Laser Microdissection applied to plants

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Laser microdissection (LM) is a process that allows the harvest of isolated cells or groups of cells of the same type resulting in homogenous and pure samples that can be used for DNA, RNA, proteins and metabolite extraction for further analysis. The use of LM in plants has been made mainly for transcript expression profiles studies through microarray analysis or, more recently, by high-throughput sequencing methods. LM is more adequate for gene expression studies than for proteins or metabolites analysis due to the fact that it is possible to amplify transcripts “in vitro” and by that, overcoming the reduced amount of material collected by LM as a limiting factor. Despite that, proteosome and metabolosome profiles in plants have been reported. Here we give an overview of LM in plants and of its potential to understand the distinct and complex functions of each cell type.

Keywords: Laser microdissection, plant, proteosome, transcriptome, single-cell

Introduction

Any multicellular organism is a collection of organs that are composed by distinct types of cells that display specific genetic, protein and metabolites profiles [1]. Researchers need to collect cells of the same type and therefore to develop protocols for isolating specific cells out of complex tissues. Manual dissection procedures under microscope control arose first, using razor blades, needles of fine glass pipettes also known as micropipetting. In this last case, the micropipettes, filled with RNA or protein extraction buffer are handled with a micromanipulator. By punching individual cells, its content is aspired entering in contact with the buffer [2, 3]. The main drawback of the manual dissection is the very time consuming and the lack of precision during the isolation process [4, 5]. In the micropillarie technique, the amplification of the extracted RNA, quite often fails enabling the microarrays analysis [3]. One other problem is the fact that only surface cells can be reached leaving internal cell types impossible to be processed [2]. This problem can be overcome using transgenic plants capable of developing accessible nonsurface cells [2]. However, transforming grown trees is almost impracticable [6].

One other approach to isolate specific cells is through the cell sorting [7] that can be achieved by protoplasts [8] or by tissue homogenization and cell fractionation [9]. Protoplasts can be obtained from transgenic lines expressing green fluorescent protein (GFP) and then, isolated by a fluorescence-activated cell sorter (FACS) [7]. This method also presents a limitation related to the expression pattern modification of a number of genes due to the generation of protoplasts [10-12].

All manual methods developed for single cells isolation are limiting to external cells or to tissues from which, protoplasts can be obtained. With the development of LM, any specific cell type can be isolated and studied. One key step for the development of laser microdissection as we know it today was made by Shibata et al. [13]. The method developed by this group relies on a negative selection of the material: SURF (Selective Ultraviolet Radiation Fractionation) where a UV laser beam is used to destroy the DNA of the area surrounding the sample to be harvested that, in turn, is protected from the laser by a dye.

The first report on the use of LM for gene expression of plant cells through a specific cDNA library construction came from Asano et al. [14]. In 2003, Nakazono [15] and colleagues published one of the first papers where laser microdissection coupled to microarrays was used for the first time in plants.

The laser microdissection (LM technique)

The method of LM was initially developed by Emmert-Buch and colleagues [16] and was licensed to Arcturus Bioscience that developed the Pixcell™, Autopix™ and Veritas™ instruments.

The complete laser microdissection process requires 4 main steps as follows: 1- specimen preparation. According to the analysis to be performed, the biological material can be histological samples (fixed and paraffin-embedded or cryosections), living cells, cell-cultures, chromosome spreads and forensic preparations. 2- Visualization. This step is performed under the bright field microscope on stained or unstained samples. Fluorescence can also be applied when secondary antibodies, acrilne-orange or FISH is used. 3- Microdissection. After the identification of the cells or group of cells, these are dissected by the laser beam in a non-contact and contamination-free manner. 4- Extraction. From the collected cells, DNA, RNA, proteins or metabolites can be extracted for subsequent studies using techniques such as PCR, microarray, expression profiles, FISH, LC-MS/MS, 2D-PAGE, SELDI or MALDI.
Today, laser microdissection (LM) technique can be divided into two main groups: laser-capture microdissection and laser cutting microdissection [17]. The first one includes the system developed by Arcturus molecular devices (http://www.moleculardevices.com/pages/instruments/arcturusXT.html) where an infrared (IR) capture is applied. The laser cutting processes includes the Leica LMD microdissection devices that use a UV laser beam for cutting (http://www.leica-microsystems.com) and the system belonging to P.A.L.M. Microlaser Technologies (http://www.zeiss.de/microbeam) that applies a catapulting strategy. From the two LM approaches, the laser cutting presents same advantages: speed and efficiency because it allows the use of hydrated and thick sections; and since the cells are isolated by laser cutting instead of being pulled out of the section by a sticky membrane, it prevents the possible harvest of undesirable cells that in theory might happen in the laser capture method [18].

Arcturus system

The Arcturus LCM system is based on a low-power infrared (IR) laser that is used to melt a specific thermoplastic film (ethylene vinyl acetate membrane – EVA) above the cells to be collected [19]. A custom-designed PCR-tube cap, coated with the EVA film is placed on the tissue section with the help of a transport arm. The film is melted by the action of the laser when this is pulsed through the cap. The melting promotes a thin protrusion enough to touch the tissue that adheres to the EVA film, being in this way, embedded by the polymer [20]. This method is called melt-stick-pull [17]. The caps with the biological sample are ready to be transferred to a centrifuge tube containing the proper extraction buffer according to the analysis to be performed. Larger target areas or irregular shapes require multiple pulses. This method permits the preservation of the morphology of the transferred cells that can then be observed under the microscope. The new system commercialized by the Arcturus, Veritas™ microdissection, combines the IR laser-based LCM system with the UV laser cutting (http://www.moleculardevices.com/pages/instruments/arcturusXT.html)

Leica System (AS LMD)

The specimen is mounted on a polyethylenaphthalate-foil (PEN) slide and the area of interest is visualized and selected on a computer screen [19]. In this system, a UV-A laser with 337.1 nm wavelength cuts the plastic film by “cold ablation” along the drawn line and the complete excised specimen falls by gravity in a PCRNtube cap that is filled with extraction buffer according to the analysis to be performed. Larger target areas or irregular shapes require multiple pulses. This method permits the preservation of the morphology of the transferred cells that can then be observed under the microscope. The new system commercialized by the Arcturus, Veritas™ microdissection, combines the IR laser-based LCM system with the UV laser cutting (http://www.moleculardevices.com/pages/instruments/arcturusXT.html).

P.A.L.M. System

The P.A.L.M. System relies on the laser microdissection and pressure catapulting (LMPC). The pressure catapulting makes use of the energy released of the microdissection that pushes the specimen against gravity, into the cap of a microfuge tube [18]. This procedure takes about 1ns, a period too short for generating any heat that could be transferred to the sample. LMPC is performed in an inverted microscope and uses a UV-A (337 nm wavelength) laser that is focused through a lens down to a spot size of < 1 μm in diameter [21]. It is in this point of focus that the laser will cut the material mounted on a glass slide coated with a polyethylenaphthalate (PEN) membrane or culture dish. The isolation of the specimen results from the ablation of the material that surrounds it in a photochemical process carried by the energy generated within the narrow laser focal point, a phenomenon called “cold ablation” (http://www.zeiss.de/microbeam). This fact allows the utilization of microdissection on living cells without the loss of viability [21].

The use of LM in plants

Immunolocalization, in situ hybridization and reporter gene observations have been the main analysis to study expression profiles in specific cells. Nowadays, with the efficiency of transcriptomic analysis and proteomics and with the advance of more and more sensitive and reliable techniques, the study of DNA, RNA, proteins and metabolites are strengthened when the samples to be analysed are of pure cell lines or tissue type. Laser microdissection (LM) is a powerful tool for such precise cell-specific studies. With LM, it is possible to generate pure and homogenous samples from which, DNA, RNA, proteins and metabolites can be extracted [4, 22, 11, 23]. Plants are amenable for the use of LM due to the regular cell organization and the presence of cell walls allowing the researcher to an easy identification of the different tissues. LM is also used in animal science, the field where the technique was developed, plant biology and in forensic investigations [21]. Another application of ultraviolet laser beam is to induce the fusion of plant protoplast and mammalian cells for antibody production [24]. It can also be applied to histological specimens, living cells and cell cultures, chromosome spreads, formalin-fixed paraffin-embedded (FFPE) or fresh frozen tissues [18]. The use of LM in plants is recent and has been applied mainly to gene expression profiles. LM was used to isolate Arabidopsis embryonic cells [25], rice male gametophyte and tapetum [26], cotton ovules [27] and maize apical
particles. It is therefore mandatory to wear powder-free gloves at all times and sterilize glassware by heat. All the paraffin embedding is a fast method because it avoids the dehydration steps that are usually carried out in ethanol. The samples are generally mounted on a membrane adjacent to the glass slide. After the sections are mounted on the slide, RNases. Solutions should be prepared with DEPC (Diethylpyrocarbonate)-treated water because it destroys the activity of the RNases.

Fixatives

The use of LM on plant tissues faces some challenges mainly due to the presence of the cell wall and the large central vacuole. Because the cells that are collected by laser microdissection are used for DNA, RNA, protein and metabolites extraction, the fixation of the material has to be in such a manner that allows maintenance of the histological integrity, prevents cross-contamination during sectioning and above all, permits extraction of intact nucleic acids and proteins. For LM preparation samples, the most used chemical fixatives are the coagulating fixatives (e.g. alcohol and acetone) and cross-linking fixatives (e.g. aldehydes) that have the capacity of cross-linking cytoplasmic proteins and lipids conferring a very good histological preservation of the material.

In plant material, several fixations were tested by different laboratories. Kerck et al. [22] made a comparison of RNA yield and quality extracted from plant material fixed with precipitative ethanol-acetic acid (Farmer’s fixative) and material treated with the cross-linking fixative formaldehyde-acetic acid-ethanol (FAA). They recovered two to three times more RNA from cells fixed with Farmer’s fixative.

Nakazono et al. [15] used samples fixed with 37-40% formaldehyde/glacial acid (95%), ethyl alcohol (10%:5%-50%, v/v) (FAA) and with ethanol/acetic acid (75%:25%) (Farmer’s fixative). They found that in the first case, morphology of the sections was better but the second fixation resulted in a much better RNA yield. Cryofixation was also tested but morphology was compromised owing to the formation of ice-crystals in the vacuoles damaging the cell. However, this problem can be overcome by subjecting the samples to sucrose solutions prior to the cryofixation [15]. Of all the analysed fixative protocols, cryofixed samples are the ones that give better RNA yields and less degraded RNA both in animals [34-36] and in plants [15, 25, 37, 38].

Another protocol that showed good results was developed by Schichnes et al. [39] with a 5h fixation with the use of a microwave paraffin embedding procedure. Inada and Wildermuth [40] modified the microwave paraffin preparation protocol by using only phosphate buffer as fixative, and obtained a good RNA yield and keeping the structure of the Arabidopsis leaves well preserved.

Concerning RNA degradation, it has been reported that paraffin embedding might be responsible of some degradation of the macromolecules [35, 40, 41, 42]. Since it was notice that the simple use of water in the stretching of the paraffin ribbons induces RNA degradation, in order to avoid this step Cai and Lasbrook [43] applied the tape transfer system (Instrumedics, Hackensack, NJ, USA) with good results. In fact, despite that, moderate RNA degradation has only a small influence on microarrays hybridization results [44] but acid nucleotides of lower quality due to fragmentation is one of the main problems of the RNA samples extracted after laser microdissection. The application of RNA later™ (200 µl, 4ºC, 1h – Ambion Austin, Tx, USA) prior to fixation has been registered to increase RNA yield in about four-fold as well as increasing its quality [45]. It was also noted that fixation with 100% acetone works very well for RNA and protein preservation in plant tissues. Acetone fixation in combination with paraffin embedding is a fast method because it avoids the dehydration steps that are usually carried out in ethanol gradients [46, 18].

Samples preparation

If RNA extraction is the goal of microdissected plant samples, all the work environment has to be ribonuclease (RNase)-free to avoid RNA degradation. The most common sources of RNase contamination are the hands, glassware and dust particles. It is therefore mandatory to wear powder-free gloves at all times and sterilize glassware by heat. All the solutions should be prepared with DEPC (Diethylpyrocarbonate)-treated water because it destroys the activity of the RNases.

The samples are generally mounted on a membrane adjacent to the glass slide. After the sections are mounted on the slide, paraffin has to be removed by xylene and it should be performed right before the LCM procedure. The laser beam
cuts the specimen together with the polyethylene film and both are collected in the centrifuge cap. After that, the caps with the microdissected material can be stored at -80°C in the initial buffer. Esposito [19] tested in animal tissue that if fixation, sectioning and microdissection were performed continuously, the RNA yield was higher than from slides mounted with cryosections that had been stored at -80°C before the microdissection.

Quantification and quality of the extracted RNA

RNA quantification should be performed on several sections scratches before the laser capture procedure in order to estimate the possible number of cells necessary for the final analysis and to check the integrity of the nucleic acids in the sample.

For samples obtained from laser microdissection, conventional electrophotometers can not be utilized for RNA quantification because they require a large amount of RNA. Lately, NanoDrop Technologies (Wilmington, DE) developed a highly sensitive spectrophotometer where the quantity assessment is the ribogreen fluorescent detection system with a sensitivity down to concentrations of 1.0 ng/µl [47, 48].

The verification of RNA integrity should be performed on microcapillary electrophoresis devices such as the ones by Agilent Bioanalyser, Agilent Technologies(Santa Clara, CA) or BioRad Experion (Hercules, CA).

Functional genomics

The ability to isolate single cells by LM combined with high throughput technologies has made a great impact in interpreting the function of a particular cell type. There is one key step that has made possible the cell transcriptome: the linear RNA amplification methods (e.g. T7 RNA polymerase based RNA amplification) because it enables analysis of very small quantities of RNA [22-15].

LM combined with microarray studies have been used with success for gene expression [48, 23]. Nakazono et al. [15] analysed gene expression in epidermal cells and vascular tissues in maize coleoptiles by cDNA microarrays with LM harvested samples. Casson et al. [25] used globular and heart stages embryos of Arabidopsis collected by LM for a gene expression comparison. Wu et al. [27] also applied LM coupled to gene expression analysis in cotton fibre initial cells and non-fibre epidermal cells from cotton seeds.

Microarray platform has been used by several groups in LM plant samples [15, 25, 27, 46]. Through microarrays it is possible to detect thousands of genes in one single experiment [49].

Microarray is called a “closed” platform because the only genes that can be found are the ones spotted on the array. On the other hand, “open” platforms such as SAGE (Serial Analysis of Gene Expression) and 454-sequencing [50] are able to give information of the transcript profile without prior knowledge of the genes creating the opportunity to unveil new genes [51]. On SAGE, non-normalized short expressed tags (ESTs) are produced permitting comparative and qualitative analysis of thousands of genes [52-55]. The 454-sequencing is a high-throughput method capable of sequencing > 200 000 fragments per 4h run [50]. This technique does not require cloning-based library construction therefore being a very appealing method for highly specialized transcripts detection [56].

Proteins

In order to have a true picture of the cellular mechanism, protein levels expression should be analysed. It has been shown that transcriptome does not correlate completely with the proteome in the same cell [57].

The need of protein expression patterns analysis in specific cell types was developed in cancer research since it is important to identify specific proteins that could act as diagnostic markers for specific carcinomes [58]. The combination of LM with several other proteomic technologies allowed the high-throughput molecular analysis [59] and identifying proteins that are specific for various cell types and tissues [29, 60]. Despite the enormous potential of the laser microdissection technique in single cell transcriptome, the use of this tool in proteome studies have not reached the same level of results. Such is due to the fact that it is not possible to make use of “in vitro” amplification steps such as the ones that are employed for the RNA. Therefore, for a stable protein analysis, the researcher needs to collect a high number of cells [61]. According to Shad et al. [28], 250 000 cells were enough to yield about 25 µg of total protein and sufficient for a well-resolved 2ND (Two Dimensional Gel Electrophoresis) gel. When applying a more sensitive protein detection method, LC-MS/MS (Liquid Chromatography- Mass Spectrometry), the number of cells necessary was reduced to 20 000 with a total protein of 2µg. Dembinsky et al. [29] isolated 200 000 maize pericycle cells with a final protein amount of 30µg. In sum, for a 2D-PAGE gel using LM samples, an elevated number of cells is required (10 000 to 100 000 in animal tissue) [62, 63]. It is reasonable to say that the use of LM extracted protein samples on 2D-PAGE gels is not very feasible since it necessitates a very high number of cells. More sensitive methods for protein detection and identification are available such as 2D-DIGE (Two Dimensional Difference Gel Electrophoresis) saturation labelling [64]. Here, the protein samples are labelled with different fluorescent dyes before performing the gel and this method is 100 times more sensitive than silver staining, thereby diminishing considerably the number of cells required for protein extraction [65].
The MALDI-TOF/MS (Matrix-assisted Laser Desorption/Ionization Time Of-Flight Mass Spectrometry) is another technique used for detection of a large number of proteins that requires a reduced number of cells from where proteins are extracted [66]. Albeit proteins are less susceptible than RNA to the handling of the samples during tissue collection and processing [59], the application of fixatives such ethanol/acetic acid or ethanol alone and further paraffin embedding leads to reduced protein yield and results in complexity on 2-DE gels such as spot duplication or multiplication as reported by Schad et al. [28]. So far, the use of LM in protein studies has worked better with cryosectioning material showing the best compromise between tissue morphology and protein extraction yield [28].

Conclusions

LM is a reproducible technique for sample collection and is the tool that best works for single-cell or cell type harvesting. Together with the development of more sensitive methods for transcript and protein analysis, LM becomes more attractive since a lower number of cells need to be collected. Once LM becomes a fast method for cell sampling it is possible, for example, to have a compared transcriptome analysis of different cells within the same organism.

References


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