Laser Microsurgery of Filamentous Fungi: The Latest Protocol Enabling Patch-Clamp Amenable Protoplasts

K. Stevanović[1], T. Pajić[1], N. Todorović[2], A. Krmpot[3], M. Živić[1], M. Rabasović[3]

1 University of Belgrade - Faculty of Biology, Serbia
2 Institute for Biological Research, University of Belgrade, Serbia
3 Institute of Physics, University of Belgrade, Serbia
e-mail: katarina.stevanovic@bio.bg.ac.rs

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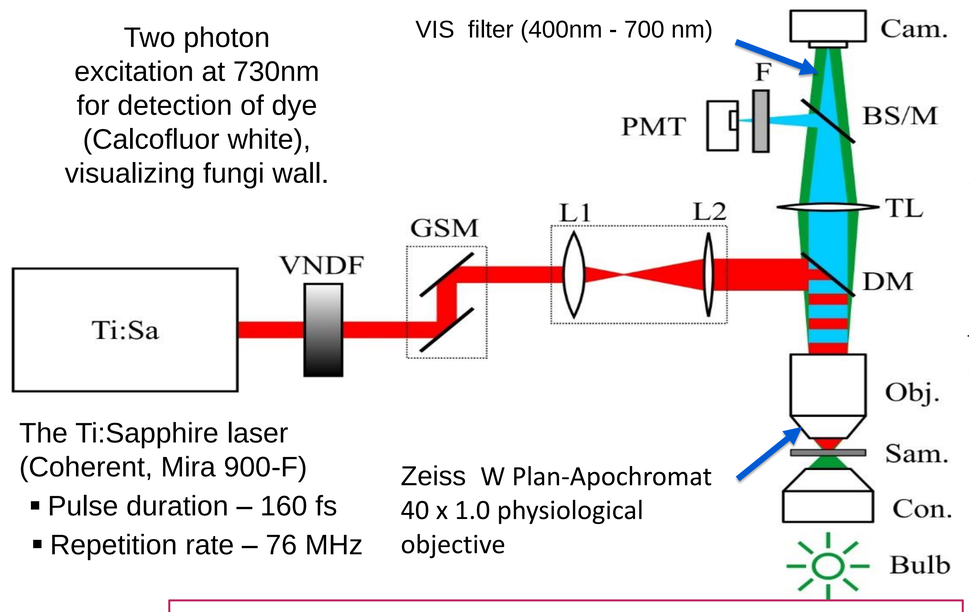
Abstract

During the last 45 years since the first gigaseal formation was made, patch clamp technique was adapted to almost every type of animal cell - meanwhile, the electrical behavior of native fungal hyphae membrane is still scarcely described due to difficulty in removing the fungal cell wall. Few attempts of cell wall removal by the means of laser microsurgery were made (Roberts et. Al. 1997, Véry and Davies, 1998), but never emerged as a routine protocol. We developed a precise protocol for femtosecond laser cell microsurgery used to free the protoplasts of P. *blakesleeanus* suitable for studying ion channels of the hyphae plasma membrane. Here we describe the protocol steps and emphasize which factors led to a crucial rise in the success rate. We are also showing the preliminary results made by analysis of 20% of the total sample size, showing factors that contribute to optimal results.



Bukara K. *et al.*, J of Biomedical Optics, 2017

Turning points for success

Calcofluor staining + pre-plasmolysis
(dye diluted by 495mOsm solution)

Inserted pre-plasmolysis step decreased the physiological shock made to the hyphae by the next solution

Short plasmolysis with second solution
(620 mOsm, 30 mM CaCl2)

High CaCl2 concentration allowed both faster plasmolysis and membrane stabilisation

Two-photon imaging

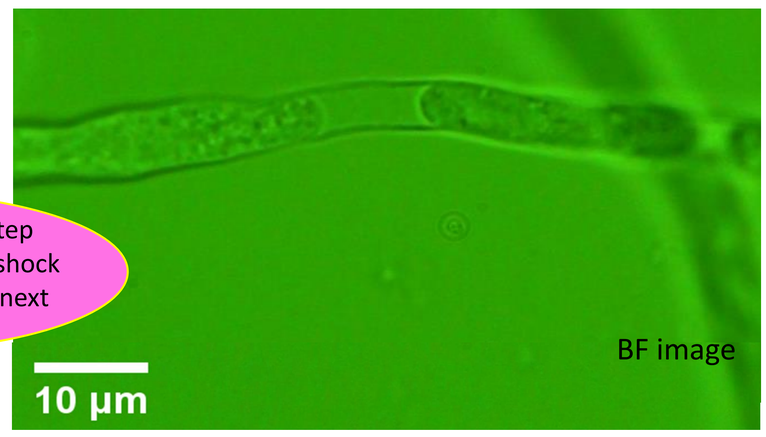
water dipping objective
Made possible to work on upright microscopic system separated from pacth clamp setup

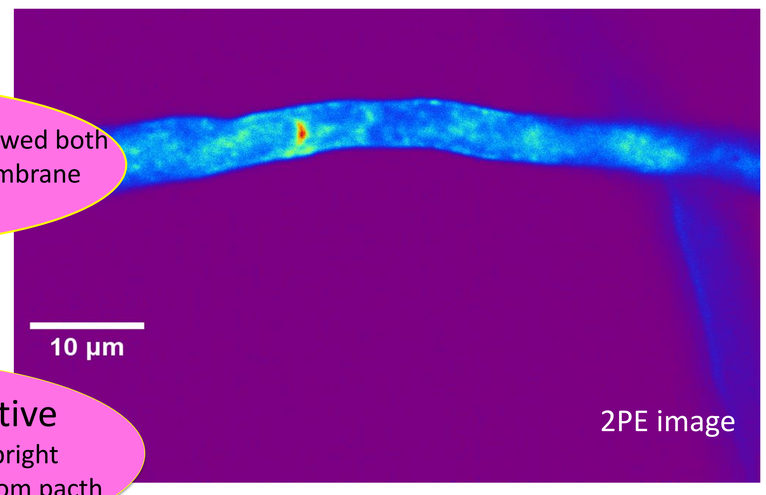
Cell surgery
fine tuning of cuting parameters

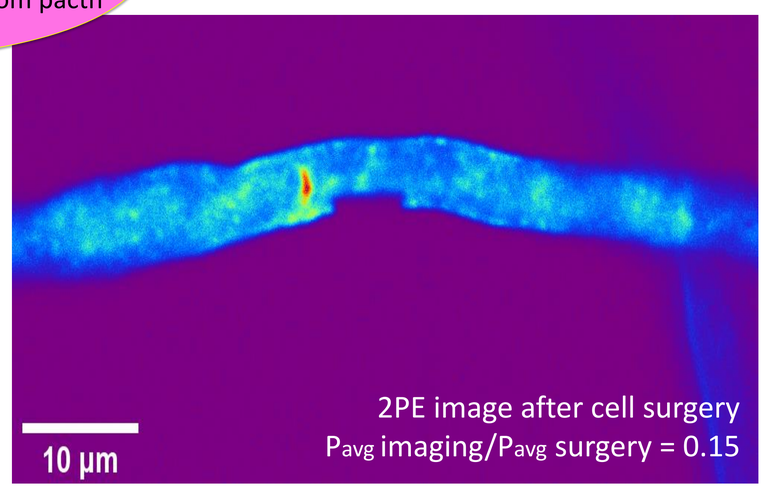
Subdle deplasmolysis
(570 mOsm + Na-azide)

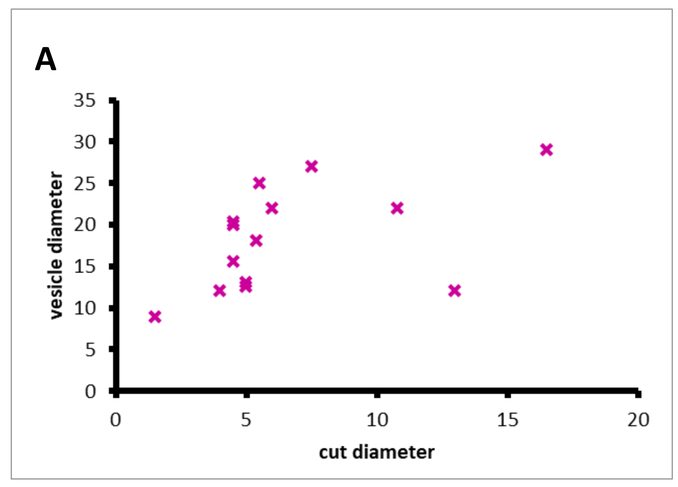
Cell wall synthesis blocking agent

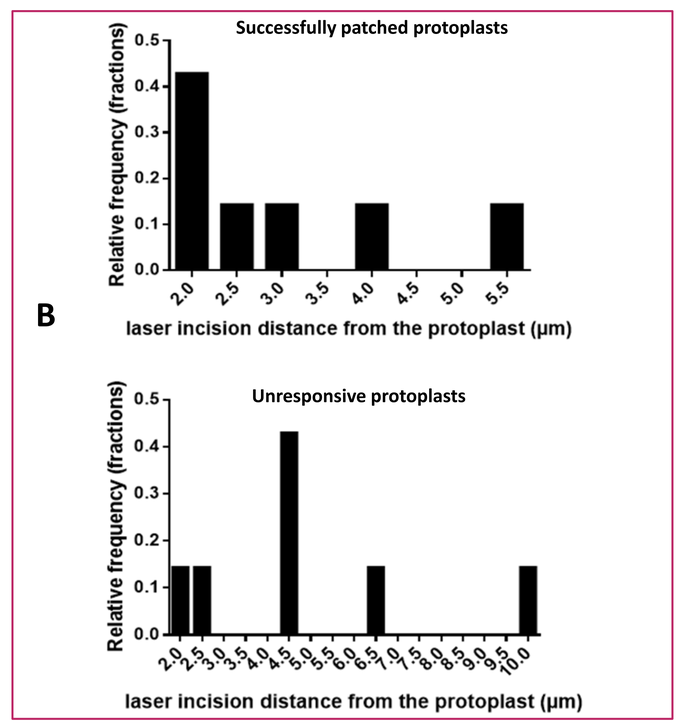
Patch-clamp
increased Ca++ in pipette solution;
size of pipette vs size of vesicle

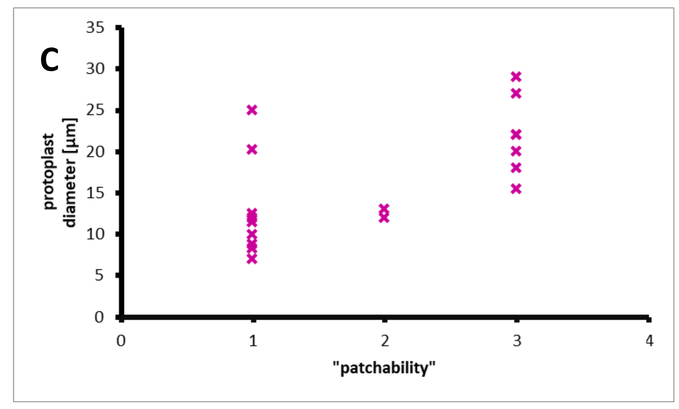












A : Relation of vesicle diameter and incision size;
B : Histogram of incision distances form protoplasts among protoplast that were successfully patched (up) and unresponsive (bellow);
C : protoplast diameters among "patchability" categories:
3 : GΩ seal obtained
2: response under 1GΩ
1: unresponsive protoplasts

Conclusions:

Preliminary data show that most factors examined may have a synergic role in maintaining physiologically stable protoplasts. Success rate for obtaining the high resistance (GΩ) contacts on patchable protoplasts was similar to the rates obtained on other fungal model systems, with a crucial advantage in providing recording of the native hyphae membrane. Both single channel and currents across the entire protoplast membrane were recorded. The newly developed protocol is expected to also be applicable on other fungi species, after modifications to allow for differences in cell wall thickness, sensitivities to plasmolysis and to wall regeneration inhibitors.

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