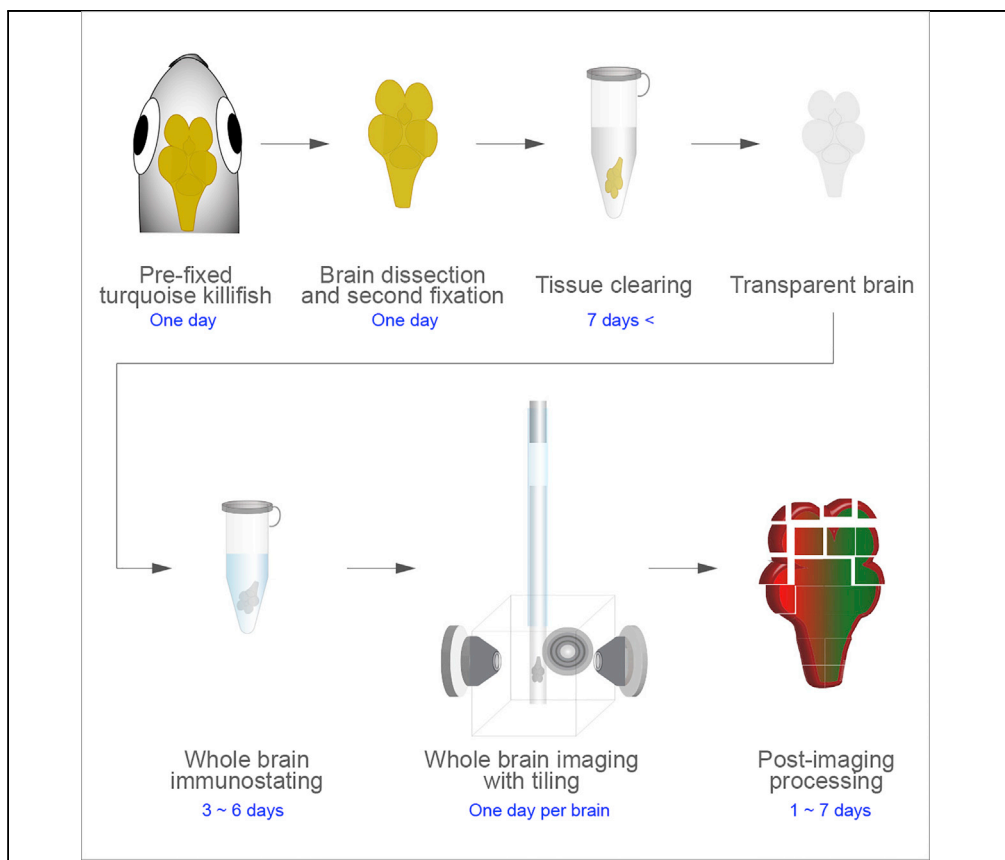


Protocol

Protocol for whole-brain immunostaining of the turquoise killifish after tissue clearing



Distribution of cells and proteins in whole organs, such as the brain, can provide another level of insight into molecular and cellular functions. Here, we describe a whole-brain immunostaining method using the turquoise killifish, an emerging model for aging research. We optimized a protocol for tissue clearing and whole-brain immunostaining to the turquoise killifish brain. This protocol provides a comprehensive procedure from brain dissection to whole-brain imaging and image processing.

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Highlights

Intact brain isolation from the turquoise killifish

Whole-brain immunostaining after tissue clearing

3D and 2D reconstruction of protein localization in the brain

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Protocol

Protocol for whole-brain immunostaining of the turquoise killifish after tissue clearing

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<https://doi.org/10.1016/j.xpro.2021.100564>**SUMMARY**

Distribution of cells and proteins in whole organs, such as the brain, can provide another level of insight into molecular and cellular functions. Here, we describe a whole-brain immunostaining method using the turquoise killifish, an emerging model for aging research. We optimized a protocol for tissue clearing and whole-brain immunostaining to the turquoise killifish brain. This protocol provides a comprehensive procedure from brain dissection to whole-brain imaging and image processing.

For complete details on the use and execution of this protocol, please refer to Eunjeong Do (2020) and Lee et al. (2021).

BEFORE YOU BEGIN**Preparation of fish for imaging**

⌚ Timing: 1 day to months

1. Raise fish in the facility until the age you need. Lifespan of the turquoise killifish is largely varied upon its strain (median lifespan ranges 9–26 weeks) (Terzibasi et al., 2008). Due to its short lifespan as a vertebrate with highly conserved aging phenotypes, the turquoise killifish gives enormous advantages to study aging. To obtain a reproducible aging phenotype, it is critical to evaluate age-dependent changes in fish that are raised for longer than a year. We used the shortest-living strain, GRZ-AD in this study and this strain is sexually matured around 3–4 weeks after hatching and has around 16 weeks of median lifespan in our facility. Male fish, wild-type, should start to show coloration between 3 and 4 weeks after hatching. In this protocol, we used 6- and 16-week-old female fish.
2. For immunostaining, you can use either wild-type fish (depending on antibody availability; e.g., specificity to the turquoise killifish proteins, and this should be validated before starting this experiment) or transgenic fish carrying a fluorescent or tagged protein. Here, we demonstrate the application of this protocol using a wild-type turquoise killifish with commercial antibodies that are validated for their specificity to endogenous turquoise killifish proteins (Bmal1 and Clockb).

Preparation of reagents

⌚ Timing: 30 min

3. Tricaine solution for sacrificing fish



- a. Dissolve 1.5 g of Tricaine (MS-222) in 1 L of tank water (pH 7.0, 700 μ S).
- b. Adjust pH to 7.0 with sodium hydrogen carbonate.

Note: This solution can be stored at 4°C for a month.

4. 1000 \times 4',6-diamidino-2-phenylindole (DAPI) stock solution
 - a. Dissolve 100 mg of DAPI in 1 mL of water.
 - b. Aliquot the solution into light-protected e-tubes (100 μ L per tube).
 - c. Store DAPI aliquots at -20° C before use.

Note: This solution can be stored up to 3 months at -20° C and 3 years at -80° C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Immunotag™ BMAL1 Monoclonal Antibody (conjugated with Alexa Fluor®647, mouse monoclonal)	G-Biosciences	ITM0071-100M-647 (RRID:AB_2868539)
CLOCK Antibody (rabbit polyclonal)	HUABIO	R1511-2 (RRID:AB_2868538)
Goat anti-rabbit IgG, cross-adsorbed, Alexa Fluor 488-conjugated	Invitrogen	A11008 (RRID:AB_143165)
Chemicals, peptides, and recombinant proteins		
Tricaine (MS-222)	Sigma-Aldrich	E10521-50G
Sodium hydrogen carbonate	Samchun Pure Chemical	S0344
4% Paraformaldehyde solution (in 1 \times PBS)	Biosesang	PC2031-100-00
DAPI	Sigma-Aldrich	D9542
NuSieve GTG Agarose	Lonza	50080
10 \times PBS	Calbiochem	LC6505
Experimental models: Organisms/strains		
<i>Nothobranchius furzeri</i>	GRZ-AD	n/a
Critical commercial assays		
Binaree Tissue Clearing Starter's Kit (High-Resolution)	Binaree	HRTI-101
Software and algorithms		
ZEISS ZEN lite	Zeiss	ZEN 2.3 (blue edition)
ZEN 2014 SP1	Zeiss	ZEN 2014 SP1 (black edition)
Lightsheet Z.1 Tile Scan	Zeiss	LS_TileScan V.14.8.0.23
Imaris	Bitplane	Imaris \times 64 7.6.0
Imaris File Converter	Bitplane	ImarisFileConverter \times 64 9.2.0
Imaris Stitcher	Bitplane	ImarisStitcher \times 64 9.2.1
Other		
Lightsheet microscope[20 \times objective lens (W Plan-APOCHROMAT 20)]	Zeiss	Lightsheet Z.1
Stereoscope M80	Leica	M80
Microwave	Samsung	MS23F301TAW
Fine tweezers	FST	11251-35
Microscissors	FST	15024-10
Transferpettor caps, 100 and 200 μ L, blue, glass capillaries color code	BRAND	701910
Piston rod for Transferpettor, Fix and Digital type 100 μ L	BRAND	701936
Mini Digital Incubator	Benchmark	H2200-HC

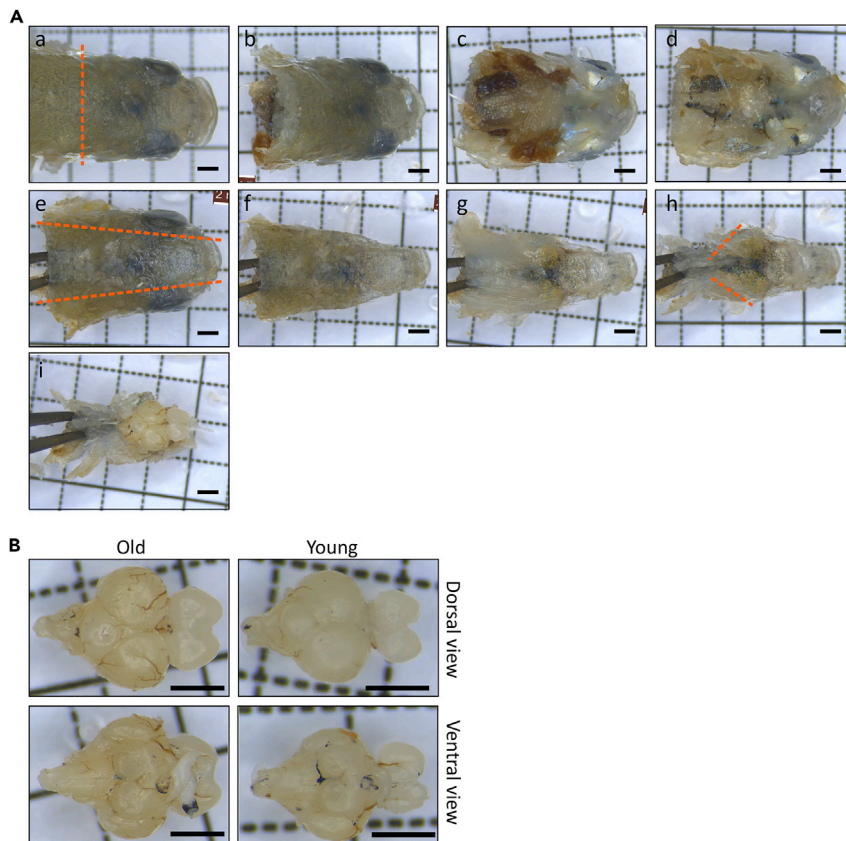


Figure 1. Dissection of the turquoise killifish brain

(A) Step-by-step images of brain dissection a. Cut the head between the gills and the pectoral fin. b. Isolate the fish head and remove the lower jaw. c. Flip the head upside-down. d. Clear the palate muscle from the upper jaw with fine forceps. e. Hold the spine to fix the head with fine tweezers and remove the eyes and gill covers. f. Remove the eyes and gill cover. g. Remove the head skin and muscle. h. Cut the skull to reveal the brain. i. Remove the upper skull to expose the brain. Red dashed lines indicate cutting lines.

(B) Isolated brains from young (6-week-old, after sexual maturation, female) and old (16-week-old, around median lifespan, female) fish. The scale bar indicates 1 mm.

STEP-BY-STEP METHOD DETAILS

Preparation of the killifish brain

⌚ Timing: 1 day

This step describes how to dissect and pre-fix the killifish brain before tissue clearing.

1. Sacrifice fish
 - a. Transfer fish to a 1.5 g/L tricaine solution.
 - b. Wait until the fish gills no longer move, and touch the caudal fin to confirm that they do not respond.
 - c. Transfer whole fish into 30 mL of 4% paraformaldehyde (PFA) in 1×PBS (pH7.4) and incubate at 4°C from 6 to 24 h.

Note: It is possible to perform brain dissection on freshly sacrificed fish, but it becomes much easier after fixation because the brain tissue hardens.

2. Brain dissection under a stereomicroscope (Figure 1)

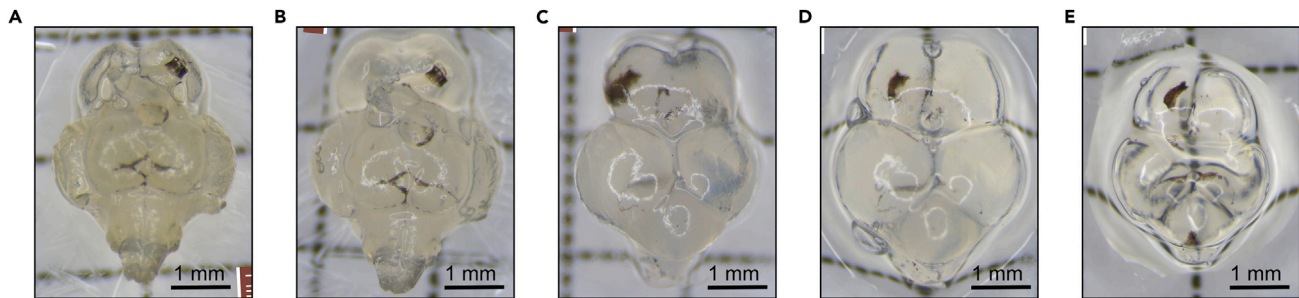


Figure 2. Brain tissue clearing

Images from the brain of an older fish are shown. (A) After treatment with tissue clearing solution A, (B) after the first wash, (C) after treatment with tissue clearing solution B, (D) after the second wash, and (E) in the mounting and storage solution. All brain images were taken under same magnification.

- a. Cut below the gills with scissors.
- b. Remove the lower jaw and soft palate muscles.
- c. Hold the spine tightly with forceps and cut out the eyes along the frontal skull.
- d. Remove skin and muscle above the skull.
- e. Cut from the infraorbital ring to the subopercle.
- f. Carefully remove the frontal skull with fine forceps.
- g. Remove the brain from the lower skull.
- h. Put the isolated brain into 500 μL of the second fixation solution in the Binarree Tissue Clearing Starter's kit and incubate without agitation for at least 6 h at 4°C.

Note: The brain will float at first but will sink to the bottom of the tube as fixation occurs.

△ CRITICAL: The brain is an extremely soft tissue, and the brains of young fish are much softer than those of older fish. Even after the brain is fixed with PFA, it can still tear easily during dissection, particularly at soft structures such as the pineal and pituitary glands and the telencephalon. Cut out the frontal skull and remove the brain with special care.

Clearing the whole brain

⌚ **Timing:** 7 days or more, depending on the amount of tissue clearing (Figure 2)

This step involves clearing the brain tissue before immunostaining for better visualization of immuno-stained or fluorescently tagged proteins. If you started with transgenic fish expressing a fluorescent protein, you can stop at optional step 5 and proceed directly to the imaging step.

3. Tissue clearing step A
 - a. Remove the fixation solution carefully with a pipet.
 - b. Add 200 μL of tissue clearing solution A (provided in the kit) to the fixed brain and gently invert the sample a few times, and discard the solution.
 - c. Add 500 μL of tissue clearing solution A and incubate the tube at 42°C for 3 days without shaking. Invert the tube gently a few times a day.
 - d. Remove tissue clearing solution A with a pipet, taking special care not to touch the brain.
 - e. Add 500 μL of washing solution (provided in the kit) and briefly wash out the tissue clearing solution A and discard the solution.
 - f. Add 1 mL of washing solution and incubate the tube at 42°C for 8 h.
 - g. Repeat the washing step (step f) twice.

- h. Invert the tube gently a few times per wash.
4. Tissue clearing step B
 - a. Remove the washing solution carefully with a pipet.
 - b. Add 200 μL of tissue clearing solution B (provided in the kit) and gently invert the sample a few times to mix.
 - c. Add 500 μL of tissue clearing solution B and incubate at 35°C for 2 days without shaking. Invert the tube gently a few times a day.
 - d. Remove tissue clearing solution B with a pipet, taking special care not to touch the brain.
 - e. Add 500 μL of washing solution and briefly wash out the tissue clearing solution B.
 - f. Add 1 mL of washing solution and incubate the tube at 35°C for 8 h.
 - g. Repeat the washing step (step f) twice.
 - h. Invert the tube gently a few times per wash.
5. **Optional:** If you are using transgenic fish carrying a fluorescent protein, you can stop at this stage and store the brain. The cleared tissue can also be stored up to a week at 4°C without further immunostaining. Mounting and storage buffer (provided in the kit) should be washed out when you want to proceed immunostaining again with the cleared tissue.
 - a. Add DAPI solution (1 μL per 1 mL solution) for nuclear staining into the washing solution at the final wash step and incubate the tube at 35°C for 8 h.
 - b. Remove the washing solution carefully with a pipet.
 - c. Add 200 μL of mounting and storage solution, and gently agitate a few times.
 - d. Add 500 μL of mounting and storage solution.
 - e. Incubate the tube at 35°C for 12 h.
 - f. Store the tube at 4°C in the dark for long-term storage up to 1 month before imaging.
6. **Optional:** If the brain tissue is not as clear as shown in [Figure 2D](#), repeat step 4 until the tissue is completely cleared.

Immunostaining of the cleared brain

⌚ **Timing:** 3 to 6 days

This step is for staining candidate proteins with appropriate antibodies to elucidate their location in the killifish brain. The antibodies for immunostaining should be tested for specificity, especially when using antibodies generated from orthologous proteins.

⚠ CRITICAL: To stain for two different proteins, it is critical to select compatible antibodies and determine the optimal staining order.

Note: If the primary antibodies are all fluorescently tagged with different emission wavelengths, the immunostaining can be performed at the same time, following the steps described below for primary antibody incubation.

Note: If only one antibody is fluorescently tagged, confirm that the antibodies were generated in different hosts or different isotypes (eg, IgG and IgM), and determine the order of immunostaining. We would recommend staining with the unlabeled antibody first, followed by the appropriate secondary antibody, and then staining with the fluorescently tagged antibody.

Note: If the two antibodies were generated in the same host species but they are labeled with different fluorescent tags (eg, anti-ProteinX-Alexa647 and anti-ProteinY-Alexa488 and both antibodies are generated in mice), both antibodies can be added to the same immunostaining solution.

Note: If the two antibodies were generated in the same host species but they are not fluorescently tagged, the two antibodies cannot be used together due to cross-recognition by the

secondary antibody. In this case, it would be necessary to fluorescently conjugate the antibodies prior to immunostaining or identify an antibody generated in a different host species.

7. Incubation with CLOCK antibody

- a. Remove washing solution carefully with a pipet.
- b. Gently wash the brain with 100 μ L of staining solution (provided in the kit).
- c. Add 100 μ L of staining solution containing antibody.
 - i. The amount of antibody to use varies. Usually 1:100 is a good starting dilution for whole brain immunostaining, but we highly recommend testing at least a few antibody concentrations.

Note: If you are comparing brains from animals at two different ages, it is always better to increase the volume of the immunostaining solution and put young and old brains together in the same tube so they are stained under equivalent conditions. Young and old brains can be identified later by the size.

- d. Incubate the brain with the anti-CLOCK antibody (1:200) at 4°C in the dark for 2 days without agitation.
 - e. Remove the antibody solution and add 1 mL of washing solution.
 - f. Incubate the tube at 35°C for 12 h without shaking and gently invert the tube from time to time during the incubation. Repeat this three times.
 - g. **Optional:** Follow this step and skip step 8 if the primary antibody is fluorescent-tagged.
 - i. Remove the washing solution carefully.
 - ii. Add 200 μ L of mounting and storage buffer.
 - iii. Add 500 μ L of mounting and storage buffer and incubate the sample at 35°C for 12 h without shaking. Gently invert the tube from time to time during the incubation.
 - iv. Store the tube at 4°C in the dark until imaging.
8. Incubation of an Alexa Fluor 488-conjugated secondary antibody to the CLOCK antibody
- a. Remove the washing solution carefully with a pipet.
 - b. Gently wash the brain with 100 μ L of staining solution.
 - c. Add 100 μ L of staining solution containing a goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (1:100).

Note: The optimal concentration of the secondary antibody also varies, same as with the primary antibody. A 1:100 dilution is a good starting point for titrating the antibody.

- d. Incubate the brain with the secondary antibody at 4°C in the dark for 2 days.
 - e. Remove the antibody solution and add 1 mL of washing solution.
 - f. Incubate the tube at 35°C for 12 h without shaking and gently invert the tube from time to time during the incubation. Repeat this three times.
9. Incubation with BMAL1 antibody conjugated with Alexa Fluor®647
- a. Repeat Steps 7a through 7f (BMAL1 antibody dilution, 1:200).
 - b. Remove the washing solution carefully with a pipet.
 - c. Add 200 μ L of mounting and storage buffer.
 - d. Add 500 μ L of mounting and storage buffer, and incubate the sample at 35°C for 12 h without shaking. Gently invert the tube from time to time during the incubation.
 - e. Check the fluorescence briefly using a fluorescence microscope (e.g., Epifluorescence microscope or stereomicroscope with fluorescence equipment).
 - f. Store the tube at 4°C in the dark until imaging.

Note: When you store brain tissue in the mounting and storage solution, the tissue will shrink a bit, but this does not affect the overall structure.

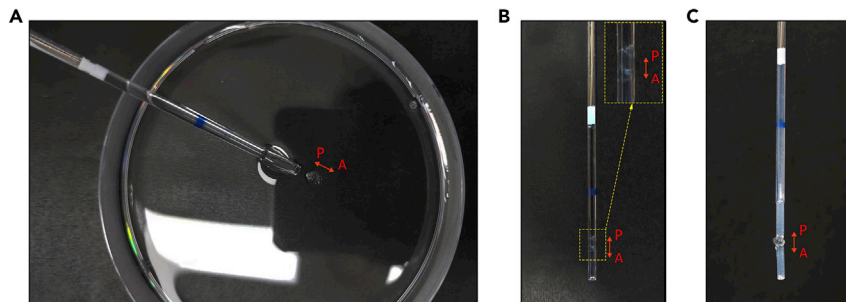


Figure 3. Embedding of cleared and stained brains for imaging

(A) Place the brain in 2% agarose solution when its temperature is around 60°C. Suck the brain into a capillary placed at the posterior of the brain.
 (B) The agarose-embedded brain. Dashed-yellow box shows enlarged image of an embedded brain in the capillary.
 (C) The embedded brain status within the sample chamber of the lightsheet microscope. The red arrow indicates the brain direction. P, posterior, and A, anterior brain.

Imaging of the stained brain

⌚ Timing: 1 day per brain

This step introduces the protocol we have used to image the turquoise killifish brain in our group, but these steps may vary depending on the microscope and imaging software used.

10. Preparation of agar blocks with stained brains (Figure 3)
 - a. Completely melt 2% low melting temperature agarose in PBS.
 - b. Cool down the 2% agarose solution to around 60°C.
 - c. Select a proper-size capillary for the prepared brain.
 - d. Pour 3 mL of agarose into a Petri dish.
 - e. Place the brain onto the Petri dish in the agarose after brief washing with PBS.
 - f. Transfer the brain into the agarose on the Petri dish and gently rotate the dish to cover the brain with the agarose.
 - g. Fill a capillary halfway with agarose, taking care to avoid bubbles.
 - h. Place the capillary at the posterior brain and suck the brain into the capillary using a plunger.
 - i. Make sure the brain is placed 0.5–1 cm from the tip of the capillary and that there are no air bubbles.
 - j. Wait until the agarose solidifies.

Note: The killifish brain mostly fits into a capillary with a 2 mm diameter. If the brains of older animals do not fit into a 2 mm capillary, place the stained brain in a custom capillary with a larger inner diameter (3 mm) or a thin agarose block with a size of 3 mm (w) × 4 mm (l) × 3 mm (h), and affix the agarose block onto the end of a 2 mm capillary using super glue. Another option is to use a 1 mL disposable syringe. This size syringe also fits to the Lightsheet Z.1 microscope.

11. Collection of raw image files with ZEN 2014 SP1 and Lightsheet Z.1 Tile Scan.
 - a. Place the sample chamber onto the microscope and fill the chamber with PBS or mounting and storage buffer.

Note: The Lightsheet microscope in this protocol is water based so we filled the sample chamber with PBS.

- b. Place a capillary tube onto the sample holder and place the brain embedded in agarose in front of the objective lens by pushing the plunger.

- c. Rotate the embedded brain so that the dorsal or ventral part of the brain is facing the objective lens.
- d. Choose a filter set with the proper excitation and emission.
- e. Check the fluorescence intensity and focus.
- f. Execute a tile imaging experiment covering the whole brain.
 - i. Define the Z-depth, capturing the region from the cerebellum to the pituitary.
 - ii. Define the corner at the top left.
 - iii. Define the corner at the bottom right.
 - iv. Save this corner-defined file as file_name.mlv in ZEN 2014 SP1.
 - v. Read file_name.mlv from Lightsheet Z.1 Tile Scan to create a pseudo tile array (usually with a 10% overlap between tiles) and save this file_name2.mlv.
- g. If the brain is too thick, flip the brain vertically and repeat steps from 11c to 11f.

△ CRITICAL: If you want to examine differences in protein expression between two brains, you must use the same parameters for imaging, including the gain, exposure time, and laser intensity. A few test trials should be done to check the general fluorescence intensity. It is always better to adjust the parameters on a sample with strong fluorescence intensity because when you set the parameters based on a sample with weak fluorescence intensity, the sample with strong fluorescence intensity may be saturated when you image it under the same settings, which prevents further analysis.

Image processing

⌚ Timing: 2 h to 7 days

12. Whole brain comparison
 - a. Using the Imaris File Converter, convert file_name.czi files generated using the same imaging conditions into Imaris files (file_name.ims).
 - b. Read files in Imaris Sticher, which automatically arrays tiled raw images.
 - c. Adjust the edges of raw images and run stitching.
 - d. Check the merged image and the individual images using the slide sections.
 - e. Adjust the minimum and maximum intensity, gamma, and opacity in each channel.
 - f. Apply the same image display parameters to the other brains, and export images.
13. Section comparison
 - a. Crop the regions of interest from whole brain images or from single tile images.
 - b. Read the files in Zen (file_name.czi) or Imaris (file_name.ims) and adjust the image display.
 - c. Apply the same parameters for image display and export images.

EXPECTED OUTCOMES

This protocol provides a comprehensive procedure for killifish brain dissection, brain tissue clearing, immunostaining, and three-dimensional brain imaging. Clearly transparent brains can be easily imaged deep into the tissue at a high resolution, and intact structural analysis is possible from various directions (Figure 4, Method videos S1, S2, S3, and S4). This type of imaging can also be used to compare differences in protein expression levels and patterns between two different brains as well as between different regions of the same brain. Using this protocol, we compared the expression and localization of Bmal1 and Clockb proteins in the brains of young and old turquoise killifish, as shown in a previous study (Lee et al., 2021) (Figure 4). Bmal1 and Clockb proteins are known to localize mostly in the nucleus and cytosol of the cell, respectively (Biernat et al., 2012; Fekry et al., 2018). However, there are limited reports of localization of these proteins in whole brain except their mRNA expression in brain sections (Weger et al., 2013). Comparing Bmal1 and Clockb protein localization in young and old brain, fluorescence intensity from Bmal1 protein was dramatically reduced throughout major brain regions in old animals, but Bmal1 protein in the pineal gland of the aged

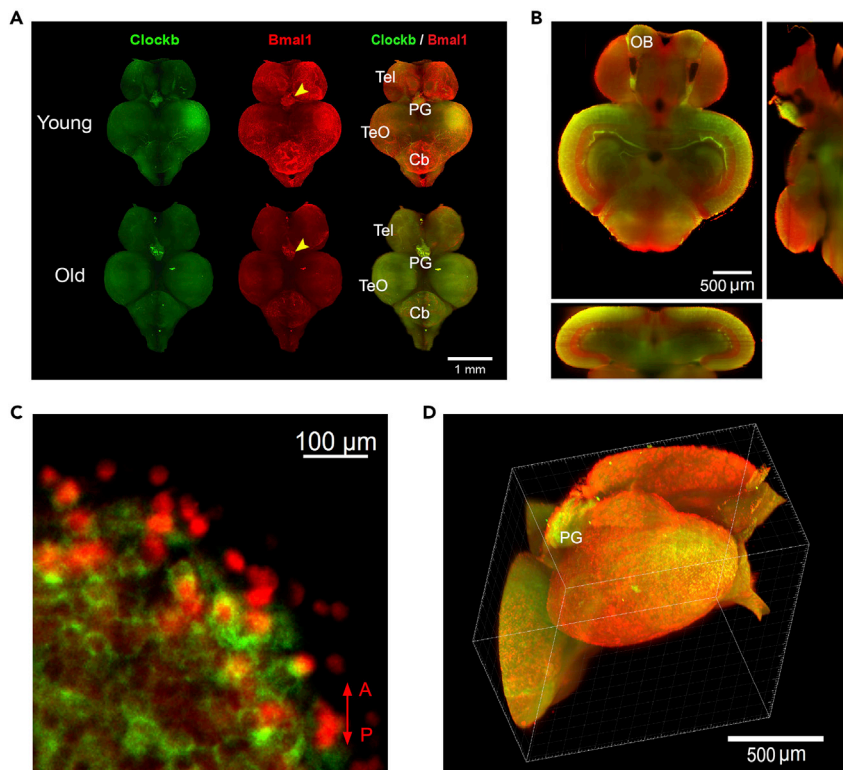


Figure 4. Diversely processed whole-brain images of the turquoise killifish

(A) Whole brain staining for Bmal1 (red) and Clockb (green) in the turquoise killifish brain. This image is reproduced from Lee et al. (2021).

(B) Whole brain section images (Center, horizontal section; Right, medial section; Bottom, frontal section).

(C) Enlarged and single image section of the telencephalon. Bmal1 (red) localized mostly in the nucleus and Clockb (green) localized mainly in the cytosol. A and P indicate anterior and posterior directions of the brain.

(D) Three-dimensional view of the telencephalon of the turquoise killifish brain. Images of Figure 4 except Figure 4A were processed from young brain of Figure 4A and old brain looked similar. Abbreviations are Tel (telencephalon), TeO (optic tectum), PG (pineal gland), Cb (cerebellum) and OB (olfactory bulb).

brain was relatively well maintained (Figure 4A). Bmal1 and Clockb proteins are distinctively expressed in specific brain regions/structures. Fluorescence from both Bmal1 and Clockb was detected in whole brain, with Bmal1 preferentially distributed at the surface of the brain as well as in the telencephalon while Clockb localized in a deeper layer of the optic tectum (Figure 4B). Bmal1 localized mostly in the nucleus and Clockb localized mainly in the cytosol (Figure 4C), which is comparable to previous studies (Biernat et al., 2012; Fekry et al., 2018). Subcellular localization of both proteins can be re-constructed into 3D (Figure 4D). As in the example shown here, this protocol can be used to gain functional insight into the roles of specific proteins within the cell as well as their spatial distribution in the brain.

LIMITATIONS

This protocol is largely dependent on antibody availability against to proteins of the turquoise killifish. Because the turquoise killifish is still a developing model organism, there is lack of turquoise killifish-specific antibodies. It may be possible to use antibodies generated from proteins from other species, but it is always recommended that several antibodies be tested to determine their specificity and the optimal antibody titer to use for staining. This protocol can be applied to other organs, but the amount of clearing solution and the incubation time may vary. For larger organs, it may not be possible to find a large enough capillary or mold to fix samples for the microscope, and even if a

proper embedding mold can be found, the bulk size of the sample may interfere with the movement of the objective lens in the sample chamber of the microscope. Microscopy also can be another limitation for imaging. The microscope that we used is equipped with a water-based sample chamber and an objective lens so that the cleared brains slowly became turbid and the numerical aperture (NA) of the objective lens did not perfectly match the cleared tissue. Thus, the images that are obtained may not be completely clear. You can generate much clearer and thicker brain images using a light sheet microscope equipped with a sample chamber filled with mounting and storage solution and an objective lens with an NA matched to that of the mounting and storage buffer.

TROUBLESHOOTING

Problem 1

Brain is torn during dissection (step 2).

Potential solution

Increasing the fixation time up to 48 h may help to harden the tissue samples.

Problem 2

Brain is not clear enough (step 3 to 6).

Potential solution

This protocol is specifically adapted for the killifish brain, and makes both young and old brains transparent. If the brain is not clear enough, you could repeat step 4 or increase the volume of tissue clearing solutions A and B.

Problem 3

There is no fluorescent signal observed (step 10 to 11).

Potential solution

Evaluate protein expression levels via protein gel blot analysis to determine whether the protein expression is sufficient. If strong protein expression is observed by this analysis, confirm that the correct antibody was used. If so, try again with a different antibody to the same protein. If one is available, choose an antibody with a low molecular weight, which will penetrate the tissue more readily.

Problem 4

Fluorescence signal detected only on the tissue surface ([Figure 5](#); step 10 to 11).

Potential solution

Antibody does not penetrate a cleared tissue so fluorescence signal glows on the tissue surface ([Figure 5](#)). In this case, it is always better to check your clearing protocol. When the tissue clearing process was not enough, this can happen. Thus, we highly recommend to increase tissue clearing step A and B. Importantly, compare the staining pattern of your candidate protein with immunohistochemistry from tissue section or previously known localization.

Problem 5

Computer freezes during image processing (step 12 to 13).

Potential solution

The raw image sizes can be as large as 300 Gb per brain in our case, and all software for imaging processing uses the Windows operating system. To process raw brain images of the turquoise killifish brain in this work, we increased the random access memory (RAM) size of the computer to 256 Gb.

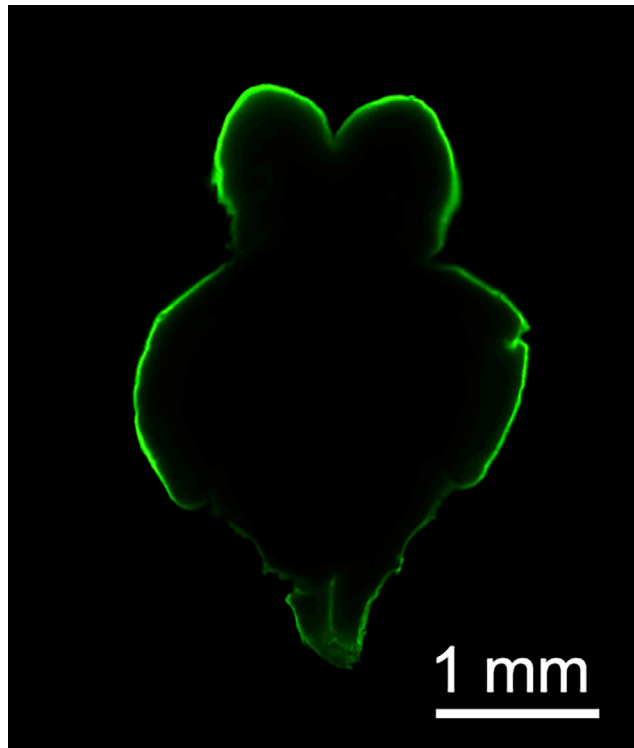


Figure 5. An example when an antibody fails to penetrate brain tissue

Green fluorescence is from Clockb protein. Clockb primary antibody and secondary antibody with Alexa-488 did not penetrate into the brain, so surface of the brain was intensely bright with green fluorescence but inside of the brain was almost black with no fluorescence signal.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yumi Kim (yumikim@ibs.re.kr).

Materials availability

This protocol does not generate new materials.

Data and code availability

The data from this study are available from the corresponding author upon reasonable request.

This study did not generate any new codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100564>.

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AUTHOR CONTRIBUTIONS

Y.K. conceived and designed this work. S.L. and Y.K. performed the experiments. Y.K. wrote the initial manuscript, and both authors read and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Biernat, M.A., Eker, A.P., van Oers, M.M., Vlaskovits, J.M., van der Horst, G.T., and Chaves, I. (2012). A baculovirus photolyase with DNA repair activity and circadian clock regulatory function. *J. Biol. Rhythms* 27, 3–11.
- Eunjeong Do, Y.K. (2020). Anatomical identification of the neuroendocrine system in the *Nothobranchius furzeri* brain. *bioRxiv*. <https://doi.org/10.1101/2020.10.15.342014>.
- Fekry, B., Ribas-Latre, A., Baumgartner, C., Deans, J.R., Kwok, C., Patel, P., Fu, L.N., Berdeaux, R., Sun, K., Kolonin, M.G., et al. (2018). Incompatibility of the circadian protein BMAL1 and HNF4 alpha in hepatocellular carcinoma. *Nat. Commun.* 9.
- Lee, S., Nam, H.G., and Kim, Y. (2021). The core circadian component, *Bmal1*, is maintained in the pineal gland of old killifish brain. *iScience* 24, 101905.
- Terzibas, E., Valenzano, D.R., Benedetti, M., Roncaglia, P., Cattaneo, A., Domenici, L., and Cellerino, A. (2008). Large differences in aging phenotype between strains of the short-lived annual fish *Nothobranchius furzeri*. *PLoS one* 3, e3866.
- Weger, M., Weger, B.D., Diotel, N., Rastegar, S., Hirota, T., Kay, S.A., Strahle, U., and Dickmeis, T. (2013). Real-time in vivo monitoring of circadian E-box enhancer activity: a robust and sensitive zebrafish reporter line for developmental, chemical and neural biology of the circadian clock. *Dev. Biol.* 380, 259–273.