NeuroExaminer: an all-glass microfluidic device for whole-brain in vivo imaging in zebrafish

Kai Mattern1,3, Jakob William von Trotha2,3, Peer Erfle1, Reinhard Wolfgang Köster2 & Andreas Dietzel1

While microfluidics enables chemical stimuli application with high spatio-temporal precision, light-sheet microscopy allows rapid imaging of entire zebrafish brains with cellular resolution. Both techniques, however, have not been combined to monitor whole-brain neural activity yet. Unlike conventional microfluidics, we report here an all-glass device (NeuroExaminer) that is compatible with whole-brain in vivo imaging using light-sheet microscopy and can thus provide insights into brain function in health and disease.
The zebrafish was the first vertebrate organism in which the neural activity of nearly the entire brain could be observed in vivo at single cell resolution approximately every second. More recently, microfluidics has discovered this small vertebrate as a suitable model system to investigate brain function and to screen for new drugs to alleviate psychiatric diseases. The ability of microfluidics to deliver chemical stimuli with millisecond precision, together with the zebrafish's optical translucency during its larval stages thus provide an outstanding opportunity to investigate the dynamics and interactions of neural circuits in a non-invasive manner in an intact living organism. Here, we report an all-glass microfluidic device that, in contrast to the more commonly used polydimethylsiloxane (PDMS), does not exhibit solvent permeability or autofluorescence, and is therefore compatible with whole-brain in vivo imaging via light-sheet microscopy.

Results
Microfluidic chip design and simulations. For computational fluid dynamics simulations (CFD software 18.0 from Ansys, Canonsburg, USA) three-dimensional models of the microfluidic system with the enclosed larva were created using computer-aided design (CAD software from SolidWorks, Velizy-Villacoublay, France). It is worth noting that horizontal mounting of the larva represents a more physiological orientation compared to the vertical mounting approach used in early whole brain-imaging studies. The larva and the glass walls were assumed as non-deforming solid bodies. Two fluid phases exhibiting the characteristic properties of water at a temperature of 28 °C were considered in the simulations as representing media and chemical stimuli. Transient state simulations of the injection of chemical stimuli over a period of 1.5 s (at 50 ms steps) were performed (Fig. 1a–f). The simulations were carried out for advancing design versions, whereby the position of the bypass opening as well as the size and positioning of stimulus and media injection channels were progressively improved to enable a fast exchange of fluids and three-dimensional flow focusing of the stimulus phase. The media inlet divides into an upper and a lower channel (Fig. 1g), which focuses the stimulus injection positioned in between. The lower media inlet channel exhibits a slightly higher hydrodynamic resistance, so that the injected stimulus is initially directed slightly downwards within the alignment chamber. The flow of medium introduced at the bottom of the alignment chamber helps to reduce the loss of stimulus that would otherwise be conducted directly out of the system via the bypass. Both of these features ensure that the stimulus reaches the larvae in a targeted manner. CFD simulations of two-system variants (Fig. 1a–f; Supplementary Movies 1 and 2) support the targeted exposure of the larva head to the stimulus under conditions of continuous supply of fresh media. The partially open system variant (Fig. 1c, d, f) exhibits a lower hydrodynamic resistance due to a recess area above the head of the larva. This additional opening deflects the current of the stimulus in an upward direction. With the additional opening the counter pressure that retains the larva is reduced which may be compensated by a higher throughput of stimului and medium. In the simulations, both NeuroExaminer variants enable a precise spatial and temporal control (with subsecond resolution) of exposure to varying stimuli. With the opening though, the flow in the bypass channel practically stops and parts of the larva are less exposed to fresh media fluid. The partially open variant is advantageous for optical imaging, since no microfabricated glass material is introduced in the imaging light path, while the completely closed variant (Fig. 1a, b, e) enables unparalleled control over stimuli exposure of the larva and is ideally suited for precise time-resolved compound stimulation. The simulations shown in Fig. 1a–f represent a total volume flow rate (with media and stimulus injection at a ten-to-one ratio) of 1.1 µl/s. The Reynolds numbers, which indicate the balance between viscous forces and inertial forces, were confirmed to stay below Re = 10. Hence, a completely laminar flow pattern without backflows or vortices is observed that allows 3D focusing of the stimulus for precise targeting of the chemical stimuli. Transient simulations revealed that a 100% concentration of stimulus reaches the larva head in <1 s after the injection into the alignment chamber in both the open and closed system (Fig. 1a–d; Supplementary Movies 1 and 2). Based on these simulations, which can very reliably predict the behavior in laminar flow experiments, it can be concluded that the desired precise spatial and temporal control of the stimulus delivery in the NeuroExaminer is indeed possible. The complex 3D chamber was designed to gently immobilize zebrafish larvae for high-resolution imaging (Fig. 1h illustrates the incoupling of the light sheet and the positioning of the larva under the detection objective) and to subsequently release them without harm (see also Supplementary Fig. 1). The CFD simulations support the chosen 3D design including microchannels used as inlets, outlets, and for stimulus application as shown in Fig. 1g. The 3D chamber design follows the concept of reversible fluidic larva immobilization (Fig. 1i–l) enabling: (Fig. 1i) zebrafish larvae loading through fluidic inlets, (Fig. 1j) flow-mediated autonomous animal alignment, (Fig. 1k) reversible larva fixation through reversed flow from the outlet, and (Fig. 1l) controlled exposure to stimuli. Small stimuli volume flows of 0.1 µl/s or less in combination with higher media volume flows of 1 µl/s or more not only considerably reduce the consumption of potentially expensive substances, but also facilitate rapid fluid exchange under laminar conditions. The microfluidic chip (NeuroExaminer) is part of a larger ensemble and embedded in a custom-made imaging chamber; Supplementary Fig. 1 provides an overview of the setup used in this study.

Next, we constructed the two variants of NeuroExaminer chips solely made from glass using femtosecond laser processing and subsequent thermal and chemical surface quality enhancement. Multi-photon-absorption in glass allows iterative ablation used for creating three-dimensional microfluidic structures (see “Methods” section for details). After structuring, the two system halves are bonded to obtain closed microfluidic chips (Fig. 1m, n).

Light sheet imaging in the microfluidic chip. To test the imaging properties of the microfluidic devices, 6 days post fertilization (dpf) zebrafish larvae expressing the genetically encoded Ca2⁺-indicator GCaMP6s under the control of the pan-neuronal elavl3 (formerly HuC) promoter in the crystal background were injected into the NeuroExaminer and oriented manually (Fig. 2a, e; Supplementary Movie 8). After an experiment the larva could easily be flushed out of the device to image another larva. For whole-brain imaging, we collected 21 optical sections every 10 µm at nearly 0.3 Hz via digital light sheet (DLS) microscopy for more than 10 min (see “Methods” section for details). We monitored and compared neuronal activity and image quality in both an open (n = 11 fish; Fig. 2b–d; Supplementary Movies 3 and 4) and a closed system (n = 15 fish; Fig. 2f–h; Supplementary Movies 5 and 6). While the open system contains a recess area just above the larva's head, in the closed system this area is enclosed by borosilicate glass (compare Fig. 2a, e). In both systems, the resulting images enabled single cell resolution of Ca2⁺-transients at about 0.3 Hz (Supplementary Movie 7). Faster volumetric imaging at about 1 Hz, however, is limited to about five optical planes on the Leica SP8 DLS (Supplementary Fig. 2 and Supplementary Movies 9 and 10). In the open system, single-cell
resolution could be obtained throughout all layers (Fig. 2c, d) enabling whole-brain 4D reconstruction of Ca\(^{2+}\)-signals at a temporal resolution of 1 brain volume nearly every 3 s (Supplementary Movies 3 and 4). The closed system provided this resolution for volume layers of about half of the brain (100 µm) (Fig. 2g, h) with Ca\(^{2+}\)-signals emanating from further ventral structures still being detectable but appearing weaker and spatially less well resolved (compare z-planes at 120 and 180 µm in Fig. 2c, d with Fig. 2g and h). Clearly, individual neurons throughout the brain could be allocated to distinct brain regions and neural clusters and their resting activity could be resolved (Fig. 2i–m; Supplementary Fig. 2; Supplementary Movies 7 and 10).
Future versions of the NeuroExaminer will be used to investigate the response of the whole brain and the interaction of neural circuits within it to specific stimuli. Although we have tested our microfluidic chip thus far only with a single commercial light sheet microscope (Leica SP8 DLS), it is worth noting that it should be compatible with a variety of different commercial and non-commercial light sheet setups that allow for even faster imaging.
volumetric imaging (several brain volumes per second). Moreover, with the gentle fluidic manipulation and fixation of zebrafish larvae in a natural upright position that does not require embedding in agarose, single larvae can even be reused at a later time for consecutive long-term monitoring. This may be especially useful to see how neural circuits are modified or adapt to a repeated application of neuromodulatory compounds over time. Furthermore, the NeuroExaminer can thus be equipped with automation for larva loading and ejection that will allow for moderate compound throughput in whole-brain physiological analysis at cellular resolution in the scale of seconds. High-throughput sequential imaging of several larva with an upscaled device will be of significant interest to screening studies. Together, our microfluidic chip will provide a unique and strong tool for neuromodulatory compound characterization, connectome analysis, as well as drug validation and quality control.

Methods

Microdevice fabrication. The structuring of glass (700 µm-thick BOROFLOAT® substrates from Schott AG, Mainz, Germany) was carried out in a laser workstation (MicroMicromac, Germany) equipped with femtosecond Yb:-KGW laser (Pharos from Light Conversion, Vilnius, Lithuania) operated at the fundamental wavelength of 1030 nm. The beam was scanned over the substrate surface at 2000 m/s focused with an F-theta lens of 100 mm focal length. The 3D-design was converted into multiple layers of 50 µm height for laser processing. The area to be filled was modeled with scan lines with a distance of 4 µm from the desired contour edge. Each of these sets was rotated 30° against the previous one. The laser was operated at a repetition rate of 600 kHz and emitted pulses of 215 fs with an energy of 1.465 µJ. After ablation, the solution was washed in an ultrasonic bath with ethanol for 15 min, then immersed for 0.5 min in a solution of 45 ml H2O, 100 ml HIPO4, and 30 ml hydrofluoric acid and finally subjected to cleaning in a spray processor unit (Fairchild Convac, Neuenstadt, Germany) using distilled water and a mixture of H2SO4 and H2O2. After alignment of both system halves (in mask aligner EGV 620 from EV Group, St. Florian am Inn, Austria) a pre-bonding force was applied manually. The final thermal bonding was carried out at 630 °C for 6 h with a pre-heating phase at 600 °C for 15 min while a force of 4 kN was uniformly applied to the entire 4” wafer surface. After separation with a wafer saw (DADS320, Disco Corporation, Tokyo, Japan), the individual chips (exemplary chip shown in Fig. 1m) were rinsed with water. Chips were dehydrated on a hotplate at 120 °C for 5 min before they underwent a heat treatment at 740 °C for 1 h in the muffle furnace. This heat treatment was performed twice to establish smooth glass surfaces in the microchannels (Fig. 1n illustrates the improvement in optical transparency). Finally, the chips were cleaned in an ultrasonic bath.

Zebrafish maintenance. Zebrafish (Danio rerio) were maintained at 28.5 °C on a 14-h light/10 h dark cycle and bred following standard procedures. Transgenic zebrafish larvae (Tg(ELAVL3:3x2H2B-GCaMP6s)) in the crystal (ab3b58, nacre56, roy59) background were raised in modified Danieau solution (~103 s; Supplementary Data 2). Graphs for calcium traces were created with Prism 8.2.1 (GraphPad, La Jolla, CA, USA) and Adobe Illustrator CC (22.1). Statistics and reproducibility. Two to four dfp elavl3:3x2H2B-GCaMP6s larvae in the crystal background were imaged between 10 a.m. and 8 p.m. on a single day in the first versions of the closed or open microfluidic chip. The imaging was repeated on several days. A total of 11 and 15 larvae were imaged in the open and closed microfluidic chip, respectively.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data generated or analyzed during this study are included in this published article and its supplementary information files.

Received: 6 November 2019; Accepted: 18 May 2020; Published online: 16 June 2020

References

1. Ahrens, M. B., Orger, M. B., Robson, D. N., Li, J. M. & Keller, P. J. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. Nat. Methods 10, 413–419 (2013).
2. Candelier, R. et al. Microfluidic device to study neuronal and motor responses to acute chemical stimuli in zebrafish. Sci. Rep. 5, 12196 (2015).
3. Lin, X. et al. High-throughput mapping of brain-wide activity in awake and drug-responsive vertebrates. Lab Chip 15, 680–689 (2015).
4. Yang, F., Cao, C., Wang, P., Zhang, G.-J. & Chen, Z. Fish-on-a-chip: microfluidics for zebrafish research. Lab Chip 16, 1106–1125 (2016).
5. Nady, A., Peitani, A. R., Zoidl, G. & Rezai, P. A microfluidic device for partial immobilization, chemical exposure and behavioural screening of zebrafish larvae. Lab Chip 17, 4048–4058 (2017).
6. Khaliili, A. & Rezai, P. Microfluidic devices for embryonic and larval zebrafish studies. Brief Funct. Genom. 18, 419–432 (2019).
7. Toepke, M. W. & Beebe, D. J. PDMS absorption of small molecules and consequences in microfluidic applications. Lab Chip 6, 1484–1486 (2006).
8. Piruska, A. et al. The autofluorescence of plastic materials and chips measured under laser irradiation. Lab Chip 5, 1348–1354 (2005).
9. Vladimirov, N. et al. Light-sheet functional imaging in fictively behaving zebrafish. Nat. Methods 11, 883–884 (2014).
10. Antinucci, P. & Hindges, R. A crystal-clear zebrafish for in vivo imaging. Sci. Rep. 6, 29490 (2016).
11. Aleström, P. et al. Zebrafish: housing and husbandry recommendations. Lab. Anim. 45, 672–699 (2011). https://doi.org/10.1177/0023677211409473.
12. Cheng, R.-K., Krishnan, S. & Jesuthasan, S. Activation and inhibition of pH2 serotonergic neurons operate in tandem to influence larval zebrafish preference for low over darkness. Sci. Rep. 6, 20788 (2016).

CONTACT A. P. Orger \& M. B. Ahrens \& X. Liu, Department of Molecular and Visual Neuroscience, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA. E-mail: orger@caltech.edu

COMMUNICATIONS BIOLOGY | (2020) 3:311 | https://doi.org/10.1038/s42003-020-1029-7 | www.nature.com/commsbio
Acknowledgements
We are grateful to Misha Ahrens for the Tg(elavl3:H2B-GCaMP6s) line and to Paride Antinucci for providing the crystal fish. We thank Jomo Walla and Manuel Hohgardt for their help to determine the point spread function (PSF), and Timo Fritsch for excellent animal care. The work was funded in part by the DFG—Deutsche Forschungsgemeinschaft (KO1949/7-2, Project No. 241961032). K.M. was financed through the EXIST program (Grant No. 031L0149) supported by the Deutsches Bundesministerium für Wirtschaft und Energie. P.E. was financed by QUANOMET (Programm der Niedersächsischen Wissenschaftsallianz, Grant No. ZN3378) provided by the Ministerium für Wissenschaft und Kultur des Bundeslandes Niedersachsen. We acknowledge support by the German Research Foundation and the Open Access Publication Funds of the Technische Universität Braunschweig.

Author contributions
K.M., J.W.v.T., R.W.K., and A.D. conceived the basic idea of this interdisciplinary project and continuously refined the NeuroExaminer concept together. K.M. performed the microfluidic simulation, the chip design as well as the microfabrication. J.W.v.T. suggested to combine microfluidics with light sheet microscopy and performed the whole-brain in vivo imaging and image analysis. P.E. developed the glass surface smoothening. The manuscript was jointly written by K.M., J.W.v.T., R.W.K., and A.D.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-1029-7.

Correspondence and requests for materials should be addressed to A.D.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020