 mL
1 M citric acid can be stored at 20°C–25°C for one year.		

1 M $MgCl_2$		
Reagents	Final concentration	Amount
$MgCl_2 \cdot 6H_2O$	1 M	20.322 g
ddH_2O	N/A	Up to 100 mL
Total	N/A	100 mL
1 M $MgCl_2$ can be stored at 20°C–25°C for one year.		

5 M NaCl		
Reagents	Final concentration	Amount
NaCl	5 M	73.05 g
ddH_2O	N/A	Up to 250 mL
Total	N/A	250 mL
5 M NaCl can be stored at 20°C–25°C for one year.		

1 M Tris-HCl (pH9.5)		
Reagents	Final concentration	Amount
Tris-HCl	1 M	30.35 g
ddH_2O	N/A	250 mL
Total	N/A	250 mL
1 M Tris-HCl can be stored at 20°C–25°C for one year.		

Note: Adjust pH to 9.5 by slowly adding in concentrated HCl.

⚠ CRITICAL: Concentrated HCl is corrosive and highly volatile, and should be handled safely with protective clothing, gloves and mask in a fume hood.

1× PBS		
Reagents	Final concentration	Amount
PBS powder	0.01 M	19.6 g
ddH_2O	N/A	Up to 2 L
Total	N/A	2 L
1× PBS can be stored at 20°C–25°C for one year.		

1× PBST		
Reagents	Final concentration	Amount
Tween 20	0.1% (volume/volume)	0.5 mL
1× PBS	1×	Up to 500 mL
Total	N/A	500 mL
1× PBST can be stored at 20°C–25°C for weeks.		

4% PFA		
Reagents	Final concentration	Amount
PFA	4% (weight/volume)	4 g
1× PBS	1×	Up to 100 mL
Total	N/A	100 mL
4% PFA can be stored at 4°C for about one week.		

Note: Place PFA into 1× PBS with heating at 60°C for about 2 h to make PFA completely dissolved, and then cool it down to 20°C–25°C.

⚠ CRITICAL: PFA is toxic, flammable and potentially carcinogenic, and should be handled safely with protective clothing, gloves and mask.

20× SSC		
Reagents	Final concentration	Amount
NaCl	3 M	87.65 g
Citric acid trisodium salt	300 mM	44.1 g
ddH_2O	N/A	Up to 500 mL
Total	N/A	500 mL
20× SSC may be stored at 20°C–25°C for several months.		

2× SSC		
Reagents	Final concentration	Amount
20× SSC	2×	10 mL
ddH_2O	N/A	Up to 100 mL
Total	N/A	100 mL
2× SSC is prepared fresh on the day of experiment, and can be left at 20°C–25°C.		

Hybridization Mix (HM)		
Reagents	Final concentration	Amount
Formamide deionized	50%	25 mL
20× SSC	5×	12.5 mL
5 mg/mL Heparin	50 μg/mL	0.5 mL
50 mg/mL tRNA	500 μg/mL	0.5 mL
Tween 20	0.1%	0.05 mL
1 M citric acid	~9.2 mM	~0.46 mL
ddH_2O	N/A	Up to 50 mL
Total	N/A	50 mL
Hybridization mix can be stored at -20°C for weeks.		

Note: Adjust pH to 6.0 with 1 M critic acid. The HM used for washing does not contain Heparin and tRNA.

⚠ CRITICAL: Formamide is toxic, corrosive and potentially carcinogenic, and should be handled safely with protective clothing, gloves and mask.

Blocking buffer		
Reagents	Final concentration	Amount
BSA	2 mg/mL	0.8 g
Normal sheep serum	2% (volume/volume)	8 mL
1× PBST	1×	Up to 400 mL
Total	N/A	400 mL
Blocking buffer can be stored at -20°C for weeks.		

Alkaline Tris (AT) buffer		
Reagents	Final concentration	Amount
1 M Tris-HCl pH9.5	100 mM	70 mL
1 M $MgCl_2$	50 mM	35 mL
5 M NaCl	100 mM	14 mL
Tween 20	0.1%	0.7 mL
ddH_2O	N/A	Up to 700 mL
Total	N/A	700 mL
AT buffer needs to be prepared fresh on the day of experiment, and can be left at 20°C–25°C.		

Labeling solution		
Reagents	Final concentration	Amount
50 mg/mL NBT	0.45% (volume/volume)	225 μL
50 mg/mL BCIP	0.35% (volume/volume)	175 μL
AT buffer 1×	1×	Up to 50 mL
Total	N/A	50 mL
Labeling solution needs to be prepared fresh on the day of experiment, and can be left at 20°C–25°C.		

Note: Labeling buffer should be kept in the dark during experimental period.

30× Danieau buffer (PH = 7.6)		
Reagents	Final concentration	Amount
NaCl	1.74 M	101.7 g
KCl	21 mM	1.56 g
$MgSO_4 \cdot 7H_2O$	12 mM	2.96 g
$Ca(NO_3)_2 \cdot 4H_2O$	18 mM	4.25 g
HEPES	150 mM	35.75 g

Note: Add 800 mL of ddH_2O. Adjust the pH to 7.6 using HCl and NaOH. Add ddH_2O to a volume of 1 L, and 30× Danieau buffer can be stored at 4°C for 6 months. 0.3× Danieau buffer solution: Dilute 30× Danieau buffer with ddH_2O to obtain a 0.3× concentration. 0.3× Danieau buffer solution can be stored at 20°C–25°C for months.

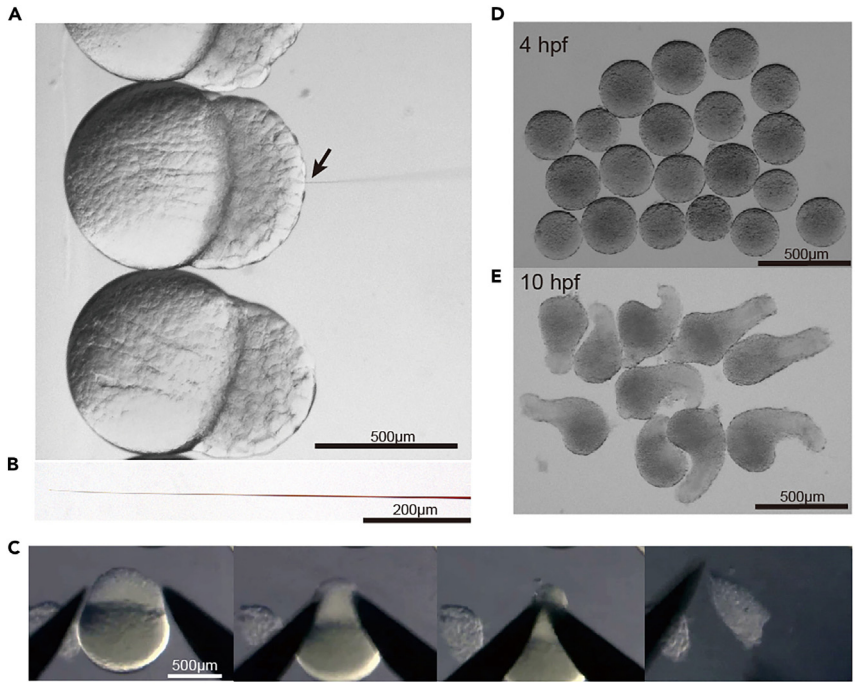


Figure 1. Representative images showing manipulations for generating head-like structure (Nodal explants)
(A) Bright field image showing how to inject mRNA mixed with fluorescein dextran into one animal pole blastomere of zebrafish embryos at 128-cell stage. The embryos were aligned with corrected orientation in the groove made in the agarose.
(B) Image showing the injection needle which was filled with ndr2 mRNA mixed with fluorescein dextran.
(C) Bright field image showing how to cut off the zebrafish animal pole explants.
(D and E) Bright field image showing the Nodal explants at 4 hpf (D) and 10 hpf (E). Scale bar: 500 μm (A, C, D and E), 200 μm (B).

STEP-BY-STEP METHOD DETAILS

Generation of zebrafish explant with Nodal injection

Timing: 1 day

This section describes the generation of Nodal explants. Completion of this step will achieve Nodal explants, which can develop into head-like structure (troubleshooting 1).

1. Dechorionate zebrafish embryos by pronase.

   Note: Pronase powder is dissolved in 0.3× Danieau at concentration of 10 mg/mL. The duration time of treatment is about 1–2 min.

2. Put the dechorionated zebrafish embryos on an agarose-coated plate (2% agarose) with 0.3× Danieau buffer.
3. Inject *ndr2* mRNA (10 pg) mixed with dextran-fluorescein or dextran-rhodamine B into one zebrafish animal pole blastomere at the 128-cell stage (Figures 1A and 1B).

   Note: The *ndr2* injected explants are defined as Nodal explants and the un-injected control explants are defined as wild-type explants hereafter. When we identify Nodal downstream genes in the following section (bulk RNA-seq analysis), the Nodal explants with different dosage of Nodal injection (such as: 2 pg, 6 pg Nodal mRNA) or

different Nodal duration (different developmental stages, such as: 4 hpf, 5 hpf and 6 hpf) are used.

⚠ CRITICAL: Needles should be generated by needle puller with appropriate parameters to get extra long tapers as previous study reported.[15]

4. Cut off the animal pole region (1/3–1/2) of the embryo using a syringe needle at 1k-cell stage (Figure 1C and Methods video S1).
5. Culture the animal pole explants in an agarose-coated plate (2% agarose) with zebrafish cell culture medium at 29°C until harvest (Figures 1D and 1E).

Note: At most 30 explants are cultivated in each culture dish. If the samples are used to perform single-cell RNA-seq, these explants are dissociated into single cells immediately. Otherwise, these explants are fixed by 4%(w/v) paraformaldehyde (PFA)/PSB (The PBS solution is prepared using RNA-free water) at 4°C for 12 h–16 h for WISH or HCR. After fixing the embryos or explants in 4% PFA for 12 h–16 h, discard the liquid and rinse once with 100% methanol. Then add 1 mL of 100% methanol and store the samples at -20°C for dehydration.

Cell dissociation and single-cell RNA sequencing

Timing: 2–3 days

This section describes the steps of performing single-cell RNA sequencing of explants. Completion of this step will achieve single-cell sequencing raw data of explants.

6. Prepare the cell washing solution and cell resuspended solution.

Cell washing solution	
Reagent	Amount
NaCl	116 mM
KCl	2.9 mM
HEPES	5 mM
BSA	1% (w/v)

Cell resuspended solution	
Reagent	Amount
NaCl	116 mM
KCl	2.9 mM
HEPES	5 mM
BSA	0.05% (w/v)

7. Dissociate the explants into single cells by dissociation reagent (TrypLE Select).

Note: The explants can be dissociated into single cells by pipetting up and down several times through a P200 tip.

8. Centrifuge the dissociated cells at 200 g, 4°C, for 5 min.
9. Remove the supernatant, and then resuspend the cells by cell washing solution.
10. Centrifuge the dissociated cells at 200 g, 4°C, for 5 min.
11. Remove the supernatant, and resuspend the cells by cell resuspended solution.

12. Count the cell number and assess the cell viability by Trypan blue staining and Propidium iodide (PI) staining.
13. Construct the cDNA libraries using the Chromium Controller and Chromium Single Cell 3′ Library & Gel Bead Kit per the manufacturer's protocol.
14. The libraries can be sequenced by Illumina NovaSeq with paired-end at 150 bp read length.

Note: When the explants are transferred into 1.5 mL microcentrifuge tube, the tube should be pre-coated with 10% BSA/PBS for 15 mins at 20°C–25°C.

Bulk RNA sequencing analysis

Timing: 1 day

This section details the steps of bulk RNA-seq analysis. Completion of this steps will identify the downstream targets of Nodal (or other molecular signals).

15. Pre-process bulk RNA-seq data.
    a. Align reads from Fastq files to the reference (*Danio rerio* reference transcriptome Ensembl Release 92) using the *STAR* function.
    b. Sort aligned reads using the samtools *sort* function.
    c. Assign aligned reads to each gene using the *featureCounts* function, generating gene counts of each sample.
16. Pre-process data and construct DESeqDataSet object for differential expression (DE) analysis.
    a. Read in gene count files of each sample using the *read.table* function.
    b. Merge gene counts of each sample using the *multimerge* function, returning a count matrix.
    c. Construct a metadata with a sample name column and a condition column merging time and sample treatment.
    d. Construct DESeqDataSet object using the *DESeqDataSetFromMatrix* function with the count matrix and the metadata as inputs and the condition column as design.
17. Perform DE analysis for the identification of downstream targets (troubleshooting 3).
    a. Perform DE analysis on DESeqDataSet object using the *results* function, setting the groups for comparison as contrast.
    b. Both Nodal explants and Cycloheximide (CHX)-treated Nodal explants are compared to wild-type explants at each time point respectively, and appropriate thresholds of p-value and absolute log2FC are used to identified DE genes. Here a p-value <0.05 and absolute log2FC ≥ 1 are used.

Note: CHX is a protein-translation-blocking inhibitor, which is used to reduce the effects of cascading reactions caused by Nodal for the identification of Nodal immediate-early genes. CHX-treated Nodal explants are generated using CHX-treated embryos with Nodal injection, which are obtained by treating the embryos with 50 μg/mL CHX after 20 mins of Nodal injection.

Note: Here we are focusing on Nodal targets. Other manipulations can be applied instead for investigating the downstream targets of other molecular signals.

    c. Select overlapped DE genes in Nodal explants and CHX-treated Nodal explants at each time point, generating three overlapped DE gene sets.
    d. Define common genes in three overlapped DE gene sets as Nodal targets. Here 105 Nodal targets are identified.
18. Cluster downstream targets and perform functional analysis.
    a. Scale the expression of downstream targets (105 Nodal targets) using the *scale* function.
    b. Cluster downstream targets into four clusters using the *kmeans* function.

Note: Here different cluster numbers can be tested for achieving an optimal clustering performance.

c. Perform a heatmap showing scaled expression of Nodal targets within 4 k-means clusters using the *Heatmap* function with sample treatment annotated on the top and time annotated on the bottom.
d. Perform Gene ontology (GO) enrichment analysis on each gene cluster using the *enrichGO* function.

19. Visualize correlations between downstream target clusters and treatment concentration/duration time.
    a. Calculate mean of scaled gene expression in each cluster using the *mean* function.
    b. Use Pearson method to calculate correlation coefficients between scaled expression mean of each cluster and treatment concentration/duration time using the *cor.test* function with p-values calculated by Student's t test.
    c. Perform a heatmap using the *pheatmap::pheatmap* function with color indicating correlation coefficients and p-values displayed in each box.

Single-cell RNA sequencing analysis

Timing: 1 day

This section provides the steps of single-cell RNA sequencing analysis. Completion of this section will identify the cell types existing in the explants.

20. Align sequencing reads in FASTQ files to the reference (*Danio rerio* reference transcriptome Ensembl Release 92) using cellranger count function, generating the output used for Seurat.
21. Create Seurat objects and pre-process scRNA-seq data.
    a. Read in the output of the cellranger pipeline using the *Read10x* function, returning a unique molecular identified count matrix.
    b. Use the count matrix to create a Seurat object using the *CreateSeuratObject* function.
    c. Check the level of unique feature counts, total molecule counts and mitochondrial counts.
    d. Filter out low-quality cells using the *FilterCells* function. Here we filter out cells with unique feature counts less than 200 or over 5000 as well as >5% mitochondrial genes.
    e. Normalize data using the *NormalizeData* function with the default settings ("LogNormalize" method with a scale factor as 10,000).
    f. Identify highly variable features using the *FindVariableFeatures* function.
    g. Scale the data using the *ScaleData* Function with regression of nUMI and mitochondrial genes.
22. Cluster cells and annotate clusters (troubleshooting 4).
    a. Perform PCA on scaled data using the *RunPCA* function with the identified variable features as input.
    b. Determine the optimal component numbers (dimensionality) for data representation using the *JackStraw* function.
    c. Construct a KNN graph using the *FindNeighbors* function with the defined dimensionality as input.
    d. Cluster cells using the *FindClusters* function with a resolution ranging from 0.5 to 2.
    e. Run non-linear dimensional reduction using the *RunUMAP* function with the defined dimensionality.
    f. Visualize the dataset using the *DimPlot* function with UMAP as reduction.
    g. Identify top marker genes of each cluster using the *FindMarkers* function.
    h. Annotate each cluster based on the expression of cellular markers (Figure 2).

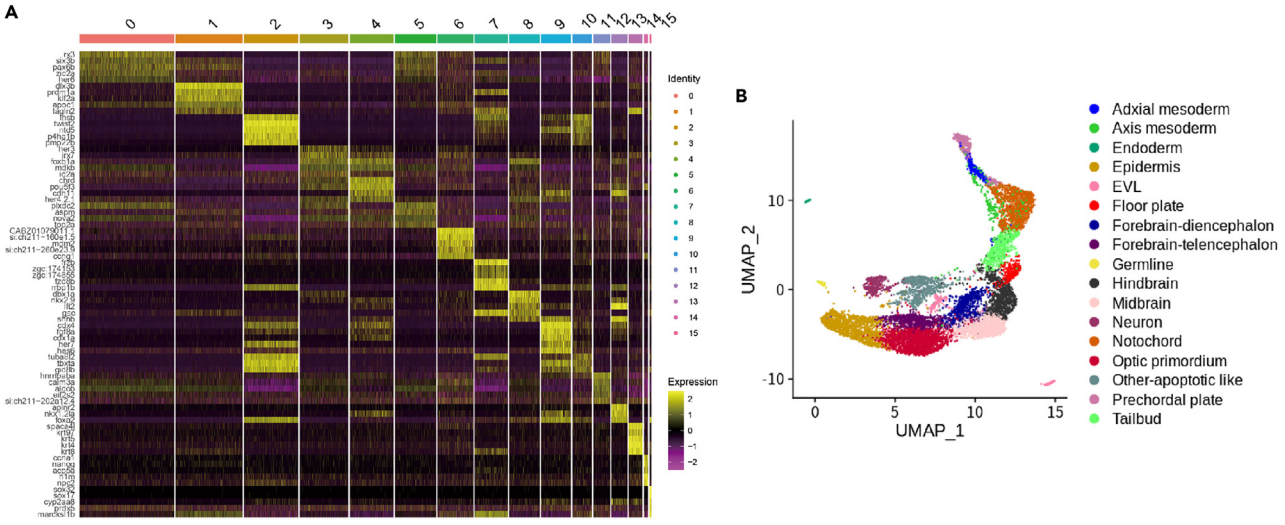


Figure 2. Single-cell RNA-seq analysis of Nodal explant at 10 hpf

(A) Heatmap showing the expression levels of top 5 markers in each cluster of Nodal explant at 10 hpf.

(B) UMAP plot showing identified cell types of Nodal explant at 10 hpf. Cells are colored according to their cell-type annotations. Cell type annotations and their corresponding colors are shown on the right.

WISH (Whole-mount *in situ* hybridization)

Timing: 3 days

This section describes the steps of WISH. Completion of this step will enable the assessment of gene expression patterns on explants. The process of this section mainly follows the published protocol.[16] As the assessment of expression patterns is important, we provide the detailed steps below (troubleshooting 2).

23. The explants or embryos which are older than 24 h post-fertilization (hpf) should be decolorized by H_2O_2.
    a. Decolorization solution preparation: prepare the decolorization solution by mixing 30% H_2O_2 solution and 5% KOH solution at a volume ratio of 1:9.
    b. Remove the fixative and place the explants or embryos that need to be decolorized in a 35 mm plastic culture dish.
        i. Add 2–3 mL of the decolorization solution
        ii. Observe under a microscope until the pigment on the embryos' eyes (or explants' pigments in epidermis) disappears.
    c. Remove the decolorization solution and perform gradient dehydration through washing with 25%, 50%, 75% and 100% methanol/PBS for 5 min each at 20°C–25°C.
    d. Store the decolorized embryos or explants in 100% methanol and keep them at -20°C for later use.

Probe hybridization (Day 1)

24. Explants or embryos rehydration. (Gradient wash through 75%, 50% and 25% methanol/PBST for 5 min each at 20°C–25°C and then three times for 5 min in PBST.)
25. Digestion and permeabilization by proteinase K.
    a. Add Proteinase K (20 mg/mL) to PBST to prepare a digestion solution with a final concentration of 10 μg/mL.

b. Treat the explants or embryos with digestion solution at 20°C–25°C. The digestion time for explants or embryos at different stages is shown in the following table:

Developmental stage	Treated time
Younger than 10 hpf	None
10-24 hpf	30 s
24 hpf	5–10 min

26. Refixation.
    a. Remove the digestion solution and add 1 mL of 4% PFA for refixation. Leave it at 20°C–25°C for 20 min.
    b. Wash four times with PBST at 20°C–25°C, 5 min each time.

Note: This step is only required after proteinase K treatment.

27. Prehybridization.
    a. Prepare the HM solution in advance and preheat it in the hybridization oven at 70°C for 30 min.
    b. Pipette 300 μL of the preheated HM solution into the EP tube containing the explants or embryos from the above steps for pre-hybridization.
    c. Incubate it at 70°C for 3–5 h.

⚠ CRITICAL: HM solution contains formamide, which is toxic, corrosive and potentially carcinogenic, and thus should be handled safely with protective clothing and gloves.

28. Hybridization.
    a. Use a vacuum pump to extract the solution from the EP tube.
    b. Add 300 μL of HM solution containing 2 ng/μL probe to the tube.
    c. Hybridize for 12 h–16 h in a hybridization oven at 70°C.

⚠ CRITICAL: The HM solution containing the probe also needs to be preheated at 70°C in the hybridization oven for 30 min in this step. If performing single-color *in situ* hybridization, only probes labeled with DIG need to be added. However, if performing double-color *in situ* hybridization, probes labeled with both DIG and fluorescent need to be added, and the EP tube should be wrapped with foil to avoid light exposure.

Antibody incubation (Day 2)

29. Recycle the hybridization solution containing the probe and store it in a -20°C freezer.

Note: Generally, the probes can be reused 5–6 times.

30. Wash up the residual hybridization solution containing the probe. Quick wash with HM solution first, and then wash through 75%, 50% and 25% HM/2× SSC for 15–20 min each at 70°C. After that, wash once with 2× SSC 70°C for 15–20 min and twice with 0.2× SSC at 70°C for 30–40 min each.

⚠ CRITICAL: All washing steps are carried out in a hybridization oven, and the above solutions need to be preheated in the hybridization oven at 70°C. The HM solution in washing steps does not contain Heparin and tRNA.

31. Gradual transition to PBST buffer. Wash through 75%, 50% and 25% 0.2xSSC/PBST for 15–20 min each at 20°C–25°C and then once in PBST for 15–20 min at 20°C–25°C.
32. Block and incubate with antibody.
    a. Remove PBST, add 1 mL of Incubation Mix, and incubate at 20°C–25°C on a low-speed shaker for 3–5 h.
    b. Remove the Incubation Mix, and add 1 mL of Incubation Mix containing 0.1 μL/mL DIG AP (anti-DIG antibody).
    c. Incubate for 12 h–16 h at 4°C on a low-speed shaker.

Washing and labeling (Day 3)

33. Antibody removal and washing.
    a. Transfer the explants or embryos that have been incubated for 12 h–16 h to experimental bench at 20°C–25°C.
    b. Remove the antibody incubation solution, and quickly wash once with PBST.
    c. Wash with PBST six times at 20°C–25°C, 15 min each time (place on a low-speed shaker).
    d. Remove the PBST, and add 1 mL of freshly prepared Alkaline Tris (AT) Buffer. Wash with AT Buffer three times on a low-speed shaker at 20°C–25°C, each time for 5 min.
34. Labeling.
    a. Transfer the explants or embryos in the EP tube to the six-well plate. Mark the position of the wells in advance.
    b. Remove the AT Buffer and add 2 mL of labeling solution to the wells.
    c. Transfer the six-well plate to a dark place to avoid light.
    d. Observe the labeling under a microscope every 15–30 min to prevent over-labeling.

⚠ CRITICAL: The labeling solution is prepared as needed and stored in the dark.

35. Termination of staining.
    a. Remove the labeling solution, and wash quickly with PBST.
    b. Wash three times with PBST at 20°C–25°C for 5 min each time.
36. Storage.
    a. Remove the PBST.
    b. Add glycerol and transfer to a 4°C refrigerator for storage (this step is not performed for double-color *in situ* hybridization).
37. Photography and data processing. Use a stereomicroscope to photograph and record the results of *in situ* hybridization from different angles and positions for subsequent analysis (Figures 3A and 3B).
38. The following experimental steps are required for double-color *in situ* hybridization (continuing from Step 32 above):
    a. Treat with 0.1 M glycine-HCl (pH 2.2, 0.1% Tween 20) at 20°C–25°C for 10 min.
    b. Wash with PBST at 20°C–25°C four times, 5 min each time.
    c. Remove the PBST, add Incubation Mix and transfer to a low-speed shaker at 20°C–25°C for 5 h.
    d. Block and incubate with antibody.
        i. Add fluorescein-labeled antibody at a ratio of 1:2000 in the Incubation Mix.
        ii. Remove the Incubation Mix, and add the Incubation Mix containing the antibody.
        iii. Transfer to a low-speed shaker in a 4°C refrigerator for 12 h–16 h.
    e. Antibody removal and washing.
        i. Remove the incubation solution.
        ii. Wash quickly with PBST at 20°C–25°C once.
        iii. Wash six times with PBST at 20°C–25°C, 15 min each time (on a low-speed shaker).
    f. Remove the PBST, add 1 mL of pH 8.2, 1 M Tris-HCl, and wash three times on a low-speed shaker at 20°C–25°C for 5 min each time.

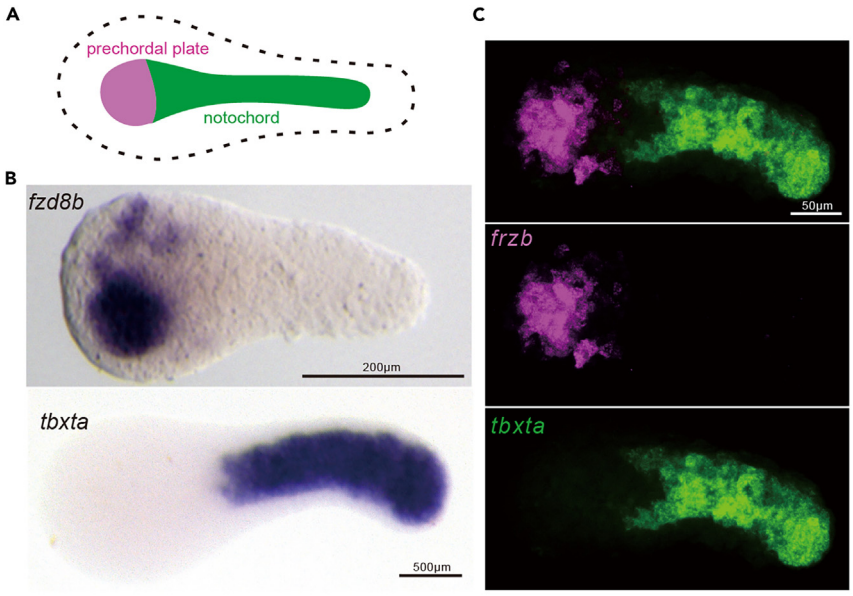


Figure 3. Whole Mount In Situ hybridization (WISH) and HCR for the marker genes of prechordal plate (fzd8b and frzb) and notochord (tbxta)

(A) Cartoon plot showing the pattern of prechordal plate (magenta) and notochord (green) in Nodal explant at 10 hpf.

(B) WISH of fzd8b (top) and tbxta (bottom) in Nodal explant at 10 hpf.

(C) HCR co-staining of frzb (magenta) and tbxta (green) in Nodal explant at 10 hpf. Scale bar: 200 μm (B, top), 500 μm (B,bottom), 50 μm (C).

g. Labeling: Remove Tris-HCl and add labeling solution. Labeling should be carried out in the dark. The labeling solution (10 mL):

Reagent	Volume
Fast red stock	100 μL (100 mg/mL)
NAMP stock	40 μL (100 mg/mL)
Tris-HCl	10 mL (pH8.2, 1 M)

h. Termination of staining: After specific regions are stained, remove the staining solution, wash once with PBST, and then wash three times with PBST at 20°C–25°C, 5 min each time (on a low-speed shaker).

i. Remove PBST and add glycerol. Store in a refrigerator at 4°C for later use.

j. Photography and data processing: Use a stereomicroscope to photograph and analyze the results of *in situ* hybridization.

HCR probe sets and amplifiers design and generation

Timing: 3 days

This section describes the steps of HCR. Completion of this will enable assessment of multiple gene expression patterns on one explant. As genes are assigned with different fluorescence, the signals of different genes will not be mixed up. And the fluorescent signals can be quantified, reflecting the gene expression patterns better (troubleshooting 5).

39. Identify the marker genes that are specifically expressed in each cell cluster by analyzing the single cell sequencing data. Find the NCBI Accession numbers of each gene for HCR probe set design.

Note: Sometimes we may find one gene that is not only expressed in a specific cell cluster but also in any other cell clusters. For this circumstance, we can use 2 or more genes to distinguish this cell cluster.

40. All the HCR probes and HCR Amplifiers are purchased from Molecular Instruments, Inc. (https://www.molecularinstruments.com/), and the probe set and amplifier information is listed below.

Note: The B isoform is a specific and unique sequence designed in HCR probes, which will initiate corresponding amplifier polymerization. Here we provide the NCBI Accession number and the required B isoform to the company, and they will design a set of HCR probes accordingly. When performing double HCR experiment, make sure different B isoforms are selected for two HCR probe sets to ensure achieving different fluorescence.

Probe sets and amplifiers		
Oligonucleotides		
Zebrafish *tbxta* HCR v3.0 probe set (B3) set size: 20	Molecular Technologies	GenBank: NM_131162
Zebrafish *frzb* HCR v3.0 probe set (B2) set size: 20	Molecular Technologies	GenBank: NM_130943
B2 647 HCR Amplifier	Molecular Technologies	N/A
B3 488 HCR Amplifier	Molecular Technologies	N/A

Note: Here we choose *tbxta* and *frzb* as examples. We always choose 20 for the probe set size, which means a probe set consists of 20 probe pairs. For most circumstances, it is enough to gain strong fluorescent signals. The production of all the reagents usually takes 1–2 weeks.

Sample preparation

We use explants for HCR RNA-FISH experiments. The preparation of explants is described in the first section.

Note: Because the explant is too small to discern with naked eye, you can take advantage of a stereo microscope when exchanging solutions. And it is better to incubate without agitation.

41. Collect 30–40 explants at the time points required into 2 mL microcentrifuge tube. Discard the extra culture media as much as possible.
42. Explant fixation. Add 1 mL 4% PFA to the tube and store at 4°C for at least 16 h but no more than 24 h.
43. Explant dehydration and permeabilization.
    a. Wash explants with 1 mL 1× PBST 3 times for 10 min per wash.
    b. Wash explants with 1 mL 100% methanol 2 times for 10 min per wash.
    c. Store explants at -20 °C at least 2 h before use.
44. Rehydration the explant with a series of MeOH/PBST for 10 min per wash.
    a. 75% MeOH/ 25% 1× PBST
    b. 50% MeOH/ 50% 1× PBST
    c. 25% MeOH/ 75% 1× PBST
    d. 3 times in 100% 1× PBST, 10 min per wash
45. Permeabilization by Proteinase K treatment.
    a. Incubate with 10 μg/mL Proteinase K at 20°C–25°C for 1 min.
    b. Fix explants with 4% PFA for 10 min.
    c. Wash explants with 1× PBST 4 times for 10 min per wash.

Note: Proteinase K treatment can dramatically increase the HCR fluorescent signal for explants.

HCR hybridization

In this step, the HCR probe will hybridize with its target mRNA in explants. All the incubation at 37°C in this section is carried out by water bath.

46. Pre-hybridize the explants.
    a. Pre-warm the probe hybridization buffer to 37°C.
    b. Transfer 30–40 explants to a 1.5 mL centrifuge tube.
    c. Rinse explants with 100 μL probe hybridization buffer.
    d. Discard the excess probe hybridization buffer, add 100 μL probe hybridization buffer, and incubate for 30 min.

Note: The probe hybridization buffer is provided by the manufacturer who makes the HCR probe.

47. Probe hybridization in explants.
    a. Prepare probe hybridization buffer by adding 0.4 μL probe stock (1 μM) to 100 μL probe hybridization buffer (2 pmol for final concentration).
    b. Pre-warm the probe hybridization buffer to 37°C.
    c. Incubate the explants with 100 μL probe hybridization buffer at 37°C for 12 h–16 h.

HCR signal amplification

In this step, the fluorescent signals are gained by amplifiers undergoing hybridization chain reaction with HCR probes.

48. Stop the hybridization by repeated washing.
    a. Carefully remove the hybridization buffer.
    b. Remove excess probe by washing 4 times for 20 min per wash with 100 μL pre-warmed probe buffer.

Note: Keep at 37°C during washing.

49. Pre-amplify the samples with 200 μL probe amplification buffer at 20°C–25°C for 30 min.
50. Prepare the hairpin solution.
    a. Transfer 2 μL hairpin h1 and 2 μL hairpin h2 to separate centrifuge tubes.
    b. Snap cool the hairpins by heat at 95°C for 90 s and cool for 30 min at 20°C–25°C in the dark
    c. Add the snap cooled hairpins to 100 μL amplification buffer.
51. Incubate the explants with 100 μL hairpin solution at 20°C–25°C in the dark until appropriate strength of fluorescent signals is gained.

Note: Incubate with hairpin solution at least 1 h but no more than 16 h.

52. Stop amplification by using 5× SSC 0.001% tween-20 to wash several times at 20°C–25°C.

⚠ CRITICAL: This process should be performed as fast as possible to minimize light exposure.

53. HCR samples are stored at 4°C and protected from light. The fluorescent signals will not reduce significantly after as long as 2-month storage.
54. Finished HCR samples are photographed by confocal at specific channels (Figures 3A and 3C).

Secondary axis induction

Timing: 4 day

This section describes the induction of secondary axis by Nodal downstream targets. Completion of this step will assess the Nodal targets on their capability of inducing secondary axis.

55. *In vitro* synthesis of specific mRNA downstream of Nodal which were identified by bulk RNA-seq.
56. Inject the mRNA into one random blastomere at the margin of the 32-cell stage zebrafish embryos.

⚠ CRITICAL: About 4 pL of the mixture composing the mRNA and fluorescent dye was injected. The appropriate dose of injected mRNA needs to be determined by injecting different dosage of the mRNA and checking the morphology of the secondary axis.

Note: The mRNA *in vitro* synthesis and injection take one day, and the following WISH takes 3 days.

57. Select the embryos with fluorescent signal in ventral side at shield stage using a fluorescence stereoscopic microscope, and transfer them to a new culture plate.
58. Photograph the selected embryos, and then fix them for 12 h–16 h by 4% PFA at 12 hpf or 24 hpf (Figure 4).
59. Perform WISH of *six3b*, *egr2b*, *shha*, *pax2a* and *cdx4* for the fixed embryos.

Note: The WISH images of these genes are available in our published article.[1]

60. Photograph the embryos, and analyze the WISH results.

EXPECTED OUTCOMES

The head-like structure of zebrafish will be generated, and its transcriptomic and patterning landscape will be constructed. This protocol mainly contains 4 steps to generate and assess the HLS. In the first step, a Nodal signaling gradient was constructed in the animal pole of zebrafish embryo by injecting *ndr2* mRNA into one blastomere of the embryo at 128-cell stage. The explant was obtained by cutting off 1/3–1/2 animal pole region of the embryo, and then was cultured in the medium (zebrafish cell). The explant was spherical before 6 hpf, and was extended to maximal length at 10 hpf. The explant developed into HLS at 10 hpf. In the second step, transcriptomic landscapes of Nodal explants were constructed by performing single-cell RNA-seq. The specific marker of each cell type was identified by conducting differential expression analysis between all cell clusters. In the third step, the DIG-labeled probe or HCR probe of the specific marker gene in each cell type was synthesized or ordered to perform WISH or HCR experiments. Cell patterning landscapes were constructed by analyzing those WISH/HCR results. In the last step, ventral overexpression assay was used to assess the axis-induction ability of Nodal downstream genes (master genes of inducing HLS formation). This protocol can be used to generate and assess other zebrafish explant systems induced by other molecular signals.

LIMITATIONS

The first and the last step of this protocol was optimized for zebrafish embryos. While, the second and third step can be used for other model systems, such as: mouse gastruloid or trunk-like structure, human gastruloid or organoid (The experimental condition should be optimized for these systems).

TROUBLESHOOTING

Problem 1

The Nodal injected explants do not extend well at the end of gastrulation (related to *Step 1–5*).

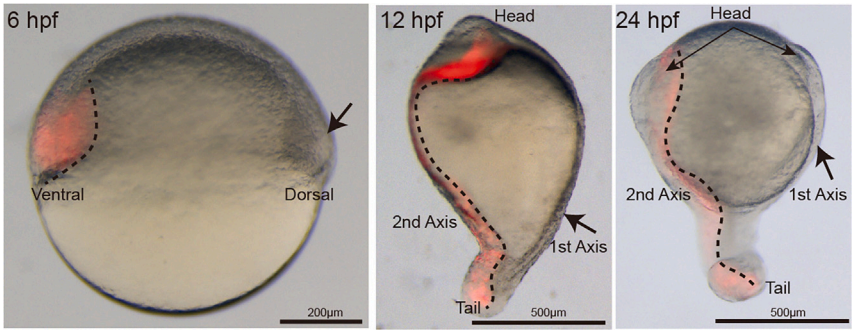


Figure 4. Representative images of ventral injection of ndr2 mRNA in zebrafish embryos

Merged image showing a zebrafish embryo with ventral injection of ndr2 mRNA at 6 hpf (Left). Black arrow indicates the shield region of the embryo, and the dotted line indicates the ventral region with fluorescence labeling. Merged image showing a zebrafish embryo with a secondary axis at 12 hpf (Middle). Black arrow indicates the first axis (Primary axis) of the embryo, and the dotted line indicates the secondary axis with fluorescence labeling. Merged image showing a zebrafish embryo with a secondary axis at 24 hpf (Right). Scale bar: 200 μm (Left), 500 μm (Middle and Right).

Potential solution

The efficiency of injected mRNA (*ndr2*) is essential for the induction of the explants. The new synthesized mRNA should be packaged into small volumes and stored at -80°C, and the maximum time for storage is 6 months. If a decrease in the extension efficiency of the explant is observed, it may be considered to re-synthesize the mRNA.

Problem 2

The results of *in situ* hybridization show low signal intensity or high background staining signals (related to *Step 23–38*).

Potential solution

In several steps of *in situ* hybridization, proper pH is essential for the reactions, and thus it is essential to ensure each solution is at proper pH and is not stored for a long time (Freshly made solutions are recommended). On the other hand, providing 5% Dextran Sulfate to hybridization Mix in the hybridization step can significantly enhance the signal intensity without increasing background signals. Else, a ~5° temperature increase in this step can enhance the specificity of probes and reduce background staining signals.

Problem 3

Too many DE genes are identified in bulk RNA-seq analysis, so that it is hard to find the most relevant genes (related to *Step 17*).

Potential solution

Setting a threshold for gene fold changes helps identify genes showing large extent of expression changes. In our cases, we further took the overlapped DE genes in Nodal explants and CHX-treated Nodal explants to narrow down Nodal downstream candidate genes. We achieved three overlapped gene sets corresponding to the developmental time points, and only the common genes of these three overlapped gene sets were defined as Nodal targets.

Problem 4

In the analysis of scRNA-seq data, cells are not clustered well, which may lead to either under-clustering or over-clustering issues (related to *Step 22*).

Potential solution

The cell clustering is performed under the *FindClusters* function implemented in Seurat, and the resolution parameter in this function can be used to adjust the number of clusters. Finding an optimal resolution is essential for the downstream analysis. Often the optimal resolution increases for larger

datasets. In this step, we tested the resolution parameter in the range of 0.5–2 to check the clustering performance, and optimal resolution parameters were selected for each sample.

Problem 5

In HCR experiments, the fluorescent signals may not strong enough, especially for the double or triple experiments (related to *Step 39–54*).

Potential solution

Increasing the probe concentration and prolonging the incubation time with amplifier will help to gain higher signal. But for our experience, permeabilization by 10 μg/mL Proteinase K treatment for 30 s is the most effective way to increase the signal.

RESOURCE AVAILABILITY

Lead contact

Further information and reasonable requests for resources should be directed to and will be fulfilled by the Lead Contact, Dr. Peng-Fei Xu(pengfei_xu@zju.edu.cn).

Materials availability

All the materials used in this protocol are commercially available.

Data and code availability

This study did not generate any unique data sets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102553.

ACKNOWLEDGMENTS

We thank the members of the Laboratory of Development and Organogenesis (LDO) at Zhejiang University for helpful suggestions and discussions. We also thank Mr. Guang-Xu Zhang from AccuraMed Technology (Shanghai) Limited (https://www.accuramed.com/) for the technical supports of the single-cell transcriptomic sequencing (10×). This work was supported by grants from the Chinese National Key Research and Development Project (2022YFA1103100, 2019YFA0802402), the National Scientific Foundation of China (32050109, 31970757, 31900576).

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing of the STAR protocol. T.C. composed figures from data and generated graphical images. Y.-Y.X. and T.C. performed experiments. T.C. and Y.D. perform the data analysis. P.-F.X. revised and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Cheng, T., Xing, Y.Y., Liu, C., Li, Y.F., Huang, Y., Liu, X., Zhang, Y.J., Zhao, G.Q., Dong, Y., Fu, X.X., et al. (2023). Nodal coordinates the anterior-posterior patterning of germ layers and induces head formation in zebrafish explants. Cell Rep. *42*, 112351. https://doi.org/10.1016/j.celrep.2023.112351.

2. de Jong, M., Rauwerda, H., Bruning, O., Verkooijen, J., Spaink, H.P., and Breit, T.M. (2010). RNA isolation method for single embryo transcriptome analysis in zebrafish. BMC Res. Notes *3*, 73. https://doi.org/10.1186/1756-0500-3-73.

3. Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. *36*, 411–420. https://doi.org/10.1038/nbt.4096.

4. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell *177*, 1888–1902.e21. https://doi.org/10.1016/j.cell.2019.05.031.

5. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550. https://doi.org/10.1186/s13059-014-0550-8.

6. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner.

Bioinformatics *29*, 15–21. https://doi.org/10.1093/bioinformatics/bts635.

7. Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., and Li, H. (2021). Twelve years of SAMtools and BCFtools. GigaScience *10*, giab008. https://doi.org/10.1093/gigascience/giab008.

8. Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*, 923–930. https://doi.org/10.1093/bioinformatics/btt656.

9. Yu, G., Wang, L.G., Han, Y., and He, Q.Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS A J. Integr. Biol. *16*, 284–287. https://doi.org/10.1089/omi.2011.0118.

10. Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics *32*, 2847–2849. https://doi.org/10.1093/bioinformatics/btw313.

11. Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis.

12. Kolde, R. (2019). Pheatmap: Pretty Heatmaps.

13. Wagner, D.E., Weinreb, C., Collins, Z.M., Briggs, J.A., Megason, S.G., and Klein, A.M. (2018). Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo. Science (New York, N.Y.) *360*, 981–987. https://doi.org/10.1126/science.aar4362.

14. Farrell, J.A., Wang, Y., Riesenfeld, S.J., Shekhar, K., Regev, A., and Schier, A.F. (2018). Single-cell Reconstruction of Developmental Trajectories during Zebrafish Embryogenesis. Science (New York, N.Y.) *360*. https://doi.org/10.1126/science.aar3131.

15. de Olivera-Melo, M., Xu, P.F., Houssin, N., Thisse, B., and Thisse, C. (2018). Generation of Ectopic Morphogen Gradients in the Zebrafish Blastula. Methods Mol. Biol. *1863*, 125–141. https://doi.org/10.1007/978-1-4939-8772-6_7.

16. Thisse, B., and Thisse, C. (2014). In situ hybridization on whole-mount zebrafish embryos and young larvae. Methods Mol. Biol. *1211*, 53–67. https://doi.org/10.1007/978-1-4939-1459-3_5.