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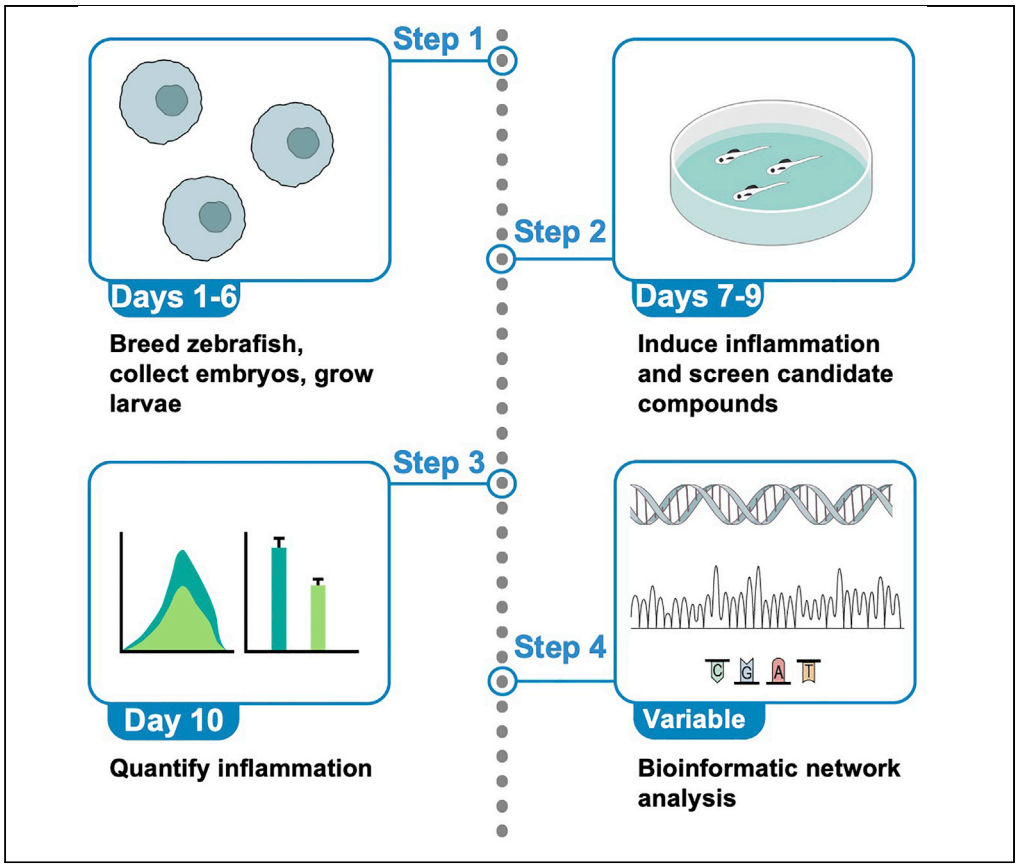
Protocol

Protocol for inducing inflammation and acute myelin degeneration in larval zebrafish



This protocol details the induction of inflammation and acute myelin degeneration in larval zebrafish with a duration of <10 days. We describe the use of this model to screen the effects of candidate compounds on inflammation, followed by RNA isolation, and qPCR-based quantification of gene expression. We then outline the steps for bioinformatic analysis of the mechanisms associated with the compounds. This protocol can be used in combination with drugs and genetic targeting to identify pathways that contribute to neurodegeneration.

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Highlights

Protocol for rapid screening of candidate compounds on inflammation in zebrafish

Protocol enables the identification of transcriptional changes

Bioinformatic analysis of the pathways associated with drug candidates

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Protocol for inducing inflammation and acute myelin degeneration in larval zebrafish

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SUMMARY

This protocol details the induction of inflammation and acute myelin degeneration in larval zebrafish with a duration of <10 days. We describe the use of this model to screen the effects of candidate compounds on inflammation, followed by RNA isolation, and qPCR-based quantification of gene expression. We then outline the steps for bioinformatic analysis of the mechanisms associated with the compounds. This protocol can be used in combination with drugs and genetic targeting to identify pathways that contribute to neurodegeneration.

For complete details on the use and execution of this profile, please refer to Wheeler et al. (2019).

BEFORE YOU BEGIN

This protocol describes a zebrafish model of systemic inflammation and whole-animal qPCR. However, we have also used this protocol for isolation of specific cell types, such as astrocyte-related radial glia (Bernardos and Raymond, 2006; Linnerbauer et al., 2020; Sanmarco et al., 2021; Wheeler and Quintana, 2019), by increasing the number of zebrafish used for the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Cuprizone (Bis(cyclohexanone) oxaldihydrazone)	Sigma-Aldrich	#C9012
LPS-EK	InvivoGen	#tlrl-eklps
TRIzol LS	Thermo Fisher Scientific	#10296010
PBS	Thermo Fisher Scientific	#14190250
0.5M EDTA	Amresco	#E177-100ML
Bovine serum albumin (BSA)	MilliporeSigma	#A3294
Critical commercial assays		
Direct-zol RNA kit	Zymo Research	#R2050
High-Capacity cDNA Reverse Transcription Kit	Life Technologies	#4368813
Taqman Fast Universal PCR Master Mix	Life Technologies	#4367846

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zebrafish: actb1 probe	Life Technologies	#Dr03432610_m1
Zebrafish: ccl20 probe	Life Technologies	#Dr03431608_m1
Zebrafish: il1b probe	Life Technologies	#Dr03114369_m1
Zebrafish: il10 probe	Life Technologies	#Dr03103209_m1
Zebrafish: il17a/f1 probe	Life Technologies	#Dr03096843_g1
Zebrafish: mpz probe	Life Technologies	#Dr03131914_m1
Zebrafish: nos2a probe	Life Technologies	#Dr03124753_m1
Zebrafish: actb1 probe	Life Technologies	#Dr03432610_m1
Experimental models: Organisms/strains		
Zebrafish: *Danio rerio*/AB	Wheeler et al., (2019)	ZFIN: ZDB-GENO-960809-7; RRID:ZIRC_ZL1438
Zebrafish: *Danio rerio*/ Tg(*gfap*::*egfp*)	Bernardos and Raymond (2006)	ZFIN: ZDB-TGCONSTRCT-070117-154

MATERIALS AND EQUIPMENT

E3 water		
Reagent	Final concentration	Amount
NaCl	5 mM	14.61 mg
KCl	170 μM	0.634 mg
$CaCl_2$	433 μM	2.403 mg
$MgSO_4$	675 μM	4.062 mg
HEPES	80 μM	0.953 mg
ddH_2O	n/a	To 50 mL
Total	**n/a**	**50 mL**
Store at 20°C for >1 month.		

Cuprizone stock solution		
Reagent	Final concentration	Amount
Cuprizone	40 μg/mL	0.02 g
E3 water	n/a	500 mL
Total	**n/a**	**500 mL**

Note: Prepare cuprizone solution fresh immediately before use. Dissolve cuprizone in zebrafish system water by warming the solution to 42°C.

⚠ CRITICAL: Cuprizone is a hazardous chemical which can cause irritation, brain and liver damage. Handle in a ventilated fume hood. Use gloves, a lab coat, and goggles when handling.

LPS stock solution		
Reagent	Final concentration	Amount
LPS-EK	1 mg/mL	5 mg
Endotoxin free water	n/a	5 mL
Total	**n/a**	**5 mL**

Note: Prepare LPS stock solution in endotoxin free water. Aliquots can be stored at 4°C for 1 month or −20°C for 6 months. Avoid repeated freeze-thaw cycles.

FACS buffer		
Reagent	Final concentration	Amount
EDTA 500 mM	500 μM	500 μL
BSA	0.5%	2.5 g
PBS	n/a	to 500 mL
Total	n/a	**500 mL**
Store at 4°C for >1 month.		

Cuprizone + LPS treatment solution		
Reagent	Final concentration	Amount
LPS-EK stock solution	150 μg/mL	900 μL
Cuprizone stock solution	12.5 μg/mL	1875 μL
Zebrafish system water	n/a	3225 μL
Total	n/a	**6000** μL
Store at 20°C for 1 day.		

Target gene	qPCR probe ID
ccl20	Dr03431608_m1
il1b	Dr03114369_m1
il10	Dr03103209_m1
il17a/f1	Dr03096843_g1
mpz	Dr03131914_m1
nos2a	Dr03124753_m1
actb1	Dr03432610_m1

STEP-BY-STEP METHOD DETAILS

Zebrafish breeding

Timing: 2 h

In this step, we describe the parameters used to collect zebrafish for these experiments

1. Prepare the E3 water (see "materials and equipment" for detailed recipe).
2. Breed the zebrafish to acquire the embryos and larvae for the experiment. (If the number of embryos is inadequate, see troubleshooting 1).
   a. To maximize embryo production, females and males can be kept in separate tanks before breeding.
   b. Prior to the day when you wish to obtain embryos, transfer adult male and female zebrafish into the same tank, at a 1:2 ratio, respectively. Use breeding tanks or use marbles to cover the bottom of the tank.

Pause point: Allow the fish to stay in the breeding tank for next ~12 h

3. After the beginning of the next light cycle, collect embryos into a fresh petri dish by siphoning and place the adult fish back in their home tanks.

a. Remove unfertilized and dead embryos from the petri dish and place 50 live embryos per dish in E3 water at 28°C for 7 days to develop.

Pause point: Allow larvae to develop at 28°C for 7 days.

b. During the growth period, remove dead embryos daily and change E3 water if needed.

Induction of inflammation and demyelination

Timing: 1 h

In this step, we describe the use of cuprizone and LPS to induce inflammation and demyelination

4. Prepare cuprizone stock solution (see "materials and equipment" for detailed recipe).
5. Thaw or prepare LPS stock solution (see "materials and equipment" for detailed recipe).
6. Prepare Cuprizone + LPS treatment solution (see "materials and equipment" for detailed recipe) and control (zebrafish system water) conditions in a 12-well plate and transfer the 7 dpf fish into the plate with 3 fish/well and 3 wells/condition.
7. To screen the effects of additional compounds on inflammation (such as environmental pollutants as described in (Wheeler et al., 2019)), additional groups can be included which include compound only, and Cuprizone + LPS + compound.
8. Place the 12 well plate containing larvae into a 28°C incubator for 48 h.

Pause point: Keep larvae in the Cuprizone + LPS solution at 28°C for 48 h.

Sample collection

Timing: 1 h

In this step, we describe how to isolate zebrafish samples.

Note: Animals have to be euthanized according to the permission and ethical rules of local authorities.

9. Euthanize larvae by placing the 12-well plate on ice.
10. Collect at least n=3 larvae per group for the desired end point measurement.

RNA isolation from zebrafish

Timing: 1–2 h

In this step, we describe how to isolate RNA from zebrafish larvae

11. Isolate RNA from at least n=3 larvae per group with TRIzol LS per the manufacturerś protocol.

⚠ CRITICAL: TRIzol is a hazardous chemical, process samples in a ventilated hood and use gloves, a lab coat, and goggles when handling.

12. Purify RNA with Direct-zol RNA kit according to the manufacturer's protocol.

Measuring inflammation and demyelination markers by qPCR

Timing: 3–4 h

In this step, we describe how to quantify gene expression in zebrafish

13. Synthesize cDNA with High-Capacity cDNA Reverse Transcription Kit according to manufacturerś protocol.

Reverse transcription thermocycling conditions

Steps	Temperature	Time	Cycles
Pre-primer extension	25°C	10 min	1
DNA polymerization	37°C	120 min	1
Enzyme deactivation	85°C	5 min	1
Hold	4°C	forever	

14. Measure gene expression by qPCR with TaqMan Fast Universal PCR Master Mix according to manufacturerś protocol.

Quantitative PCR thermocycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	20 s	1
Denaturation	95°C	1 s	40–60 cycles
Annealing/Extension	Variable	20 s	

15. Analyze qPCR data using the DDDDCt method by comparing the expression of target genes of interest (*ccl20*, *il1b*, *il10*, *il17a/f1*, *mpz*, *nos2a*) relative to *actb1*. (troubleshooting 2).

Bioinformatic identification of environmental compound mechanisms

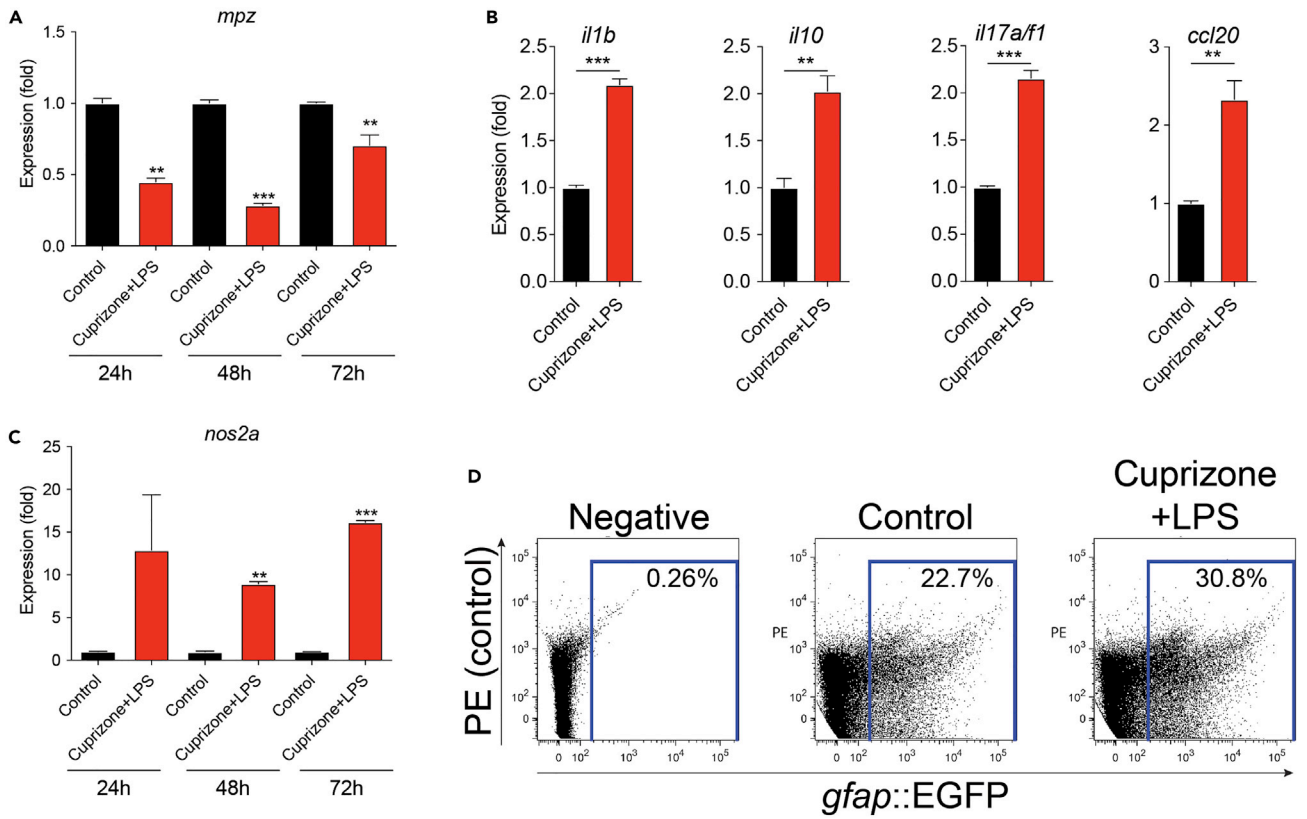
Timing: Variable

In this step, we describe how to bioinformatically analyze the pathways regulated by each compound

16. To identify mechanisms associated with compound hits from the EPA ToxCast inventory, go to the Dashboard, here. These data are derived from the EPA ToxCast compound inventory studies (Dix et al., 2007; Sipes et al., 2013).
17. Search for a compound of interest.
18. Identify the molecules which interact with the compound of interest using the "Bioactivity" ↪ "ToxCast: Summary".
19. To construct interaction networks, go to https://www.networkanalyst.ca/ and input the gene set of interest derived from the compound's interactions.
20. Select the species of interest as well as the database of interest to identify interactions between the interacting molecules (Figure 1E).

EXPECTED OUTCOMES

After induction of inflammation and demyelination as described above, there should be a clear downregulation of myelination associated genes (Figure 1A) and upregulation of inflammation related genes (Figures 1B and 1C) as measured by qPCR. Tested compounds should show effects on canonical markers of cellular activation specific to each experimental system (Figures 1C and 1D). When analyzing CNS inflammation for example, we focused on cytokines and chemokines



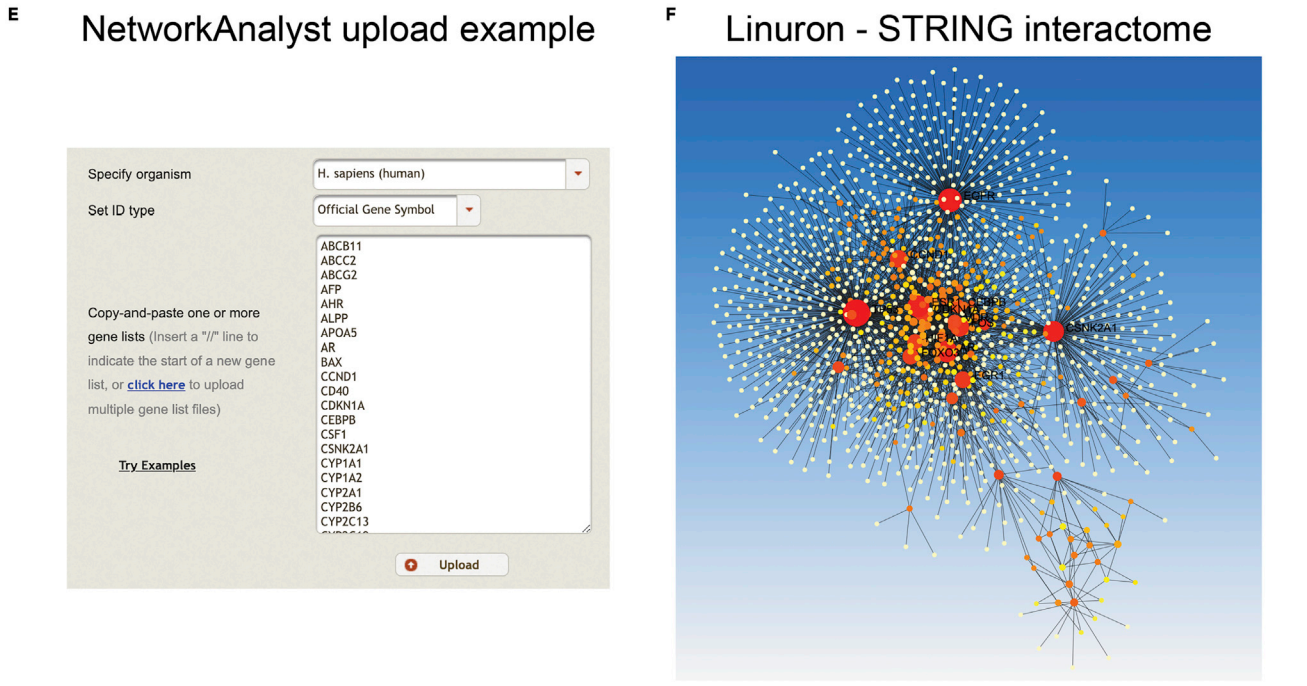


Figure 1. Expected outcomes of screening environmental factors in zebrafish

(A) Expression of myelin protein zero (*mpz*) in different exposure time points. LPS + cuprizone exposure results in significant demyelination in all measured time points.

(B) LPS + cuprizone induces expression of inflammatory markers. Expression of *il1b*, *il10*, *il17a/f1*, and *ccl20* is significantly increased after 48 h cuprizone exposure.

(C) Expression of inducible nitric oxide synthase (*nos2a*) in different exposure time points.

Figure 1. *Continued*
(D) LPS + cuprizone causes astrocyte activation. Dot plot analysis of *gfap*::EGFP expression after 48 h LPS + cuprizone exposure demonstrates increased *gfap*::EGFP expression. Expression of *nos2a* in *gfap*::EGFP+ cells is increased after 48 h LPS + cuprizone exposure.
(E and F) Example upload and (F) visualization in NetworkAnalyst of geneset identified by compound screening. **p<0.001; *** p<0.0001. Data shown as mean ± SEM.

il1b, *il10*, *il17a/f1*, *ccl20*, *nos2a*, as well as GFAP when studying astrocytes (Figures 1B–1D). Putative gene expression networks can be constructed using a combined analysis of the EPA ToxCast Dashboard and any bioinformatic tools of interest, for example, Network Analyst (Figures 1E and 1F) (Xia et al., 2015).

> *Note:* For details on cell isolation and sorting, including transgenic lines (Figure 1D) please see the original paper (Wheeler et al., 2019).

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: Variable

All qPCR data should be analyzed by comparing treatment condition to controls. For bioinformatic network analysis, only genes with significant effects in the EPA ToxCast database should be included.

LIMITATIONS

Other brands and types of LPS reagents have been demonstrated not to result in a similar inflammation and demyelination as seen in Figures 1A–1D. To avoid this, use only reagents listed in the Key Resources table.

TROUBLESHOOTING

Problem 1

Zebrafish are not producing the desired number of embryos for experiments (step 2).

Potential solution

This problem might arise from zebrafish husbandry. To resolve the issue, pay attention to proper and reproducible husbandry and well-being of the adult fish. Another option is to pair more fish by setting up additional spawning tanks. Ensure that adult females are not spawned more frequently than once weekly, with 2–3 week breaks between spawnings being ideal.

Problem 2

Upregulation of the inflammation and demyelination markers by qPCR are not observed at the end point (step 15).

Potential solution

Ensure that cuprizone is completely dissolved (step 3). If needed, a heated shaker can be utilized.

Problem 3

cDNA concentration is low (step 13).

Potential solution

Ensure that RNA purification is performed in an RNase free environment. Combine more zebrafish to increase RNA yield.

Problem 4

No interactions between compound target genes detected bioinformatically (step 20).

Potential solution

Select a different network analysis and incorporate more genes into your network.

Problem 5

A large number of interactions are detected in the bioinformatic networks (step 20).

Potential solution

Decrease the number of genes used as input or increase the threshold criteria defining interactions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Francisco Quintana, fquintana@rics.bwh.harvard.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

M.J., M.A.W., and F.J.Q. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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