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OpenFluo: A free open-source software for optophysiological data analyses

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ABSTRACT

Optophysiological imaging methods can be used to record the activity *in vivo* of groups of neurons from particular areas of the nervous system (e.g. the brain) or of cell cultures. Such methods are used, for example, in the spatio-temporal coding and processing of sensory information. However, the data generated by optophysiological methods must be processed carefully if relevant results are to be obtained. The raw fluorescence data must be digitally filtered and analyzed appropriately to obtain activity maps and fluorescence time course for single spots. We used a Matlab® environment to implement the necessary procedures in a user-friendly manner. We developed OpenFluo, a program for people inexperienced in optophysiological methods and for advanced users wishing to perform simple, rapid data analyses without the need for complex, time-consuming programming procedures. This program will be made available as stand-alone software and as an open-source Matlab® tool. It will therefore be possible for experienced users to integrate their own routines. We validated this software by assessing its ability to process both artificial recordings and real biological data corresponding to recordings of the honeybee brain.

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1. Introduction

Since the demonstration that action potentials are accompanied by changes in light scattering, birefringence and fluorescence (Cohen et al., 1968), interest has increased in the use of optical imaging techniques for recording nervous activity (Cohen et al., 1978; Galizia and Vetter, 2005). Various dyes sensitive to different ions or to membrane potential have been successfully used for the recording of neural activity (Galizia and Vetter, 2005). This technique has provided considerable insight in many areas, including molecular cascades, the mechanisms of neurotransmitter release, and the functional architecture of the cortex (Grinvald et al., 1986), since its initial application to studies of brain slices and cell cultures (Mason, 1993).

In the 1990s, optical imaging techniques were adapted for the recording of nervous activity in the insect brain *in vivo*. These techniques were used, in particular, to study the treatment of olfactory information by the antennal lobe of honeybees (Joerges et al., 1997; Galizia et al., 1998). This technique was found to be particularly useful for simultaneously measuring neural activity in different areas of the antennal lobe (Galizia et al., 1997; Joerges et al., 1997). It was subsequently successfully applied to studies of the olfac-

tory systems of other insects, such as moths (Hansson et al., 2003; Carlsson et al., 2005), flies (Fiala et al., 2002; Wang et al., 2003) and ants (Galizia et al., 1999). Optophysiological methods have since been extended to other areas, including studies of the spatial coding of mechanosensory stimuli in the terminal abdominal ganglion of crickets (Ogawa et al., 1996; Ogawa et al., 2005), the visual system of flies (Borst and Egelhaaf, 1992; Haag et al., 2004; Kurtz et al., 2008; Elyada et al., 2009) and the auditory system of crickets (Baden and Hedwig, 2007). The transparency of larval zebrafish has been exploited for studies of neuronal activity using calcium-sensitive fluorescent markers (Fetcho and O'Malley, 1995; Brustein et al., 2003). The coding of olfactory signals (Tabor et al., 2004) and embryonic development (Ashworth and Bolsover, 2002; Ashworth, 2004; Brennan et al., 2005) have also been investigated by optophysiological methods in zebrafish. Techniques are now also available for calcium imaging on *in vivo* preparations of the mammal's brain, such as in mice (Stosiek et al., 2003; Dombeck et al., 2007).

This method is not so widely used in neurophysiology as expected from its undoubted utility, because its application requires technical expertise, not only for sample preparation, but also for data processing, which is particularly challenging for *in vivo* recordings, for which the signal-to-noise ratio tends to be very poor. Once digitized, the data are usually recorded as large matrices, the processing of which requires an appropriate, efficient, automated software environment. Many research groups currently use custom-made software for these analyses. However, such software has the drawbacks of being adapted to a single type of preparation

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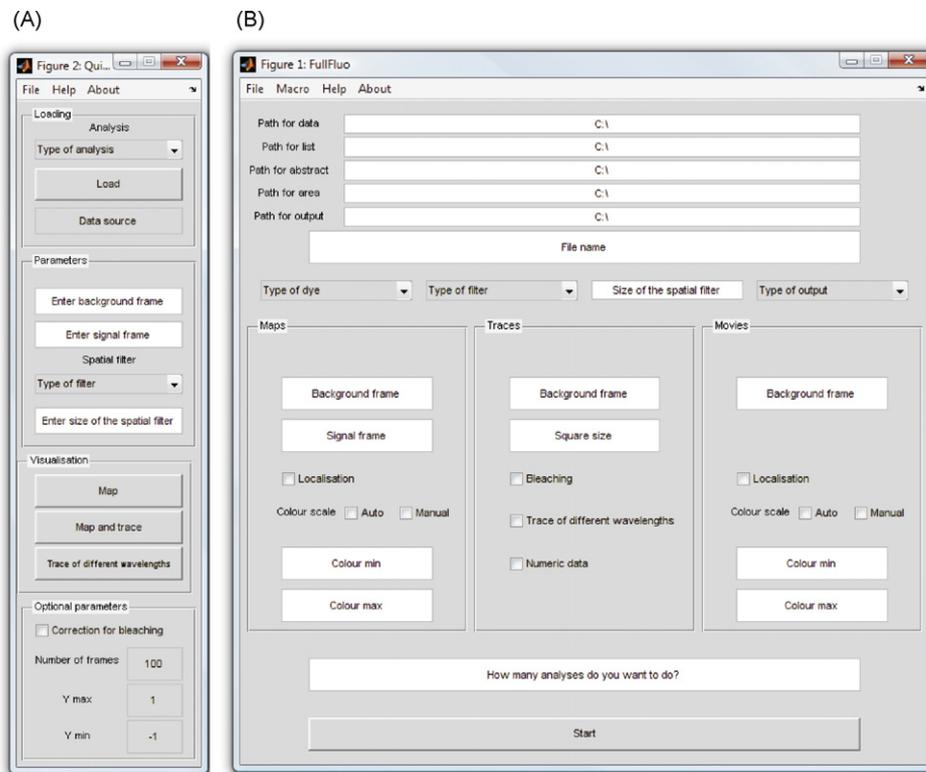


Fig. 1. Software interfaces: (A) *QuickFluo*, interface, for rapid processing of individual recordings during experiments. (B) *FullFluo*, for simultaneous processing of series of recordings, from one or several experiments.

and not being freely available in most cases. Other freely available open-source programs, such as ImageJ (Wayne Rasband, National Institutes of Health, USA), can be used to process data of this type, but require the development and integration of appropriate modules for automated analysis. Consequently, no appropriate program is currently freely available to users without the time or know-how to develop their own software.

We present here OpenFluo, an open-source software for the analysis of optophysiological data specially designed for analyses of the spatial and temporal patterns of nervous system activity. This software is suitable for use in experiments on different animal models, using different preparations and dyes (e.g. ratiometric and non-ratiometric dyes). It processes data rapidly, making it possible to visualize optical activity in single recordings during the course of an experiment. It can also be used for the overall processing of data obtained in one or several experiments. OpenFluo provides output in the form of fluorescence time curves, activity maps and movies. It also generates standard tables of results for further statistical analysis.

2. Materials and methods

2.1. Optical imaging signals

Optical imaging involves recording changes in the intensity of light of a particular wavelength emitted by a fluorescent dye. Fluorescent dyes display sensitivity to particular ions (ion-sensitive dyes) or to membrane potential (voltage-sensitive dyes). Light emission by the dye depends on both the wavelength of the exciting light and the concentration of the particular ion or the membrane potential of the cell. Optophysiological methods thus involve measurement of the changes in light emission (fluorescence) associated with cellular activity. During experiments, the recording system (CCD camera) outputs a series of monochromatic images consisting

of pixels and representing the intensity of the light emitted at each point on the recorded area, before, during and after stimulation. The amount and complexity of the data to be managed depend on the spatial resolution (number of pixels) and the gray scale used by the camera, together with the frame rate and the duration of the recording.

The images generated are subjected to a series of calculations. The basic treatment involves the removal of background fluorescence and normalization of the data. These operations yield the relative variation in fluorescence during stimulation, which can then be compared between experiments. In some cases, such as in studies of *in vivo* preparations, this basic data processing procedure is insufficient for the detection of physiological signals. In such cases, the detection of small variations in fluorescence intensity triggered by the stimulus also requires the elimination of background noise and movement artifacts. Diverse filters and correction procedures must therefore be applied to the data, to reveal the biological signal.

We used the Matlab® environment to implement these procedures for the analysis of optophysiological data obtained with two types of dye: ratiometric (dyes responding differently to excitation at different wavelengths) and non-ratiometric (dyes excited by a single wavelength only).

2.2. User modules

OpenFluo contains two modules that may be used independently, according to the type of experiment and analysis desired. The first module can be used to process one recording at a time (*QuickFluo*, Fig. 1A). It was designed for the rapid inspection of data from a single experiment. The second module (*FullFluo*, Fig. 1B) can be used to analyze a large number of recordings, from single or different experiments, simultaneously. Both analyses are based on the same mathematical procedures, but they render different amounts

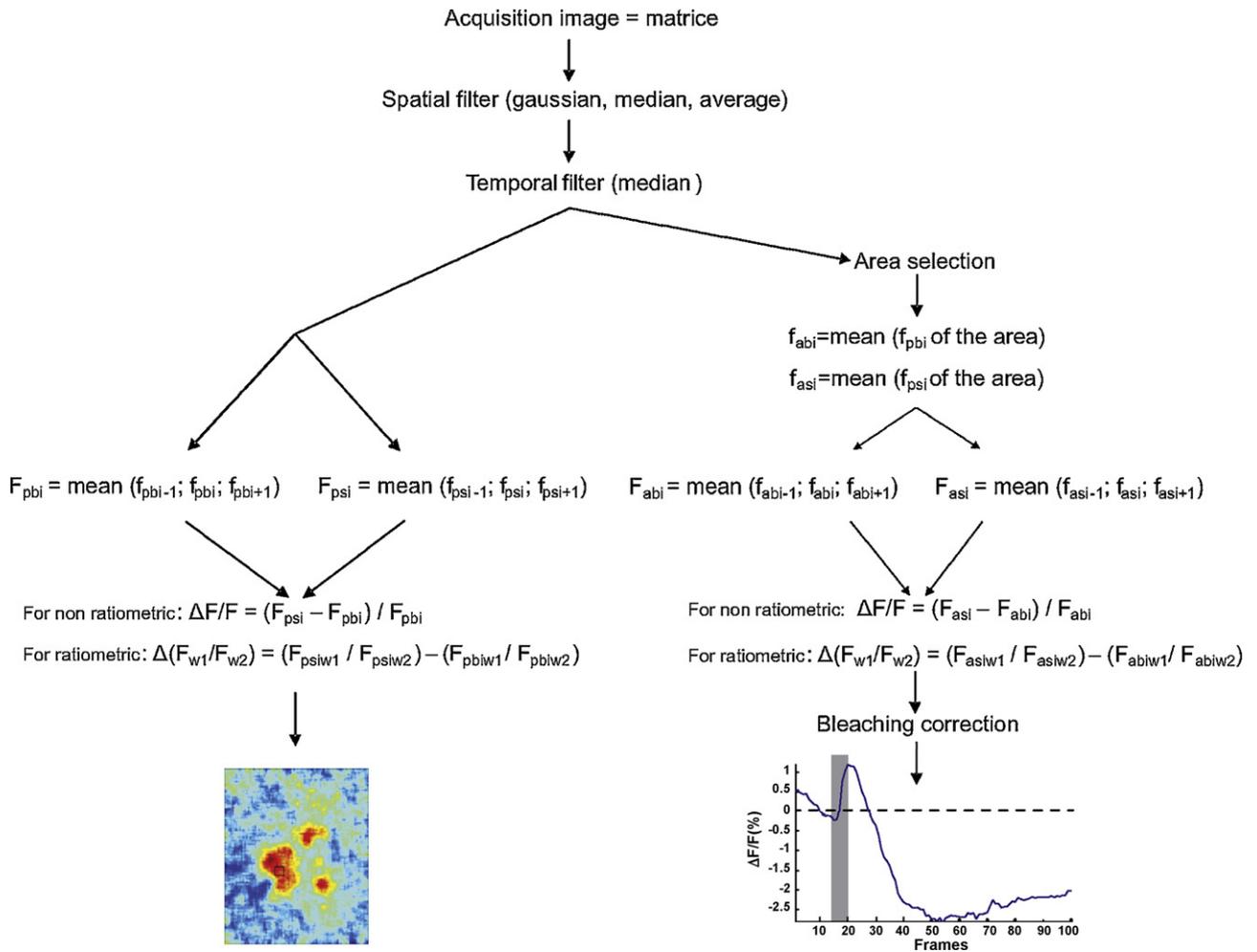


Fig. 2. Schematic diagram of the various steps in data processing. f_{abi} , fluorescence of a given area for the background image; f_{pbi} , fluorescence of a given pixel for the background image; f_{asi} , fluorescence of a given area for the signal image; f_{psi} , fluorescence of a given pixel for the signal image; F , mean of several f values, indices of F follow the same code as for f ; $w1$ corresponds to wavelength 1 and $w2$ to wavelength 2.

of information. FullFluo can also be used to establish standard procedures for automatic application to different datasets.

Each module is organized as a GUI (graphical user interface), facilitating the use of the software and the selection of parameters for the analysis.

2.3. Data processing

Data processing is summarized in Fig. 2. Background noise and artifacts were reduced by applying two filters and a correction, the exact mathematical procedures for which are described below. The two smoothing filters remove high-frequency noise in the temporal and spatial dimensions. Spatial noise is further reduced by adjusting the value of a given pixel as function of that of the neighbouring pixels, the number of which can be specified. Temporal noise is further reduced by adjusting the value of given pixel as function of the value obtained for the same pixel one frame before and one frame after the focal frame.

2.3.1. Spatial filters

The raw data generated by the CCD camera (Fig. 3A), are first spatially filtered with a low-pass filter. Three spatial filters are implemented in the program: median, average and Gaussian filters, the size of which may be modified by the user. This treatment generates spatially smoothed images (Fig. 3B).

2.3.2. Temporal filter

The second data processing step involves applying a median filter in the temporal dimension. In this case, a new value is calculated for each pixel of a given frame, by calculating the median intensity of that pixel in three consecutive frames (previous, focal and subsequent), for every frame. This filter smooths the data over time, removing high-frequency noise (Fig. 3C).

2.3.3. Analysis and visualization of fluorescence changes

QuickFluo generates a sequence of images showing variations in fluorescence with respect to a reference image corresponding to a stimulus-free background selected by the user. The program then calculates the mean fluorescence intensity (F_{pbi}) of each pixel in the reference image, in the focal frame (f_{pbi}) and in the previous (f_{pbi-1}) and subsequent frames (f_{pbi+1}) (Fig. 2). The same procedure is applied to three images corresponding to stimulation conditions (i.e. F_{psi}), with the focal frame selected by the user (it is not necessary at this stage to have identified the precise moment at which the stimulus produces a response). The software then carries out pixel-to-pixel subtraction (ΔF) of the two sets of values and normalizes the result obtained by dividing by the background value ($\Delta F/F$), thereby generating a new image (the “primer image”; Fig. 2) depicting relative changes in fluorescence at a given moment after stimulation in a false-colour code (JET-code).

The primer image is then used to visualize changes in fluorescence in the area of interest during the recording period. The user

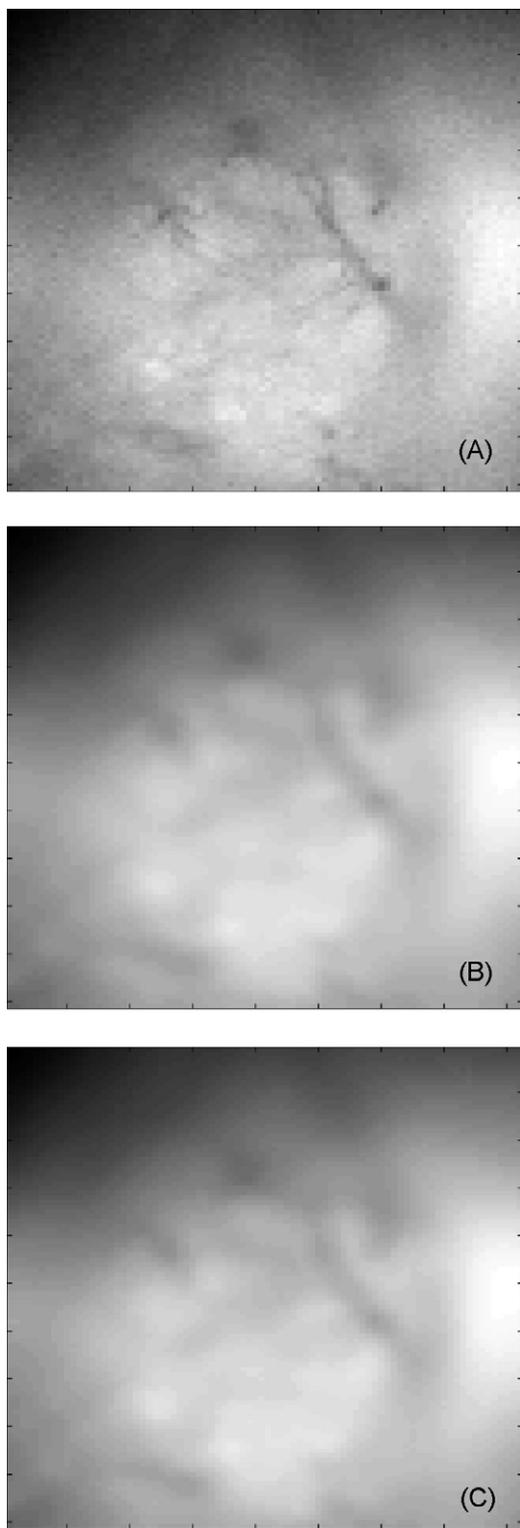


Fig. 3. Processing of the data for a single biological recording for honeybee antennal lobe. (A) Raw data. (B) Spatially smoothed data. (C) Spatially and temporally filtered image.

defines a square zone of interest in the primer image in which activity should be analyzed. The software then applies a computation routine similar to that used to obtain the primer image. However, in this case, fluorescence changes are determined for the entire designated square area, rather than for individual pixels, as follows. The mean fluorescence intensity is calculated for the defined area in the same three previously defined images. This procedure calculates

the background, averaging the values of the pixels of the square over space and time (in the three images). The same procedure is applied automatically to every image taken during the recording period (averaging pixel values for the area and then over the three images). Once this procedure has been completed for all the images, a time-curve is constructed in which ΔF is plotted as the percentage change in fluorescence due to stimulation (i.e. $\Delta F/F \times 100$).

Examples of the curves obtained are shown in Figs. 4B and 5B. Provided that fluorescence changes over time are plotted for the entire experiment, the user may now visualize the time point (i.e. the frame) at which the activity induced by the stimulus is maximal. The initial procedure used to generate the primer image can then be used to obtain an activity map for that time point and to readjust the recording parameters if necessary.

Finally, once the parameters for the analysis and the time course of activity have been determined, FullFluo can be used for the automatic processing of a dataset and the production of a false-colour video sequence of the spatial and temporal changes in fluorescence during a single experiment. FullFluo can also export fluorescence variation data in the form of an ".xls" table, for quantitative and statistical analyses.

FullFluo also generates movies showing the changes in fluorescence in false colours for the whole recorded area of interest over the course of an experiment. The procedure for obtaining a map at a particular time point is automatically repeated for each frame, the results being presented as an AVI sequence of images. The various outputs of FullFluo are stored automatically.

2.3.4. Using ratiometric dyes

For ratiometric fluorescent dyes, OpenFluo calculates the ratio of the fluorescence emitted for two different excitation wavelengths, w_1 and w_2 . The procedures described above are applied, for both w_1 and w_2 . The software calculates F_{bi} and F_{si} for each wavelength and then $\Delta(F_{w1}/F_{w2}) = F_{siw1}/F_{siw2} - F_{biw1}/F_{biw2}$ (F_{asiw1} for area or F_{psiw1} for pixel in Fig. 2).

2.3.5. Correcting for bleaching and photoisomerization

Both these phenomena may decrease fluorescence, by destroying the dye molecules or by inducing transient or permanent structural changes induced by light. Thus, during the course of an experiment, total fluorescence emission (both background and signal) may decrease exponentially as a function of time and excitation conditions, such as pulse duration, light intensity and wavelength (Galizia and Vetter, 2005). No correction is required for spatio-temporal maps, but these artifacts must be corrected for time-course plots. Once the area of interest has been selected by the user and fluorescence between frames has been calculated, the software carries out a correction in which the first 10 and the last 10 $\Delta F/F$ values of the recording are used to fit the following function to the data: $a * e^{b * x} + c$ (Galizia and Vetter, 2005). The values of the three parameters a , b and c are estimated by least mean square adjustment. This function corresponds to the changes in fluorescence due to bleaching and photoisomerization and is subtracted from the original values. A time-course plot is generated showing changes in the fluorescence intensity of the area of interest resulting exclusively from nervous system activity.

2.3.6. Output

The software output consists of the following elements:

- A map of changes in fluorescence intensity, with increases and decreases indicated in a false-colours code (Figs. 4 and 5).
- Curves of changes in fluorescence intensity over time for the area or areas selected by the user (Figs. 4 and 5).

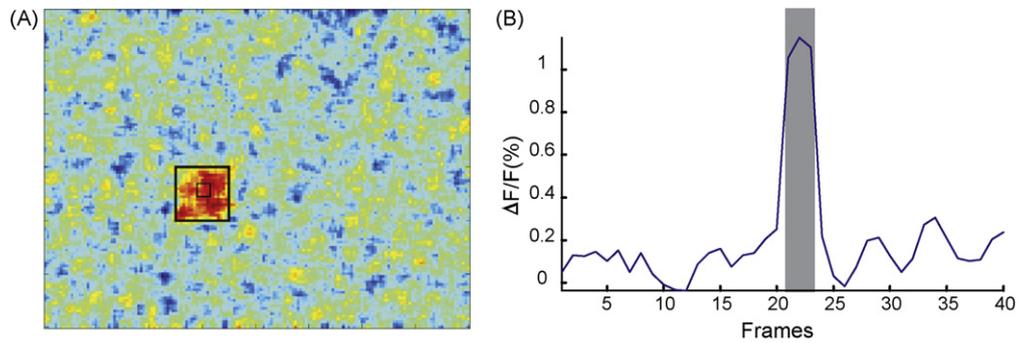


Fig. 4. Analysis of an artificial recording. The recording has a noisy background, with random values around a mean of 1700 and a standard deviation of 20 over 40 frames. The activation area presents random values with a mean of 1720 and standard deviation of 20 (large black square) from the 20th to the 22nd frame (gray area). Sample frequency: 5 fps. The small black square indicates the area used for time-course analysis.

- (c) AVI movies showing changes in fluorescence as a function of time, also in false colours.
- (d) Tables of $\Delta F/F$ over time for the various areas selected.
- (e) For ratiometric dyes, changes over time in fluorescence for one or two excitation wavelengths, and changes in the fluorescence ratio over time.

2.4. Input file

OpenFluo requires two types of data provided by the acquisition software as input. We use TILLvisION v4.00 (TILL Photonics GmbH Imaging System Software), but other programs can be used, provided that they supply recordings (sequence of frames) in AVI format and a text LOG file containing information about the experiment. A typical LOG file is presented as an example in the Help Menu. By default, OpenFluo is parameterized for understanding TILLvisION LOG files, but information can be organized in other ways, by editing and modifying the ABSTRACT module. OpenFluo obtains the following information from the LOG file: the frame dimensions in pixels, the total number of frames recorded, the duration and wavelength of excitation, horizontal and vertical binnings, the name of the storage file, and the name of the associated files for ratiometric dyes. Other experimental parameters of importance to the experimenter are usually saved, but not used directly by the software (date, time, etc.). A first routine called “ABSTRACT” recovers the information required to construct a table of parameters for subsequent use by OpenFluo. This routine remains accessible for consultation by the user, through Matlab[®]. It differs for the two types of dyes – non-ratiometric and ratiometric – and provides specific information, such as the name and location of the associated files, for later use.

2.5. Testing the software on simulated data

For software validation, we assessed the ability of the program to process artificially generated data correctly. We generated artificial recordings, in which an intensity value was assigned to each pixel. Different simulated recordings were built in the form of 40 matrices of different pixel values, with each matrix corresponding to one frame. In one case, we introduced areas of higher or lower intensity of different sizes and durations (frames) and with different distances between activity areas on a uniform background (i.e. in the absence of noise). Another group of simulated recordings consisted of matrices simulating random noise constructed from random values around a mean of 1700 arbitrary units (range from 1 to 4096) and a standard deviation of 20 (i.e. 0.5% of the whole range). The activation area was a square of 20×20 pixels, with random intensity values with a standard deviation of 20 around a mean of 1720, during frames 21–23.

2.6. Application of the software to biological data

We tested the software on real biological data. We obtained recordings of calcium activity in the olfactory system of the honeybee, a well established model system for calcium imaging, from the *Centre de Recherches sur la Cognition Animale* (Toulouse, France). These recordings were obtained with standard recording methods (Galizia et al., 1998). *Apis mellifera* bees were anesthetized by cooling and fixed in a Plexiglas chamber. The head was immobilized and a window was opened in the frontal cuticle to expose the antennal lobes. The tracheae and the neurolemma were gently removed. We dissolved Calcium-Green-2AM (50 μg) dye in 50 μl Pluronic F-127 (20% in dimethylsulfoxide) in 800 μl of Ringer solution. Bees were

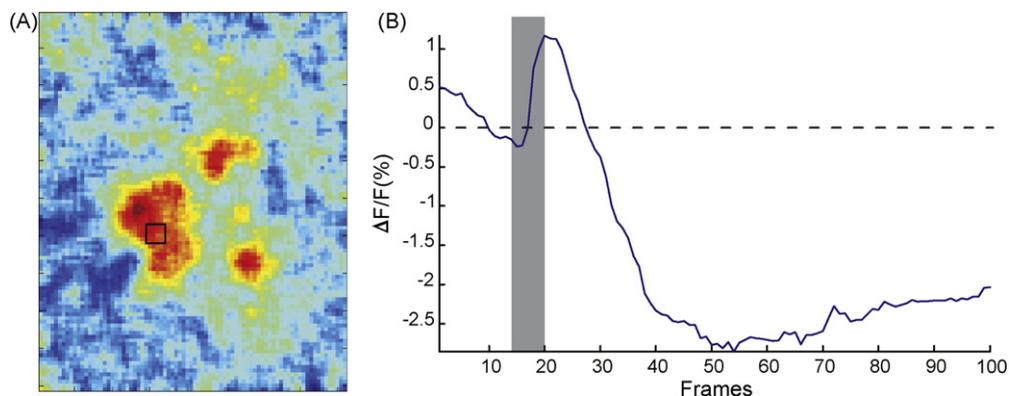


Fig. 5. Analysis of a biological recording from the antennal lobe of the honeybee *Apis mellifera* incubated in Calcium-Green-2AM for 1 h. The antenna of the honeybee was stimulated with 4 μl of octanol for 5 frames (1 s; gray area). Three areas of activity are shown on the map. Sample frequency: 5 fps. The small black square indicates the area used for time-course analysis.

subjected to bath incubations, in the dark, in a humid atmosphere, on ice beds. The brain was thoroughly rinsed with Ringer solution.

Optical recordings were carried out with TILLvisION v4.00 (TILL Photonics GmbH Imaging System Software). The bee was placed under an epifluorescence microscope (Olympus BX-70WI) with a 10 \times , NA 0.3, water immersion objective (UMPlanFL Olympus). Recordings were taken with a 640 \times 480 pixel 12-bit monochrome CCD-camera (TILL Imago) cooled to -12°C . Each recording consisted of 100 frames, at a rate of 5 double frames per second. The antennae of the bees were stimulated by an air-puff loaded with octanol.

3. Results

3.1. Performance of the software with simulated data

The two types of output provided, together with the results obtained with OpenFluo for the processing of artificial recordings are shown in Fig. 4. A map is presented, together with a time course for a noisy recording with a variation of fluorescence of 1.1%, a low level of variation for biological recordings (Sachse and Galizia, 2003; Sandoz et al., 2003). Even weaker signals were clearly identified in both activity maps and time-course plots. For example, a signal covering around 20 pixels was still clearly identifiable for a maximum of $\Delta F/F$ of 0.7% within the activated area in conditions of 0.2% background variation (data not shown). In further tests, not described here, OpenFluo was able to distinguish two active points separated by only 1 pixel, using average or Gaussian filters two or three pixels in size, in a noise-free background. Moreover, signals lasting only two frames were also clearly detected.

3.2. The performance of OpenFluo with biological data

We applied the software to calcium activity measurements on honeybee antennal lobes, which we analyzed with OpenFluo (Fig. 5). Three different areas of activity were observed. The time course shown in Fig. 5B relates events for the square area marked on Fig. 5A. A short activation phase occurred between frames 16 and 20, when $\Delta F/F$ reached about 1.5%, followed by a long inhibition phase in which $\Delta F/F$ was negative. At the end of the recording, fluorescence intensity returned to basal levels. The results obtained were consistent with the typical activity maps obtained for studies of bee antennal lobes using a bath-applied calcium-sensitive dye (Sandoz et al., 2003).

4. Discussion

We have developed free open-source software for optophysiology, OpenFluo, which presents several advantages over other currently available programs. It is particularly appropriate for people seeking an easy-to-use, simple platform suitable for use in various types of experiment (*in vitro* and *in vivo*).

OpenFluo has two user-friendly interfaces, QuickFluo and FullFluo, for analyses of different degrees of complexity. QuickFluo can be used for rapid, interactive analyses of changes in fluorescence in any region of the image selected by the user. Thus, the analytical parameters (area of interest) can be established with precision and the recording conditions adjusted during the experiment (e.g. duration of excitation). Once parameterized, FullFluo automatically performs a complete analysis and saves data from single and multiple experiments, without the need for user intervention in the processing of individual datasets.

The software was tested with both artificially generated and real data. OpenFluo performed efficiently, resolving small changes in fluorescence (0.7%), over a small area (1 pixel) and lasting only a

short time (2 frames). The analysis of biological data yielded results similar to those obtained with other methods of analysis.

In some of the more extreme conditions tested, but for which the results are not presented, very large spatial filters were found to generate red areas (i.e. high $\Delta F/F$) on maps that did not correspond to activated areas. Close observation of the time course is required to exclude these false activation areas. Increasing the size of the square used to calculate fluorescence changes decreased both noise and signal. Selection of the most appropriate values of these parameters therefore appears to be very important.

The software is available as a stand-alone application, and no Matlab[®] license is therefore required, and in versions for Windows, Linux and Macintosh systems.

The development of OpenFluo in Matlab[®] makes it possible to adapt this software, modifying it for specific tasks. As Matlab[®] is a very popular system, many modules are available, and there is an active community of users and discussion forums. Users needing help to solve specific problems should therefore be able to find the tools they need rapidly, or to discuss the problem with Matlab[®] users.

We could have used other systems, such as Java. Indeed, another open-source program for image analysis, ImageJ, is written in Java. However, Matlab[®] has several advantages over Java, including its ease-of-use and the simplicity of programming. Furthermore, the multitude of Matlab[®] toolboxes available makes it possible for programmers to choose from a large number of prewritten functions when trying to accomplish time-consuming or difficult tasks. These tools facilitate effective software development. Our version of OpenFluo is already quite rapid, but its performance should gradually improve, with the addition by users of new Matlab[®] adapted for a quicker treatment of the different functions.

Optophysiological methods are frequently used in neurosciences and by many laboratories. In many cases, data are acquired and analyzed by custom-made software that is not freely available. The open-source and the stand-alone (Windows, Linux and Macintosh operating systems) versions of our software can be downloaded freely from <http://www.mathworks.com/matlabcentral/fileexchange/24729> and the open-source version also from the Matlab[®] website (<http://www...to be completed in proofs>). This, together with its open-source nature, makes it possible to envisage continual refinement for the benefit of all, with potential improvements and the incorporation of new analysis methods being made available to the entire scientific community. One of the more interesting features of OpenFluo is the possibility of adapting the software to new types of analysis and recording. Thus, OpenFluo is a program capable of performing many basic and fundamental data processing procedures and is both easy-to-use and amenable to modification.

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