Effects of micro and nano structured surfaces on the cell adhesion using single cell force spectroscopy

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Introduction

Cochlea implants (CI) restore hearing in profoundly impaired patients using electrical stimulation of the auditory nerve in the inner ear (Clark, 2003). To achieve this, sound waves are detected via a microphone, which is placed near the outer ear. The sound is than processed and translated into electrical signals by a speech processor and directed through an electrode array (made of platinum) into the inner ear, where the electrodes come in close contact with the auditory nerve (Fig. 1). The electrode contacts are embedded in an electrode carrier made of silicone (Fig. 2).

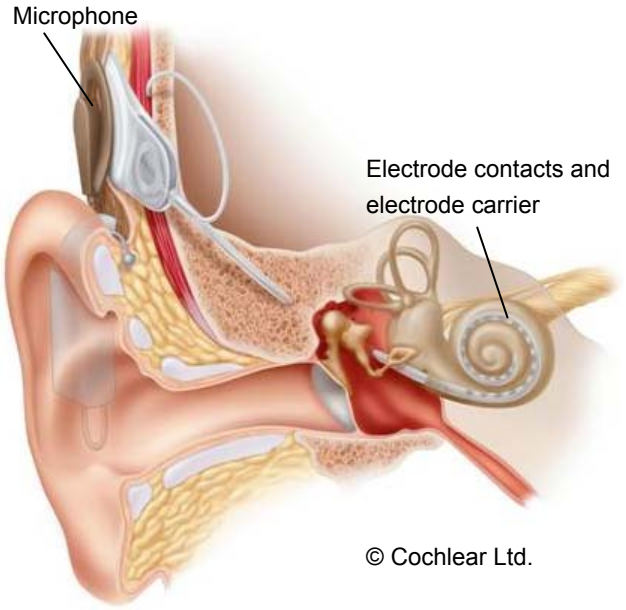

Fig. 1: Components of a Cochlea Implant and their position inside and outside the ear.

As a result of the surgical insertion of the electrode array, an increase of the contact impedance between the electrodes and the nerve is observed 2-3 weeks post operative. Investigations show that this increase of impedance may be due to a growth of the connective tissue around the electrode array of the CI. Thus, there is a need of new electrode surfaces which significantly inhibit fibroblast (main part of the connective tissue) growth.

Micro- and nanostructuring of substrate surfaces affect the interaction of cells with the substrates (Biggs et al. 2007a, 2007b, Reich et al. 2008). For example, surface topographies with hydrophobic properties have an inhibitory effect on the cell growth and the cell morphology is changed (Schlie et al. 2009). This can be used to control cell adhesion and cell growth on implant surfaces.

Fig. 2: CI-electrodes contain contact rings made of platinum and a silicon electrode carrier.

Investigation of interactions between cells and surfaces on a molecular level may lead to answers for two main questions in implant-related research: first, how does the micro- and nanostructuring of the surfaces affect the cell adhesion? And then, is it possible to generate surface topographies which have an inhibitory effect on the adhesion and growth of the connective tissue to the substrates?

Single Cell Force Spectroscopy (SCFS) of Atomic Force Microscopy (AFM) represents a unique method to investigate the interactions between cells and substrates. This method allows the investigation of the cell adhesion using different quantitative parameters, such as the maximum adhesion force (F_{max}), the work of the de-adhesion ($W_{de\text{-}ad.}$) and the number and formation of single

cell substrate binding sites (t and j events) (Fig. 3) (Franz CM et al. 2007).
In the following report we will share our experimental experiences and some results with other research groups working with the same method.

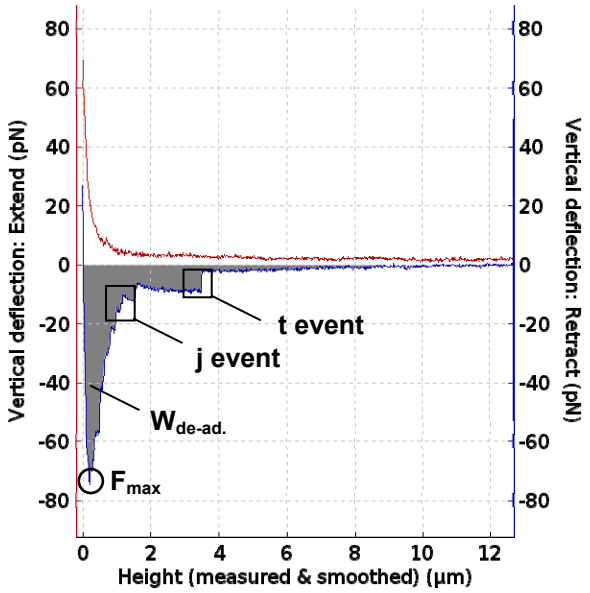

Fig. 3: Different quantitative parameters of a force displacement curve for the characterization of the cell adhesion.

Material and Methods

We used NIH3T3 cells lentivirally modified to express GFP for fluorescence labelling as a model for our *in vitro* tests. To attach the cells to the cantilever (Arrow TL1 tip less cantilevers) we functionalized the cantilevers with a collagen mixture (Calbiochem, Germany, #234112). A JPK-AFM (NanoWizard II) with the CellHesion module was used to measure the cell adhesion. The BioCell module allowed us to measure the cell substrate interactions at 37°C in serum free Medium (DMEM, Biochrom AG, Germany, # FG0445). For the optical control over the whole investigation we used an inverse light microscope (Axio Observer 200, Zeiss).

Cell adhesion was measured on several surface topographies, which were generated using nanosecond and femtosecond lasers in *Laserzentrum* in Hannover (LZH) (Fig.4). Non-structured surfaces made of the same materials (silicone and polished silicon) were used as controls. The roughness of the control surfaces (silicone ~ 150 nm and Si ~ 20nm) was determined using AFM (data not shown here).

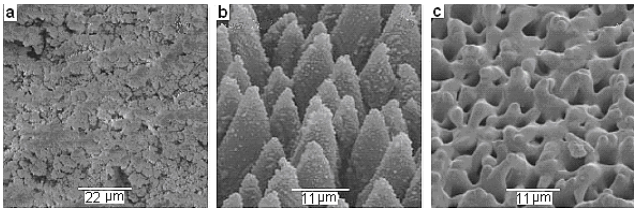

Fig. 4: SEM image of the investigated micro structures. Silicone structured with nanosecond laser (a), silicon (Si) structured with femtosecond laser (Si-spike-stucture) (b) and the silicone mold of the Si-spike-structures.

Protocols

1. Sample preparation

Since the BioCell chamber has a limited area, in which the cantilever could be safely moved, there are some points to be considered.

- We recommend a sample size of 3*3*0.5 mm^3 (length*width*height).
- Place the samples in the left top side of the BioCell chamber (Fig. 5A, B) in order to have enough space for the cantilever calibration and for catching a single cell.
- For calibration and thermal stabilization of the cantilever place the cantilever at the right side of the BioCell chamber. This allows the applicator to manoeuvre without any collision between hard samples and the cantilever glass holder..

For best results thoroughly clean the sample with gentle air pressure and install it over the glass plate in the cleaned BioCell using a drop of industrial fat with high viscosity (Molykote, H. Costenoble GmbH, Eschborn, Germany). Fill the BioCell with medium and set the temperature to 37°C. To maintain the BioCell chamber clean and dust free, it can be covered until the cantilever is inserted into the medium.
After the BioCell reaches a temperature of 37°C, the cantilever can be inserted into the medium.

2. Cantilever preparation

- Functionalization

Give a drop of collagen into a Petri dish. Place a clean cantilever carefully into the collagen drop and incubate for 30-45 min at 37°C.

In order to rinse the collagen-coated cantilever dip it in a drop of PBS. A functionalized cantilever can be stored at 4°C in PBS for several days.

- *Cleaning* after use

Piranha solution (sulphuric acid 30% + water peroxide 30% 1:1) was used to clean the cantilevers. The cantilevers were placed for approximately 30 min in the piranha solution and were afterwards washed with pure distilled water for several times.

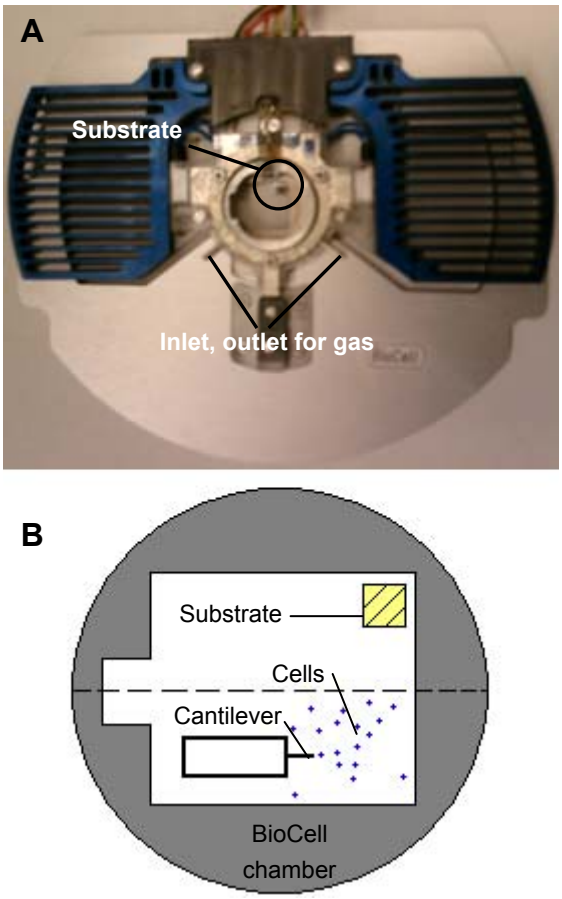

Fig. 5: A) BioCell module and the optimal position for samples. B) Schematic drawing of the BioCell chamber for the cell attachment procedure.

3. Cell Culture

Cell suspensions at a cell density of about 50 000 to 100 000 cells/ml were used for cell adhesion investigations. At this cell density a single cell can easily be caught avoiding the attachment of more cells to the cantilever tip. After removal of the medium cells were incubated with 0.3 ml trypsin for 4 min at 37°C. Then 1 ml of serum free medium plus 4 ml of PBS was added to the cells in order to stop the effect of the trypsin. Cells were then transferred into a falcon tube, which was stored on ice-water mixture. This avoided cell adhesion to the falcon tube so that they could be easily transferred into the BioCell chamber. The cells survived more than 2 hrs for the investigations.

4. AFM setup and cantilever calibration

After the BioCell was filled with 0.6 ml of serum free medium the medium was heated to 37°C. Then the JPK software was started in the CellHesion mode.

The functionalized cantilever was then installed on the AFM head using the extra long glass holder from JPK and was placed over the BioCell. In order to avoid air bubbles formation around the cantilever it was moistened with a drop of PBS before dipping it into the medium. The laser point was then positioned on the very front edge of the cantilever (Fig. 6) and was aligned in the middle of the four segment photo diode.

In order to measure forces, the cantilever has to be calibrated and the spring constant has to be estimated. In the JPK software (SPM desktop), which we used to calibrate the cantilever, the spring constant was estimated using the thermal noise method. This method is based on the equipartition theorem (Butt and Jaschke 1995). For good results before the cantilever was calibrated, the thermal fluctuations of the cantilever had to be stabilized. This is of importance, since changes of the environmental temperature cause a strain resulting to an alteration of the spring constant of the cantilever. We recommend a time of at least 45 minutes for the stabilization of the cantilever.

One can use the real time scan oscilloscope to observe the stabilization of the cantilever fluctuations. A draft of the vertical deflection in the negative direction shows that the cantilever is stretched over time after it is inserted into an

environment with higher temperature than the room temperature. After stabilization of this draft the spring constant of the cantilever can be estimated.

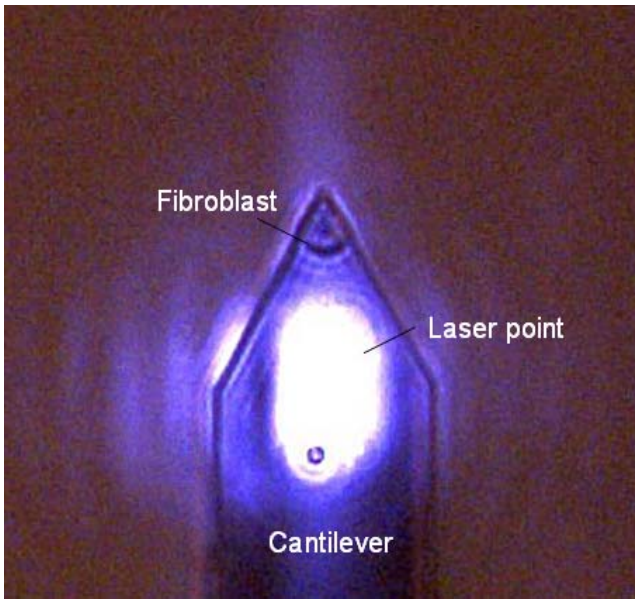

Fig. 6: Position of the laser point and the attached single cell on the Cantilever (100X magnification).

In order to measure the sensitivity and the spring constant of the cantilever, a single force displacement curve has to be recorded on a hard substrate, for example on glass. Extension/retraction velocities of 5 µm/s without any extended delay were very useful parameters for the cantilever calibration. Due to the fact that just the very last linear section of the extension curve was needed for the calibration, the pulling length was set to 10 µm.
First, the sensitivity of the cantilever was measured using the linear part of the extension curve. After the sensitivity was saved, the thermal noise behaviour of the retracted cantilever was recorded for 30 seconds and then took the second peak for the curve fitting due to the lower noise ratio. This peak occurred for the mentioned cantilevers in the used medium at a frequency between 5-10 kHz (in liquid). There is a correction factor for different peaks. For the second harmonic it is 0.251. For more information about the cantilever calibration, please, read Butt and Jaschke 1995 (Nanotechnology 6: 1-7) and the AFM user manual supplied by JPK.

5. Attaching a single cell to the cantilever
For inserting the cells into the BioCell chamber, the cantilever is elevated using the piezo motors so that it just does not leave the medium but allows a little gap between the glass holder and the BioCell chamber, where the pipette tip can be placed. Then 100 µl of the cell suspension was injected into the BioCell. For the insertion of the cell suspension, it was much more convenient to use the inlet/outlet for liquid on the sides of the BioCell chamber (Fig. 5). Just put the pipette at the inlet and press the cell suspension gently into the BioCell chamber. It is important to control the situation optically, in order to avoid unwanted attachment of any dirt or cells to the cantilever.
Now position the very front tip of the cantilever over a round, intact cell and press the approach button after you set the relative setpoint to 1 nN. After contacting the cell, the cantilever is allowed to interact with the cell for 3 seconds before separation using the cancel button. In order to catch a cell, you can also set the retract delay to 5 seconds and use the constant force mode with a relative setpoint of 1 nN. After recording a single curve, the cell should be attached to the cantilever (Fig. 6). It's crucial to catch the cells before they adhere to the glass plates, which takes just a few seconds. In our investigations we did not need any anti adhesive covering of the glass slides for example with DMSO. But as mentioned before the cell attachment to the cantilever had to occur very fast and before the cell adhered strongly to the glass.

6. Setup parameters and force displacement (FD) curves
To record FD curves, the cantilever with the attached cell is moved from a certain distance (pulling length) towards the surface with a constant velocity (extension speed) and is kept on the surface for a certain time (extended delay). The relative setpoint is the force, with which the cell is kept in contact with the substrate during the extended delay. The contact mode defines how the force is applied on the cell during the interaction time. There are two modes available in the SPM desktop software of JPK. Constant force, which means that the cantilever is keeping the cell in contact with the surface by applying a defined relative setpoint on it over the whole extended delay. The disadvantage within this mode is that the cell is pressed between the cantilever and the substrate during the whole

extended delay. By contrast, in constant height mode the cantilever is moved towards the surface, the relative setpoint is reached after the cell is in contact with the surface and the piezo motors are kept at the same position on the vertical axis. Therefore, the cell is not stressed over the whole interaction time by the cantilever. After the cell interacted with the surface for a certain time, it is then retracted from the substrate with a defined velocity (retraction speed) and is lifted back to the starting position.
It is crucial to find optimal setup parameters for different cell types with different adherence capabilities. For example, using too long extended delays or too high relative setpoints for strong adhering cells may lead to a partial retraction of the cells from the substrate after the interaction time, so that several binding sites between the investigated cell and the substrate would remain after the cantilever reached a maximum pulling length of 100 µm.
For each surface structure 3-5 cells were investigated and at least 5 curves were recorded with every single cell.

Setup parameters:
Extended mode: Constant height
Relative setpoint: 200-500 pN
Extended and retracted speed: 3µm/s
Extended delay: 120s
Pulling length: 30-100 µm

7. Data processing and analyse
To analyse the saved FD curves, we used the JPK data processing software (version 3.4.14). First, the offset was set to zero on the vertical axis and the zero point of the x axis was found. Second, the maximum adhesion force, the work of de-adhesion and the number of the cell substrate bindings (CSB) were observed using the related functions and curve fittings in the software. We found a significance factor of 0.001 and a smoothing factor of 3 very useful for the curve fitting. This allowed bypassing very little jump-like noises (Fig. 7).
Nevertheless, some noises would have been calculated as CBSs. Because the noise is related to the background noise, the curve fitting parameters for processing the data of a certain day should be selected properly and independently from the data of other days.

There are two different sections observed in the FD curves. The very first part of the retraction curve, which contains very small jumps, and a section, which occurs at a higher distance to the x-axis offset. We recommend the fitting of these two different parts separately not with the same significance and smoothing factors. The JPK data processing software allows the export of files for statistical analysis in other software programmes such as excel.

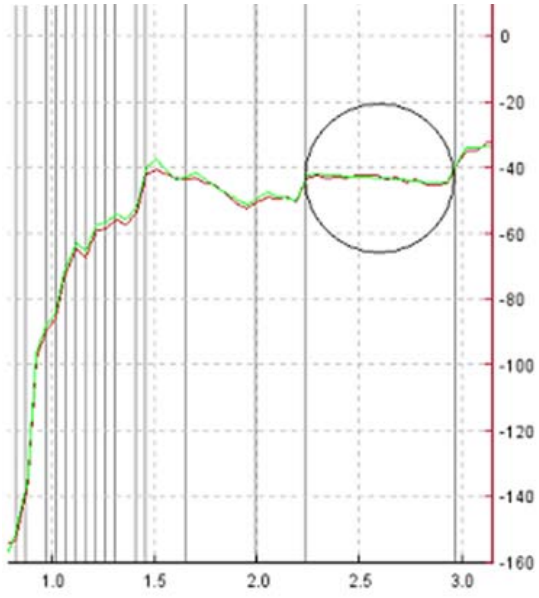

Fig. 7: Optimizing the curve fitting using the JPK data processing software. Compared with a smoothing factor of 5 at a significance of 0.001 (default settings), the use of a smoothing factor of 3 leaded to significantly better results regarding the counting of single CSBs.

Results and Discussion

In our experiments using the mentioned setup parameters 80% of the cells could be detached from the substrate at a distance of 60-100 µm from the substrate. The detachment of the remaining 20% did not take place so that the curves could not be used for interpretation. Investigation of longer cell-surface interaction time periods than two minutes requires a higher pulling length than 100 µm.
For the quantitative characterization of the cell adhesion, three main parameters (F_{max}, $W_{de\text{-}ad}$ and single cell to

substrate bindings (CSBs)) can be measured and processed using the SCFS. Because the results did not mach to the normal distribution, we used the median, the median absolute deviation and the Mann-Whitney U-test for the statistical interpretation.

In order to correctly interpret the data, the most useful parameter for characterization of the cell-substrate-adhesion needs to be identified. It is shown that the number of the adhesion complexes can be strongly reduced using the so named low-adhesion nano structures (Biggs et al. 2007a, 2007b). $F_{max.}$ and $W_{de\text{-}ad.}$ could be interpreted as values, which give a general idea about the strength of the cell adhesion. Because the values for the number of CSBs and the $F_{max.}$ showed more stability and more correlation with each other than with the $W_{de\text{-}ad.}$, we decided to take these values for the interpretation of the data.

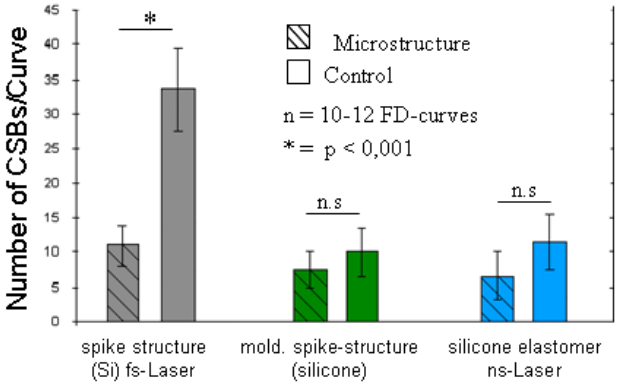

Fig. 8: Diagram of the observed numbers of cell substrate bindings. Data is shown as median ± median absolute deviation (mad). Paired bars show the observed values over the structure and the flat non-structured surface of the same material.

On silicone, no significant changes in the number of the CBs per curve or in the maximum force of the adhesion (Fig. 8, 9) were observed. Also the maximum force of the adhesion measured on the silicon (Si) spike structure was not significantly changed compared with the measured values on the control (polished Si). The only significant change was observed comparing the number of CBSs on the silicon spike structure and the silicon control.

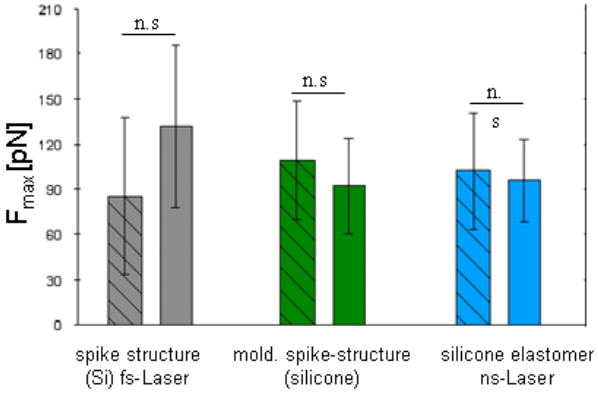

Fig. 9: Diagram of the maximum adhesion forces observed over different micro structures and the corresponding control surface (Aliuos et al. 2010).

In order to measure the strength of the interaction between the cells and the topography, we measured just once over a certain point over the substrate and did not repeat the measurement at the same point. So, the manipulation of the micro structures for example with proteins of the extracellular matrix was avoided. Since the local topographies on the micro structures vary a lot from point to point, the contact area between a certain cell and the substrate can vary dramatically just by changing the measurement point. This accounts for the high standard deviation of the medians.

Conclusion and Outlook

Single cell force spectroscopy introduces a unique method, which allows the quantitative characterization of the cell substrate adhesion. In vitro tests for implant surfaces could be established using SCFS. In the otolaryngology department of the MHH, new surface topographies were investigated concerning the adhesion of fibroblasts. Our data did not reveal statistically significant changes, in the maximum force of the adhesion and also not in the work of de-adhesion but in the number of the cell substrate bindings.

The work of de-adhesion data showed the highest standard deviation and did not correlate with the maximum force of the adhesion or the number of CSBs (data not shown here).

Depending on the underlying topography of the measurement points, the strength of adhesion may vary dramatically. This may probably explained by the variation of the contact area between cells and micro structured surfaces. Another reason for the non significancy of the data was the too small number of investigated cells. Thus, there is a need of additional methods for the investigation of the actual contact area between the cells and the local topographies. Here, methods such as Scanning Electron Microscopy (SEM) or Laser Scanning Microscopy (LSM) could be helpful. In addition, the proliferation and the morphology of growing cells on the structured surfaces should be investigated in order to find correlations between the effects of such micro structures on both adhesion and proliferation of the cells.

Friedrichs et al. (2009) showed that the functionalization of the cantilever using extracellular matrix proteins leads to a stimulation of the associating receptors. For example cells which were attached to cantilevers that were functionalized with fibronectin showed lowered adhesion to the fibronectin coated substrates. This effect let them suggest that the receptor (integrins) density at the opposite site of the cell, where it is interacting with the substrate is strongly lowered because the most adhesion receptors for, for example, fibronectin are activated at the site which is interacting with the cantilever.

These results have to be taken in account for the data interpretation and would be a reason for the use of different protein-based (f.e. fibronectin) and non protein-based (f.e. ConA) adhesion molecules for the attachment of cells to the cantilever in order to find possible differences in the adhesive behaviour of cells on micro structures.

According to the goal of our investigation we used uncoated substrates for the experiments. Nevertheless it would be interesting to find out which adhesion molecule is the most appropriate for such applications and if the stimulation of certain receptors is not intended. In further studies we are looking forward to answer these questions.

Acknowledgements

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