Cell

Resource

Whole-Brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis

Etsuo A. Susaki,[1,2,3,4,14] Kazuki Tainaka,[1,3,4,14] Dimitri Perrin,[2,14] Fumiaki Kishino,[5] Takehiro Tawara,[6] Tomonobu M. Watanabe,[7] Chihiro Yokoyama,[8] Hirotaka Onoe,[8] Megumi Eguchi,[9] Shun Yamaguchi,[9,10] Takaya Abe,[11] Hiroshi Kiyonari,[11] Yoshihiro Shimizu,[12] Atsushi Miyawaki,[13] Hideo Yokota,[6] and Hiroki R. Ueda[1,2,3,4,*]

[1]Laboratory for Synthetic Biology, RIKEN Quantitative Biology Center

[2]Laboratory for Systems Biology, RIKEN Center for Developmental Biology

2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

[3]Department of Systems Pharmacology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[4]CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan

[5]Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[6]Image Processing Research Team, RIKEN Center for Advanced Photonics, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan

[7]Laboratory for Comprehensive Bioimaging, RIKEN Quantitative Biology Center, OLABB, Osaka University, 6-2-3, Furuedai, Suita, Osaka 565-0874, Japan

[8]Bio-Function Imaging Team, RIKEN Center for Life Science Technologies, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe City, Hyogo 650-0047, Japan

[9]Division of Morphological Neuroscience, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan

[10]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan

[11]Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

[12]Laboratory for Cell-Free Protein Synthesis, RIKEN Quantitative Biology Center, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

[13]Laboratory for Cell Function Dynamics, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

[14]Co-first author

*Correspondence: uedah-tky@umin.ac.jp

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SUMMARY

Systems-level identification and analysis of cellular circuits in the brain will require the development of whole-brain imaging with single-cell resolution. To this end, we performed comprehensive chemical screening to develop a whole-brain clearing and imaging method, termed CUBIC (*c*lear, *u*nobstructed *b*rain *i*maging *c*ocktails and *c*omputational analysis). CUBIC is a simple and efficient method involving the immersion of brain samples in chemical mixtures containing aminoalcohols, which enables rapid whole-brain imaging with single-photon excitation microscopy. CUBIC is applicable to multicolor imaging of fluorescent proteins or immunostained samples in adult brains and is scalable from a primate brain to subcellular structures. We also developed a whole-brain cell-nuclear counterstaining protocol and a computational image analysis pipeline that, together with CUBIC reagents, enable the visualization and quantification of neural activities induced by environmental stimulation. CUBIC enables time-course expression profiling of whole adult brains with single-cell resolution.

INTRODUCTION

Whole-brain imaging with single-cell resolution is one of the most important challenges in neuroscience, as it is required for system-level identification and analysis (Kitano, 2002) of cellular circuits in the brain. Various cross-sectional tomography methods have been developed to obtain high-resolution images throughout the brain (Gong et al., 2013; Li et al., 2010). Such section-based methods require expensive, specialized equipment, and the detailed structure between sections can be lost during sample processing. Alternatively, brain-transparentizing and -clearing techniques have been combined with rapid three-dimensional (3D) imaging using single-photon excitation microscopy, such as light-sheet fluorescence microscopy (LSFM) or specific plane illumination microscopy (SPIM). The 3D imaging can enable the study of systems from cells to organisms, such as cellular behavior in developing embryos and neural circuits in the adult brain (Dodt et al., 2007; Hägerling et al., 2013; Tomer et al., 2011). Such studies require a highly transparent sample for both illumination and detection.

Transparency is achieved by minimizing the light scattered by an object. Light is scattered at the boundary between materials with different refractive indices (RIs). Because lipids are a major source of light scattering in the fixed brain, the removal of lipids and/or adjustment of the RI difference between lipids and the surrounding areas are potential approaches for increasing brain

sample transparency. In an early trial of whole-brain imaging with macrozoom LSFM (Dodt et al., 2007), the brain tissue was cleared with BABB, a mixture of benzyl alcohol and benzyl benzoate. Because of BABB's hydrophobicity and because its high RI (1.56) (Becker et al., 2012) matched that of the fixed tissue, the sample was markedly cleared and suitable for observation with LSFM. Unfortunately, the clearing medium caused the rapid quenching of fluorescent signals. Although other organic chemical-based mixtures, such as tetrahydrofuran (THF) and dibenzyl ether (DBE) (Becker et al., 2012), performed somewhat better, some frequently used fluorescent proteins such as yellow fluorescent protein (YFP) were still quenched (Ertürk et al., 2012).

A hydrophilic chemical mixture called Sca*l*e was developed to clear brain samples (Hama et al., 2011). This urea-based reagent reduced quenching, and the resulting sample was suitable for both single-photon and multi-photon-based 3D tissue observation. Other issues, including relatively long sample treatment periods (weeks or months) and tissue swelling, were resolved by the development of another clearing reagent, SeeDB, which renders brain samples transparent within a few days by adjusting RI differences between the sample lipids and the surrounding tissue (Ke et al., 2013). SeeDB, a hydrophilic reagent lacking detergents or denaturation agents, preserves detailed structures as well as fluorescence signals. The remaining issue was that such simple hydrophilic reagents do not render whole-brain samples transparent enough for single-photon-based rapid whole-brain imaging. CLARITY, a recently reported technique for brain clearing (Chung et al., 2013), rendered brain samples transparent by aggressively removing lipids using electrophoresis. The resulting sample was highly transparent and suitable for LSFM imaging; however, use of a specialized electrophoresis device with a narrow range of optimal parameters makes parallelization and comparison among different samples difficult. Thus, although previous protocols have addressed some of the issues required for the efficient clearance of brain samples, an improved protocol was still required for whole-brain imaging with single-cell resolution.

The anatomical annotation of images is also critical. In conventional human brain imaging techniques such as computed tomography (CT) (Cormack, 1973), magnetic resonance imaging (MRI) (Lauterbur, 1973), and functional magnetic resonance imaging (fMRI) (Ogawa et al., 1990), anatomical annotation consists of two steps: (1) acquisition of whole-brain structural and specific signal (or functional) images and (2) computational analysis of these images. For example, fMRI, which visualizes neural activity, is almost always accompanied by MRI, which acquires a structural image of the whole brain. This image is then used for image registration and alignment to a standard whole-brain image ("reference brain") so that signal images can be compared across individuals. Because computational image analysis is so essential, informatics tools have been developed and implemented to facilitate this process in conventional human brain imaging (Avants et al., 2011). However, this kind of computational image analysis is rare for fluorescence imaging of whole mammalian brains due to the lack of whole-tissue histological counterstaining of cell nuclei, which can be used for anatomical orientation. 3D images acquired with sectional tomography or tissue-clearing protocols provide information on fluorescently labeled cells, but not on the overall shape and characteristic anatomical structures that are required for the registration and alignment steps. Even in a trial in which the image registration step was successful, manual determination of landmark structures was necessary (Gong et al., 2013). The larger number of images generated in time-course experiments makes the need for automated processing even more acute. Thus, efficient anatomical annotation will be required for whole-brain imaging with single-cell resolution.

We have developed a simple, efficient, and scalable brain-clearing method and computational analysis pipeline, CUBIC (*c*lear, *u*nobstructed *b*rain *i*maging *c*ocktails and *c*omputational analysis), that enables rapid whole-brain imaging with single-photon excitation microscopy. CUBIC can be applied to whole-brain imaging of various fluorescent proteins and 3D imaging of immunostained adult brain samples. CUBIC is scalable from primate brain imaging to subcellular structures such as axons and dendritic spines. In order to facilitate interindividual comparisons, we have also developed an anatomical annotation method using a whole-brain cell-nuclear counterstaining protocol and a computational image analysis pipeline. CUBIC enables the time-course expression profiling of adult whole brains with single-cell resolution.

RESULTS

Development of CUBIC Reagents by Comprehensive Chemical Screening

To develop a simple, efficient, and scalable tissue-clearing protocol for whole-brain imaging, we started by rescreening the chemical components of the hydrophilic brain-clearing solution Sca*l*eA2 (Hama et al., 2011). We started with this protocol because of its ease and its ability to preserve fluorescent signals. We considered 40 chemicals, including polyhydric alcohols, detergents, and hydrophilic small molecules (Table S1 available online), corresponding to the components of the Sca*l*eA2 solution: glycerol, Triton X-100, and urea. To evaluate the clearing efficiency of these chemicals, we first sought to improve the experimental throughput of clearing measurements. In a typical evaluation procedure, we first fix, isolate, and immerse a whole mouse brain in one chemical mixture and then evaluate the clearing efficiency of the chemical by measuring the transmittance of the fixed, cleared whole brain. For a more efficient evaluation protocol, we exchanged the order of the fixation and isolation steps and introduced a homogenization step so that many chemicals can be tested using only one mouse brain (Figure 1A and Extended Experimental Procedures). This "solubilization" assay using a homogenized suspension instead of a whole brain enabled the reproducible, quantitative, and comprehensive evaluation of chemicals for their ability to dissolve brain tissue (Figure 1B). We also evaluated the quenching effect of each chemical on EGFP fluorescence in this first chemical screening (Figure 1C). We noted that a series of aminoalcohols (#4, #8, #9, #10, #15, #16, #17) showed considerable brain tissue solubilizing activity,