Title: TI Workbench, an integrated software package for electrophysiology and imaging

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Abstract

TI Workbench is a software package that serves as a control and analysis center for cellular imaging and electrophysiological experiments. It is unique among general-purpose software packages in that it integrates the control of cellular imaging and electrophysiological devices, as well as sophisticated data analyses, which provides superior usability in imaging experiments combined with electrophysiology. During the development over the last 20 years, the range of supported image acquisition devices has expanded from cooled CCD cameras to multi-photon microscope systems. In this review, I outline the concept of TI Workbench together with its unique functions and features derived from ideas emerging during daily experiments in my own lab and in those of my collaborators over the last 20 years. TI Workbench includes standard functions required for time-lapse multicolor fluorescence imaging and electrophysiological experiments, in addition to specialized functions such as random-scan or conventional raster-scan two-photon microscopy packages and fluorescence life-time imaging (FLIM) utilities. Data analysis modules, e.g., digital data filters for temporal waveforms of time-lapse image data and electrophysiology and for 2-D image data, and fluorescence correlation spectroscopy (FCS) analysis functions, are well integrated with data acquisition functions. A notebook function holds formatted text, graphs, image, and movie data altogether, which are linked to the actual data files. TI Workbench uses Igor Pro software as a back-end output for publishing. In addition, TI Workbench imports several different formats of image and electrophysiology data, serving as a general-purpose data analysis software package.

Introduction

Digital microscopy was introduced in biology when scientific-grade digital charge coupled device (CCD) cameras were introduced in the mid 1980's. CCD cameras were used in measurement of fast calcium dynamics in neurons [1,2]. Calcium imaging from patch-clamped single neurons required control and monitoring of both imaging acquisition and electrophysiological control and recording, and there were no software packages that supported integrated control and recording of the both modalities until recently. The solution was either running two independent computers for imaging and electrophysiology that were connected by a synchronizing trigger line, or custom building an integrated software program. Performing patch-clamp in neurons requires intense concentration by the experimenter, who keeps close attention to the integrity of patch-clamp conditions, cell health and viability, and the completion of scheduled procedures within a limited time of recording. Imaging patch-clamped neurons adds further duties: the experimenter has to adjust the focus and field of view, select regions of interest within the image frame, and define the exposure parameters. For a single person performing all of these procedures, having two separate computer systems is impractical and prone to human errors. Due to these challenges, I began building a software package that will handle everything needed for simultaneous imaging and electrophysiology in the mid 1990's, which I named TI Workbench.

Monitoring intracellular calcium signals in neurons requires temporal resolutions above 50 frames/second. A typical fast frame-transfer type CCD camera with 512x256 pixel format yields more than 5 mega-pixels/sec (10 Mbytes/sec). In the 1990's, it was not easy to handle more than 1 Mbyte of RAM programmatically with the DOS operating system (OS) or the early Windows OS. The Macintosh OS was a better choice because it handled Mbytes of

image data without troubles. I built TI Workbench to run in the Macintosh OS first with the Motorola 68x CPUs in the C++ language, then moved it to the Power PC series CPUs, and finally to the Intel 64 bit CPUs using the Objective C++ language in the current Mac OS-X operating system.

During the past 3 decades, time-lapse live cell imaging has become widespread used and critical for all biological fields, and imaging software packages have expanded their functionality. An open-source development, NIH Image running in the Mac OS, evolved to ImageJ as a Java-based environment independent of the OS [3] and, further, to Fiji [4] is widely used with open-source plugins shared by a world-wide community of users. A commercial software package, MetaMorph (Molecular Devices, Sunnyvale, CA, USA), is also quite popular, although rather expensive for individual labs. Many imaging experiments on confocal and two-photon microscopes are performed using built-in software provided by the microscope companies. However, none of these imaging software packages fulfill the need for acquisition of both image and electrophysiological data at the same time in the same computer. Thus, TI Workbench is a unique and powerful tool for the neuroscience community.

In this review, I outline the concept of TI Workbench together with its unique functions and features derived from ideas emerging during daily experiments in my own lab and in those of my collaborators over the last 20 years.

Imaging

Digital cameras

TI Workbench can handle a wide range of image acquisition devices. Scientific-grade CCD and complementary metal-oxide semiconductor (CMOS) cameras used as a main camera in TI Workbench achieve high-speed and low-noise image acquisition. More affordable CCD cameras can be used as a second camera with different configurations from the main camera, e.g., video-rate monitoring of wider field of view with a microscope adapter of different magnification from that of the main camera. Although the hardware specifications of different CCD and CMOS cameras vary, the basic principles of software instructions on the image acquisition device are consistent across designs, manufacturers, and OS platforms. Software elements dealing with image data structure definitions, pixel formats (pixel bit depth and color setting), and temporal parameters (exposure time of each frame, frame interval and number of frames to take) are all shared by different image acquisition devices. Thus, new image acquisition devices are integrated into TI Workbench with a minimum coding effort only for device-specific features. The range of image acquisition device is not limited to CCD and CMOS cameras. Until the mid 1990's when analog video cameras were popular, frame grabber computer interfaces were a major source of image acquisition. Analog video cameras coupled to a computer through a digital frame grabber are handled in TI Workbench in the same way as digital CCD cameras. More recently, photo-multiplier tubes (PMT) in laser-scanning two-photon microscopes have been added to the image acquisition device list supported by TI Workbench. Although the operation principle of laser-scanning microscopes is completely different from that of CCD or CMOS cameras, most of the software and user interface

elements in TI Workbench did not have to be rewritten. It is beneficial in terms of user experience that the same user interface is used in a CCD-equipped microscope system and in a two-photon microscopy in the same laboratory, and experimenters who have already learned to use TI Workbench with CCD cameras do not have to learn about another image acquisition software to use a TI Workbench-driven two-photon microscope.

Excitation light sources, shutters, and excitation light switchers

Not only the image acquisition devices but also other optical devices are important factors in image acquisition experiments. Synchronized and precise control of shutters and switching devices in excitation light sources and other optical elements in the light path are necessary for multicolor fluorescence imaging during time-lapse imaging experiments. In time-lapse fluorescence microscopy, the extent of illumination with high-energy excitation light must be tightly controlled to reduce cellular photo-toxicity and unnecessary photo-bleaching of the reporter fluorescent dye or protein. A computer-controlled shutter in front of a high-power light source that is synchronized with the image acquisition device is the minimum required configuration, which allows time-lapse imaging with a single wavelength excitation, e.g., time course of calcium signals reported by the organic calcium indicator dye Fluo-4 or the calcium sensitive protein GCaMP6. For quantitative calcium measurements, the dye Fura-2 requires two excitation wavelengths, which are provided by a computer-controlled filter exchanger in combination with a mechanical shutter between the light source and the microscope. In addition, excitation light sources containing different light emitting diodes (LEDs) can be controlled by sequences of transistor-transistor logic (TTL) pulses to each LED, which don't require shutters because they can be turned on and off within fractions of a

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millisecond. On the other hand, imaging of Förster (or fluorescence) resonance energy transfer (FRET) [5] requires the acquisition of two different emission wavelength lights, for which typically a computer-controlled filter- or light path- switcher is inserted between the microscope and the camera. For simultaneous multi-color fluorescence imaging using multiple fluorescence tracers, a computer-controlled beam splitter exchanger can be used, which is supported by motorized microscopes. Thus, a universal fluorescence-imaging configuration would equip two filter switchers in the light source and the camera sides, a shutter at the light source, and/or a motorized microscope that is capable of computer control of the beam-splitter and other optical elements in the microscope. In many situations, the imaging software sends a sequence of commands to the peripheral devices one by one. In a typical calcium imaging experiment using Fura-2 as a dual-excitation wavelength indicator, the command sequence is shown in fig. 1B. The execution of each step can be achieved as a user-defined macro in a general-purpose imaging software, such as ImageJ or MetaMorph, in which different software modules that control the camera, the filter exchanger, and the shutter work independently of each other. But in the case of imaging fast calcium dynamics in neurons using Fura-2 and alternating excitation filters without temporal gap between exposures at 10 frames/second, the rotating wheel-type filter exchangers are too slow, and a fast filter/optical path switching device with millisecond response must be precisely synchronized with camera exposure timing. Such a temporally tight and synchronized control of several devices is too complex for a software architecture handling each optical device as an independent module, and can only be achieved by an integrated control of multiple hardware modules at the source code level as TI Workbench does.

<Fig. 1 insertion here>

Microscope control

Integration of computer-controllable microscopes with image acquisition software increases the capabilities of automated image acquisition sequences. TI Workbench controls focus, objective lens selection, filter positions, and fluorescence mirror cube selection of electronic-controlled microscope models such as IX-81 and BX-61 from Olympus (Tokyo, Japan) through serial connection. For example, control of microscope focus devices allows synchronized image acquisition at different focal planes for deconvolution of wide-field images or in spinning disk confocal microscopes that use CCD or CMOS cameras. In addition, focus control is a necessary function in confocal and two-photon microscopy for obtaining 3-D image stacks. TI Workbench controls focus control devices for two-photon microscopy. Focus controllers using piezoelectric actuators in the microscope objective nosepiece (e.g., Physik Instrumente (PI), Karlsruhe, Germany) provide fast focus changes that is suitable for fast z-stack scanning synchronized with the timing of camera exposures. Focus controllers based on stepping motors controlling the focusing knob of the microscope are slower but more widely used while still useful in most applications, such as those in motorized microscopes (IX-81 and IX-61, Olympus).

Light path control

The type of optical devices used in automated sequential microscopic imaging depends on the requirements of individual experiments. The combination of a xenon or mercury arc lamp light source with a computer-controlled shutter and filter exchanger has been the

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standard excitation light source for fluorescence microscope over the last three decades. Light switching devices that are capable of changing excitation light wavelength within a few milliseconds are used in multiple fluorescence recording of > 1 Hz frame rate, e.g., dual wavelength imaging with the ratiometric calcium indicator Fura-2, or fast imaging of multiple fluorescent probes such as combination of calcium and sodium-sensitive probes [6]. For example, the Lambda DG-4/5 (Sutter, Novato, CA, USA) and the Polychrome series (TILL Photonics, Graefelfing, Germany) are examples of fast light switchers supported by TI Workbench. LED array light sources are becoming more popular due to their extended lifetime, intensity stability (rivaling lasers), modularity for specific excitation wavelengths, ease of intensity control, and extremely fast on-off cycles, which save the need for mechanical shutters. In a study measuring calcium dynamics in swimming sperms, pulsed outputs from a customized LED flash device with 0.5 msec duration were synchronized to the frame interval of a CCD camera acquiring images at a rate of 50 frames per second [7].

Slave / master control of devices designed for Windows OS

Devices used in imaging and electrophysiological experiments were adapted for Macintosh computers initially during the early 1990's when TI Workbench was launched. The situation totally changed during early 2000's when most of manufacturers stop developing and supporting device driver software for Mac OS, but only for Windows OS. Two measures have been taken to overcome this limitation. One is to customize device driver software for Mac OS in-house. Another way is to use a Windows PC as a slave with a simple interfacing software running in the Windows PC that communicates between a peripheral device connected to the Windows PC and a Mac PC in which TI Workbench is running. The interface program

receives commands from TI Workbench and sends out control commands to the peripheral device, and, in turn, transfers data from the device to TI Workbench. The connection between the master Mac PC and the slave Windows PC is through standard gigabit Ethernet, which is fast enough for most applications. Virtual Windows PCs running on a Mac PC can be used for this purpose. Window OS running in VirtualBox (Oracle Corp., Redwood Shores, CA, USA) on Mac OS can take over USB ports of the host Mac PC. For example, the slave program running in virtual Windows OS can communicate with an ORCA-Flash CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan) connected to the host Mac PC by a USB cable, and TI Workbench running on the host Mac PC works as if the CMOS camera is directly connected to the host Mac OS.

User interface concept (on-line)

Simultaneous time-lapse image acquisition and electrophysiological control and recording are the main target application of TI Workbench. Since the experimenter must be concentrated on the state of target cells, electrophysiology status, and progress of sometimes complex experimental protocols within a limited time, the concept of the user interface of TI Workbench for data acquisition is to minimize the steps to access and change parameters and to offer high visibility of control and acquisition parameters (Fig. 2). Image data are displayed in two different windows: the "full" window is used for adjusting the field-of-view (FOV) and focus, as well as for monitoring the sample by continuously updating images or by taking a single snapshot, while the "seq" window displays the frames of a time-lapse sequence of images (sequential). Specific image formats, e.g., the full-area or a sub-region of the image sensor, binning factor and expose time, usually differ between these two image windows. TI

Workbench keeps displaying the essential parameters of the two image modes in the main panel (the "camera" window) for imaging (Fig. 2) so that the user does not have to click and open menus and dialog windows to confirm or change these parameters. Real-time monitoring of changes in fluorescence intensity is a very important feature of any useful time-lapse software. Temporal changes in fluorescence intensity is calculated by averaging within small areas called region-of-interest, ROI (red box in Fig. 2e), and plotted over time. Quick selection of ROIs and their placement within the FOV is immediately followed by the recalculation of the time course plots, which allows the user to keep their full attention on the experiment. Such addition and/or movement of ROIs are done without having to access a ROI selection dialog window, but rather by dragging the mouse pointer in image windows.

<Fig. 2 insertion here>

Custom laser-scanning microscope, and raster-scanning two-photon microscope

Since the 2000's, two-photon excitation microscopy has been widely used in *ex vivo* brain slices and *in vivo* imaging experiments. Imaging of intracellular calcium levels and the fine structure of neurons in living neurons *in vivo* were first made possible with two-photon excitation microscopy [8]. The optical design of a standard two-photon microscope derives from confocal microscopy, and all commercial two-photon microscope systems are built on confocal microscope optics. The laser scanning mechanism is the same in the two systems: a pair of mirrors mounted on galvanometers steer the laser beam in the x- and y- axes, and fluorescence intensity from each illuminated point in a 2-D plane is read out in a pixel-by-pixel manner by a light detector device, typically a photomultiplier tube (PMT). The main difference between the optics in the two systems is that the confocal pinhole in the back focal

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plane in the confocal microscope is removed in the two-photon microscope, which yields "confocality" in the z-plane by the localized excitation of the fluorophores within the plane of highest photon concentration, i.e., the "2-photon" effect. Fine and precise adjustment of the position and size of the pinhole is necessary in confocal microscopy, and all modern commercial confocal microscopes come equipped with automated adjustment mechanisms. Without necessity of the confocal pinhole, building and adjustment of optics in two-photon microscopy is much simpler. In collaboration with Olympus Co. for the optical design, two-photon microscopes have been built using TI Workbench for control of image acquisition. This two-photon microscope on TI Workbench has all the standard functions of two-photon microscopy, such as selection of the scanning area with optical zoom, adjustment of laser intensity, gain control of PMT detectors, as well as line-scanning mode. Since the primary imaging structure of TI Workbench is conserved, i.e., independent "full" and "seq" imaging windows (Fig. 2), the main imaging panel is shared with TI Workbench versions for conventional CCD and CMOS cameras, which reduces the learning curve of experimenters that use both versions of TI Workbench for cameras and two-photon microscopes.

The maximum frame rate of a typical pixel format of 512 x 512 in the TI Workbench-driven two-photon microscope is about 0.7 Hz, which is limited by the speed of the galvanometer-mounted mirrors. In this condition, one of the mirrors has to go and back within 2.5 msec, and the resulting dwell time for a single pixel is less than 4 msec. Thus, the readout time of analog voltage signals from PMT for each pixel must be less than 4 msec. Thanks to technological advances, general purpose analog/digital interface devices (data acquisition (DAQ) cards) provide 1 MHz temporal resolution for input and output signals. National Instruments (Austin, TX, USA) provides numerous DAQ card models with such performance.

Although the performance of the hardware is outstanding, the device driver software for the

Mac OS provided by the company is only a subset of the fully functional device driver

provided for Windows OS, and unfortunately does not meet the needs for laser-scanning

microscopy. To overcome this limitation, a custom-made device driver software for the PCIe-

6259 and PCIe-6363 models of DAQ cards from National Instruments was built for Mac OS.

These cards support 500 and 1,000 kHz input/output frequency, respectively. These interface

cards work as a central control device for the laser-scanning mechanism: output analog

command to the two galvanometer-mounted mirrors, input analog signal reporting mirror

position, and input analog signal from the PMT detectors. All these output commands and

input readouts are synchronized in TI Workbench to yield precise pixel localization.

Random-scan two-photon microscope

Since galvanometer-mounted mirrors do not stop their motion instantly due to inertia,

laser-scanning must track the individual lines repeatedly. Alternative line scan mode tracks a

single line, and planner scan mode tracks parallel lines of equal interval. Acousto-optical

deflector (AOD) devices enable instantaneous deflection of the laser beam, thus enabling

discontinuous laser beam tracking. Placing two AODs orthogonally in the laser beam path,

scan positions can be chosen randomly in the scanning area [9,10]. This type of random access

two-photon microscope using AODs for laser beam deflection was recently implemented on

the TI Workbench software base. By reducing the number of recording points, random-access

two-photon microscopy enables 1 kHz sample rate with 50 recording points. Such sampling

frequency is fast enough to follow single action potentials reported by a fluorescent voltage-

sensitive indicator [11]. Fast CCD cameras capable of >1 kHz frame rates are used for voltage

imaging, but fast bleaching of the indicator dye is a problem due to the continuous illumination of high-power light. When neurons were stained with the DiO/dipicrylamine voltage sensor [12], there was no apparent photobleach using the random scanning method after more than 60 minutes of imaging [11], because it only illuminates a single point within each neuron, and the bleached dye in the scan points is rapidly recovered by dye molecules diffusing from the surrounding cell membrane.

In this random-scan two-photon microscope, we adopted a time-correlated single photon counting (TCSPC) module (SPC-150, Becker & Hickl, Berlin, Germany) [13] for signal processing after the PMT detectors. The optical signals are interpreted as photon counts by the TCSPC module, which is beneficial in discarding the majority of photon noise. In addition, the TCSPC module enables fluorescence lifetime imaging (FLIM) as described below. Since device driver software for the TCSPC modules is provided only for Windows OS, the master/slave control scheme described above is used to control the TCSPC module connected to a Window PC.

Fluorescence Lifetime Imaging

FLIM detects nanosecond-order lifetime of fluorescence, the time elapsed between excitation of fluorophore and photon emission, which is very sensitive to the environment of the fluorophore. To detect FRET efficiency, the combination of FRET and FLIM is more advantageous than conventional measurements of fluorescence intensity because lifetime changes are independent of the cross-talk of fluorescence spectra of donor and acceptor fluorophores, and of photobleaching, which reduces quantitative accuracy in measurements of

spectral intensity change [13,14]. The random-scanning configuration is better for FLIM because FLIM requires much longer photon accumulation time for each recording point than conventional fluorescence intensity imaging, and random-scanning is capable of recording time increase within smaller number of selected points without increasing the dwell time by scanning unnecessary pixels. TI Workbench implemented FLIM on the random-scanning two-photon microscope using the TCSPC module described above. In the FLIM mode, fluorescence lifetime information for every photon event is acquired and stored for both 2-D image recording and temporal multi-point recording. Fluorescence lifetime information is displayed as a temporal decay plot of lifetime with curve fitting to single- or double-exponential functions in real-time, i.e., lifetime display is updated with the 2-D image updates during on-line acquisition.

Data analysis, online and off-line
Image data

Image data must be processed before it is displayed: TI Workbench performs smoothing (noise reducing) and sharpening of images, adjustment of pixel intensity ranges to display color/gray scale (contrast adjustment), selection of different color look-up tables, to name a few. Fluorescence images are almost always acquired at low light level, which results in high photon shot noise [15]. Noise reduction filters by convolving raw image data with mask functions, typically a Gaussian mask, are effective for such noisy images, where otherwise meaningful fluorescence readouts are hidden behind the noise. In time-lapse imaging, relative changes in fluorescence intensity are displayed as ratio images obtained by dividing each image frame in a pixel-by-pixel manner by an image taken at time zero (F_0, F/F_0), or by

creating a differential image by subtracting the F_0 image from each image in the sequence (ΔF). In ratiometric imaging, two image frames obtained with different excitation light are divided, i.e., Fura-2 ratiometric calcium imaging or FRET dual color imaging, which are preceded by background subtraction using values calculated from a user-defined ROI, an image frame taken as the background image, or any user-defined values. All these calculations are performed in real-time every time ratio images are displayed. Users do not have to worry about the actual calculation steps, they only select options for ratio calculation, background method, and smoothing filter, etc., and these options are stored in the header of data files, which means that image files will be displayed using the same calculation options the next time they are opened.

Particle tracking

Particle detection and tracking analyses are popular in many imaging fields such as motion analysis and single-molecule tracking. Automatic particle detection and tracking can be performed if the shape of the target is simple, and the contrast of the image is high. TI Workbench implements an automatic particle tracking function (Fig. 3D) [16]. When motile cilia were targeted for motion track, several automatic motion detection algorithms were not effective due to the low contrast of differential interference contrast (DIC) microscopy and the difficulty in detecting the cilia head; manual tracking of cilia head was adopted in TI Workbench, in which manual steps that users have to repeat are minimized [17].

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3-D data display

When the data structure is three-dimensional or more, 3-D display helps comprehension of the data. TI Workbench has several 3-D display modes. Simple 3-D projection mode displays the projection of a 2-D pixel stack of time series or along the z-axis to the monitor 2-D screen (Fig. 3A). The surface tracing 3-D mode displays a 3-D view of reconstructed 3-D elements whose surfaces are rendered by thresholding the pixel density (Fig. 3B). 3-D display of pixel intensities in a 2-D image is a vivid presentation (Fig. 3C). In Fig. 3D, single particle tracking results are displayed in a 3-D space, in which the trajectory of each particle is shown in a 3-D space consisting of x-y image axes with time in z-axis.

User-controlled movement of the 3-D display by changing the angle or distance facilitates the understanding of the displayed structure. In all these 3-D display modes, images are rotated by dragging the mouse. Sequences of user-controlled movements of the 3-D display can be recorded in a movie file, which is saved in a standard movie format or stored in the "Lab note" described below.

Electrophysiology

Electrophysiology is a well-established technique to control and record the properties of excitable cells, such as neurons and muscle cells. To assess the electrical properties of excitable cells, the experimenter imposes arrays of one-dimensional command outputs while recording the temporal changes of membrane potential and current. Typical experiments require 1-4 input and output independent channels at frequency of at least 20 kHz and durations between seconds and minutes, and sometimes hours. Devices handling these tasks in

the mid 1990's were equipped with a FIFO (first-in, first-out) memory buffer and exclusively designed and built for electrophysiological experiments. TI Workbench was adapted for such devices, like ITC-16 and ITC-18 (HEKA, Lambrecht/Pfatz, Germany). In addition, TI Workbench works with some models of DAQ cards for electrophysiology including those used for the two-photon microscopes described above (e.g. National Instruments models), because the performance of general purpose DAQ cards have exceeded the requirement of electrophysiological use since 2000's, and are cheaper than electrophysiology-specialized devices.

TI Workbench provides most functions typically required for cellular electrophysiological experiments. The stimulation pattern generator uses a simple command syntax to generate temporal patterns of analog outputs, from simple rectangular step pulses to complex combinations of different functions such as ramps and sine waves (the "Stimulator" window, Fig. 4A). The digitized readout of membrane potential and current are displayed in an oscilloscope-style panel (the "Oscillo" window, Fig. 4B). User-defined stimulation and recording parameter sets (i.e. protocols) are stored in a hierarchical list, which can be easily accessed by a mouse click for quick changes during a recording session.

<Fig. 4 insertion here>

Averaging and overlapping waveforms of electrophysiological traces, temporal voltage and current changes, are also required functions for a software package. Adding and removing waveforms to and from averaged and/or overlapped waveform groups are done simply by drag-and-drop tags in the waveform viewer next to the off-line oscilloscope window (Fig. 5). More sophisticated and off-line analyses, as well as creation of publishing-ready graphs and

figures are performed in Igor Pro (WaveMetrics, Portland OR, USA), following an automated

data transfer function from TI Workbench as described below.

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Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) measures the dynamics of molecules in

solution by focusing the excitation light within a femtoliter-sized volume and detecting the

fluctuations in the emission intensities of target molecules tagged with fluorophores as they

enter and exit the focused spot by molecular diffusion. By correlating the fluctuation,

molecular concentration and diffusion rate of the target molecule species are deduced [13,18].

When two types of molecules are tagged with fluorophores of different emission wavelengths,

the cross-correlation of the individual fluctuations of the two fluorophores reflects associations

and interactions of the two molecular species in the excitation spot [18], a technique called

fluorescence cross-correlation spectroscopy (FCCS). Confocal and two-photon microscopy

systems are capable of FCS and FCCS because both methods focus the laser beam to a

diffraction-limited spot. FCS and FCCS can be performed not only in cell-free media, but also

in intracellular environment of living cells imaged by confocal or two-photon microscopy. TI

Workbench implemented analysis of FCS/FCCS on a confocal microscopy and on the

random-scan two-photon microscope described above. In the first implementation, two high-

sensitive GaAsP-type PMTs (H8631-40, Hamamatsu Photonics) were added to a confocal

microscope (FV-300, Olympus). The confocal microscope is set to point-scan mode: it detects

fluorescence emission from a single spot in the sample during FCS acquisition. The output of

the PMTs set in photon counting mode is fed into the digital counter port of a DAQ card (PCIe-6363, National Instruments), which is connected to a Mac computer running TI Workbench. The oscilloscope module of TI Workbench displays the output of the PMTs at a sampling rate between 0.5 and 1 kHz. In the implementation using the random-scan two-photon microscope, FCS from multiple points is possible owing to the fast laser beam switching mechanism. The ideal temporal resolution required for FCS relates to the diffusion rate of the target molecule: a 10 kHz sampling rate is suitable for FCS/FCCS of macromolecules such as proteins and RNAs. The random-scan two-photon microscope is capable of performing FCS/FCCS from up to 3 or 4 different spots in the sample, which is effective in measuring molecular dynamics in different cellular compartments, such as dendritic spines, dendritic shafts, and cell bodies of neurons simultaneously. All calculations of correlation and cross-correlation are performed and displayed in real-time, showing fitting results to specific diffusion models.

Lab notebook

Experimenters need to take detailed notes of every step during complex and time consuming electrophysiology and imaging experiment. For example, the time of events, specific observations, and the names of data file all need to be written in a notebook; however, most of that information is already present and managed by the acquisition software. TI Workbench has a log event window that stores such information (Fig. 2k), including the time of start and finish of each sequences of steps, file names of saved data, and analysis results. Users can save the content of log window as a text file. In addition, TI Workbench has a

separate "lab note" function, in which any text and graphic information can be placed in notebook-style pages of unlimited horizontal and vertical dimensions. Users can drag-and-drop contents of the log window or snapshots of images onto the "lab note" window in real-time during experiments. Even movie data are stored in "lab note", which are played by a mouse double click. The "lab note" file is stored in its original format, and a PDF file is automatically stored in the same disk location of the original format file to guarantee the future readability. The contents of the "lab note" format file and the associated PDF format file are automatically updated. Meta information accompanying image graphics or electrophysiological traces (e.g., location of the original data file), are also attached to the images and plots placed in the "lab note" file. When a 3-D image is reconstructed from 2-D image data, every parameter needed for the display of the 3-D image is stored as meta data, such as selection of sub-area and range of z-stack, rotation angles, color options, and thresholding levels to separate signal from background. By just double-clicking the 3-D image in the "lab note", the same 3-D image is reproduced from the original data file.

Postproduction with Igor Pro

The appearance of the final output of imaging and electrophysiology data is very important and needs to be easily modifiable by the user. Different commercial graphical presentation programs designed for scientific purposes meet these demands, serving as back-end data processors. General file formats, such as text file format and Excel format, are sometimes used for data transfer to graphical presentation back-end software, in which the appearance of graphs must be rebuilt by setting multiple parameters, including specifying data

sets for each axis, graph type, ranges of axes, etc. TI Workbench uses Igor Pro as a back-end processor for sophisticated data analyses and creating images for publishing. The contents of graphs and images in TI Workbench are transferred to Igor Pro by simple operations that preserve all relevant signal and time-base scales. Typical calcium imaging data with electrophysiological traces contain one or more image data, time-dependent plots of temporal changes in raw fluorescence intensity and of ratio values of F/F_0 or ratio of two different wavelength calculated from several ROIs, plus one or more waveforms of electrophysiological traces and stimulation pulses. All of these plots, image data, and electrophysiology traces are transferred to Igor Pro and arranged in a "Layout" window in Igor Pro by a simple operation in TI Workbench (Fig. 6). Users further arrange the appearance of graphs and images, and add text in the "Layout" window in Igor Pro. This workflow effectively reduces time-consuming postproduction steps and user errors. TI Workbench communicates with Igor Pro through Apple Event, a standard process-to-process communication method in Mac OS.

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External data formats

TI Workbench has been developed not only for on-line data acquisition but also for off-line data analyses. TI Workbench reads different data formats: TIFF and other standard image formats, Olympus confocal microscope data formats, Zeiss LSM format, Pulse and pClamp electrophysiology data formats, etc. Once imported, image and electrophysiological data within external format files can be analyzed as standard TI Workbench format files. TI Workbench serves as a useful unified data analysis solution for laboratories where experimenters handle various data formats produced by different data acquisition systems.

In a laboratory microscopy course, students learn to use a confocal microscope and a CCD camera-based fluorescence microscope. The former system is controlled by built-in software provided by Olympus, and the latter by TI Workbench. Later, students analyze data obtained in both systems using TI Workbench. They do not have to learn the details of different software programs about data manipulation and can concentrate themselves on learning the basics of microscopy. After data analysis in TI Workbench, graphs of temporal fluorescence changes are transferred to Microsoft Excel through Apple Event by a simple operation in TI Workbench, while image data are saved in PDF format files. Students take the data back home as Excel format files and image data as PDF files for further adjustments of graphs and images on their own Windows or Mac PCs to write the final reports.

Performance with C++

The primary core language of TI Workbench is C++, which is suitable in optimization for speed. Some operations require high CPU power, e.g., particle detection by fitting to a Gaussian distribution through many stacks of images [16]. A prototype code written on MatLab (The MathWorks, Inc, Natick, MA, USA), which is a popular programming and execution environment in image data analysis, took several hours to detect particles in hundreds of image stacks on standard PCs in the mid 2000's. The same algorithm was ported to C++ in TI Workbench, and the time to perform the same task was only several minutes after optimization of the code. Optimization consists of many features: multi-threading, use of the vector operation of CPUs, efficacy in filling CPU operation pipeline, etc. Although the current CPUs are much more powerful than those only a decade ago, faster operations, e.g.,

instantaneous reconstruction of complex data analysis results on opening a data file in response to user operations to change parameters, etc., are still much beneficial to keep the workflow smooth and to reduce the workload of users, which reduces user errors.

Conclusions

The development of TI Workbench started from the need of synchronized imaging and electrophysiological recordings to be performed by the same computer. After two decades of development, TI Workbench has supported the majority of necessary software functions for synchronized or independent imaging and electrophysiological experiments. Additional special functions have been added to meet specific requirements of my laboratory and those of national and international collaborators. Routines have been polished through loops of daily feedback from users, modifications and debugging of the source code over many years. I believe that TI Workbench is a superior solution as a user friendly and stress-free interface for complex imaging and electrophysiological experiments.

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Figure Legends

Fig. 1 Device control flow in Fura-2 calcium imaging.

A. Diagram of light path and optical device control. Data acquisition software controls shutter open/close, selection of excitation and emission filters and beam splitter, focus control, and camera exposure. Electrophysiological devices, an amplifier and a computer interface, are also indicated, which are also controlled by the software for simultaneous electrophysiology and imaging experiments. **B**. Workflow of a dual-wave length time-lapse imaging with a calcium indicator dye, Fura-2.

Fig. 2 Appearance of TI Workbench in image acquisition

A snapshot of windows of TI Workbench on time-lapse image acquisition. In this example, a two-photon microscope and a CCD camera are used together with an electrophysiological recording system. A cerebellar Purkinje cell in a slice preparation from a mouse was filled with a calcium indicator dye, Oregon Green 488 BAPTA-1 by the patch-clamp thechnique. The image control panel (**a**) is shared by every image display window, which is used for selection of image frame, color look up table, smoothing filter, image display modes and for contrast adjustment. Through the "camera" panel (**b**), image formats for snap-shot or focusing ("full" image (**d**)) and for time-lapse image frames ("seq" image (**e**)) are set and executed. Because formats of time lapse images are usually different from those of focusing/snap-shot images in binning factor, sub-area of camera, and exposure time, keeping the two different image windows on the monitor screen is useful. In this example, time-lapse recording was performed in line-scan mode, and fluorescence intensity along the line is

displayed horizontally with time along the y-axis. In the full frame image (**d**), the position of the line scanned is indicated as a pink line over the 3-D stacked image of the Purkinje cell. Temporal changes of image intensity in each ROI (red box in (**e**)) are shown in the plot windows (**f**), which are updated with the progress of the frames or changes of the ROI shape or addition of ROIs by the user. The upper plot shows raw average of fluorescence intensity within the ROI and the lower plot displays F/F_0 values. An additional panel (**c**) is attached to the "camera" window (**b**) for setting parameters specific to the two-photon system. Another camera window (**i**) is used to control the CCD camera for monitoring FOV. The "full" image window for the CCD camera (**j**) displays real-time video. In this example, a DIC view of the brain slice with a stimulation glass pipette is shown. Small panels attached to the left sides of the two camera windows (**b** and **i**) are used for control of the focus control device. The "Simulator" (**g**) and the "Oscillo" (**h**) panels are for electrophysiology (refer to Fig. 4). The log window (**k**) holds event logs. Most of the parameters for both imaging and electrophysiology can be glanced, so that the user does not have to peek them by selecting menus or opening other windows.

Fig. 3 Several 3-D data display modes.

3-D image stacks of pyramidal cells in murine neocortex are shown in **A** and **B**. **A**. A simple 3-D projection mode displays projection of 2-D pixel stacks along z-axis with rotated angles to the monitor screen. **B**. The surface tracing 3-D mode displays reconstructed surfaces of elements traced by thresholding pixel intensity. **C**. Calcium imaging shows a localized calcium increase in a cerebellar Purkinje cell. Fluorescence image of a Purkinje cell is overlaid with a pseudo colored relative fluorescence changes (**left**). The relative

fluorescence change pattern is transformed to a 3-D space (**right**). **D**. Single particle tracking results are displayed in a 3-D space, in which trajectory of each particle (colored spheres) is shown in the 3-D space consisting of x-y image axes and z-axis as time.

Fig. 4 Stimulator and Oscillo windows

Electrophysiological experiments require command output to an amplifier and to a stimulator and data acquisition from the amplifier. Waveforms of output command are typically consisting of rectangular pulses, and sometimes ramp or sine functions are used. **A**. The "Stimulator" window of TI Workbench generates such wave forms by a simple syntax. **B**. Recording parameters are set in the "Oscillo" window which displays recorded potential and current traces. User-defined stimulation and recording parameter sets are stored in a hierarchical list in the left-bottom box, which can be easily accessed by a mouse click for quick changes during a recording session.

Fig. 5 Off-line electrophysiology data analysis

Electrophysiological data are displayed and analyzed with the off-line oscilloscope window. Waveforms of current (**green**) and potential (**red**) can be averaged and overlaid simply by dragging and dropping data tabs on another tab in the wave list displayed in the drawer window (**right**).

Fig. 6 Export to Igor Pro

A. Image data with plots of the temporal changes in each ROI. **B**. Images and graphs in **A** were transferred to Igor Pro software by a single operation. Note that graph formats are preserved in Igor Pro. User can further adjust the exterior of them in Igor Pro for final publishing.

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A

focusing

objective lens

Electrophysiology: amplifier

shutter

high power lamp

excitaton filter wheel

beam splitter wheel

computer interface

emission filter wheel

camera

B

(1) Placement of a beam splitter and an emission filter in the light path

(2) Placement of a 340 nm filter

(3) Open a shutter

(4) Start exposure of a camera

(5) Stop exposure of the camera

(6) Placement of a 380 nm filter

(7) Start exposure of a camera

(8) Stop exposure of the camera

(9) Close the shutter

(10) Readout image data from the camera

(11) Wait for next loop

fig. 1, T. Inoue

Menu bar: TI Workbench File Edit Layout Window ImageOption(Global) ImageOption(Local) Misc-Option Help   16:14:41 inouelab

full frame (a)
100 % 25452/25452
LUT
filt none
z-stack
anim 1 / 20
0.00 / 38.00 um
max 20
z(1/e) ofs
F1 0 0
F2 0 0
Raw
1185/1185
info comment
TIW8 format,10/17/2017
16:10:35.004,,expose
5000ms,16bits,back:none,full
pixel (10000 10000),binning
20x20,sub register:(0 0 9999
9999),z-pos
0.00/38.00um,disp range
(1185 25452),line dwell 1.58
ms, line interval 0.00 ms,

focus control (b)
focus control cur.pos
reset to 0   41.6
z stack
full seq
from 0.0 get cur
to 41.6 get cur
interval 2.0
slice num 20
variable exp

camera [mirror-2P] (c)
full frame save
seq
focus 1500 ms
F1
take 5000 ms
full seq
f1 f2 ex1
ex2 ex3 dark
bin 20 full
autoGain 0/100
min/max 2489/25508
actual duration: ~1 ms
-back
segment 1710170017
frame num (F1) 2001
frame num (total) 1
duration [sec] 4.0
frame:156KB/seg:156KB
0 KB free in disk flim
plotAutoGain mark
Record save
take back link Oscillo
easy Timelapse
ROI clear line
subregion clear
2.0 ms
interval dark focus
measure minimum
pixel/clock: 31.2
assumed min/max:3/51
camera settings...
exp.set
filter config
1st frame dark
backImgAs1stFr
extra light source
ex1 ex2 ex3
F2 1st last
alternate F1/F2
F1/2/3 F1/2/3/4
segments
file 171017 1st" Li
auto run log
free train
interval 2
seq autoSave
interleave 1
filePlot autoGain
link full frame
fullAutoSave
scan start delay [ms]
PMT digital 1 c
options

2P control
PMT analog 1 gain: 55.0
PMT analog 2 gain: 50.0
AOM
40 %
adjust to z depth
from 0 get cur
to 0 get cur
mirror galvano resonar
OSP-EXA
close F1 F2
channel assignment
F1 PMT analog 1
F2 PMT analog 2
ext 1 PMT analog 3
ext 2
dark

full frame (d, e)
full frame
100 0.0°

Oscillo (h)
file 171017 1st" Line b data id:7AH1518148
record stop save auto save
trigger mark
auto extern
free 3885
train
freq. 0.130 ms
duration 7.00 s
interval 0.10 s
hardware gain
auto track
event detect
event detect config
window s
experiment set
name of exp. exp set
update   Stim
new group   Imaging
new param.   patch
delete   Yuho
sealing
Vm_check
10/21/2016 18:26:41.013
multiple 5 peak
mean 5 layout
mean -> multi
ch 1
-9465.0 pA   -69.8 mV
78.4/div [pA] gain ofs
-172.188
scroll
auto scroll
optimize find
1.719   700.0 msec/div full 2.335

Stimulator (g)
output record
x
y
c for disp 0
z
DA0 0,2000;[1,.2;0,9.8],7;0
DA1
DA2
DA3
DD0 0,2000;[1,.2;0,9.8],10;0
DD1 0,20;1,.2;0
DD2 0,40;1,.2;0
DD3
ch 1 set to comment ch -
400.0/div [mV] gain ofs
0.000
optimize find
0.0000 700.01 msec/div full 7.000

camera... (i)
focus control cur.pos
reset to 0   41.6
z stack
full seq
focus 60 ms
F1
take 60 ms
full seq
f1 f2 ex1
ex2 ex3 dark
bin 1 full
autoGain 0/100
min/max 390/864
from 0.0 get cur
to 62.8 get cur
interval 2.0
slice num 31
actual duration: ~1 ms
-back
variable exp
avg
easy Timelapse
ROI clear
subregion clear

full2 (j)
full2
40% 0.0°

seq (e)
50% 0.0°

1710170016 raw1 plot[but... (e)
8000
7000
6000
5000
4000
0 1 2 3 [sec]

1710170016 ratio plot[but... (f)
1.2646
0.0168
2.5
2
1.5
1
0 1 2 3 [sec]

Log (k)
[10/17/2017 15:38:49] exposure
time too short
[15:39:16 seq start]
[15:39:22 seq save '171017 1st"
Line before.dts' (1710170011)]
[15:39:24 ad save '171017 1st"
Line before.dta' (7AH1518142)]
[15:39:58 seq start]
[15:40:04 seq save '171017 1st"
Line before.dts' (1710170012)]
[15:40:06 ad save '171017 1st"
Line before.dta' (7AH1518143)]
[15:42:07 seq start]
[15:42:14 ad save '171017 1st"
Line before.dta' (7AH1518144)]
[15:42:20 seq save '171017 1st"
Line before.dts' (1710170013)]
[15:43:03 seq start]
[15:43:11 ad save '171017 1st"
Line before.dta' (7AH1518145)]

A

[dendrite and cell zstack depth 0-700 bin5 AOM 45-75 int1.dtf] shining dot 3D

B

3D:zstack0001

C

131302.dts

3D:1313020016

D

3D:7film

fig. 3, T. Inoue

A Data in TI Workbench

B Exported data in Igor Pro

fig. 6, T. Inoue