Benchmarks

Isolation of guard cells from fresh epidermis using a piezo-power microdissection system with vibration-attenuated needles

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The Eppendorf Piezo-Power Microdissection (PPMD) system uses a tungsten needle (MicroChisel) oscillating in a forward-backward (vertical) mode to cut cells from surrounding tissue. This technology competes with laser-based dissection systems, which offer high accuracy and precision, but are more expensive and require fixed tissue. In contrast, PPMD systems can dissect freshly prepared tissue, but their accuracy and precision is lower due to unwanted lateral vibrations of the MicroChisel. Especially in tissues where elasticity is high, these vibrations can limit the cutting resolution or hamper the dissection. Here we describe a cost-efficient and simple glass capillary-encapsulation modification of MicroChisels for effective attenuation of lateral vibrations. The use of modified MicroChisels enables accurate and precise tissue dissection from highly elastic material.

The Piezo-Power Microdissection (PPMD) system (Eppendorf, Hamburg, Germany) is used to separate and isolate tissue regions and/or particles from circumjacent material (1–3). In biological applications, the system is used to dissect groups of cells from tissue as sample inputs for further analysis. The system consists of a tungsten-needle (MicroChisel) oscillating in ultrasonic frequencies in a forward-backward mode to dissect samples, and a piezo-driven micropipet to collect the dissected samples (1,2). PPMD systems do not work as accurately and precisely as laser microdissection systems (4). However, laser systems may negatively affect the samples by both heat and UV irradiation (5). Hence, dissection by means of PPMD systems generally offers a higher integrity of the dissected samples which is particularly important for biological applications (e.g., sampling cells for recovery of RNA

and proteins). Furthermore, in contrast to laser systems, which are limited to fixed tissue (6), the mechanically operating PPMD allows the dissection of samples from living tissue (7). While dissection of biological samples from relatively less elastic material is also described (7–9), single-cell dissection from difficult tissues (e.g., living plant tissue), has not been reported yet. The root cause for this is the high elasticity of turgescient cells in plant tissues in conjunction with highly stable cell walls (10). Dissection of samples from such tissue by means of the PPMD system would require the highest frequency of vertical oscillation and highest amplitude to cut this tissue without shearing. However, it is a general feature of the PPMD that high-frequency vertical oscillation of the MicroChisel is accompanied by strong lateral vibrations that prevent accurate dissection. To overcome

this limitation without compromising the advantages of the PPMD system, we developed a simple protocol for an encapsulation of the MicroChisel that effectively attenuates lateral vibrations.

A glass capillary (Cat. no. GC150TF-15; Harvard Apparatus, Holliston, MA, USA) was pulled using a 2-step vertical puller (Cat. no. PC-10; Narishige, Tokyo, Japan) using the program: 4 weights, step 1: 62.7, stop in position 5, step 2: 49.6, and then shortened to a length of ~3 mm by means of a diamond glass cutter. The fine tip of the glass capillary was carefully opened out to a diameter of 110–150 μm by forceps under a dissecting microscope. Note that the tip diameter of the shortened glass capillary was slightly bigger than the diameter of the tungsten tip (100 μm) and the glass capillary diameter (1.17 mm) slightly exceeded the diameter of the aglet of the MicroChisel (1 mm). By means of forceps, the glass capillary was placed manually over the tungsten tip and the aglet of the MicroChisel and was loosely fixed due to the taper of the pulled glass capillary such that the initial 400 μm of the tungsten tip remained uncovered (Figure 1). Subsequently, the space between glass capillary and tungsten tip was filled with a UV-hardening adhesive (Vitalit 1605; Panacol, Oberursel, Germany) while avoiding bubbles. The MicroChisel, together with the glass capillary, was oriented vertically with the tip opening facing upward. Subsequently, the adhesive was applied to the lower end of the glass capillary and drawn into the space between aglet and glass by capillary action. The adhesive was then hardened by incubation at 120°C for 30 min. The encapsulated MicroChisel was used with a PPMD system (1) provided by Eppendorf. The system was mounted on a vibration-dampened table. An upright microscope (Axioskop; Zeiss, Jena, Germany) equipped with a high-speed camera (Sensicam; PCO, Kelheim, Germany) was used to monitor the dissection process. The dissector unit was fixed at an angle of 40–45° for dissection, or parallel to the microscope table to investigate lateral and vertical oscillation. Manual control by means of a Transferman micromanipulator (Eppendorf) allowed the movement of the dissector unit in all directions. The dissection process was triggered by a pedal control. Analysis of dissection behavior was performed on fixed mammalian tissue sections (human mammary carcinoma, provided by Olaf Bartsch, Eppendorf AG, Germany). The accuracy (i.e., the slitting width) was determined microscopically by means of a calibrated eyepiece micrometer.

The precision, defined as the width of the zone of disrupted tissue alongside the slit, was also measured. With forceps, an epidermis layer was manually removed from the underside of a *Phaseolus vulgaris* leaf and placed on a microscope slide bottom-side up. Stomata were isolated by means of the encapsulated MicroChisels. Amplitude and frequency were set to 100% and 45–54 kHz, respectively (maximal settings allowed by the system). These settings were found to be optimal for dissection of this tissue material. Dissection was performed by pulling (cutting in direction of MicroChisel holder) and avoiding pushing, since pushing the MicroChisel was often accompanied by uncontrolled disruption of the tissue.

By applying the standard operation settings (36 kHz frequency, 50% amplitude; suggested by the Eppendorf PPMD user manual), the lateral vibrations were relatively small. However, when frequency and amplitude were set to 54 kHz and 100%, respectively, MicroChisels showed very pronounced lateral vibrations [Figure 1A, panel (i)] which disappeared almost completely

upon attenuation [Figure 1B, panel (i)]. Determining the accuracy (slit width) and precision in dissection experiments revealed mean values of 4 μm and 12 μm , respectively, for encapsulated MicroChisel [Figure 1B, panel (ii)], whereas mean values of 6 μm and 23 μm , respectively, were observed for untreated MicroChisels [Figure 1A, panel (ii)]. The described adverse side effects, which are connected to the application of high frequencies and amplitudes, were clearly reduced by use of the encapsulated MicroChisels, and the dissection performance was consistently increased. This was also demonstrated by the separation of guard cells from surrounding epidermis cells of fresh *P. vulgaris* leaves (Figure 2). Whereas one pair of guard cells could easily be isolated within an operating time of 20 s, control experiments using untreated MicroChisels led to the disruption of the tissue independently from the chosen system settings (in control experiments the following settings were tested: 36 kHz, 45 kHz, or 54 kHz frequency, and 50% or 100% amplitude).

PPMD is a low-cost alternative to laser dissection systems offering distinct advantages

by dealing with living biological samples. However, the significantly lower accuracy and precision (4) reduces the attractiveness of this mechanical system drastically. In this article, we show that application of an easy-to-use protocol can substantially broaden the application spectrum of this method. Use of an encapsulated MicroChisel reduces the lateral vibration of the MicroChisel and leads to a better visual control of the experiment. Reducing the lateral vibrations makes high frequency and amplitude settings applicable and thereby enables these system configurations to be exploited for the dissection of difficult tissue.

The performance increase observed for dissection of epidermis tissue is probably also transferable to other types of elastic tissues and broadens the application of this method. The need for alternative micro-preparation techniques became obvious recently when Rottloff et al. developed a new method to isolate single glands directly from fresh *Nepenthes* tissue (11). The modification of MicroChisels that we have described and the advantages offered by its application should be of interest not only to plant physiologists and biotechnologists

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Figure 1. Lateral/vertical displacement and application in microdissection of untreated and encapsulated MicroChisels. Panels in row A correspond to untreated MicroChisels while panels in row B correspond to encapsulated MicroChisels. Schematic illustrations of the corresponding MicroChisels are shown at left. In column (i), photographs of activated MicroChisels demonstrate their vibration patterns and their maximal spatial displacement. Amplitude of vertical oscillation is unaffected by the encapsulation. Note that visual inspection of the photographs may be misleading in this comparison, as capture time was set slower than vibration frequency (maximal settings: 54 kHz and 100%). However, comparison of (A) and (B) clearly demonstrates that lateral vibration is strongly attenuated by the encapsulation. Column (ii) shows the cutting accuracy of MicroChisels using frequency and amplitude settings of 45 kHz and 100%, respectively. Data represents the average of three slit values from each microchisel type, where slit value is the average of eight measurements along the slit. Column (iii) shows typical microphotographs of slits in fixed mammalian tissue using both configurations of MicroChisels, demonstrating the improved accuracy and precision of encapsulated MicroChisels. Bars in (i) and (iii) correspond to 20 μm .

Figure 2. Microphotographs of the isolation of a pair of guard cells from epidermis tissue (*Phaseolus vulgaris*, leaf) by use of a vibration-attenuated MicroChisel. (A–D) Sequence of the dissection process. (A) Stoma cell located in the epidermis tissue. (B and C) Dissection. (D) Completely isolated stoma cell. The vibration frequency and amplitude were set to 45 kHz and 100%, respectively. Bar corresponds to 10 μm .

(12) but also to researchers in other disciplines using microdissection systems (3).

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Competing interests

The authors declare no competing interests.

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