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FORCE CALIBRATION OF MICRO PIPETTES FOR SINGLE-CELL PROBING

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Abstract: Force calibration of micro pipettes is described used for the application of mechanical stress to isolated ventricular cardiomyocytes which are immobilized on glass substrates. For this purpose two methods have been developed based on a nano-Newton compensation balance at PTB and cantilever-type silicon sensors used as transferable force standards, respectively.

Keywords: micro pipette, force-calibrated single-cell probe, transferable micro force standard.

1. INTRODUCTION

Increasing attention has been paid over the last decade in biomedical engineering to the application of forces on cells giving rise to phenomena such as cellular motility and morphogenesis or intracellular trafficking. Dilation of human heart ventricle is under study to find out mechanisms of stretch-induced arrhythmias followed by ventricular fibrillation and sudden cardiac death [1].

Conversion of mechanical forces into biochemical signals and integration into cell response is referred to as mechanotransduction [2]. In these experiments stress of typically some hundred Pascals is applied on either a group of cells or on single cells eliciting strain levels around 10 % at a typical Young's modulus of a cell of 1 kPa. Groups of cells are cultured on membranes and cantilevers or embedded in a gel [2 - 4]. Optical and magnetic traps, microprobes or aspiration through micropipettes are prominent techniques for applying mechanical stress on single cells [2, 5 - 7]. Correspondingly, methods have been developed to measure forces in the nN-mN range at the cellular level and correlate it with biochemical responses [5, 8].

Glass micro pipettes manipulation has been used to investigate cell-matrix adhesion of cells attached to a substrate [9]. Alternatively, mechanical stress can be applied locally to single cardiomyocytes using attached carbon fibers or micro pipettes [1, 10]. Cell elongation or contraction upon movement of the attached micro pipette can be visualized by confocal laser scanning microscopy (CLSM) with the cell membranes or other organelles stained using a marker substance [1]. For measuring the applied forces or the cell response to manipulation the probe can be mounted on a piezoelectric or piezoresistive force sensor [8, 11, 12]. Alternatively, the applied force can be calculated from the deflection of the cell-attached micro pipette. However, fabrication of micro pipettes which is done by pulling of glass styli may result in considerable tolerances of the pipette radius, i.e. the pipette stiffness k_{pip}. Therefore, before proper application of such micro force sensors an individual calibration of each probe is mandatory.

Values of k_{pip} amounting typically to several tens of nN/µm can be estimated by measuring the deflection of the pipette tip loaded by wires, small crystals or polystyrene beads of known mass [7, 9, 13, 14]. However, such methods appear to be rather indirect, tedious and prone to error owing to drift or variation of ambient conditions. To overcome these drawbacks we developed computer-controlled calibration procedures which are based on a compensation balance and a silicon cantilever-type force sensor (Fig. 1).

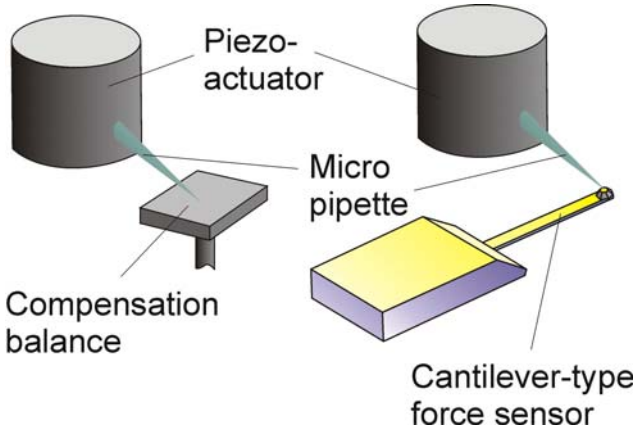

Fig. 1. Micro pipette calibration using a nano balance (left) and a transferable cantilever-type force standard (right)

2. CALIBRATION SETUP

For calibration we used two techniques: (1) In the nano-force measuring setup shown in Fig. 1 (left side), the pipette

is mounted on a piezo-actuator and incrementally moved against a compensation balance with nN resolution [15]. (2) In the right part of this figure a silicon cantilever comprising a piezoresistive strain gauge is shown which is employed as a transferable force standard operating in the µN range at an uncertainty of sub-µN [16].

2.1 Nano balance

Calibration of micro pipettes using a compensation balance is performed by incrementally moving it with its tip against the weighing pan of the balance (Fig. 2). The pipette is roughly positioned to the pan using a 2D piezoactuator (Picomotor actuator 8302, Newfocus, San Jose, USA). Subsequently, it is lowered until it is in contact with the pan. Now calibration starts by incremental movement of the pipette against the pan in steps of 250 nm at a resolution of 1 nm and a reproducibility of 5 nm using a piezoactuator with capacitive feedback (P 721, Physik Instrumente (PI), Karlsruhe, Germany). The procedure is stopped when a maximum force of 1 µN is attained as measured using a compensation balance offering a resolution of 1 nN and a reproducibility of 2.5 nN (SC2, Sartorius, Göttingen, Germany). The pipette is tilted to the pan by an angle of 30°. Therefore, the pipette stiffness is given by the slope of the load-deflection curve multiplied by cos(30°) = 0.866. The complete setup is mounted in a thermally isolated box ensuring a temperature drift of less than 10 mK/h. During one calibration run (typically 30 min) a temperature drift within 2-5 mK can be maintained. The stiffness of the balance (10^4 N/m) is much higher than the cantilever stiffness. Therefore, its effect on the calibration can be neglected.

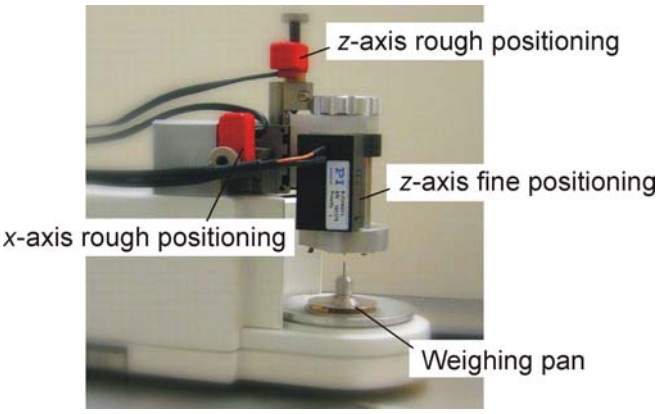

Fig. 2. Force calibration setup based on a nano balance

2.2 Cantilever-type force sensor

Cantilever-type force sensors are realized using a bulk micromachining process based on standard photo-lithography, wet etching and diffusion from a spin-on-source (Fig. 3, [15]). The process is started by etching of an *n*-type silicon wafer (2-5 Ωcm) using TMAH solution (tetra methyl ammonium hydroxide, 20%, 80 °C) through a mask of thermal oxide to obtain a membrane structure. Subsequently, *p*-type resistors are realized by boron diffusion from a spin-on silica emulsion source (Emulsitone Borofilm 100, 1100°C). For improved contact formation p^+-type regions are fabricated in a second diffusion step (1200°C). Compared to other doping techniques like implantation and deposition diffusion neither induces crystal damage nor requires toxic gases or hazardous materials.

Subsequently, the probing area is defined at the cantilever free end. By undercut etching of a circular oxide mask using TMAH a truncated pyramid is generated having an octagonal base and sidewalls represented by the fastest etching planes, i.e. {133} facets. During this step, simultaneously, the membrane is thinned to its final thickness.

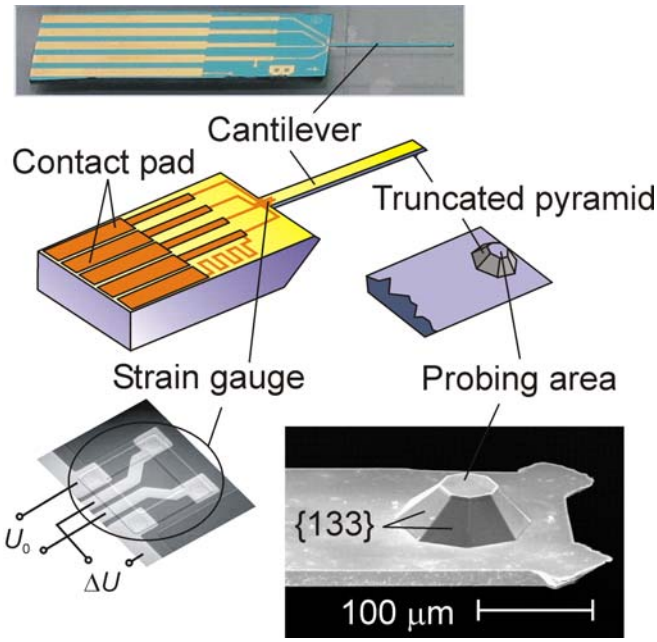

Fig. 3. Transferable force standard based on a silicon micro cantilever with integrated strain gauge and probing tip

After probing area definition the wafer is oxidized and patterned for contact holes to the p^+-type regions of the piezoresistors. A gold/chromium metallization is deposited by e-beam evaporation. The cantilever is released wet chemically using potassium hydroxide solution (KOH, 30 %, 60°C). We prefer KOH during this final etching step due to its lower mask undercut compared with TMAH. A protection of the connecting lines during this step is not necessary. The resulting cantilevers are 5 mm long, 200 µm wide and 26-28 µm thick.

For calibration the sensor is mounted into a connector designed for simultaneous load-deflection and load-strain gauge output voltage measurement. Electrical connection is attained by pressed contacts formed between the connector pins and the Au/Cr pads on the sensor chip. Thus wire bonding can be omitted. The bridge output voltage is measured at a supply voltage of 1 V using a low-noise bridge amplifier (ML10B, HBM Mess- und Systemtechnik, Darmstadt, Germany).

In Fig. 4a typical force-deflection and strain-gauge-output-voltage characteristics of a cantilever sensor (D47B) are displayed. We observe linear increase of force and simultaneous decrease of output voltage with increasing z immediately after the probing area has touched the weighing pan. By least-squares fitting within 1-5 µN we obtain the slopes which correspond to the cantilever stiffness and the sensor sensitivity, respectively. A large number of calibration runs is performed and analyzed correspondingly. The results are shown in Fig. 4b. By averaging we find values of 1.383 ± 0.003 N/m and -0.0826 ± 0.001 mV/µN for the stiffness and the sensitivity, respectively.

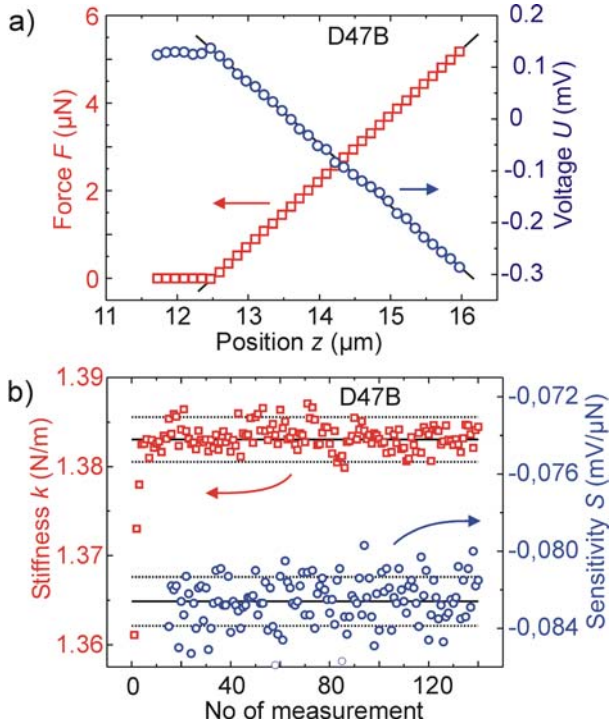

Fig. 4. Typical calibration curves of cantilever sensor D47B (a) and stiffness and sensitivity extracted from 140 calibration runs (b)

The calibration results obtained for two sets of cantilever sensors are summarized in Table 1. The cantilever thickness is between 25 µm and 28 µm resulting in a stiffnesses between 1.3 N/m and 1.6 N/m and sensitivities between -0.075 mV/µN and -0.086 N/m. These values are obtained by averaging over typically 30-100 calibration runs performed for each sensor. The residuals of the linear fitting averaged over all calibration runs with each sensor are taken as a measure of the force resolution F_{res}. We find values between 0.04-0.1 µN in the range of 1-5 µN. In the higher force range (10-50 µN, sensor B1) resolution amounts to 0.1 µN.

Table 1. Calibration data of cantilever sensors.

Sensor	h (µm)	k (N/m)	S (mV/µN)	F_{res} (µN)
D46A	27.6	1.426	-0.0752	0.07
D46B	27.2	1.416	-0.0793	0.06
D46C	26.4	1.302	-0.0825	0.05
D46D	24.5	1.037	-0.0606	0.1
D47A	26.9	1.383	-0.0826	0.04
D47B	27.7	1.493	-0.0818	0.04
D47C	28.1	1.557	-0.0863	0.06
B1	44.5	6.215	-0.0299	0.1

3. MICRO PIPETTE CALIBRATION

Micro pipettes fabricated by pulling (DMZ Universal Puller, Zeitz Instrum., Munich, Germany) glass styli (borosilicate glass capillaries, Hilgenberg, Malsfeld, Germany) of diameters typically within 20-40 µm are investigated (Fig. 5).

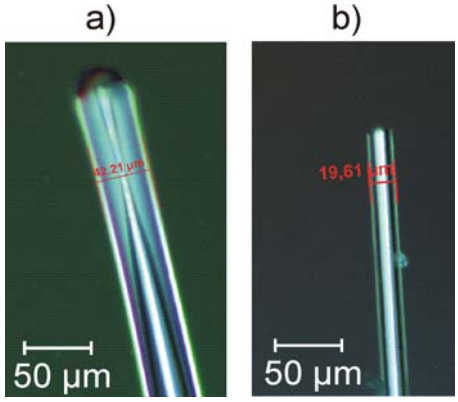

Fig. 5. Photographs of a micro pipettes with closed (a) and open (b) tip, respectively

3.1 Nano balance

Slight deviation from the optimum pulling parameters can be expected to result in considerable variations of the pipette stiffness which depends on the radius to power of four. This is confirmed by calibration of six pipettes using the described nano-balance setup. A typical force-deflection curve measured with a pipette is shown in Fig. 6. The pipette stiffness which results from its slope multiplied by cos(30°) amounts to 0.0663 ± 0.0002 N/m.

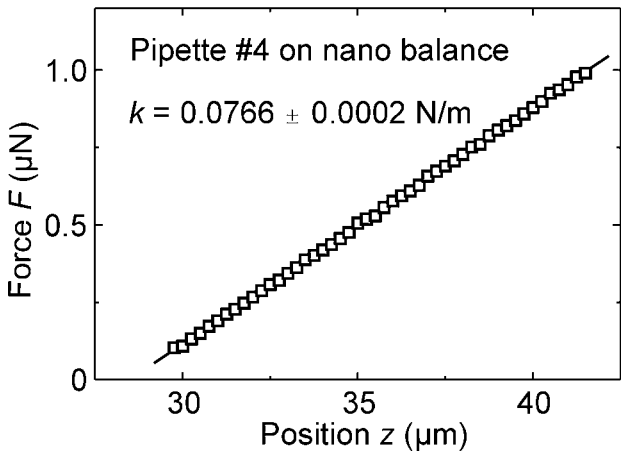

Fig. 6. Typical load-deflection curve of a micro pipette

The results obtained with additional pipettes are presented in Table 2. Variations of the measured pipettes stiffness from 0.07 N/m to 0.33 N/m are observed confirming that to be used as a force sensor individual

calibration of each pipette is mandatory. Repeated measurements show a reproducibility of calibration of 5 %. Values of 4-5 nN are found as the average of the residuals to the linear fit corresponding to the achieved force resolution between 0.1-1 µN.

Table 2. Calibration data of micro pipettes.

Pipette	*k* (N/m)	no. of meas.
#1	0.1895	87
#2	0.2160	61
#3	0.2073	124
#3	0.2078	48
#4	0.0663	31
#4	0.0675	58
#4	0.0710	46
#5	0.3305	98

3.2 Cantilever-type force sensor

Force calibration of a micro pipette using a cantilever sensor is performed by probing the cantilever at the top plane of a truncated pyramid (probing area, Fig. 3). Positioning and movement of the pipette is performed using a micro manipulator (Sutter MP 285, Novato, CA, USA). The measured deflection depends on both cantilever and pipette stiffness:

$$\frac{1}{k_{\mathrm{m}}} = \frac{1}{k_{\mathrm{pip}}} + \frac{1}{k_{\mathrm{cant}}} \qquad (1)$$

Simultaneously, the force applied to the cantilever is monitored by the piezoresistive strain gauge output of the cantilever sensor which is amplified (GSV-2ASD, ME Messsysteme, Hennigsdorf, Germany) before read out. A typical calibration procedure with a micro pipette (#4) using a cantilever sensor is shown in Fig. 7.

The pipette positioned just above the probing area of the cantilever and after touching the cantilever are displayed in the left and right parts, respectively, of Fig. 7a. We find a linear dependence of the cantilever force read out on the pipette position *x*, yielding k_{m} = 0.067 N/m (Fig. 7b). With k_{cant} = 1.383 N/m of cantilever D47A (Table 1) we obtain a bending stiffness of the pipette of k_{pip} = 0.071 N/m using eq. (1). This value compares very well with the nano-balance calibration results obtained with pipette #4 (Table 2). A maximum pipette deflection of 150 µm was applied corresponding to a force of 10 µN.

The uncertainties of the pipette manipulation Δx and the force read-out ΔF are the dominant contributions that have to be considered for an estimation of the uncertainty of the derived pipette stiffness:

$$\frac{\Delta k_{\mathrm{pip}}}{k_{\mathrm{pip}}} = \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta F}{F}\right)^2} \qquad (2)$$

The uncertainty of the cantilever stiffness is negligible since $k_{\mathrm{cant}} >> k_{\mathrm{m}}$. By repeated measurements we find reproducibilities of $\Delta x/x \approx \Delta F/F \approx 5$ % leading to $\Delta k_{\mathrm{pip}}/k_{\mathrm{pip}} \approx 7$ % using eq. (2).

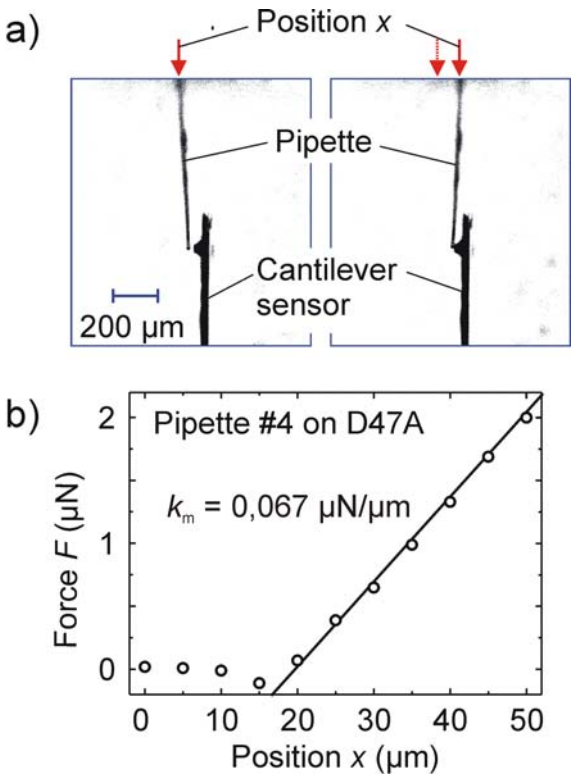

Fig. 7. Calibration of a micro pipette (#4) by probing a cantilever-type force sensor (a). The corresponding force-deflection curve with D47A on pipette #4 is shown in (b)

In Table 3 the results of micro pipette calibration obtained with the nano balance and the cantilever force sensor are collected. The described tools offer a force range of 100 nN to 50 µN. Depending on the selected range a resolution of 0.1 µN down to 4 nN is achieved. The reproducibility amounts to 5-7 %. The main advantage of the cantilever force standard is its small weight and volume. which means that it can easily be transferred to the setup used for cell manipulation. Calibration of micro pipettes can thus be performed immediately before and after each manipulation experiment.

Table 3. Comparison of force calibration methods.

Parameter	nano balance	cantilever	
Range (µN)	0.1-1.0	1-5	10-50
Resolution (nN)	4-5	40-100	100
Reproducibility (%)	5	7	-

4. PROBING OF CARDIOMYOCYTE

Probing experiments are performed with isolated ventricular cardiomyocytes immobilized on glass by poly-L-lysin. Fixing is done by a patch pipette of high stiffness positioned at one end of the cell. This pipette has an open tip patch and may be filled by an electrode solution for whole cell clamp recordings [1]. At the other end the movable micro pipette is attached to the cell at an angle of 45° (Fig. 8a).

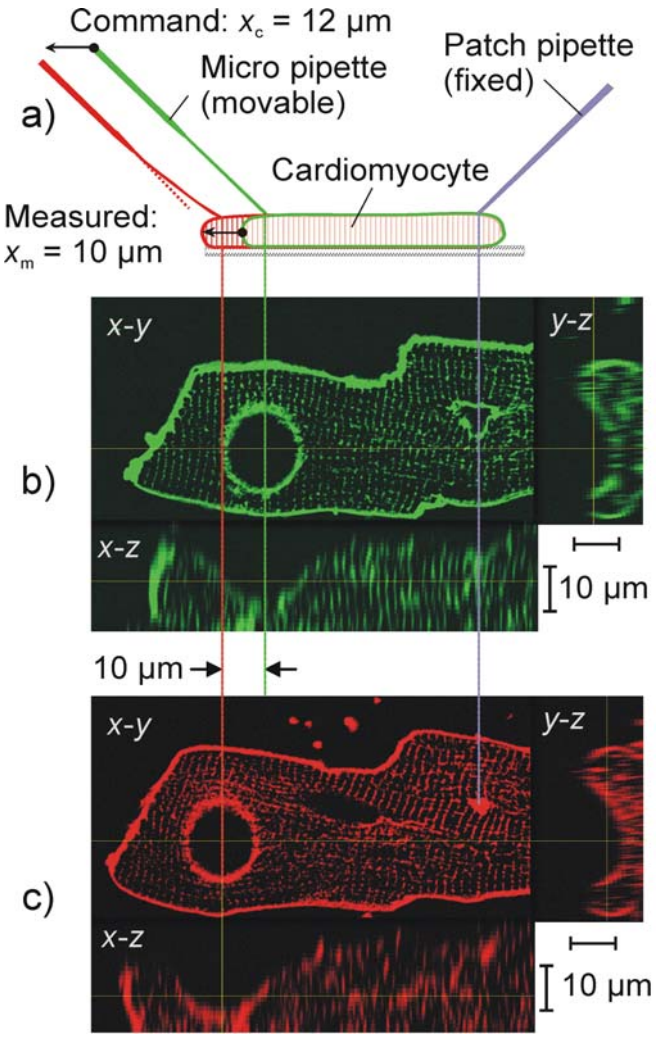

Fig. 8. Stretching of a cardiomyocyte using a calibrated micro pipette (#4). Schematic of the procedure (a), cell before (b) and after stretching (c)

For fixation and movement of the pipette a micromanipulator is employed as described above. For visualizing the cell by CLSM (Bio-Rad Radiance 2000, Hertfordshire, UK) it is stained with DI-8-ANEPPS yielding pseudo color images of the cell membranes and the transversal tubuli marking the sarcomeres. The lateral resolution amounts to 0.1 µm. Figures 8b and c show cell images after probing with a micro pipette (b) and horizontal stretching (c). The cell area attached to the pipette is indicated by the full circles in *x*-*y*-images and the open segments in the vertical cross sections (*x*-*z*, *y*-*z*). From the measured position difference x_m with respect to distance between the movable and the fixed pipettes we find a stretching of 20 %. The sarcomere length in peripheral regions increases from to 1.7 µm to 1.8 µm corresponding to a local strain of 6 %. This deviation can be assigned to a spatially non-uniform distribution of the cell deformation in lateral as well in vertical directions [1].

The force applied to the cell by the calibrated pipette during horizontal stretching can be computed from its bending:

$$F = (x_c - x_m)\frac{k_{pip}}{\cos(45°)} \qquad (3)$$

which is given by the difference of the movement command to the manipulator x_c and the measured elongation of the cell x_m. With the values given in Fig. 8 and k_{pip} = 0.071 N/m we obtain F = 0.2 µN for the horizontal stretching force.

The micro pipette can also be used to apply vertical stress to the cell. For a spherical tip geometry the contact formation is described by the Hertzian model and the force-indentation dependence is given by [6]:

$$F = c\frac{16E}{9}\sqrt{R\delta^3} \qquad (4)$$

with the Young's modulus E, the tip radius R and the indentation δ. As safe for most biological samples incompressibility, i.e. $\nu \approx 0.5$ is assumed for Poisson's ratio. In our experiment with a tip of $R \approx$ 12-13 µm on a 25 µm-thick cell we find $\delta \approx 4$ µm at an indentation force of 0.1 µN and $\delta \approx 4$ µm at $F \approx 0.2$ µN. Force values are estimated from the vertical deflections of the pipette. The constant c amounts to 1.3-1.5 if the sample is bonded to the substrate or not, respectively [6]. Inserting these values into eq. (4) we obtain $E \approx$ 900-1600 Pa in agreement with the expectation [2].

5. CONCLUSION

In this study force calibration of micro pipettes is described which are used for the application of mechanical stress to isolated cardiomyocytes. For this purpose two methods are employed based on a nano-force measuring setup at PTB and transferable cantilever-type force standards, respectively. Using the nano balance calibration was performed in the sub-µN range at a resolution of some nN. The cantilever sensor which can be transferred directly to the cell probing setup enables a calibration immediately before and after a cell probing experiment. The resolution here amounts to better than 0.1 µN in a range of 1-50 µN. The reproducibility of both methods is between 5-7 %. As an application example stress is applied on single cardiomyocytes using a force-calibrated micro pipette.

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