Characterization of cytoplasmic viscosity of hundreds of single tumour cells based on micropipette aspiration

K. Wang\textsuperscript{1,2,†}, X. H. Sun\textsuperscript{3,4,†}, Y. Zhang\textsuperscript{1,2}, T. Zhang\textsuperscript{1,2}, Y. Zheng\textsuperscript{5}, Y. C. Wei\textsuperscript{1}, P. Zhao\textsuperscript{6}, D. Y. Chen\textsuperscript{1,2}, H. A. Wu\textsuperscript{4}, W. H. Wang\textsuperscript{6}, R. Long\textsuperscript{3}, J. B. Wang\textsuperscript{1,2} and J. Chen\textsuperscript{1,2}

\textsuperscript{1}State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Beijing, People’s Republic of China
\textsuperscript{2}School of Electronic, Electrical and Communication Engineering, University of Chinese Academy of Sciences, Beijing, People’s Republic of China
\textsuperscript{3}Department of Mechanical Engineering, University of Colorado, Boulder, CO, USA
\textsuperscript{4}CAS Key Laboratory of Mechanical Behavior and Design of Materials, Department of Modern Mechanics, University of Science and Technology of China, Hefei, Anhui Province, People’s Republic of China
\textsuperscript{5}The Affiliated High School of Peking University, Beijing, People’s Republic of China
\textsuperscript{6}Department of Precision Instrument, Tsinghua University, Beijing, People’s Republic of China

Cytoplasmic viscosity ($\mu_c$) is a key biomechanical parameter for evaluating the status of cellular cytoskeletons. Previous studies focused on white blood cells, but the data of cytoplasmic viscosity for tumour cells were missing. Tumour cells (H1299, A549 and drug-treated H1299 with compromised cytoskeletons) were aspirated continuously through a micropipette at a pressure of $-10$ or $-5$ kPa where aspiration lengths as a function of time were obtained and translated to cytoplasmic viscosity based on a theoretical Newtonian fluid model. Quartile coefficients of dispersion were quantified to evaluate the distributions of cytoplasmic viscosity within the same cell type while neural network-based pattern recognitions were used to classify different cell types based on cytoplasmic viscosity. The single-cell cytoplasmic viscosity with three quartiles and the quartile coefficient of dispersion were quantified as $16.7$ Pa s, $42.1$ Pa s, $110.3$ Pa s and $74\%$ for H1299 cells at $-10$ kPa ($n_{cell} = 652$); $144.8$ Pa s, $489.8$ Pa s, $1390.7$ Pa s, and $81\%$ for A549 cells at $-10$ kPa ($n_{cell} = 785$); $7.1$ Pa s, $13.7$ Pa s, $31.5$ Pa s, and $63\%$ for CD-treated H1299 cells at $-10$ kPa.
The mechanical behaviour of biological cells is largely determined by their cytoskeletons [1,2]. As tumour progresses, abnormal cellular functions of cancer cells can alter cytoskeletons, leading to increases in cell deformability and invading capabilities [3–5].

Well-established techniques that are being used to measure the mechanical properties of single tumour cells mainly include atomic force microscopy (AFM) and micropipette aspiration [4,6–8]. In AFM, a pyramidal or spherical probe tip attached to a flexible cantilever is pressed onto the cellular surface for a set distance and then the deflection of the cantilever is measured using a laser beam, which is used to estimate the stiffness of the probed surface based on contact mechanics models [9–19]. Although powerful, AFM has limited throughput and thus compromised performances for the acquisition of large data volume [20] (e.g. less than 10 cells per sample from patient pleural fluids [21]).

On the other hand, in micropipette aspiration, a portion of a single cell is aspirated into a small glass tube with the leading edge of its surface tracked and translated to cellular elastic and viscous properties [22,23]. Compared with AFM, micropipette aspiration involves deformations of larger cellular portions, and it can characterize the mechanical properties of single cells in a more global manner [24–28]. However, in conventional micropipette aspiration, a low pressure was used to aspirate a single tumour cell partially into the pipette and after that, the cell being measured would be expelled out of the pipette. This procedure takes a considerable amount of time and thus suffers from the limitation of low throughput.

In order to improve the detection throughput, a few previous studies achieved continuous micropipette aspiration by using high aspiration pressure and pipette diameters that are marginally smaller than the cell size [29–31]. Based on image processing, the aspiration lengths as a function of time were obtained and translated to cytoplasmic viscosity using a theoretical Newtonian fluid drop model. However, these previous studies only reported cytoplasmic viscosity of blood cells while the data of cytoplasmic viscosity of tumour cells were missing.

In this study, the same approach was adopted to characterize single tumour cells continuously and the corresponding values of cytoplasmic viscosity from hundreds of single tumour cells were reported for the first time. These values of cytoplasmic viscosity, which formed a statistically important dataset, were then used to classify two different cell types (A549 versus H1299) and the same cell type but with different status (H1299 cells with and without compromised cytoskeletons) in order to demonstrate the potentials of using cytoplasmic viscosity in cell type classification and cell status evaluation.
ATCC (The Global Bioresource Center, USA) and cultured with RPMI-1640 media supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Immediately prior to an experiment, cells were trypsinized, centrifuged and resuspended in the supplemented culture medium with a concentration of 1 million cells ml$^{-1}$.

Cytochalasin D (CD), a cell permeable fungal toxin that can depolymerize action filaments, was used in this study to compromise cytoskeletons following previously reported procedures [32]. More specifically, CD with a concentration of 1 mg ml$^{-1}$ was added into the supplemented culture medium of H1299 for 30 min and then H1299 cells with compromised cytoskeletons (CD-treated H1299 cells) were trypsinized and characterized by micropipette for the quantification of cytoplasmic viscosity.

2.3. Experimental operation and raw data extraction

The experiments of micropipette aspiration were summarized as follows. A glass pipette with a diameter of 10 μm was fabricated by a micropipette puller (P-1000, Sutter Instruments, USA). During operation, single cells in suspension at a concentration of 10^6 cells ml$^{-1}$ were applied on a glass slide and the glass pipette was preloaded with culture medium. A negative pressure of 10 or 5 kPa was generated from a pressure controller (DPI-610, Druck, USA) to aspirate cells into the glass pipette, and the aspiration process was monitored by a high-speed camera (M320S, Phantom, USA) at a speed of 400–800 frames s$^{-1}$.

In order to quantify cellular radius ($R_c$), and aspiration length ($L_p$) as a function of time, a sequence of image processing steps were used to track the process of cell elongation inside the glass pipette [33]. Briefly, the video of cell entry was firstly divided into multiple images at different time frames, and then key steps of frame differencing, thresholding, particle removal using erosion, edge detection and contour fitting were conducted sequentially.

2.4. Quantification of cytoplasmic viscosity

In this study, similar to most previous studies on micropipette aspiration [34], the theoretical Newtonian fluid model was used to model a single cell where the one-dimensional cellular aspiration into a micropipette was represented by the following equation [34]

$$\frac{R_p(\Delta P - P_{cr})}{\mu_c(dL_p(t)/dt)} = 6 \left(1 - \frac{R_p}{R_c}\right),$$  \hspace{1cm} (2.1)

where $\Delta P$, $P_{cr}$, $R_p$ are the aspiration pressure, critical pressure and pipette radius, respectively. In this study, $\Delta P$ and $R_p$ were operational parameters in experiments and $L_p(t)$ and $R_c$ were derived from image processing. As to $P_{cr}$ it can be determined by the following equation:

$$P_{cr} = 2T_0 \left(\frac{1}{R_p} - \frac{1}{R_c}\right),$$  \hspace{1cm} (2.2)

where $T_0$ is the cortical tension of the cell. In our experiments, $R_p$ is about 5.0 μm and $R_c$ is in the range of 6.0–12.5 μm. The cortical tension of tumour cells $T_0$ was found to be of the order of 10^{-5} N m$^{-1}$ in the
literature [34]. Therefore, the critical pressure $P_{cr}$ was estimated to be of the order of approximately 10 Pa. Unlike previous experiments where low aspiration pressure of the order of 10 Pa was used, here a high aspiration pressure of 5 or 10 kPa was used, which was 3 orders of magnitude higher than $P_{cr}$. Therefore, $P_{cr}$ was neglected in equation (2.1), which is consistent with previous publications where high aspiration pressures were used to characterize cytoplasmic viscosity of white blood cells [29–31]. It should be noted that the model above approximates the cell as a homogenized droplet enclosed by the cell membrane, and thereby the cytoplasmic viscosity is an averaged viscosity of the cytoplasmic portion, neglecting the complex internal structures such as the nucleus.

Neural network-based pattern recognitions were conducted based on a ‘Neural Network Pattern Recognition App’ (Matlab 2010, MathWorks, Natick, MA, USA) to differentiate the cytoplasmic viscosity of (1) H1299 cells and A549 cells, (2) H1299 cells with and without compromised cytoskeletons, and (3) H1299 cells under the aspiration pressure of 10 or 5 kPa.

The app employs a two-layer (hidden and output layer) feed-forward neural network, with sigmoid hidden and softmax output neurons. In this study, for the classification of two cell types, the values of cytoplasmic viscosity were used as inputting datasets and the corresponding 0/1 matrix (0 represents cell type one and 1 represents cell type 2) was used as the output matrix. For each classification, the complete dataset was divided into training data (70%, percentage of dataset presented to the network for its adjustment based on generated errors in the training step), validation data (15%, percentage of dataset used to measure network generalization, which can be halted if no further improvements can be made) and testing data (15%, percentage of dataset used to independently measure the network performance after training). As to the results, the neural network-based pattern recognition generated an equation capable of translating the values of cytoplasmic viscosity to 0/1 matrix with the corresponding accuracies shown in a confusion matrix with three kinds of data (training, validation and test) combined. More specifically, the red, green and blue squares represented incorrect responses, correct responses and the overall accuracies (successful classification rates in this study) [35].

Note that in neural network-based pattern recognition, a successful classification rate of 50% means that two datasets cannot be classified at all while a successful classification rate of 100% means that two datasets can be differentiated with a 100% confidence. Thus, as the successful classification rate increases from 50 to 100%, more and more significant differences between the two datasets can be located.

In comparison to conventional approaches for data classification, neural network-based pattern recognition was used in this study for the following two reasons. (1) Neural network-based pattern recognition is capable of providing a successful classification rate to differentiate two types of datasets. For an incoming cell without a pre-known cell type, the successful classification rate can function as a confidence level for us to determine its cell type. Although many statistical approaches (e.g. Student’s $t$-test) can evaluate the distribution differences between two cell types, they cannot produce successful classification rates, and thus they cannot be used for further differentiations of incoming new cells. (2) Neural network-based pattern recognition can classify samples without strict distribution requirements such as normal distributions required by the Student’s $t$-test.

3. Results and discussion

Cytoplasmic viscosity is a key intrinsic cellular mechanical parameter describing the cytoskeleton status of single cells [34], which can be mainly determined by (i) viscosity sensitive fluorescent probes as a biochemical approach, and (ii) micropipette aspiration as a biophysical approach. For the biochemical approach, probes with fluorescent intensities regulated by the viscosity of surrounding medium are injected within biological cells, and the corresponding images are obtained by fluorescent microscopy to determine viscosity distributions within single cells [36,37]. However, due to the lack of calibration approaches (i.e. cells with pre-determined values of cytoplasm viscosity), the biochemical approach cannot effectively correlate raw fluorescent intensities with the actual values of cytoplasm viscosity. On the other hand, micropipette aspiration has been used to characterize single-cell cytoplasmic viscosity from the perspective of biophysics [38]. Based on this approach, actual values of cytoplasm viscosity from various types of blood cells were obtained [29–31,39–41] while the corresponding values of tumour cells were unknown. Thus, in this study, the cytoplasmic viscosity of single tumour cells was characterized and compared to investigate the possibility of using it in tumour cell type classification and status evaluation.
Figure 2. Raw experimental data include microscopic pictures of cellular entries into glass pipettes with quantified aspiration lengths as function of time where (a)–(d), (e)–(h), (i)–(l) and (m)–(p) show representative cells of H1299 at –10 kPa, A549 at –10 kPa, CD-treated H1299 at –10 kPa and H1299 at –5 kPa, respectively.

Figure 2 shows raw experimental data including microscopic pictures of cellular entries into glass pipettes with quantified aspiration lengths as function of time where Figure 2(a)–(d), (e)–(h), (i)–(l) and (m)–(p) show representative cells of H1299 at –10 kPa, A549 at –10 kPa, CD-treated H1299 at –10 kPa and H1299 at –5 kPa, respectively.

By interpreting the curves of the aspiration length as a function of time, the cellular entry processes can be divided into two sections. In section I, \( L_p(t) \) was shown to increase rapidly in response to aspiration, which can be attributed to the elastic response of cells. This behaviour was investigated in many previous studies [22,42] and is not the focus of this work. In section II, a linear increase in \( L_p(t) \) as a function of time was noted, suggesting the viscous properties of cytoplasm. By conducting a linear curve fitting for section II of the \( L_p \) versus time curve, the aspiration rates, \( \text{d}L_p(t)/\text{d}t \), were obtained.

Figure 3 shows the scatter plots of the aspiration time \( (T_c, \text{the duration from the instant when the cell first contacted the micropipette to the instant when the cell fully entered the micropipette}) \) versus aspiration rate \( (\text{d}L_p(t)/\text{d}t) \) for H1299 cells at –10 kPa \( (n_{\text{cell}} = 652, \text{figure 3a}), \) A549 cells at –10 kPa \( (n_{\text{cell}} = 785, \text{figure 3b}), \) CD-treated H1299 cells at –10 kPa \( (n_{\text{cell}} = 651, \text{figure 3c}) \) and H1299 cells at –5 kPa \( (n_{\text{cell}} = 600, \text{figure 3d}) \). More specifically, \( T_c \) was quantified as 0.06 ± 0.11 s for H1299 cells at –10 kPa, 0.19 ± 0.33 s for A549 cells at –10 kPa, 0.04 ± 0.10 s for CD-treated H1299 cells at –10 kPa and 0.15 ± 0.24 s for H1299 cells at –5 kPa. When the time gaps among incoming cells were taken into consideration, the approach can produce a throughput in the range of 1 cell s\(^{-1}\). Further optimization of cell suspension densities may potentially increase the throughput to the order of 10 cells s\(^{-1}\).

As intermediate parameters, \( T_c \) and \( \text{d}L_p(t)/\text{d}t \) depend on cell sizes, mechanical properties and aspiration pressures. When untreated H1299 cells possessing similar cell sizes and mechanical properties but under different aspiration pressure (–10 versus –5 kPa) were compared, the H1299 cells under a higher aspiration pressure (–10 kPa) exhibit lower \( T_c \) and higher \( \text{d}L_p(t)/\text{d}t \) than the H1299 cells under –5 kPa. In addition, when untreated H1299 cells were compared with CD-treated H1299 cells, both with similar cell sizes and under identical aspiration pressures (–10 kPa), higher \( T_c \).
Figure 3. Scatter plots of aspiration time ($T_o$), the time duration from the instant when the cell first contacted the micropipette to the instant when the cell fully entered the micropipette versus aspiration rate ($dL_p(t)/dt$) for H1299 cells at $-10$ kPa ($n_{cell} = 652$, (c)), A549 cells at $-10$ kPa ($n_{cell} = 785$, (b)), CD-treated H1299 cells at $-10$ kPa ($n_{cell} = 651$, (c)) and H1299 cells at $-5$ kPa ($n_{cell} = 600$, (d)).

Figure 4. Scatter plots of $\mu_c$ versus $R_{cell}$ of H1299 cells at $-10$ kPa ($n_{cell} = 652$) (a), A549 cells at $-10$ kPa ($n_{cell} = 785$) (b), CD-treated H1299 cells at $-10$ kPa ($n_{cell} = 651$) (c) and H1299 cells at $-5$ kPa ($n_{cell} = 600$) (d). Distribution percentages of $\mu_c$ with different peaks located in $10–50$ Pa s for H1299 cells at $-10$ kPa (e), $20–50$ Pa s for A549 cells at $-10$ kPa (f), $1–10$ Pa s for CD-treated H1299 cells at $-10$ kPa (g) and $20–50$ Pa s for H1299 cells at $-5$ kPa (h). Confusion matrix of neural network produced successful classification rates of 76.7% for H1299 versus A549 (i), 67.0% for H1299 versus drug-treated H1299 (j) and 50.3% for H1299 at $-5$ and $-10$ kPa (k).

and lower $dL_p(t)/dt$ of the untreated H1299 cells were observed in comparison to CD-treated H1299 cells with compromised cytoskeletons and decreased mechanical properties.

As shown in figure 2, a linear increase of $L_p(t)$ as a function of time was observed, indicating that the cellular aspiration process can be effectively captured by a viscous droplet model. Therefore, the theoretical Newtonian fluid model was used to model the cell during the aspiration process, which translated the aspiration rate $dL_p(t)/dt$ into the cytoplasmic viscosity $\mu_c$. Figure 4a–d shows the scatter plots of $\mu_c$ versus $R_{cell}$ of H1299 cells at $-10$ kPa ($n_{cell} = 652$, figure 4a), A549 cells at $-10$ kPa ($n_{cell} = 785$, figure 4b), CD-treated H1299 cells at $-10$ kPa ($n_{cell} = 651$, figure 4c) and H1299 cells at $-5$ kPa ($n_{cell} = 600$, figure 4d), respectively. Figure 4e–h shows distribution percentages of $\mu_c$ with
different peaks for the four sets of data, i.e. 10–50 Pa s for H1299 cells at −10 kPa, 200–500 Pa s for A549 cells at −10 kPa, 1–10 Pa s for CD-treated H1299 cells at −10 kPa and 20–50 Pa s for H1299 cells at −5 kPa, respectively. Furthermore, three quartiles and the quartile coefficient of dispersion were quantified as 16.7 Pa s, 42.1 Pa s, 110.3 Pa s and 74% for H1299 cells at −10 kPa; 144.8 Pa s, 489.8 Pa s, 1390.7 Pa s, and 81% for A549 cells at −10 kPa; 7.1 Pa s, 13.7 Pa s, 31.5 Pa s, and 63% for CD-treated H1299 cells at −10 kPa; and 16.9 Pa s, 48.2 Pa s, 150.2 Pa s, and 80% for H1299 cells at −5 kPa. These results reveal the significant variations of cytoplasmic viscosity within the same cell types.

As to comparisons between different cell types, significant differences in cytoplasmic viscosity were noted. More specifically, values of cytoplasmic viscosity for H1299 versus A549 cells were 16.7 versus 144.8 Pa s for the first quartile, 42.1 versus 489.8 Pa s for the median of the data and 110.3 versus 1390.7 Pa s for the third quartile, respectively. Furthermore, neural network-based pattern recognition produced successful classification rates of 76.7% for H1299 versus A549 (figure 4i), further indicating the potential role of cytoplasmic viscosity in cell type classification. Note that since the values of cytoplasmic viscosity obtained in this study cannot meet normal distributions, Student’s t-tests and other statistical approaches were not used for the cell type classification.

As to the comparisons of H1299 cells with and without the treatment of CD, significant decreases in cytoplasmic viscosity were noted for the H1299 cells following the treatment of CD. More specifically, cytoplasmic viscosity of H1299 cells with and without the treatment of CD was quantified as 7.1 versus 16.7 Pa s for the first quartile, 13.7 versus 42.1 Pa s for the median of the data and 31.5 versus 110.3 Pa s for the third quartile, respectively. Furthermore, neural network-based pattern recognition produced successful classification rates of 67.0% for H1299 cells with and without the CD treatment (figure 4j), further confirming cytoskeleton compromises due to the CD treatment.

As to the comparison of H1299 cells under −10 or −5 kPa, a successful classification rate of 50.3% was collected (figure 4k). In addition, comparable values of cytoplasmic viscosity for H1299 cells under −10 and −5 kPa were noted, which were 16.7 versus 16.9 Pa s for the first quartile, 42.1 versus 48.2 Pa s for the median of the data and 110.3 versus 150.2 Pa s for the third quartile, respectively. These results suggested that based on the current approach of micropipette aspiration, the characterization of cytoplasmic viscosity was independent of the aspiration pressures and thus the quantified values of cytoplasmic viscosity were trustworthy.

4. Conclusion

In this study, the cytoplasmic viscosity of tumour cells was characterized by micropipette aspiration. High quartile coefficients of dispersion (approx. 70%) were observed, indicating significant variations of cytoplasmic viscosity within the same cell type. Successful classification rates based on neural network (approx. 70%) were quantified between H1299 and A549 cells, H1299 cells with and without the treatment of CD, suggesting the potential role of cytoplasmic viscosity in cell type classification and cell status evaluation. Future developments may focus on the applications of micropipette aspirations to characterize tumour cells from patients continuously. In order to meet this demand, technical developments in processing throughput and stability of micropipette aspirations have to be made.

Data accessibility. Data are available from Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.46kk44r [43]. Authors’ contributions. K.W., R.L., J.B.W. and J.C. designed experiments; K.W. and Y.Z. conducted experiments; K.W., Y.Z. T.Z., Y.Z. and D.Y.C. conducted data analysis; Y.C.W. conducted cell culture; X.H.S., H.A.W. and R.L. contributed to the development of theoretical models; P.Z. and W.H.W. contributed to the preparation of glass pipettes; K.W., R.L., J.B.W. and J.C. drafted the manuscripts.

Competing interests. The authors declare no conflicts of interest.

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