COMBINED CELL SURVIVAL AND INJECTION SUCCESS RATE IN MICROINJECTION OF LIVING ADHERENT CELLS

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Abstract: For *in vitro* cell testing reliable techniques are needed. Capillary pressure microinjection (CPM) is one of the promising techniques. However, present microinjection systems still require lot of manual work and possess defective characteristics. Furthermore, a systematic method and extensive studies on a combined cell survival and injection success rate have not been reported. In this work, a method for evaluating the combined cell survival and injection success rate in capillary pressure microinjection is presented. The method is used for comparing two CPM methods. Both methods show the combined cell survival and injection success rate of approximately 30 – 35 %.

Keywords: adherent cells, capillary pressure microinjection, injection success rate, cell survival rate, green fluorescence protein, semi-automatic microinjection.

Introduction

Capillary pressure microinjection (CPM) is a mechanical microinjection method where the cell membrane is penetrated mechanically by means of a capillary, usually made of glass. The capillary is filled with the injection substance which then enters the cell by applying pressure to the capillary.

Since its inception in the early 1900's, CPM has become a prominent experimental approach in biological research [1]. CPM is used in many areas such as molecular biology, tumour biology, developmental biology, virology, pharmacology, physiology, toxicology and genetic engineering.

The main benefit of CPM is the possibility to selectively inject single living cells and even macromolecules can be transported through the glass capillary.

However, at present, CPM does not fully answer the demands of scientists working with adherent cells, mainly due to the repeatability problems. The bottleneck still is the capillary itself. As the number of injected cells rises, the probability of the capillary to get clogged or broken is rising too. Other challenges in CPM, like variability of the injected volume, have been discussed previously [2].

The automation of CPM adds reliability, repeatability and precision to the CPM procedure. This has been seen also with previous developments in CPM, with added computer control [3, 4, 5, 6, 7], standardisation of experimental conditions, microinjection protocols [8, 9, 10] and also reproducible production of capillaries [11, 12]. While moving towards automated CPM for adherent cells, the different aspects of CPM have to be known. The cell survival rate is depending on the injection success rate and experiment conditions. From literature, the best injection success rate is reported to be as high as 70-80% [13], while the cell survival rate is around 50% [14] using traditional CPM methods. As high cell survival rates as 90 % has been reported using a SLAM (Soft Lipid Assisted Microinjection) method [15]. This paper proposes a method for evaluation of the combined success rate, and provides results for two traditional CPM methods.

Materials and Methods

Cells: Experiments were performed on the adherent human breast cancer cell line MCF-7. All cell culture media and supplements were from Gibco Invitrogen Life Sciences (Paisley, UK). Before experimental studies, cells were cultivated 2-3 passages in phenol red free DMEM/F12 supplemented with 5% dextran-coated, charcoal-stripped treated fetal bovine serum, penicillin-streptomycin, 10 ng/ml insulin, and 1nM 17β-estradiol. The day before microinjection, cells were plated in 12-well plates at a density of 5×10^4- 7×10^4 cells per well. Cells were allowed to attach overnight. For transportation and experiments the medium was replaced with L-15 Leibovitz medium (Sigma-Aldrich, Munich, Germany), which requires no pH adjustment with carbon dioxide, supplemented with 5% dextran-coated, charcoal-stripped treated, FBS, penicillin-streptomycin, 10ng/ml insulin, 1nM 17β-oestradiol and 2mM L-glutamine.

Microcapillaries: Capillaries Femtotip II (Eppendorf, Hamburg, Germany), with an outer diameter of 0.7µm and an inner diameter of 0.5µm (± 0.1µm), were used.

Injection substance: Plasmid DNA (pBabe-Gem2; kindly provided by Dr. Dimitri Pestov, University of Illinois) containing a green fluorescence protein (GFP)

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gene was used as an indicator of cell survival. GFP will be expressed in those cells, which survive the injection. Expression kinetics is different for different cells, but after approximately 24 hours the cells, which survived the injection, expressed GFP.

Semi-automatic microinjection system: The system consists of a micromanipulator, a pressure injector, a vision system, a cell incubation system (integrated to the microscope) and software. The system uses the CPM technique.

Micromanipulator: The MANiPEN micromanipulator developed by the research group was used for the high-precision positioning of the microcapillary. The micromanipulator is fixed to a stand at such an angle that the tip of the microcapillary can be located in a well of a 12-well plate.

Pressure injector: The micromanipulator is connected with a pressure injector (MPPI-2; Applied Scientific Instruments, Eugene, OR, USA). The pressure injector has one channel, and its pressure range is 0–700kPa. Settings for the applied pressure, such as the amount and duration of the pressure pulse, and the amount of balance pressure, are changed manually by using dials.

Vision system: The vision system includes an inverted optical microscope with fluorescence option (TS100F; Nikon Corporation, Tokyo, Japan) and a charge coupled device camera (RT monochrome spot 2.1.1; Diagnostic Instruments, Sterling Heights, MI, USA). The vision system makes it possible to record the event of microinjection, as well as long-term monitoring of the cell, while the cells are in incubation conditions (Cell-IQ; Chip-Man Technologies, Tampere, Finland).

Injection methods: (i) Penetration method, illustrated in Figure 1. This is the conventional "stabbing" method used by conventional semi-automatic CPM systems [5, 13]. The capillary is lowered down until the contact between the cell membrane and capillary is detected visually. The capillary is then moved a few micrometers along its longitude axis to penetrate the cell and finally, an injection pulse is applied.

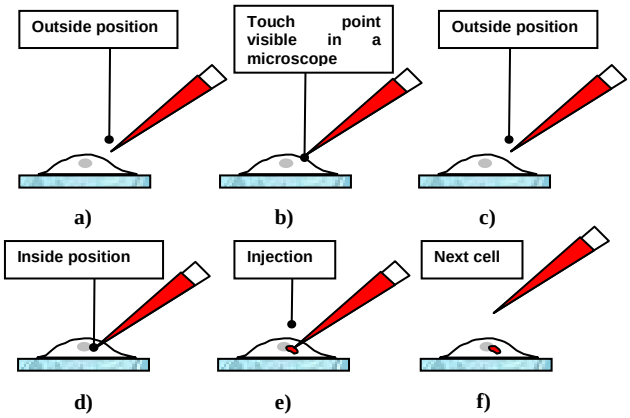

Figure 1: The penetration method

(ii) Contact method, illustrated in Figure 2. This is the method that is similar to SLAM method [16]. Our experiments have confirmed that while the contact between the cell and capillary is visually detected, the capillary is actually already located inside the cell. The pressure pulse is then applied without the penetration movement of the capillary.

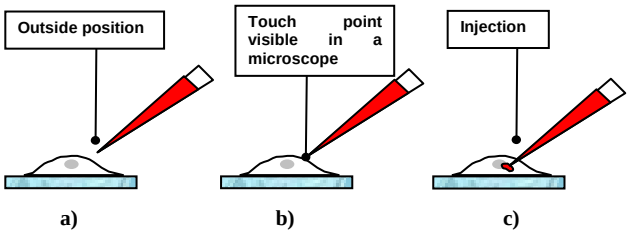

Figure 2: The contact method

Proposed evaluation method: Individual cells are injected with the injection substance (GFP). During the entire experiment, cells are kept under the microscope. This enables continuous monitoring of the injected cells. After the injection, cells are observed under the microscope in incubation conditions (controlled CO_2 and temperature) for 24 hours. After 24 hours, the number of cells expressing GFP is calculated and compared to the number of injected cells.

Results

The experiments of the penetration method and the contact method are summarised in Table 1 and Table 2, respectively.

The variations in cell numbers, in both tables in column *Number of Successful GFP Expression*, are caused by possible human errors in the analysis. First, as every cell starts to express GFP individually on different times, the weakness of GFP expression leaves the option to count the cell as a positive or negative. Second, as during the 24 hour experiment cells move around and divide, a GFP expressing cell which divides will become in two GFP expressing cells. The purpose is to count those cells as one positive only but sometimes it is difficult to judge which cells are results of division and not the actual injected cells.

The cell survival rate with the penetration method is minimum 27 % and maximum 37%, with a standard deviation of minimum 4.4 and maximum 11.7. The cell survival rate with the contact method is quite similar, minimum 31 % and maximum 37%, but with a standard deviation of minimum 11.4 and maximum 16.5. However, in general both of the methods show relatively low cell survival, below 40%.

Examples of the experiments using penetration method and using contact method are illustrated in Figure 3 and in Figure 4, respectively. On the left figures, the injected cells are marked with red dots. Yellow dots indicate those injected cells, which have divided. On the right figures, the same area is exposed by ultraviolet light to see the GFP expression inside the cell.

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Table 1: Survival rate in GFP injection using penetration method

	Number of Injected Cells	**GFP Concentration**	**Experiment Duration**	**Number of Successful GFP Expression**	**Cell Survival Rate**
Unit	***#***	***µg/µl***	***h***	***#***	***%***
	19	0.3	48	4	21
	25	0.3	48	min. 7, max. 12	min. 28, max. 48
	26	0.3	24	8	31
	9	0.3	48	min. 2, max. 3	min. 22, max. 33
	21	0.5	24	min. 6, max. 10	min. 29, max. 48
Total	**100**			**min. 27, max. 37**	**min. 27, max. 37**

Table 2: Survival rate in GFP injection using contact method

	Number of Injected Cells	**GFP Concentration**	**Experiment Duration**	**Number of Successful GFP Expression**	**Cell Survival Rate**
Unit	***#***	***µg/µl***	***h***	***#***	***%***
	52	0.3	48	13	25
	32	0.3	24	min. 9, max. 13	min. 28, max. 41
	24	0.3	48	min. 11, max. 14	min. 46, max. 58
Total	**108**			**min. 33, max. 40**	**min. 31, max. 37**

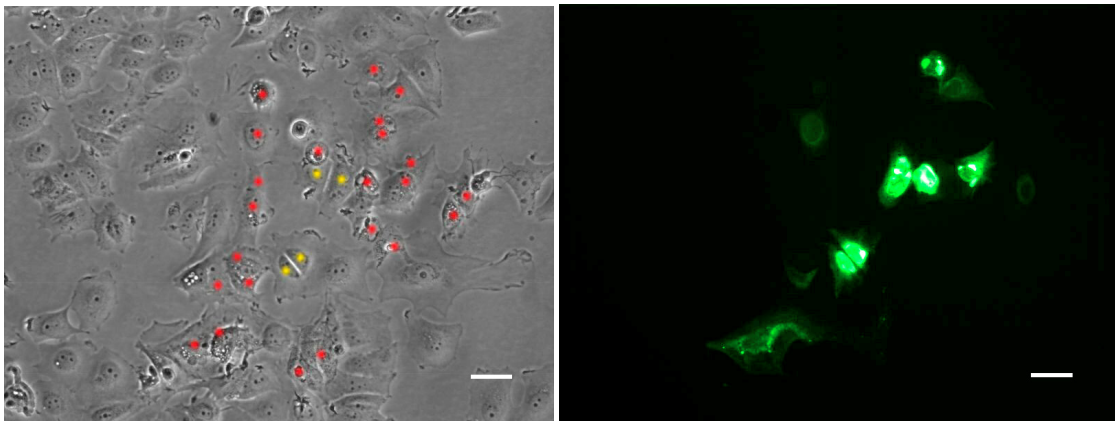

Figure 3: Illustration of the survival tests using penetration method – MCF-7 cells 24 hours after GFP microinjection. Cells with normal light (left) and the GFP expression inside the cells (right). Red dots mark injected cells and yellow dots mark injected cells, which have divided. The white bar indicates 40 µm.

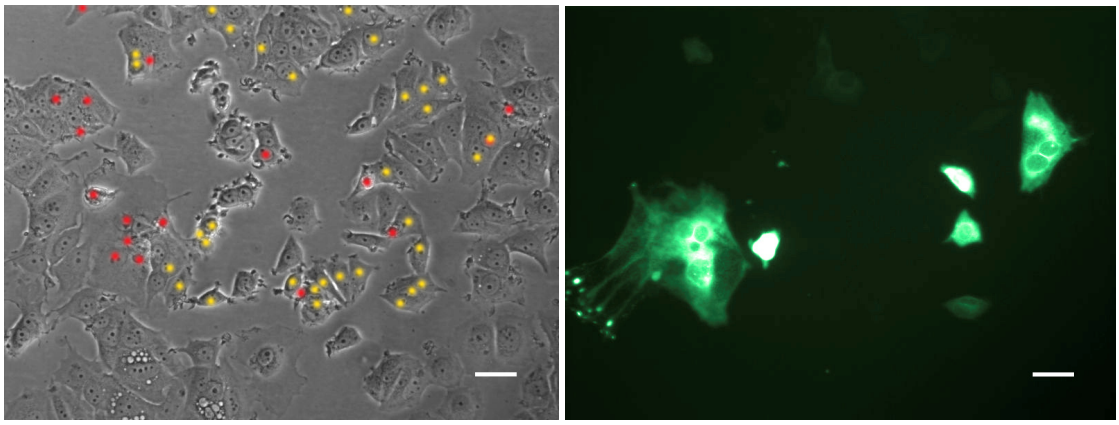

Figure 4: Illustration of the survival tests using contact method – MCF-7 cells 28 hours after GFP microinjection. Cells with normal light (left) and the GFP expression inside the cells (right). Red dots mark injected cells and yellow dots mark injected cells, which have divided. The white bar indicates 40 µm.

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Discussions and Conclusions

The proposed method allows a reliable determination of the combined success rate (the injection success rate and the cell survival rate).

The method is based on the injection of a GFP gene into cells and then incubating the cells approximately 24 hours to study the GFP expression in successfully injected cells. After the injection, the cells were incubated under the microscope. A time-lapse video generated from the experiment allows observing the activities of the injected cells at a single cell level. This significantly raises the reliability of the experiment. However, the human-based analysis still leaves space for some uncertainty.

During the determination of cell survival rate several discussion points have risen. In this paper, the definition for cell survival was combined with injection success. In other words, the positive counts where those injected cells, which were expressing GFP. The main strength of this definition is the fact, that to be able express GFP, cells have to be active, in other words they survive from the injection.

On the other hand, during the experiment there were several cases where the injected cells divided, but did not express GFP. Also dividing cell is an indication of cell survival. But as the cell did not express GFP, the injection itself was unsuccessful and so they were not included in the determination of the cell survival rate.

The cell survival rate with the penetration method is minimum 27% and maximum 37% and with the contact method around minimum 31% and maximum 37%. The relatively low cell survival rate could be explained by the injection success rate. Our experiments have shown injection success rate to be 49% in its maximum [17]. However, injection rate can be much higher, 90-100%. Such a high injection success rates are possible in experiments, where the injected sample has fluorescent co-ingredients. During the injection experiment the ultraviolet light of the microscope can be kept on to help the operator to visualize the injection event, with the aid of the fluorescent dye. However, due to the fragility of cells and samples, keeping the ultraviolet light on or using the fluorescent co-ingredients in the sample is not always an option.

The biggest bottle-necks for higher success rates and automatic microinjection are the lack of information on the contact between a cell and a microcapillary tip, and on the condition of the tip of the glass capillary. This missing information on the injection can be provided by a new impedance-based measurement system [18].

The future work includes improving the combined cell survival and injection success rate of CPM by using the impedance-based measurement system. Furthermore, the success rate of the SLAM method will be compared with that of the traditional microinjection methods. The systematic evaluation method proposed here will be used as a tool for evaluating the effect of automation on the combined cell survival and injection success rate.

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