Capillary Pressure Microinjection of Living Adherent Cells: Challenges in Automation

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Abstract. This paper is divided into two parts. The first part describes the current status and the general challenges of developing automatic microrobotics systems for microinjection of adherent mammalian cells. The discussion covers applications and the review and challenges of the components of a capillary pressure microinjection system: a micromanipulator, a microinjector, a microcapillary, a vision system and an environment control system. The second part of the paper describes the research performed on the automatic capillary pressure microinjection at the Tampere University of Technology. The advanced microinjection system includes two micromanipulators, a microinjector, a vision system and a control system. The control system comprises of motion control schemes for the micromanipulators to accurately position a microcapillary, to precisely penetrate a cell membrane and to deliver information on the injection to the operator. A novel injection guidance system being part of the control system comprises of an impedance measurement device and a user-interface which provide information on the detection of the capillary-membrane contact, capillary clogging and capillary breakage. Results show a remarkable increase of the injection success from 40 % to 65 % when the injection guidance system is used.

Keywords: Biomicromanipulation, microinjection, microrobotics, adherent cells, automation

1 Introduction

In the first phase of the Human Genome Project, the genetic sequence of human DNA was determined and all approximately 30 000 genes were identified. Knowing the gene sequence and identification of the genes is not, however, the end of the massive work. In the next phases, the functions of each gene will be determined and even further, the proteins and their functions will be identified. Microsystems technology played an important role in the acceleration of the Human Genome Project by introducing microfabricated DNA microarrays which remarkably increased the amount of experimental data. As the lessons have shown, novel tools (such as DNA microarrays) can lead to a significant leap in the research. It is expected that micro- and nanotechnology, will play even a more important role in the future biology research. Microrobotics can have an important role when the functions of the human genes and proteins are determined. Automatic injection of genes and small inhibitory RNAs (siRNAs[1]) into single cells and manipulation of protein crystals using advanced microrobotic systems can be an essential part of the functional genomics in the future. Micromanipulation techniques for single cells will also have an important role in such applications as in-vitro toxicology, cancer and HIV research, intra-cytoplasmic sperm injection and transgenics.

1. A small inhibitory RNA (siRNA) is used for the inactivation of a gene.

Micromanipulation can be performed either by touching the objects physically or without a physical contact. The sizes of objects range from about one micrometre to a few millimetres and they can be either of biological origin such as eukaryotic cells (e.g. mammalian cells), prokaryotic cells (e.g. bacteria), cellular components (e.g. DNA, protein crystals) or tissue samples, or artificial such as mechanical, electrical and optical components. In non-contact manipulation, these objects are manipulated using optic, electric, magnetic or acoustic energy, as discussed for example in [1], [2], [3], [4], [5]. Non-contact manipulation is an important way of performing micromanipulation, but this paper will concentrate only on the contact type manipulation. Therefore, the term micromanipulation will refer to the contact micromanipulation in the following sections.

In contact micromanipulation, operations are performed using an end-effector which is moved in a three-dimensional space by a micromanipulator. The end-effector can be a microcapillary, a micro gripper or a recording electrode. The tip of the end-effector has to be small enough to facilitate the handling of the minute objects and components.

The trends in many micromanipulation applications suggest that micromanipulation systems of the future must respond to the following challenges: high speed, increased flexibility, high level of automation, large information content and low cost. From the micromanipulator development point of view, this means that the performance of the micromanipulators must be improved, the micromanipulators must be miniaturised, and their automation level must be increased.

In this paper, the emphasis is on the challenges of automation but issues related to the miniaturisation of micromanipulators are briefly discussed, too. The trend towards parallel operations in biomicromanipulation and microassembly applications necessitates the simultaneous use of multiple micromanipulators and thus, their *miniaturisation*, but not at the cost of remarkably smaller work space and increased vibrations. By increasing the level of *automation*, the human involvement in tedious micro operations could be reduced and thus, the operation speed could be increased and scientists would be released to concentrate on the analysis of results. Furthermore, automation can increase the reliability and accuracy of the micromanipulation systems and thus, more reliable results.

Raising the automation level requires (i) a computer-controlled micromanipulator having a high positioning accuracy and repeatability, (ii) the development of automatic micromanipulation methods, (iii) a careful task planning which takes into account the requirements imposed by the automation and the scaling effect, and (iv) additional measurement information on the interactions between the end-effector and the micro particles. In order to obtain this information from the microworld in real-time, sensors and sensor systems, such as tactile and force sensors and machine vision systems, must further be developed. Increasing the level of automation also requires improvements in the robustness of the systems against errors and disturbances.

The focus of the discussion in this paper is on biomicromanipulation and more specifically, on the manipulation of single adherent cells. Adherent cells grow at the bottom of a cultivation dish and form an anchored cell population, while suspended cells, such as blood cells and germ cells, grow loosely in the medium, as depicted in Fig. 1. This difference poses different challenges on the micromanipulation systems. In a few recent years, the manipulation of oocytes in in-vitro fertilization and transgenics applications has gained a lot of research attention (see e.g. [6], [7], [8], [9] and [10]) in the microrobotics society, while publications on the manipulation of adherent cells have been much more infrequent. However, the despite blood cells and the germ cells, all other cells in our body are of adherent type and they are, therefore, of a very high importance in drug and disease mechanism studies, for example.

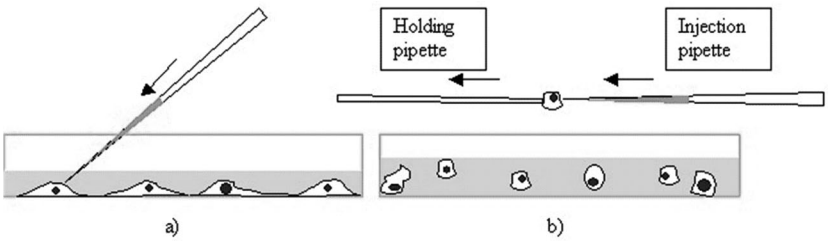


Fig. 1. Microinjection into (a) adherent cells and (b) suspended cells. Since suspended cells grow loosely in the medium, they have to be held by another capillary in order to be injected.

The most typical manipulation operations performed in an adherent cell culture include intracellular microinjection, cell isolation and microdissection, and electrophysiological

measurements of the cell membrane activities. This paper concentrates on the issues related to the intracellular microinjection and more specifically, on a capillary pressure microinjection method. Thus, this paper discusses the challenges related to the *automatic capillary pressure microinjection* of *single adherent mammalian cells*. The focus of the paper is illustrated in Fig. 2.

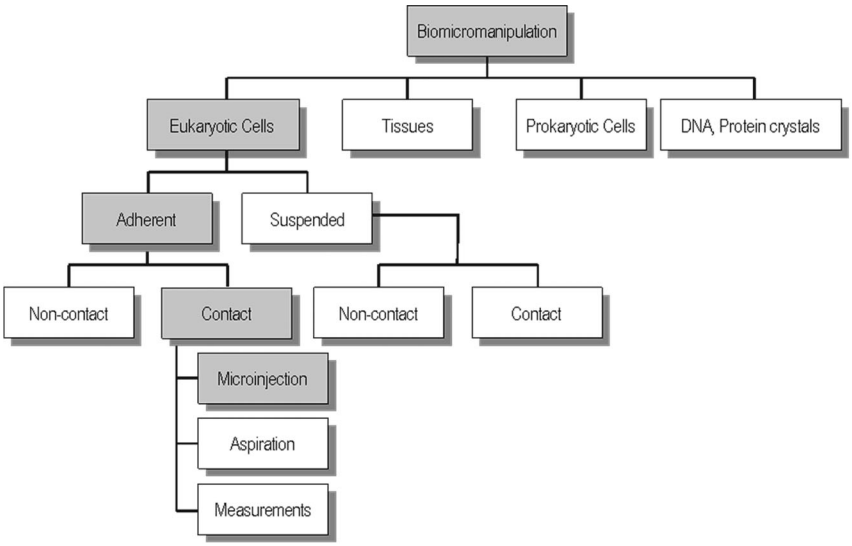


Fig. 2. Classification of biomicromanipulation and the focus of the paper.

The paper has a twofold objective: firstly, it aims to describe the current status and the general challenges of developing automatic microrobotic systems for microinjection of adherent mammalian cells, and secondly it describes the activities performed at the Tampere University of Technology in the field. The rest of the paper is organised as follows. Section 2 gives a brief overview on the application areas of adherent cell injections. Section 3 describes concisely the basic components of a general capillary pressure microinjection system and reviews the current research activities. It also analyses the automation challenges. Section 4 presents the second part of the paper. It describes two micromanipulator structures developed at the Tampere University of Technology and steps taken towards a fully automatic capillary pressure microinjection system. Cell injection experiments and the consequences of the current development on the injection success are also presented. The paper is concluded in Section 5.

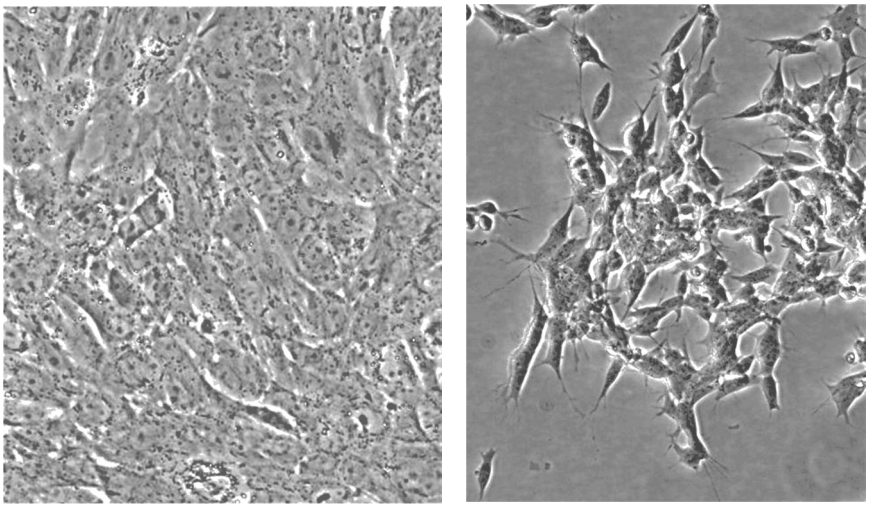
2 Applications of Adherent Cell Injections

Adherent cells are smaller in size (10--20 micrometres in diametre) than oocytes, which are the most typically microinjected suspended cells. As the size of the cells in adherent cell cultures is nearly 10 times smaller than that of the egg cells, microinjection of adherent cells requires micromanipulators of higher accuracy, both in terms of the positioning and injection accuracy as well as the preciseness of the penetration of the cell membrane. Since the cells are small and they tend to grow in population close to one another, they are difficult to detect. This imposes high requirements upon the vision and other measurement systems. The small size of the cells also results in a need for very fine injection capillaries. As thinner capillaries than one micrometre are needed, it is very difficult to visually detect the condition of the capillary, a contact with a cell, a correct injection depth, etc. On the other hand, an additional holding capillary is not needed. To summarise, the development of a microrobotic system for the automatic intracellular injection of single adherent cells is a very challenging task. However, a system that would automatically detect, manipulate and analyse a single living cell in a cell culture would provide enormous advantages over the manual systems significant leaps in the biology research.

Manipulation of micro-objects is challenging due to uncertainties in manipulation caused by the scaling effect[1]. Biomicromanipulation can be considered even more challenging because of the uncertainties caused by biology. Since each cell type has its own specific properties, slightly different detection and cultivation but also manipulation parameters are needed for each cell type. Moreover, the state of the cell population varies over the time, and cell batches and even cells are individuals which also increases the uncertainty of successful cultivation, detection and manipulation of cells. Fig. 3 illustrates

1. Relationships between physical quantities change in the micro world. For example electrostatic forces and van der Waals forces are more dominant than gravity.

differences in two adherent cell cultures: a retinal pigment epithelial (RPE) and a neuronal SH-SY5Y cell culture.



(a) (b)

Fig. 3. (a) Retinal pigment epithelial and (b) neuronal cell culture.

In the following sections, the most important application areas of adherent cell injections are briefly discussed. The discussion includes basic biological research, drug development and in-vitro toxicology.

2.1 Basic Biological Research

Capillary microinjection allows the introduction of siRNA or mRNA[1] constructs to single cells and the study of the effects of a short-time gene knock-down or gene over-expression on cells. Capillary microinjection is a better choice than lentiviral vectors, for instance, since vectors integrate to the host DNA randomly and may have uncontrolled effects on the gene expression and induce phenotypical changes. By using capillary microinjection, the cells to be injected can be accurately selected and it is also known which cells has been treated. This can increase the injection success rate and reliability of the study. Furthermore, some cells, especially primary cells and stem cells are difficult to transfect with traditional methods, and thus, an advanced microrobotic injection system could significantly aid the transfection of these cells.

For understanding the functions of genes, the gene expression analysis is an essential method. Traditional techniques require large amounts of starting RNA and therefore, it is

1. Small inhibitory RNA (siRNA) constructs silence genes and messenger RNA (mRNA) constructs activate genes.

hard to determine on which cells an individual gene is appearing. The development of microfluidics and lab-on-a-chip techniques has made it possible to develop a highly sensitive single cell reverse transcription-polymerase chain reaction (RT-PCR) method [6], [11]. The use of RT-PCR provides a unique method to investigate genetic makeup of individual cells - if an individual cell can be isolated from the population. Individual cells can be collected into a microcapillary by applying a negative pressure instead of the positive injection pressure. Later, they can be expelled to a different location to release RNA and to perform RT-PCR. Single cell RT-PCR is a promising application of microcapillary isolation but as long as the operation is manual and tedious, its importance will remain marginal. However, advances in cell injection technology will also support the development of automatic cell isolation systems.

2.2 Drug Development and Toxicology

Laboratory animals and cell lines of cancer origin are presently routinely used in drug development to study the effects of new drug compounds. Their use, however, introduces technical, ethical and economical drawbacks. Firstly, since different species are not alike, laboratory animals may not necessarily provide precise information about the effects of drug compounds on humans. Moreover, human cell lines are typically homogeneous cultures of cancer origin which do not mimic the function of normal tissues and organs. Secondly, the use of laboratory animals in drug development and toxicological tests poses ethical problems, and the European Union intends to forbid their use for instance in the cosmetic industry as soon as alternative methods become available. Thirdly, using laboratory animals is strictly regulated and expensive and therefore, companies would be ready to use alternative, technologically feasible but cost effective methods if they were available. The aforementioned reasons support the development of new cell cultures comprised of various types of cells. Heterogeneous cell cultures consisting of both healthy epithelial cells and fibroblasts[1] would mimic the function of a tissue better than cells that are of cancer origin. In addition to the heterogeneous cell cultures, it is beneficial to have primary cell[2] and in the future also stem cell cultures, which represent adequately the cell

1. A fibroblast is a cell found in connective tissues.

types from which they are derived. However, for example neuronal cells in a primary culture have a limited capability to divide. Furthermore, heterogeneous cultures and primary cells are more challenging to cultivate and they pose much more stringent demands on the cultivation and manipulation systems than cancerous cell lines.

When there is a need for more detailed information concerning either the behaviour of an individual cell in a culture, the interactions between different cell types, or cultures containing only a very small number of cells, techniques that facilitate the detection, manipulation and analysis of a single cell should be available. One step towards automatic manipulation of single cells is the development of the microrobotic cell manipulation system to be described in Section 4.

3 Automation of Capillary Pressure Microinjection

Microinjection is a technique for the delivery of small volumes of compounds into suspended or adherent cells and it has become a prominent experimental approach in biological research. A large variety of molecules like dyes, proteins, nucleic acids, drug compounds or toxins can be injected into cells and their activity can be studied [13]. Various microinjection methods exist. The most important methods are capillary pressure microinjection, laser beam injection, electroporation, iontophoresis and various endocytosis methods. [14], [15], [16], [17], [18], [19], [20]

Capillary pressure microinjection is a mechanical method, where a thin microcapillary is penetrated through a cell membrane and liquid is delivered from the capillary to the cell upon a pressure pulse. A modification of capillary pressure microinjection is Simple Lipid Assisted Microinjection (SLAM), in which a lipid fusion builds up between the microcapillary tip and the membrane [21]. The method is more gentle than sole pressure microinjection but also slower and still in the development phase.

The motivation behind choosing capillary pressure microinjection for the study is the fact that it provides the greatest potential for a highly reliable and repeatable injection of single cells and for an exact control of the volume injected. Furthermore, large molecules

2. Primary cells are taken directly from organisms and are not sub cultured.

can be injected into the cells, and the same end-effector - a microcapillary - can be used in other operations, such as single cell isolation and electrophysiological measurements. Over the last years, the development of CPM technology has enabled steps towards automation and research groups are developing automatic microinjectors [22], [23], [24], [25]. Commercial devices such as those provided by Eppendorf, Narishige and Cellbiology Trading are also available. However, even though some systems are already partly automated, a huge amount of manual work is still required by the operator. Due to the involvement of the operator, the number of cells that can be injected in a certain time is limited. This can be a problem if a large number of cells have to be injected for a biochemical assay or when microinjection is used to produce stable transfected cell lines [26]. Thirdly, the reliability, repeatability and accuracy of the method should still be improved: the operator cannot reliably detect when the tip of the capillary is in contact with the cell, cannot detect tip clogging and small breakages of the tip and therefore, does not know if the injection is successful. He/she neither knows the volume injected due to various uncertainty factors to be discussed in Section 3.2. All this particularly applies to injection of adherent cells.

By increasing the level of instrumentation and automation, (i) the speed of the method can be increased such that its usage will be feasible even in high throughput applications, (ii) the involvement of the scientist can be reduced such that he/she can concentrate on the analysis of the results and gets them much faster, and (iii) the reliability and accuracy of the system can be increased such that more reliable results will be obtained and success rates can be increased.

The components of a typical capillary pressure microinjection (CPM) system include a micromanipulator for precise positioning of the microcapillary and for the penetration of the cell membrane, a microinjector and a microcapillary for the precise delivery of a compound into the cell, a vision system for visualisation and an environment control system for maintaining cell cultivation conditions, such as temperature, pH and humidity.

This rest of this section reviews current research and analyses the challenges that are related to the automation of the capillary pressure microinjection method. The

micromanipulator positions the microcapillary next to a cell in a three dimensional space and generates a movement such that the capillary can penetrate the cell membrane without causing a cell death. Issues related to automatic positioning of the microcapillary are discussed in Section 3.1. Section 3.2 covers the challenges related to the injection event itself: the penetration movement, the delivery of the substance and the “fault diagnosis” of the microcapillary. Automation of the vision system, the environment control system and the handling of a microcapillary before and after injections are described in Section 3.3.

3.1 Positioning of End-effector

The micromanipulator should perform the given task with a certain accuracy and repeatability in a certain time and without damaging its environment (cells). In the automatic manipulation of single adherent cells, the micromanipulator must be able to position the end-effector with an accuracy that facilitates the manipulation of a single cell having a diameter between 10 and 20 micrometres. Depending on the application, accuracies from one micrometre to a few micrometres are needed. Magnifications of the optical microscope used in microinjections are such that the maximum displacement should be in the range of several hundreds micrometres to a few millimetres along each axis. In order to make automation feasible, the micromanipulator should achieve speeds of a few millimetres per second. Forces needed in the cell manipulation are in the range of micronewtons [7] and the micromanipulator should be able to carry out the manipulation tasks without disturbing the motion control performance.

The aforementioned performance requirements must be tackled in two different points of view: hardware and software. In the selection of the actuators and sensors of the manipulator and in designing its mechanical structure, the performance requirements must be taken into account. In addition to the hardware design, the motion control software and algorithms must be designed in such a way that they ensure the desired performance and possibly compensate for the shortcomings of the actuators. Motion control issues of micromanipulators will be discussed as follows. Section 3.1.1 gives an

overview of motion control. Section 3.1.2 discusses joint space control schemes, Section 3.1.3 control of piezoelectric actuators, Section 3.1.4 presents task space control schemes and Section 3.1.5 bilateral control strategies. Section 3.1.6 provides a short summary of motion control of micromanipulators.

3.1.1 General Issues in Motion Control of Micromanipulators

The role of the motion control system is to drive the actuators of the manipulator in such a way that the position and contact forces of the end-effector satisfy both transient and steady-state requirements given by the operator. The tasks can be classified into free motion tasks where no interaction between the manipulator and the environment occurs, or the interaction is negligible, and interaction tasks where the interaction between the manipulator and the end-effector must be taken into account. In biological micromanipulation, interactive forces are in a micronewton range and therefore, they are not usually taken into account. However, the measurement and control of the force applied in the penetration of a cell membrane can raise the success rates in intracellular injections indicating an increasing importance of interaction control in cell manipulation [25]. Discussion on challenges of interaction control in micromanipulation is, in this paper, limited to bilateral control strategies covered in Section 3.1.5.

3.1.2 Joint Space Control

The problem in motion control is to move the end-effector of the manipulator in free space along a desired trajectory. The trajectory is typically given in task space, whereas the control actions are performed in joint space. Therefore, the coordinates of the reference trajectory must be transformed into the joint variables. The controller can operate either on the task space coordinates or on the joint variables. If the controller operates with the joint variables, the control is called joint space control. In the joint space control methods, the task space coordinates are first transformed into the joint variables using a feedforward compensator. The feedforward compensator is typically an inverse position kinematic model, but in parallel micromanipulators it can also be an inverse velocity kinematic model, as in [27], for example. The inverse position and velocity

kinematic solutions are generally straightforward for a parallel manipulator and more complicated for a serial manipulator.

Decentralised position feedback control in joint space, or independent joint control, contains the inverse kinematic model, and *n* independent position feedback controllers, one for each active joint (either prismatic or revolute). More complicated decentralised control schemes include also velocity, acceleration and torque estimation. The joint positions are measured and the errors in the joint positions are eliminated using SISO (Single Input, Single Output) controllers. Many of today's robot systems, which use electric motors, rely on PD controllers. Since piezoelectric actuators are commonly used in micromanipulators their control is discussed separately in the following subsection.

Dynamics. In control schemes which are based on independent joint controllers, the dynamic interactions are either considered as disturbances, or they are compensated for in a feedforward path. The use of centralised multivariable control laws including manipulator dynamics has been proposed for applications where a high speed is needed but they are not very commonly applied to the control of micromanipulators.

Calibration. The control schemes that operate in joint space typically provide a high repeatability, but they suffer from a limited accuracy induced by modelling errors. Fabrication and assembly tolerances, mechanical and thermal deformations, unknown dynamics and external disturbances reduce the accuracy. Errors in the kinematic model (due to assembly and fabrication tolerances and tool exchange, for example) can be compensated for using calibration. Calibration of micromanipulators has been discussed in [28], [29], [30], for example.

3.1.3 Position Feedback Control of Piezoelectric Actuators

In many industrial manipulators, in both serial and parallel, the actuator that provides either the linear or angular displacement is an electric motor and it is controlled using a PD controller. However, in micromanipulator designs, piezoelectric ceramics have widely been used, since they have many beneficial properties for micromanipulation. Parallel piezoelectric micromanipulators have been proposed for example by Arai et. al [27], Breguet et. al [31], Gao et. al [32], Guo et. al [33], Kallio et. al [34], Lee et. al [35],

Ohya et. al [36] and Tanikawa et. al [37], while serial piezoelectric micromanipulators have been proposed by Codourey et. al [38], Fukuda et. al [39], Goldfarb et. al [40] and Morishita et. al [41], for example. Despite their wide use, piezoelectric actuators suffer from such drawbacks as large hysteresis, drift and self-heating which decrease the open-loop positioning accuracy. Therefore, feedback control is essential, if a piezoelectric micromanipulator is used in an automatic mode. Especially hysteresis is a problem, since it can cause not only positioning error but also instability. Many methods have been proposed for improving the displacement behaviour of piezoelectric actuators.

The compensation of the non-linearities is usually accomplished by means of feedback voltage control, where the displacement of the piezoelectric actuator is measured and the error is eliminated using feedback control. Other control principles include feedforward voltage control, where non-linear hysteresis models are typically used [42], [43], [44]; feedforward charge control, where the operating current is controlled in closed loop [45], [46], [47], [48] and feedback charge control, where the charge is measured and controlled [49], [50], [51].

3.1.4 Task Space Control

In the joint space control schemes, the input signal of the controller is expressed in terms of the joint variables. As was discussed, they typically provide a high repeatability, but they suffer from a limited accuracy. Errors induced by assembly and fabrication tolerances and tool exchange, for example, can be compensated for using calibration, but such error sources as thermal deformation and vibrations, cannot be eliminated. The use of the end-effector pose measurement in the control system facilitates an efficient compensation of external disturbances and inaccuracies in the manipulator structure. In micromanipulators, an optical microscope equipped with a CCD camera is the most frequently used method to detect the position of the end-effector. Visual servoing strategies for micromanipulators has been discussed for example in [25], [52], [53], [54], [55], [56], [57], [58] and [59]. The use of the optical microscope introduces such challenges as a limited depth of field which must be taken into account when visual servoing algorithms are developed for biomicromanipulation systems.

3.1.5 Bilateral Control

In teleoperation, the operator commands the motion of the manipulator using a joystick. When forces of the operation environment are reflected back to the operator, the system is called bilateral, as information flows in two directions. Several experimental studies on conventional teleoperation have shown that with the aid of this force-reflection, the performance of the operation is improved or the operator can even perform tasks that otherwise are beyond his capabilities. Same can be expected when micro parts are handled by teleoperation. The specific feature of micromanipulation is that information flows must be scaled: the master position must be scaled down and the contact forces of the micro environment must be magnified. Bilateral control of micromanipulators and the scaling effect has been discussed for example in [22], [60], [61], [62], [63], [64], [65].

3.1.6 Summary

The free motion control strategies developed for large-size manipulators are relatively well adaptable to micromanipulators and motion control of micromanipulators can be considered as a relatively well studied topic. The specific issues relate to the control of actuators, typically piezoelectric actuators, and to the use of an optical microscope as a motion sensing device and its consequences to the visual servoing strategies. The positioning challenges in automatic micromanipulation are closely related to the availability of components (sensors and actuators) which possess sufficient resolution, stroke, speed, and small size for automatic micromanipulation purposes.

3.2 Microinjection

In issues related to the automatic manipulation of biological cells, motion control of the micromanipulator is probably the most studied but not the only topic. In capillary microinjection, the precise penetration of the cell membrane and the exact delivery of a compound into a cell are crucial but still not satisfactorily automated functions. Section 3.2.1 discusses the challenges in the penetration of the cell membrane and Section 3.2.2 microinjection challenges. Section 3.2.3 describes the challenges that are related to the fault diagnosis of the microcapillary during the injection.

3.2.1 Penetration Challenges

This section discusses positioning challenges that are specific to microinjection. In addition to the three dimensional positioning, the micromanipulator related challenges include the detection of the capillary-cell contact and the penetration of the cell membrane. While the free motion control schemes are relatively well studied, the detection of the capillary-cell contact and the generation of the penetration movement have not been studied as extensively. In the current systems, the user typically controls the movements of the micromanipulator using a joystick and uses visual or a priori information to conclude when the tip touches the cell membrane. In automatic systems, the detection of the contact must be performed automatically. It can be made either using a machine vision system or electrically. An electrical method developed at the Tampere University of Technology will be presented in Section 4.

Some studies on the optimum cell membrane penetration have been performed [66]. The critical issues in the membrane penetration include the resolution, straight-line accuracy, positioning accuracy and speed of the movement. The straight-line accuracy describes the deviation of the movement from an ideal straight-line. The deviation can occur either in the horizontal or vertical plane and it should be as small as possible to minimise the cell damage. Furthermore, it is preferable to penetrate the membrane by a single high speed advancing step which must be short enough not to damage the cell. A short but high-speed step means rapid acceleration and deceleration which tend to set up tip vibrations. These after-vibrations must be eliminated, since they can damage the cell membrane if they continue inside the cell. Even though the demands on high acceleration and deceleration and eliminated after-vibrations conflict with each other, both of them must be met to penetrate the cell membrane successfully. On the other hand, controlled high-frequency small amplitude vibration of the capillary has been shown to improve the penetration success.

Force measurement and force control during the penetration of the cell membrane is a potential method to increase the success rate of the penetration. Papers discussing force measurement in the manipulation of biological cells include [7], [22], [25], [67].

3.2.2 Injection Challenges

The capillary pressure microinjection technique uses a microcapillary with a sharp tip to penetrate the cell membrane mechanically and a controlled pressure pulse for a precise volume transfer. The volume injected can be influenced with such injection parameters as:

- the amplitude of the pressure pulse (the applied injection pressure),
- the length of the pressure pulse (the duration of the injection pressure),
- the offset of the pressure pulse (the level of the balance pressure).

In an ideal case, if the pressure and time adjustments would stay the same, all cells would receive the same amount of the injection compound. However in reality, the volume changes considerably, see [68] and [69] for example. Another critical problem tightly connected with the repeatability of the injected volume is an undesired efflux from or influx into the microcapillary. In influx, the cell medium flows into the capillary resulting in less sample delivered than expected after calibration. In efflux, the filling solution leaks out from the capillary resulting in more sample delivered than expected after calibration. Furthermore, in efflux the cells can uptake the leaking filling solution and hence, change the results of the experiment. On the other hand, influx can also cause the clogging of the capillary by aggregates in the medium and thus, change the size of the capillary opening or even entirely prevent the injection. To avoid capillary clogging, most scientists prefer the efflux of the solution over the influx of the medium.

The relationship between the injection parameters and the injected volume is determined using a calibration procedure, as depicted in Fig. 4. The model describing the relationship cannot ever be determined exactly but equipment and biology related disturbance parameters affect the calibration accuracy.

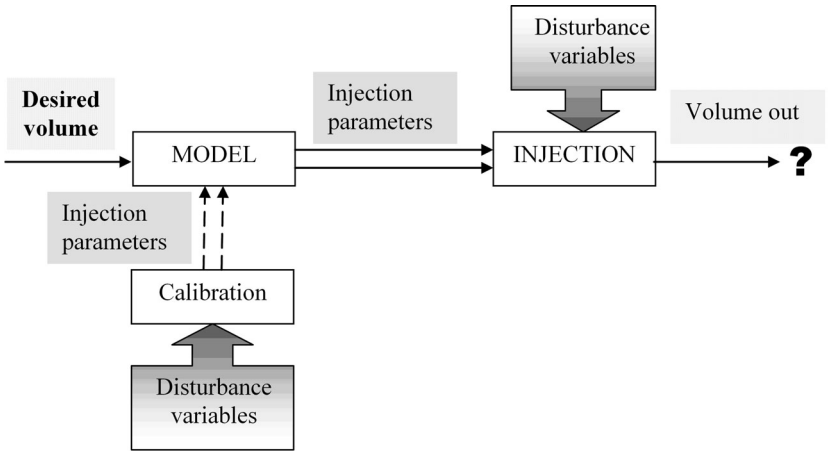


Fig. 4. Calibration of a capillary pressure microinjector.

The equipment related disturbance parameters include:

- the microcapillary tip (surface treatment, tip diameter, possible tip breakage or clogging),
- the microinjector (stability of the pressure source, accuracy of the pressure regulator and speed of the check valve) and
- the positioning and penetrating device (positioning accuracy and preciseness of the axial movement).

The biological disturbance parameters are much more difficult to compensate for and include:

- the cell (internal pressure, elasticity of the membrane, size),
- the medium (viscosity, homogeneity, aggregates) and
- the injection solution (viscosity, air bubbles).

The relationship between the injection parameters and the disturbance parameters has been analysed in [70] and is illustrated in Fig. 5.

3.2.3 Microcapillary Related Challenges

As was discussed in the previous section, one of the important factors limiting the success of capillary pressure microinjection is the clogging of the capillary. Typically a few injections can successfully be performed before the capillary gets clogged. In today's systems, the user must clean the capillary after each injection using an extensive pressure pulse and try to manually detect if a liquid jet comes out from the capillary or change the

capillary frequently. In an automatic system, a method for detecting the capillary clogging is needed. Furthermore, the other frequent problem is the breakage of the capillary tip. This is prevented in current systems by limiting the movement of the capillary not getting too close to the bottom of the cultivation well. If the tip gets broken it should be changed. Possible techniques to detect the breakage include machine vision and an electrical method. Current commercial systems do not consist of either clogging or breakage detection and no academic solutions are available according to the authors' knowledge. Section 4 will present an electrical method which facilitates the detection of the capillary clogging and the capillary breakage.

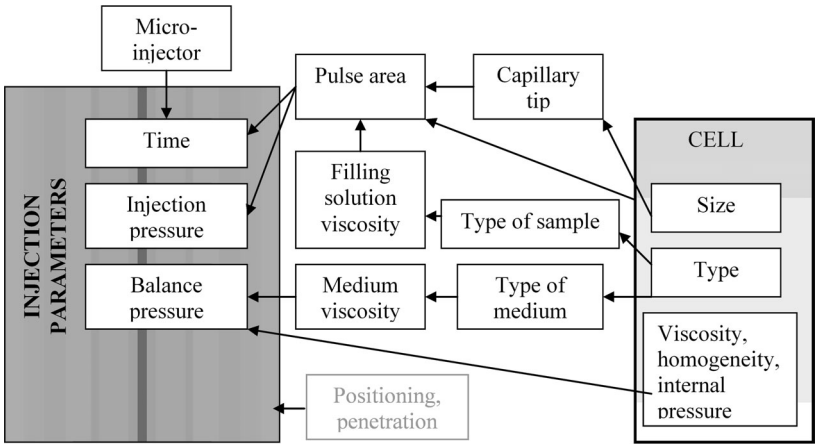


Fig. 5. Relationship between the injection parameters and disturbance variables [70].

3.3 Other Components

In a fully automatic system, other functions in addition to micromanipulation and microinjection should be automated, too. They include the handling of the end-effector - such as filling and changing the microcapillary, visualisation of the cells and the microcapillary, and cell cultivation.

If the microcapillary gets broken or permanently clogged it must be changed. This is currently made manually which, together with manual filling of the capillary, remarkably reduce the efficiency of the method. Therefore, in addition to the capillary breakage and clogging detection, an automatic CPM system should be able to change and fill the microcapillary automatically.

In automatic micromanipulation, the automation of the optical microscope plays an important role. Current commercial microscopes already provide such motorised features as zoom, change of magnification, xy positioning and illumination, all of which support the automatic cell manipulation. More advanced features include recognition of cells and their states. Examples of the research concerning cell detection algorithms include [71], [72], [73], [74], [75] and an example of a commercial system is Cell-IQ^{TM} of Chip-Man Technologies.

When developing fully automatic micromanipulation systems for living cells, issues related to cell cultivation are of primary importance: in order to perform any experiment, the cell culture must be vital. The cell cultivation system should guarantee correct temperature and pH, provide nutrition for the cells and prevent contamination. Moreover, cells should be exposed to an excitation light as little as possible in order to prevent for instance photo bleaching of fluorophores, which would result in the formation of cytotoxic radicals [26].

4 Research at Tampere University of Technology

This section presents the research carried out on the automatic manipulation of single adherent cells in the Institute of Automation and Control (ACI) at the Tampere University of Technology (TUT). Section 4.1 presents two micromanipulator structures (one parallel and one serial) developed at TUT. Both micromanipulators have three degrees-of-freedom and they are composed of piezoelectric actuators. Section 4.2 discusses the steps taken towards automatic microinjection. It first presents two motion control schemes (one in task space and one in joint space) and discusses the performance achieved with the schemes. Then a method which facilitates an automatic detection of a contact between the cell membrane and the microcapillary, a capillary breakage and a capillary clogging is presented. Finally, the benefits of the developed system in adherent cell microinjections are evaluated.

4.1 Micromanipulator Structures

4.1.1 Piezohydraulic Micromanipulator

The first micromanipulator developed in the ACI at TUT was a parallel composite-joint piezohydraulic micromanipulator [34]. It is composed of three *piezohydraulic* actuators connected in *parallel*. In the piezohydraulic actuator, the deformation of a piezoelectric disk is transformed into a linear displacement using hydraulic oil and a bellows [76]. Three bellows, which are able to elongate along their longitudinal axis and bend about the other two axes, form the kinematic chains of the micromanipulator. Since the bellows is a monolithic element and possesses both translational and rotational degrees of freedom, that micromanipulator is composed of *composite* joints.

As a bellows deforms axially and bends in two degrees of freedom, no additional prismatic, revolute, universal or spherical joints are needed. Thus, the constructed piezohydraulic micromanipulator is the first parallel structure which does not use separate joints but is composed of three composite joints. This simplifies the structure and is a beneficial feature in the fabrication and assembly of miniaturised micromanipulators.

The micromanipulator consists of three piezohydraulic actuators, a mobile platform, an end-effector and a pose measurement system. The components of the manipulator are illustrated in Fig. 6 a). By changing the lengths of the actuators, the orientation of the mobile platform, and thus the position of the end-effector, can be controlled. The micromanipulator consists of an internal pose measurement system which detects the motion of the mobile platform by means of Hall sensors [77]. In addition to the Hall sensor measurement, the micromanipulation system possesses an external vision-based measurement system to measure the position of the end-effector tip [53].

The piezohydraulic actuator consists of a piezoelectric RAINBOW® actuator, a miniaturised hydraulic chamber, hydraulic oil and a bellows, as illustrated in Fig. 6 b). The piezoelectric actuator is the active element and is placed in the chamber. When a voltage is applied to the piezoelectric element, it deforms. By buckling the actuator, the oil is moved from the fluid chamber to the bellows, which as a consequence elongates.

Since the effective area of the bellows is smaller than that of the RAINBOW® element, the displacement is amplified.

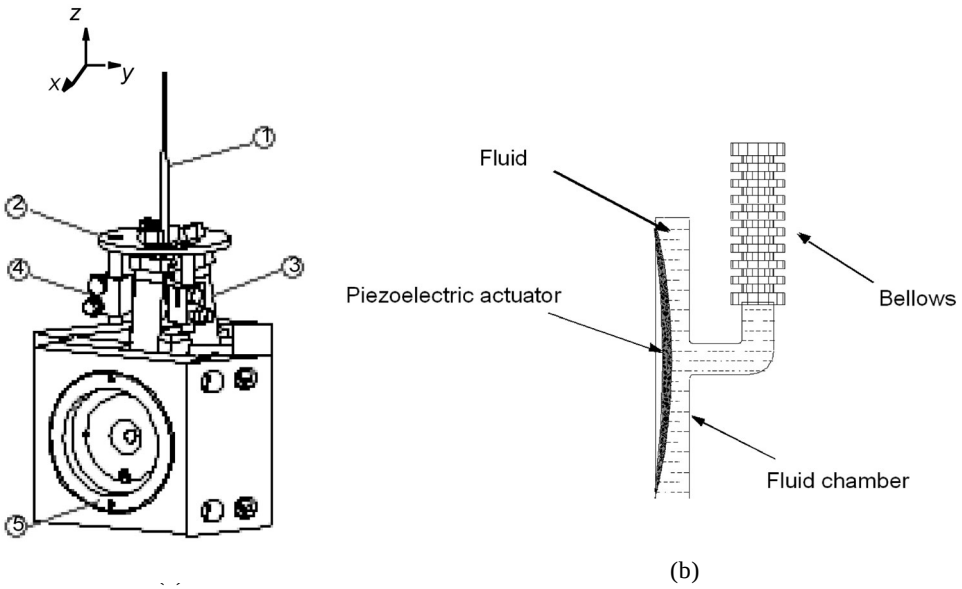


Fig. 6. (a) Structure of the piezohydraulic micromanipulator: 1) microcapillary, 2) mobile platform, 3) bellows, 4) permanent magnet of the pose measurement system, and 5) place for the piezoelectric actuator (fluid chamber). (b) Schematic of the piezohydraulic actuator.

4.1.2 MANiPEN

The second structure developed in ACI is a serial micromanipulator which is composed of two piezoelectric benders and a linear motor. The micromanipulator having three translation degrees-of-freedom is depicted in Fig. 7.

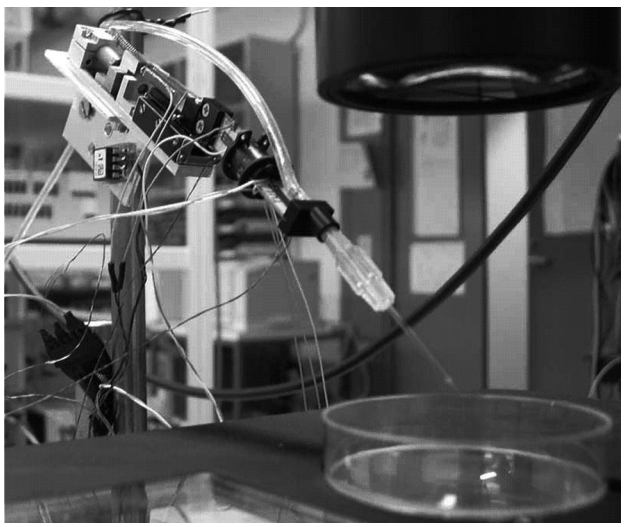


Fig. 7. MANiPEN micromanipulator.

The micromanipulator, MANiPEN, has been designed in a shape of pen such that several micromanipulators can be used simultaneously under an optical microscope. This facilitates simultaneous manipulations such as injection, isolation and electrophysiological recording. The use of several MANiPEN micromanipulators simultaneously is depicted in Fig. 8.

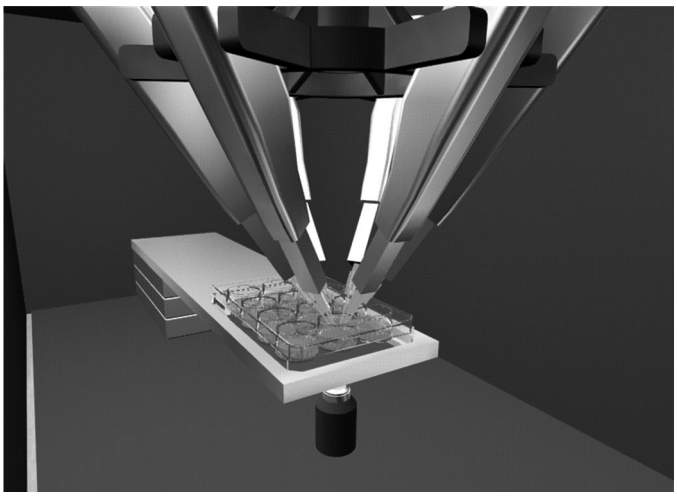


Fig. 8. Illustration of the simultaneous use of several MANiPEN micromanipulators.

4.2 Automatic Microinjection

4.2.1 Motion Control

Both task space and joint space motion control schemes have been implemented in the developed micromanipulator systems. The piezohydraulic micromanipulator has been controlled with a decentralised position feedback control scheme in task space, where the position of the capillary is measured using a vision system. The used visual servoing scheme belongs to the position-based strategies. The structure of the controller is depicted in Fig. 9.

The controller essentially consists of three independent single-input / single-output (SISO) joint controllers and a static nonlinear decoupling block (an inverse Jacobian matrix). In addition, it includes a coordinate transform element which transforms the task space variables (the position of the end-effector*: x*, *y*, *z*) to the control variables (the pose

of the mobile platform: α, β, Δz_m) of the micromanipulator, and a machine vision system which measures the position of the microcapillary.

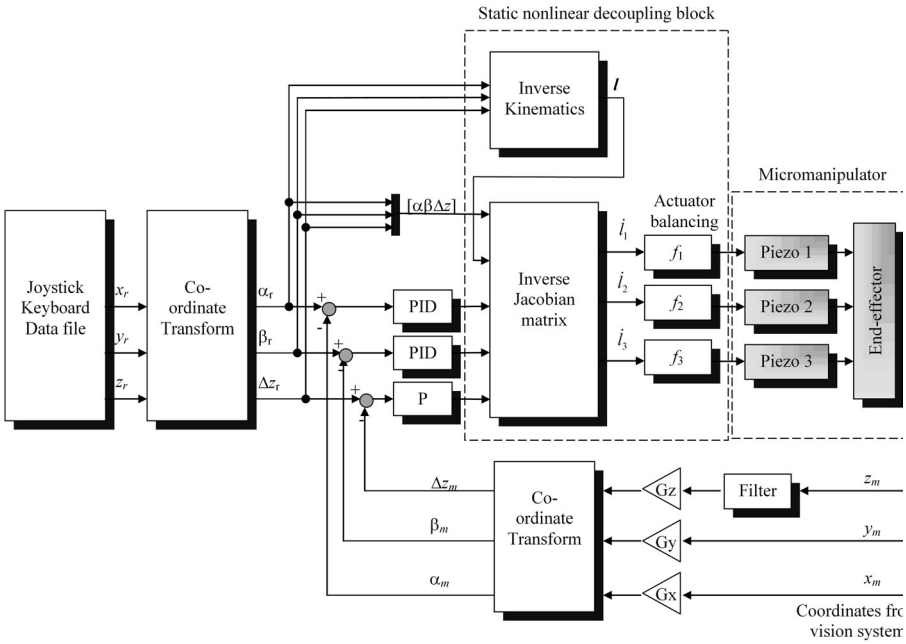


Fig. 9. Structure of the decentralised task space position feedback controller.

The steady-state accuracy of the piezohydraulic micromanipulator using the proposed task space controller is ± 1 pixel in the *xy* plane. Since the measurement is based on the vision system, the magnification used has an influence on the accuracy. With a 100x magnification, the ± 1 pixel accuracy corresponds to the spatial accuracy of 1,7 micrometres along the *x* axis and 3,3 micrometres along the *y* axis. If the magnification is 250, the accuracies are 0,7 micrometres and 1,3 micrometres, correspondingly. To demonstrate the steady-state accuracy using the different magnifications, the micromanipulator was moved along two rectangular trajectories in the *xy* plane. The larger trajectory shown in Fig. 10 is measured using the magnification of 100 and the

smaller using the magnification of 250. The accuracy is ± 1 pixel in both experiments. The performance of the piezohydraulic micromanipulator is summarized in Table 1.

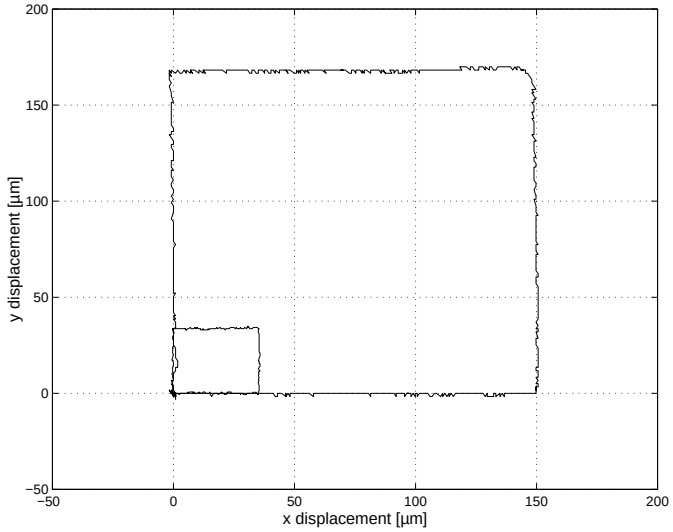


Fig. 10. Accuracy of the piezohydraulic manipulator using different magnifications. The smaller rectangle is measured using the magnification of 250x and the larger using 100x magnification.

Table 1: Performance of the piezohydraulic micromanipulator.

Quantity	**Value**
Accuracy	Steady-state accuracy: ± 1 pixel in the xy plane. With the 100x magnification: 1,7 micrometres and 3,3 micrometres along the x and y axis, respectively.
Repeatability	Depends on the magnification. With the 100x magnification: 1 and 2,5 micrometres along the x and y axis, respectively
Resolution	Better than 10 nm
Workspace	An ellipsoid, the length of the semi-axes of which are 250 x 250 x 100 μm^3.
Maximum speed	120 µm / s

The MANiPEN micromanipulator is controlled using a decentralised position feedback control scheme in joint space. The control scheme is composed of an inverse kinematics block and three independent SISO controllers as depicted in Fig. 11. The

parameters of the inverse kinematics model are derived by calibration for each micromanipulator.

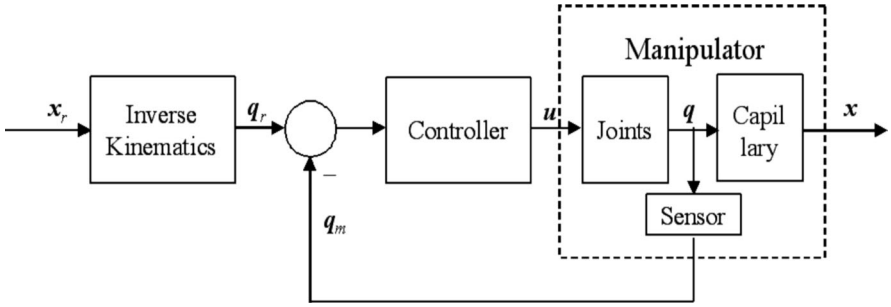


Fig. 11. Decentralised position feedback control in joint space for the MANiPEN micromanipulator.

The control software, MART, of the MANiPEN micromanipulator runs in a real time Linux environment in a desktop PC to ensure a successful execution of the control operations at a frequency of several kilohertzs. On the highest level, the software has been divided into two parts: a user interface and a controller. The controller is a real time task running in a Linux kernel and it is responsible for executing the time-critical control operations. The user interface interacts with the user and relays the commands given by the user to the controller. [78]

The repeatability performance of MANiPEN controlled using MART and the proposed joint space control scheme is shown in Fig. 12. The overall performance of the system is summarised in Table 2.

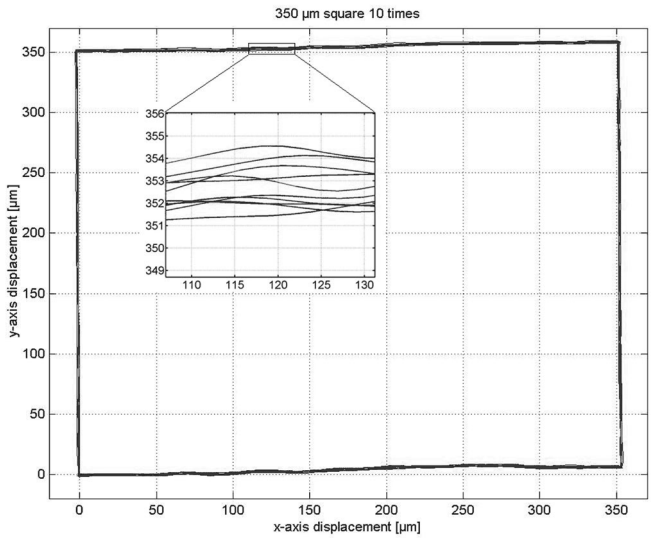


Fig. 12. Repeatability of MANiPEN when a 350 µm square is driven 10 times.

Table 2: Performance of MANiPEN.

Quantity	Value
Accuracy	3 µm with calibration
Repeatability	3 µm
Resolution	200 nm
Workspace	5x3x11 mm^3
Speed	5 mm/s

4.2.2 Microinjection

In addition to the free motion control, the research group has studied the automation of the capillary pressure microinjection method. Traditionally, the operator controls the micromanipulator using a joystick. The vision system provides visual information about the microcapillary and cells. When the operator detects a contact between the tip of the capillary and the cell membrane, he/she stops the micromanipulator and pushes a button to automatically perform intracellular injection. The micromanipulator first advances the microcapillary through the cell membrane. A precise, fast and straight penetration movement is crucial for successful microinjection. Then, the microinjector generates a pressure pulse to eject a desired amount of substance into the cell and finally, the micromanipulator removes the capillary from the cell.

The biggest bottle-neck for a versatile and fully automatic microinjection system is the lack of information on when the capillary tip is in contact with a cell. Due to the extremely small size of the capillary tip (less than one micrometer in outer diameter in adherent cell injections), the operator cannot reliably detect when the tip of the capillary is in contact with the cell, cannot detect small breakages of the tip and tip clogging, does not know if the injection is successful and does not know the amount of the injected compound.

To provide the operator with information which assists him/her in the cellular injections, an injection guidance system has been developed. Currently, the system provides information about a contact between the cell and the capillary, it detects a broken capillary, a clogged capillary, an aged measurement electrode and a faulty injection solution. The injection guidance system utilises a measurement signal provided by an impedance measurement device, which measures the impedance of the capillary by

supplying a known square-wave voltage signal between a measurement electrode placed inside the injection capillary and a reference electrode placed in a cell culture well and by measuring the current. A block diagram of the contact detection device is depected in Fig. 13. The *Stimulus Processing and Scaling* block includes adjustable scaling and buffering of the stimulus signal. The stimulus signal is generated with the control software of the manipulator system. The *Current Measurement* block consists of a sensitive current-to-voltage converter and a differential amplifier. The *Signal Conditioning and Amplification* block includes a low-pass filter and an adjustable output amplifier. The actual system implementation of the impedance measurement device is separated into two parts: a head stage and a control unit. The head stage placed next to the capillary includes the current-to-voltage converter circuit and the control unit includes all the other functions of the device. A more detailed description of the device can be found in [79].

The MART software determines the contact and the capillary condition using two indicators: a change in the square-wave signal amplitude and a change in its offset [80]. The user-interface of the software guides the operator by informing about the contact and giving suggestions for changing the electrode and the capillary and cleaning the capillary [81]. An example of the measurement signal recorded in a cell-capillary contact is shown in Fig. 14. .

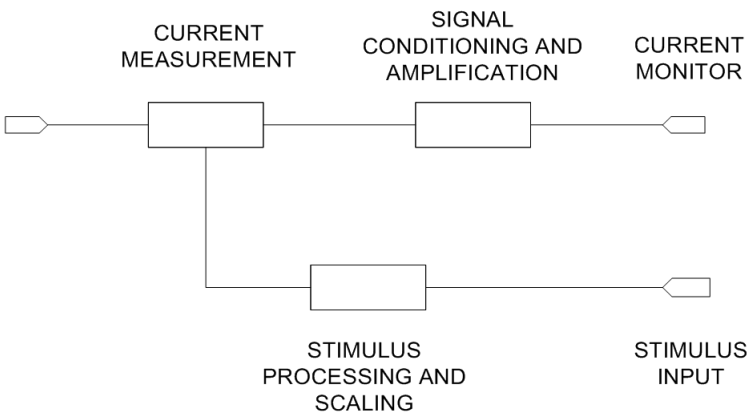


Fig. 13. Block diagram of the contact detection device.

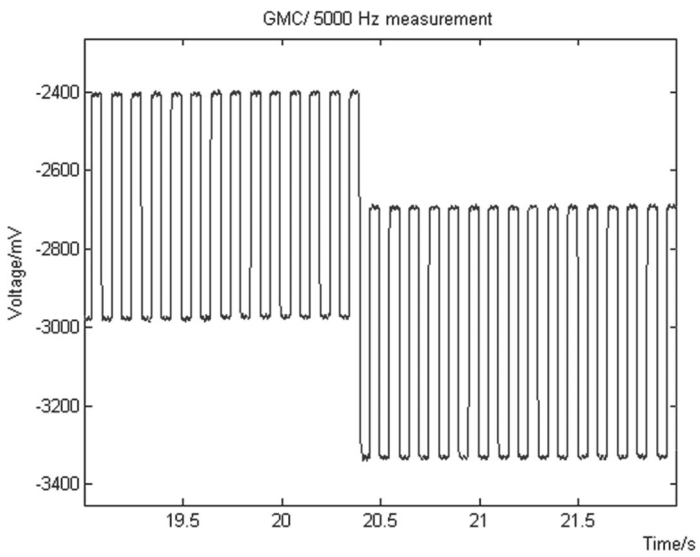


Fig. 14. Change in the measurement signal in a cell-capillary contact.

4.3 Evaluation of Injection Success

The final goal of the research is to develop a high-throughput reliable and repeatable cell micromanipulation system. Before the system can be used by cell biologists, the harmlessness of the injection method must be proved. Furthermore, the success rate[1] of the injection must be known. Thus, the goals of the evaluation study are to determine (i) the success rate of the developed microinjection methods and (ii) the cell survival rate.

A procedure to reliably evaluate the cell survival and injection success rates in capillary pressure microinjection is needed to compare different methods and to quantify the improvements of the systems to be developed. An evaluation process for capillary microinjection has been proposed in [82]. It includes a study on the success rate of delivering the injection substance inside the cell and a study of the combined injection success and cell survival rate. In the evaluation of the injection success rate, the cell survival is not studied but only the delivery success is evaluated.

In the injection success experiments, a fluorescent dye FITC (Fluorescein Isothiocyanate) has been injected into MCF-7 cells. 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,

1. The success rate describes the probability that the substance is successfully delivered into the cell.

penicillin-streptomycin, 10 ng/ml insulin, and 1nM 17 -estradiol. The day before microinjection, cells were plated in 12-well plates at a density of 5 × 104- 7 × 104 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 17ß-oestradiol and 2mM L-glutamine. The fluorescent dye FITC was used as an injection substance, since a successful injection is easily detected using a fluorescent light after the injection. Two injection methods have been tested. A “penetration method” is the conventional “stabbing“ method used by conventional semi-automatic CPM systems [83], [84]. In the method, 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. The second method is a “contact method”, which is similar to the SLAM method [21]. Our experiments have confirmed that while the contact between the cell and the 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. In the experiments, both methods were first used without the injection guidance system and the method which showed a lower performance was used with the injection guidance system. Table 3 summarizes the results of the experiments.

Table 3: Comparison of success rates of different injection methods.

Method	Number of injected cells	Number of successful injections	Injection success rate
Penetration injection without IGS*	88	35	40 %
Contact injection without IGS*	58	17	30 %
Contact injection with IGS*	55	36	65 %

*)IGS = the developed injection guidance system

As can be seen, by using the injection guidance system, the injection success rate was raised to 65 % which is significantly higher than 30 % and 40 % success rates without the system.

The combined cell survival and injection success rate is studied by injecting plasmid DNA (pBabe-Gem2) containing a Green Fluorescence Protein (GFP) gene. After approximately 24 hours, the cell starts to express GFP if the injection has been successful and if the cell has survived the injection. Fig. 15 illustrates MCF-7 cells expressing fluorescent light after injection of GFP genes. The combined cell survival and success rate is approximately 40 % without the injection guidance system [85].

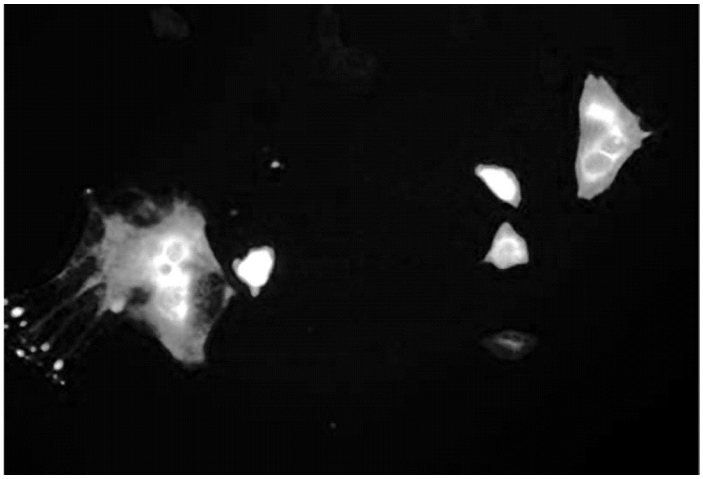


Fig. 15. GFP injected MCF-7 cells 24 h after the injection.

5 Conclusions

The development of automatic manipulation techniques for single adherent cells will be of increasing importance in the future. In such applications as functional genomics, in-vitro toxicology, cancer and HIV research and drug development, advanced microrobotics systems that facilitate parallel operations in a fast and precise manner will be needed.

Current micromanipulation systems for adherent cells are still too tedious for a large-scale use by scientists and industry. Therefore, performance enhancement and miniaturisation of the micromanipulators and automation of the operations are important development issues. The performance enhancement includes the raise of the speed but not at the expense of the accuracy. This might require the application of more advanced

motion control schemes in the micromanipulation systems. Miniaturisation is necessary to facilitate the use of several micromanipulators simultaneously under an optical microscope. Automation brings in several challenges not only in the positioning of the end-effector but also in microinjection, visualisation, automatic detection and cell cultivation. In capillary pressure microinjection, for example, the accuracy and repeatability of the injected volume must be improved. Current open loop systems are not sufficient but microsystem-based closed loop systems should be developed. Related issues include influx of the medium and cell aggregates into the microcapillary causing clogging, and efflux of the injection substance from the capillary into the medium in between the injections.

The research work in the Institute of Automation and Control at the Tampere University of Technology aims at the development of a fully automatic microinjection system for adherent cells. The system consists of two micromanipulators - a serial structure and a parallel structure - and their control system, a microinjector, a machine vision system developed by the Technical Research Centre of Finland (VTT) and a cell cultivation system. The control system includes motion control algorithms for precise positioning of the microcapillary and penetration of the cell membrane and an injection guidance system to guide the operator during the injections. Cell injection experiments have shown that the use of the injection guidance system can significantly increase the success rate of injections. The future work includes increasing the number of injections and to study the combined injection success and cell survival rate when using the injection guidance system.

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