APPLICATION NOTE I No. 442

A “Shear” Mystery – Uncovered

Minimizing Shear Stress when using Liquid Handling Systems

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Abstract

Very few publications exist that address the influence of liquid handling induced shear stress on the integrity of functional units like cells, DNA, and proteins. In this study we examined the shear stress of different liquid handling systems in theory through computer simulation and in practice through laboratory experiments. Building an example under extreme conditions, we were, indeed, able to show that functional units experience shear stress through liquid handling.
This shear stress stimulus has a very short duration as this occurs on the tip’s orifice. In our cell culture test system, we determined that the number of pipetting steps was the most important influencing factor because with only one pipetting the shear stress effects on cell death and proliferation, even at maximum piston speed, were negligible for HEK 293 cells.
With iPSCs we were able to define a more differentiated picture underlining the fact that the instrument and the tip constitute a system. It is neither the tip’s orifice nor the instrument’s piston speed alone that influences shear stress, but rather the system comprising both that influences flow velocity. We strongly recommend using electronic devices when pipetting functional units because they achieve a low flow velocity. This delivers results independent of the user, thus improving reproducibility.

Introduction

Shear force is a natural force in many biological systems. It impinges on molecules, cells and tissues as well as on whole organisms. Such functional units respond differentially to shearing as their sensitivity and natural environment and/or biological function shape their response. As shown in table 1, they can respond positively or negatively.

Regardless if the functional unit's response on shear stress is positive or negative it has the power to change the result of an analysis. For this reason, shear stress should be considered when handling functional units. An understanding of the main principles of fluid mechanics is essential.

Tab. 1: Examples of shearing influences on different functional units

Particle	Positive effect	Negative effect
Molecule: Genomic DNA		Breakage showing bands with a characteristic length on the electrophoresis gel [1, 2, 3].
Molecule: Proteins		The existence of negative effects is controversially discussed. Protein unfolding and aggregation as well as alteration of the three-dimensional structure of globular proteins is described by Bekard et al. [4]. Dobson et al. [5] found BSA aggregating in extensional flow. Whether aggregation appears is protein dependent (e.g. silk spinning or blood clotting).
Cells: Cell culture	Mechanic stimulation is an important regulator for the bone metabolism [7]. Accordingly, shear force enhances the cell differentiation of bone cells being wanted in cell therapy, tissue engineering and bone regeneration [8, 9].	Cells have shown different negative effects like cell death by membrane rupture, decreased proliferation, differentiation, or influences on morphology, gene expression or membrane permeability [6].
Cells: Erythrocytes	Physiological shear stress improves the deformability of red blood cells [13].	Increased levels of free hemoglobin by laboratory processing becomes induced by shear stress [10].
Tissues	Physiologic shear stress has a protective effect on the vascular endothelium to limit inflammation, thrombosis, and endothelial turnover [11]. Physiologic laminar flow furthermore was shown to prevent endothelial cells from apoptotic cell death [12, 13].	In contrast to effects by laminar flow, turbulent flow was shown to promote endothelial permeability and proinflammatory responses [11].
Organisms	Dinoflagellates have been shown to respond with bioluminescence causing marine phosphorescence [14].	Low shear stress induces apoptosis of embryos [15].

Fluid mechanics

Fluid mechanics distinguishes between two different liquid movements: Turbulent flow and laminar flow. To employ an explanatory image: White water coming down a mountain has a turbulent flow while straightened rivers have a rather laminar flow. A turbulent flow produces more shear stress for functional units than a laminar flow. In laboratory experiments, Abu-Reesh and Kargi [16], for example, proved this correlation for mouse hybridoma cell culture in continuously stirred tank reactors. A turbulent flow increases the shear force through an oscillation of the stream and, in the worst case, through eddies that evoke a sudden change in the flow direction and speed [6]. The Reynolds number expresses whether a flow is laminar or turbulent:

The higher this number, the more likely the flow is turbulent. Generally, a flow in a pipe is understood to be turbulent with a Reynolds number above 2300.

Shear stress is not, however, bound to turbulent flows. It can also occur in laminar flows. Returning to the image of the two types of river: Shear stress is created because the liquid's speed is highest in the middle of the river and lowest toward the shore – or a tube's wall. The steepness of this velocity gradient directly influences the shear stress impinging on functional units being carried with the liquid. In the fluids of a tube, the following factors come into play (table 2):

Tab. 2: Physical influencing factors on the dimension of shear stress and description of shear stress

Factor	Unit	Description
Viscosity	Pa*s	The resistance of a liquid to flow.
Tube diameter	mm	The diameter of the tube through which the liquid flows. Tips for liquid handling systems do not have complex geometries. Here, the narrowest point (the tip orifice) is of most interest.
Volume flow	µL/s	A volume of liquid being moved within a certain period of time. Often described as "piston speed" in operating manuals for liquid handling devices.
Theoretical flow velocity	m/s	The speed of a liquid calculated from the tube's sectional area and the volume flow. In this publication we focus the narrowest point: the tip's orifice
Effective flow velocity (CFD)	m/s	The effective speed of a liquid in a tube calculated by computer simulation. The effective flow velocity may differ from the theoretical flow velocity as, e.g., flow separation decreases the diameter available for the flow. In this publication we focus the narrowest point: the tip's orifice.
Shear rate	1/s	Velocity gradient of a liquid across the radius of a tube.
Shear stress	N/m^2 or Pa or dyn/cm^2	The force developed by the velocity gradient of a liquid from the wall (zero) to the middle of the tube (maximum).
Wall shear stress	dyn/cm^2 or N/m^2 or Pa	Special case of shear stress where only the area close to the wall is affected and examined. This is the case with the tips of liquid handling systems.
Reynolds number	No unit	A figure that describes at which point a flow changes from laminar to turbulent. In a tube, a Reynolds number below 2300 suggests laminar flow.

Bioreactor applications involving cells, tissue engineering, cell therapies and, most recently, 3D bioprinting are primarily driving research into shear force. Although all types of functional units are handled in daily laboratory routines, only a few publications exist that describe shear stress caused by liquid handling and its effects. In addition, conditions in the bioreactor and most of the published experimental setups – such as plate-to-cone viscometers – have only limited transferability to liquid handling systems. However, individuals who handle functional units as part of their daily liquid handling routine need to consider the functional unit's response to shearing as it may impact the results of their analyses. Exposing stem cells to shear stress, for example, may initiate spontaneous differentiation of the stem cells into a variety of cell types that represent all three germ layers. This shows that shear stress should not vary during an experiment. But what happens when "pipetting" the functional units?

Different functional units do, of course, demonstrate different levels of resistance to shear stress. Table 3 provides some examples of factors that influence the sensitivity of cells to shear. It becomes clear that not just one "critical" figure is suitable for all cells or for all functional units. The user should, however, understand the influencing factors to be able to assess their effects. This application note explains how shear stress arises and what to look out for in laboratory routines to minimize these factors and their influence as much as possible. We do this using the example of cell cultures with less sensitive (HEK 293 cells) and sensitive (iPSC) cell types.

Tab. 3: Examples of biological factors that influence sensitivity to shear stress

Biological factors that influence sensitivity to shear stress
Cell type [6]
Elasticity of membrane [6]
Age [17]
Size [6]
Treatments like thawing or antigen-antibody reactions [6]
Growth phase of the cell [6]
Medium: for example, with/without serum [6], the viscosity itself and number of cells in the medium
Growth phase of the cell [6]

Materials & Methods

A) General materials

Liquid handling systems:
Only electronic versions of air-cushion pipette and positive displacement dispenser were used because they offer a defined and reproducible setting of piston speeds.

a) Air-cushion pipette:
The Eppendorf Xplorer® plus 50-1,000 µL electronic pipette was used with epT.I.P.S.® 50-1,000 µL in purity grade Biopur®. Additionally, sterile 1,000 µL standard and wide-bore tips from competitor C and E were used.

b) Positive displacement instrument:
The electronic Multipette® E3x from Eppendorf was used with Combitips® advanced 10 mL, 5 mL and 2.5 mL, all in purity grade Biopur®.

c) Pipette controller:
Easypet® 3 from Eppendorf and Drummond® Portable Pipette-Aid® XP have been used with Eppendorf Serological Pipets 5 and 10 mL, sterile, and with 5 mL and 10 mL sterile serological pipettes of competitor F and G.

Applying shear stress:
Two different cell types were applied to shear stress by successively and completely emptying and filling the respective tips. The number of repetitions and the instrument's speed levels were adjusted according to cell sensitivity (table 4).

After the shearing experiment the HEK 293 cells were cultured in medium without serum because a lot of applications require the lack of serum and the cells are more sensitive [6] to the consequences of shear stress.

Estimation of orifice diameter for competitor pipette tips and serological pipettes:
Two sets of metric pins from Vermont Gage® (diameters from 0.20 to 1.28 mm, increment 0.02 mm) and Hoffmann Group® (diameters 1.0 to 5.0 mm, increment 0.5 mm) were used. To determine the diameter, the operator progressed from one pin to the next at the point it perfectly entered the tip. To calculate the flow velocity, the mean results of five passages were used.

B) HEK 293 experiments

Cell culture:
Human Embryonic Kidney 293 cells (HEK 293, DSMZ #ACC 305) were cultured in EMEM medium (ATCC®, 30-2003) following the supplier's recommendations and supplemented with 10% FBS in a T-75 TCT flask (Eppendorf). After a visible check with the microscope, cells were washed with PBS and detached by adding 2 mL of 0.25 % trypsin-EDTA. After inactivation of trypsin-EDTA, cells were centrifuged and diluted in fresh culture medium.

Cell proliferation:
After cell culture in T-75 flasks, HEK 293 cells were suspended in EMEM medium without serum to generate a cell suspension at 3.105 cells/mL. From this cell suspension, triplicates were prepared for each pipetting condition to be tested. To evaluate the impact of the condition evaluated on cell proliferation, 300,000 cells per well were seeded into 24 well cell culture plates (Eppendorf). Cell proliferation was examined microscopically after 2 d of culture in absence of serum.

DNA release measurement:
this assay, 500 µL of medium was taken directly after applying shear stress. DNA quantification was performed using the Quant-iTTM PicoGreen® dsDNA assay kit (Life Technologies®). This kit is based on a molecule inducing a high fluorescence once it is bound to DNA. The fluorescence intensity is directly linked to the amount of nucleic acid.
After 5 min of incubation in the presence of dye, the fluorescence generated is read in each sample at two wavelengths (excitation at 485 nm, emission at 535 nm) using a Fluoroskan™ Ascent (Thermo Fisher Scientific®). As a negative control, 500 µL of the medium with unsheared cells was taken. For evaluation, the ratio of DNA release of sheared to unsheared was calculated.

Tab. 4: The shear stimulus applied during the experiments was adapted to the cell type being tested

Cell type	Number of successive tip filling and emptying steps	Selected instrument speed
HEK 293	5	Maximum / Medium
iPSC	2 x 3 every 5 passages	Medium (air-cushion pipette) Low (pipette controller)

C) iPSC experiments

Equipments:
CO_2 Incubator New Brunswick Galaxy® 170 R (Eppendorf), EVOS™ FL Cell Imaging System (Thermo Fisher Scientific), Inverse-microscope DM IL LED (Leica®) and Vi-CELL™ XR 12-sample carrousel Cell Viability Analyzer (Analis).

Cell proliferation and passage:
Human iPSCs (Thermo Fisher Scientific, A18943PIS) were cultured in a complete Essential 8™ Flex culture medium (E8™ Flex medium, Thermo Fisher Scienific) on freshly Matrigel®-coated (Corning® hESC qualified matrix, LDEV-free) 6-well TCT plates (Eppendorf). After two washings with 2 mL/well of PBS (Thermo Fisher Scientific), cells were incubated 4 min at 37 °C in presence of 1 mL/well of versene solution (Thermo Fisher Scientific) for dissociation. Before cell colony detachment, the solution was removed and replaced by 2 mL/well of complete E8™ Flex medium. Cell colonies were then washed off the plate by gentle pipetting (pipetting aid with lowest speed).
The cell clump suspension obtained was then diluted in E8™ Flex medium and distributed in new wells accordingly to the split ratio of interest (1:6 or 1:8 depending on the confluence level in the initial well).
From 24 h post-seeding, a close microscopic inspection and medium refreshment with complete E8™ Flex medium were performed daily until the confluence level of interest was reached. About 3 d were required between successive passages. Photomicrographs were generated from representative areas.

Immunostaining:
To highlight the expression of several key pluripotent markers in expanded hiPSCs, immunostainings were performed using the Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher Scientific). This kit allows a specific fluorescent staining of two nuclear proteins (OCT4, SOX2) and two cell surface proteins (SSEA4 and TRA-1-60) in cells counterstained with a classical nuclear stain (DAPI):
Cells were fixed within 15 min at room temperature in a fixative solution. They were then incubated 15 min in a permeabilization solution followed by 30 min in a blocking solution. After blocking, primary antibodies were directly added to the blocking solution accordingly to supplier's dilution recommendations for an overnight incubation at 4 °C. After three successive washing steps of 3 min each, cell nuclei were counterstained by 5 min incubation in the presence of NucBlue® Fixed Cell Stain (DAPI, Thermo Fisher Scientific). Fluorescent-stained cells were then observed with the EVOS™ FL Cell Imaging System.

D) Computer simulation

CFX® was used to evaluate the flow in the tips the Computational Fluid Dynamics (CFD) Software Ansys®. The model used was a 3D half-model for capturing possible 3D effects in the flow with an acceptable calculation time. The mesh has more than 2 million cells consisting of tetrahedra with 20 prism layers. Using the SST model with automatic wall function formulation and a fine mesh that ensures a y-plus value of less than 1 leads to a high resolution of the boundary layer.

The simulation focused on calculating the conditions inside the tip, and a steady flow of water through the tip was simulated. The dynamic effects found at the beginning and end of the aspiration and the dispensing operation were not simulated. For the simulations, we followed the recommendations of such authors as Tanzeglock [18], who call for applying the characteristics of water instead of cell culture medium, and applied the standard values for viscosity and density of water at 25 °C.

Results and Discussion

In preliminary studies, we investigated which cell type and which treatment were suitable for determining the influence of shear stress by liquid handling systems. HEK 293 cells have shown to be sensitive to shearing (fig. 1). Freezing and thawing did not impact their sensitivity to shear stress (data not shown). In addition, we also tested different methods of defining the negative impact of shearing on cell function. The influence was determined at two points in time:
1) Directly after the shearing stimulus via DNA release into the supernatant of the medium. This method was already shown to be a good marker for shear-induced cell death [18].
2) In a second analysis 2 d after the shear stimulus, by microscopy of cell proliferation in medium without serum. This assay design was chosen as two different types of reaction by cells to shear stress are known in the literature: If the shear stress stimulus is too high, the membrane ruptures and the cell lyses. This can be determined by the DNA release assay. In addition, it has also been proved that smaller shear stress stimuli can provoke a reaction. This does not lead to lysis, but it does induce changes on the cellular level, which can, for example, be a change to membrane permeability, gene expression or cell morphology. According to the results of Mulhall et al. [19] and other authors, these second-level reactions take a certain amount of time for expression. Consequently, decreased cell proliferation determined 2 d after shearing is an indirect, unspecific but nonetheless suitable marker for negative shear-stress effects.

Refining the results with HEK 293 cells, we also conducted shear-stress experiments with the more sensitive induced pluripotent stem cells (human iPSC). Here, shear stress can trigger an unwanted differentiation.

Influence of the number of pipetting steps on the integrity of HEK 293 cells
In a previous publication [20] we demonstrated that all three liquid handling systems (Eppendorf Xplorer plus, Multipette, Easypet 3 with serological pipettes) securely transfer HEK 293 cells when careful handling was applied: The cells were only subjected to one slow, complete filling and emptying of the tip and grown in a medium with serum. However, in laboratory practice cells are not always cultured in medium with serum. And, depending on the expertise of the lab worker or the protocol being followed, cells are not always handled gently. In our present study we wanted to determine cell reaction when liquid handling was harsh and the medium did not support the cells because it lacked serum. By examining “worst case scenarios” we could derive recommendations.

The Multipette E3x is constructed to support a large number of different applications. For this reason, it offers a broad range of piston speeds – from very slow to very fast. Not all of its speed levels are suitable for cell-culture applications. It is possible to choose very high speeds with this device so we chose it for examining development of shear stress in liquid handling. To convert the high piston speeds into a great volume flow rate that would generate a high flow velocity, we equipped the instrument with the 10 mL Combitip advanced. Using HEK 293 cells, it was indeed possible to stress the cells by shearing in a way that led to a portion dying off and another portion exhibiting decreased proliferation. This response was only possible when we set the instrument to its maximum piston speed, repeated the pipetting steps and cultured without serum afterward. As fig. 1a and 1b show, decreasing the number of pipetting steps does indeed result in reduced DNA release (and thus a reduction in the portion of cells that die) and improved cell proliferation. No cell reaction was observed with just one complete filling and emptying cycle of the tip, even though the flow velocity was high.

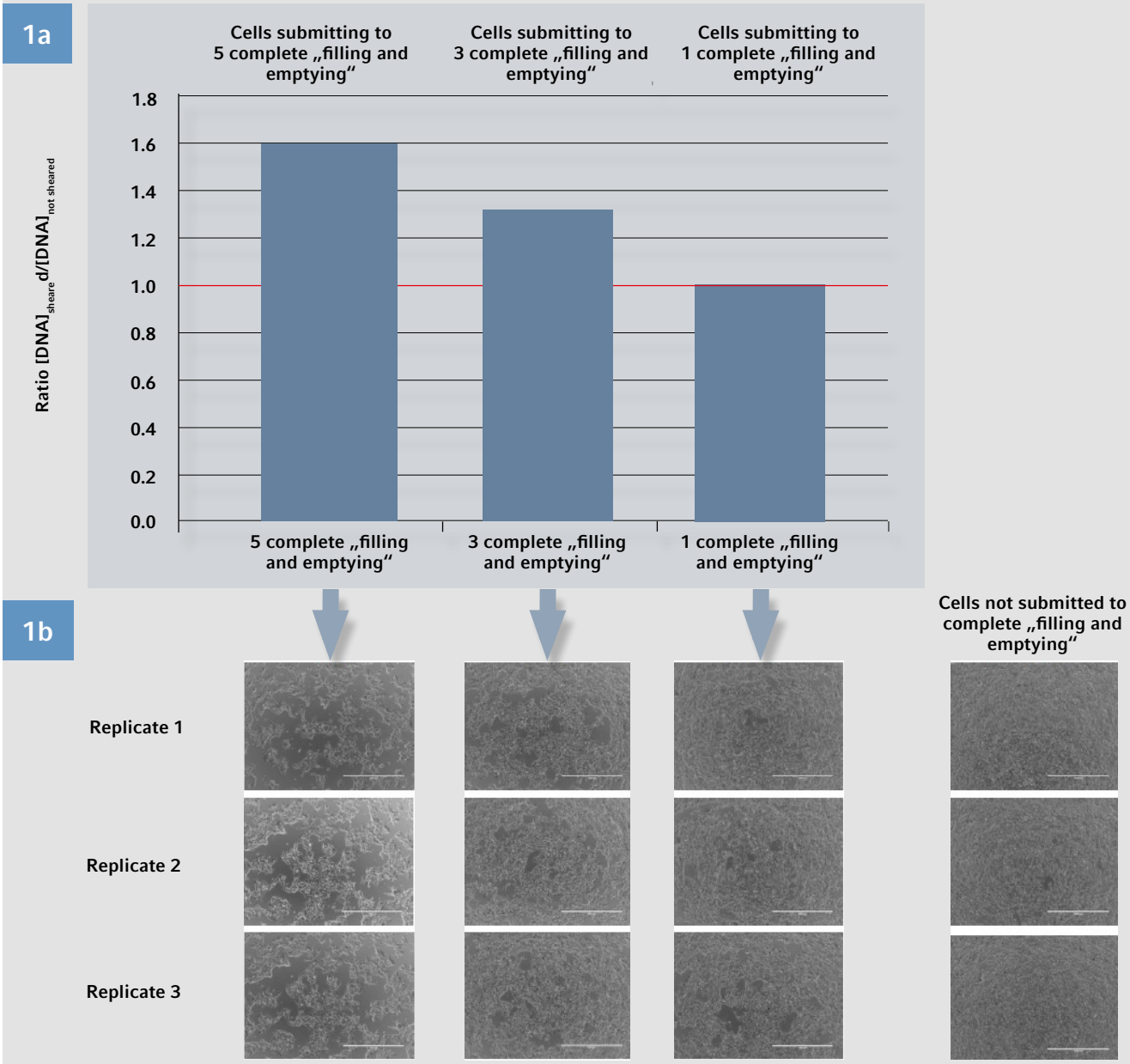


Fig. 1: Influence of the number of pipetting steps on cell death (A) and proliferation of HEK 293 cells

a) DNA release according to the number of times the tip was completely filled and emptied. Here, DNA release is presented as the ratio of mean DNA concentration in the supernatant of sheared cells divided by the mean DNA concentration in the supernatant of unsheared cells.
b) Cell proliferation according to the number of times the tip was completely filled and emptied. Each condition was performed in triplicate. Shearing was provoked by completing five, three, and one successive filling and emptying cycles of the HEK 293 cells using the Combitip advanced 10 mL and setting the instrument to its highest piston speed. The shearing design was chosen to provoke shear stress. It does not reflect normal laboratory handling. Magnification 4x.

This is an important finding, and we will see in the following that "processing stress" is not directly connected to flow velocity. It always influenced cell proliferation following completion of five successive cycles of filling and emptying the tips – even when the flow velocity (and thus the shear stress) had been reduced by applying wide-bore tips.

In the literature, the influence of shear stress duration is widely and controversially discussed [6, 10, 21, 22]. However, more authors speak for than against an influence of stimulus duration. In our experiments the shear stress duration was very short as the point of interest was only the narrowest point – that is, the tip's orifice – and we found a clear relationship between the number of times the shear stress stimulus was repeated and cell death and decreased cell proliferation. This finding is well in line with Zoro et al. [23] who report a decrease in the number of intact cells and cells able to grow with an increasing number of capillary passes. Veriatch et al. [24] found repeated exposure of undifferentiated embryonic stem cells to repeated fluid flow in capillaries to result in a reduced proliferation capacity. Our results are also confirmed by the findings of Rossetti et al. [25] that examined neuronal stem cells protected by vehicles. In their experimental design they found that five or fewer pipetting cycles yielded very little difference whereas 10 pipetting steps proved harmful to the cells. Unfortunately, these authors worked with a mechanical pipette so the flow velocity (and thus the shear stress) applied during pipetting remains unknown. We do, however, clearly learn this: In general, pipetting steps should be decreased as much as possible by concentrating work and optimizing the workflow – for example, by reducing the pipetting steps for resuspending cells. Rossetti et al. [25] concluded from their results that pipetting once was sufficient to maintain a single-cell solution. It is worth the effort to determine how many processing steps are really needed to sufficiently resuspend cells for a specific cell culture.

Influence of the speed level set on the liquid handling instrument

Following the unwritten law "use a wider tip whenever possible," many scientists who work with functional units focus on bore size. Protocols followed by various scientific fields, but especially those in molecular biology, recommend cutting pipette tips to widen the orifice and thus reduce shearing [26, 27]. As will be discussed later, it is correct that bore size plays a role in shear stress. However, the magnitude of the influence from the piston speed is all too often underestimated.

Electronic air-cushion pipettes and positive displacement instruments, regardless of their manufacturer, offer different piston speed levels. As described previously, the Multipette E3x, for example, is constructed to support a wide variety of applications and thus offers a broad range of piston speeds. However, not all speed levels are suitable for dispensing fragile functional units like cell cultures. The user's expertise is crucial for selecting the appropriate flow velocity for an application. Here, it is not enough to simply work with the speed levels described in the operating manual as the effective flow velocity will differ with the tip size.

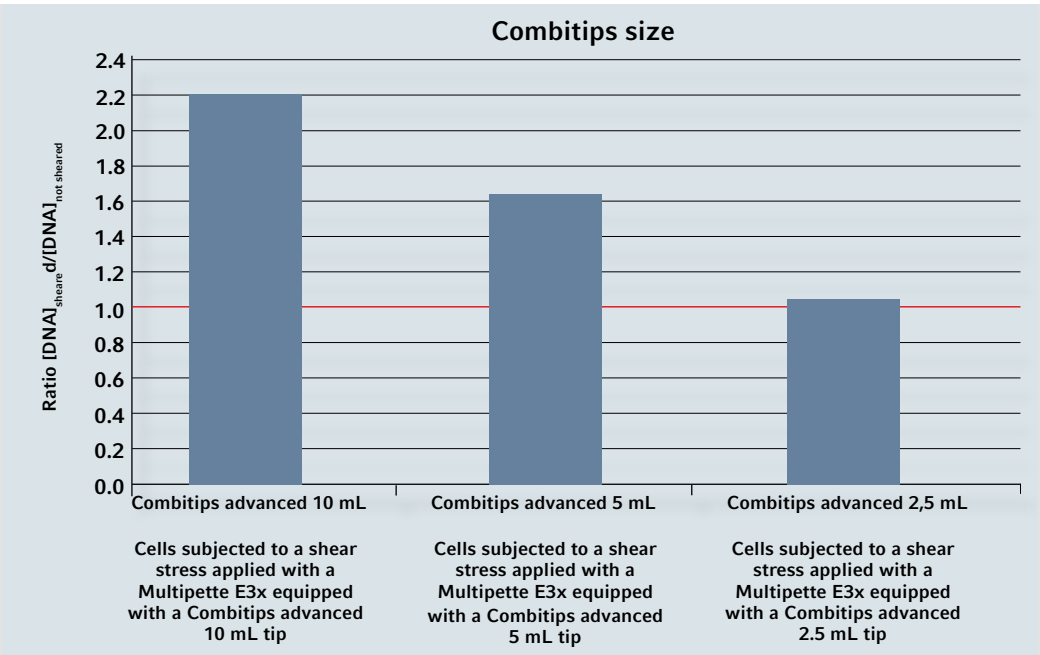
To explain these differences, we conducted a shearing experiment with five successive cycles of completely filling and emptying 10 mL, 5 mL and 2.5 mL Combitips advanced, each time running the instrument at its maximum speed. Again, cells were cultured in medium without serum. The results are shown in fig. 2a and 2b. While the 10 mL Combitip advanced with Multipette's E3x maximum speed provoked cell death and decreased proliferation, the 5 mL Combitip advanced improved the situation. With the 2.5 mL Combitip advanced no cell death could be detected. Cell proliferation slightly decreased, which was – as described in the previous section – due to the "processing stress" resulting from five successive pipetting steps. This shows that, although the instrument was set to the same speed, different tips resulted in different shearing stress. But these differences were not primarily caused by the Combitips advanced having a different shape, but rather predominantly driven by the tip's filling volumes and thus the different volume flows, which ranged from 0.83 mL/s (2.5 mL) and 1.67 mL/s (5 mL) to 3.33 mL/s (10 mL). What we learn for the laboratory routine: The bigger the tip size, the lower the speed level to be selected on the instrument in order to obtain proper conditions for the functional units.

To further investigate these results, we performed a computer simulation using the Combitips advanced mentioned above and applied the instrument's maximum speed. The results for aspiration are shown in fig. 2c and 2d. All three Combitips advanced sizes have similar orifice diameters, so this factor did not come into play much. However, we did see a significantly higher flow rate than with the other tips due to the larger volume flow of the 10 mL Combitips advanced tip with the higher volume flow resulting in an overall higher flow velocity (fig. 2c).

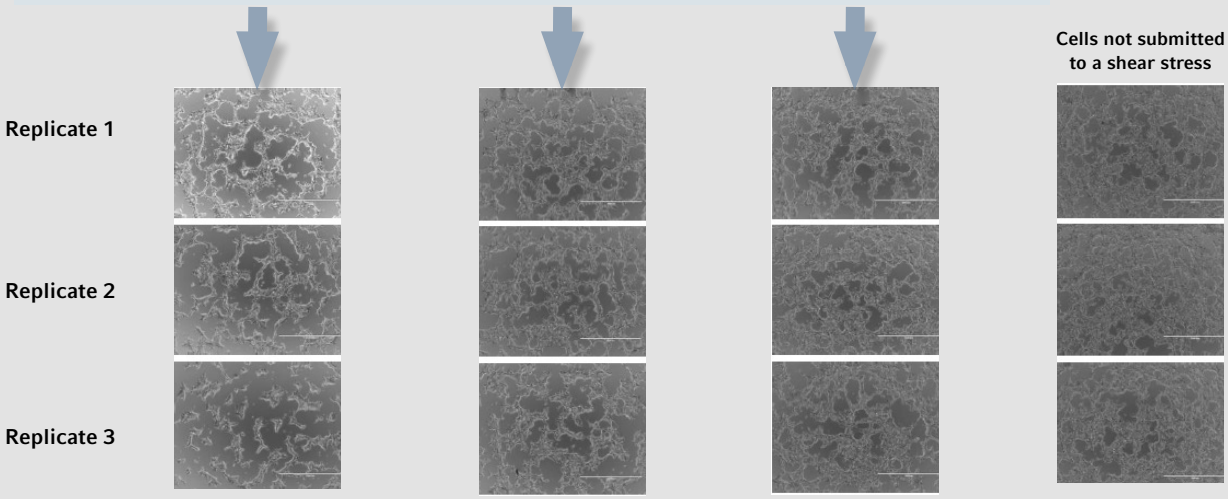
Since flow velocity at the tip's wall is zero, a higher flow rate means the velocity gradient across the radius, called the shear rate, becomes steeper. It is this velocity gradient that generates the shear stress. To condense this into an easy correlation: The higher the flow velocity, the steeper the velocity gradient and the higher the shear stress.

We found the computer simulation results confirmed the laboratory results: The smaller the magnitude of the calculated flow velocity and thus the lower the shear stress values, the less significant the damage to the cells.

2a



2b



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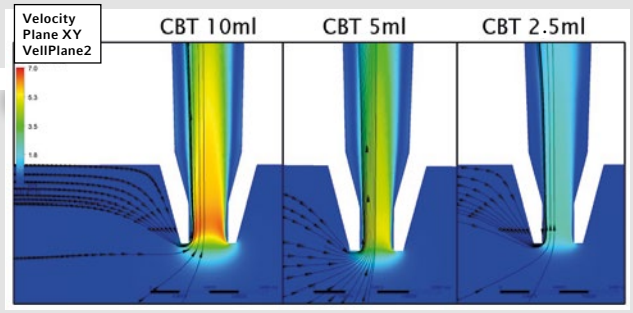


2d

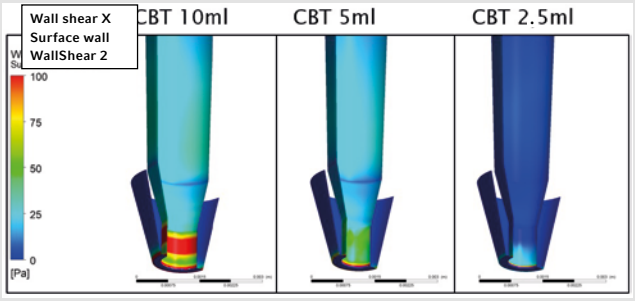
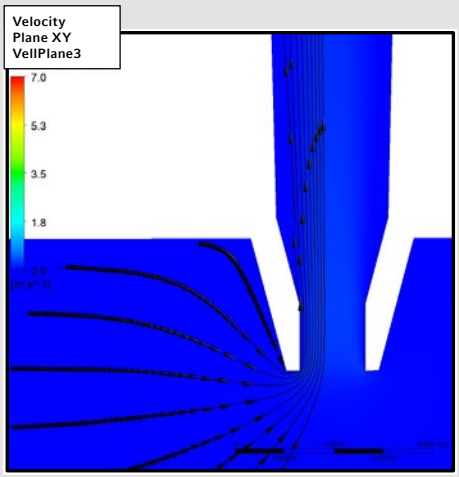


Fig. 2a through 2d: A smaller volume flow reduces shear stress: Actual response of HEK 293 cells after shearing and calculated shear stress.
Liquid handling system examined: Multipette E3x set to maximum speed and equipped with Combitips advanced 10 mL, 5 mL and 2.5 mL.
a) DNA release into the supernatant of medium directly after the shearing experiment (ratio of sheared to unsheared).
b) Proliferation of the HEK 293 cells after 2 d of growth in medium without serum. Each condition was performed in triplicate. Shearing was provoked by completing five successive filling and emptying cycles of the HEK 293 cells using the respective tips and setting the instrument to its highest piston speed. The shearing design was chosen to provoke shear stress. It does not reflect the normal laboratory handling. Magnification: 4x.
c) Results of computer simulation of flow velocity (aspiration).
d) Results of computer simulation of shear stress (aspiration).

As explained previously, the computer simulation results for the shear stress of different liquid handling systems can only be compared with data from the literature to a limited extent. Shear-stress-related cell responses are strongly influenced by different factors such as cell type, type of shear treatment (for example, plate-to-cone viscometer, capillary), stimulus duration, and the presence or absence of serum in the medium. It should be noted that the stimulus duration for pipetting is extremely short. Fig. 2d illustrates that the highest shear stress with >100 N/m2 (10 mL Combitips advanced tip at instrument's highest speed) only occur at the tip's inlet port. Functional units like cells experience this for only a very short duration. If the mean shear stress of the inlet were to be calculated instead, this value would be 82 N/m^2. Calculated over all surfaces of the Combitips advanced tip 10 mL at maximum speed, shear force is only 0.112 N/m^2. This finding not only relativizes the increased shear stress that functional units are exposed to for a very short duration when entering the tip. It also shows that the Hagen-Poiseuille equation, which has been used by several authors to estimate the shear stress of their experimental setup, is not suitable for liquid handling systems. The equation describes the shear stress of a liquid with a readily formed flow – not a developing flow, which is the situation at the tip's orifice. This means the equation can be applied to the tube-like regions of a dispenser tip or a pipette tip but cannot be used to describe the conditions near the orifice.
Returning now to the influence of the stimulus duration, a years-long discussion has ensued about stimulus duration having an influence on cell response. Amer et al. [22] postulate that the magnitude of the shear stimulus and the duration of its exposure should be balanced. With respect to the liquid handling systems we studied, this means increased shear stress was permissible as long as the stimulus duration was short and the threshold for the maximum stress on the cell membrane was not exceeded [6].

In this study, we created an extreme example by stressing HEK 293 cells with our fivefold filling and emptying and use of the liquid handling system's maximum speed. These are not conditions found in laboratories when sensitive functional units are pipetted. When we reduced piston speed, we were able to decrease the flow velocity of the Multipette E3x and 10 mL Combitips advanced system to 0.32 m/s and shear stress to 1.8 N/m^2 (fig. 3a and 3b). If needed, the flow velocity and shear stress can also be reduced even further by changing the tip to a smaller one like the 2.5 mL Combitips advanced tip. This system results in a minimal effective flow velocity of 0.15 m/s and a shear stress of only 0.27 N/m^2 (see table 6 of the conclusion).

3a



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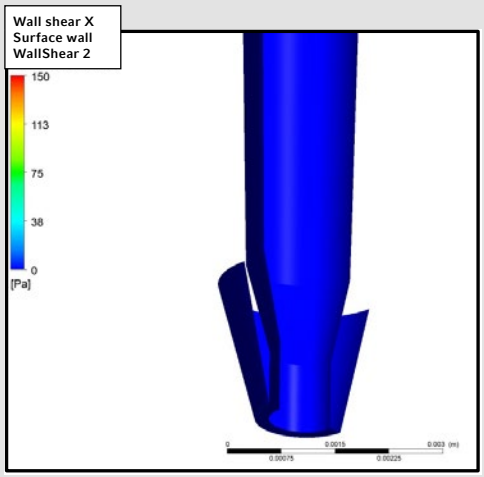


Fig. 3: Effective flow velocity and wall shear stress with 10 mL Combitips advanced tip and Multipette E3x set to minimum speed.
a) Flow velocity during liquid aspiration.
b) Shear stress during liquid aspiration.

Influence of the flow type and liquid movement direction (aspiration / dispensing)

As already discussed, there are two types of flow, laminar and turbulent, and the transition from one to the other is smooth. Turbulent flows are found in certain regions or bioreactors or in vortexing, where eddies develop. This type of mixing is known to even lyse erythrocytes, which are rather mechanically stress resistant, and to break DNA [10, 28]. In contrast, less turbulent flows have no swirls or eddies, and instead oscillating streamlines occur.

Determining if a flow is turbulent or laminar is interesting from two points of view:

First, with the exception of erythrocytes, most cells are known to be less resistant to shear stress in turbulent flows [6]. Second, turbulent flows create high velocity gradients near the wall. In addition, the flow separation at the inlet of a tip reduces the effective cross-section area for the flow and thus increases the effective flow velocity. The small image in fig. 4 shows a flow separation (blue area) occurring at the inlet of the Combitips advanced 10 mL tip with the Multipette E3x set to maximum speed. As the flow velocities are different at the different positions of the radius, a velocity gradient develops that leads to the development of shear stress.

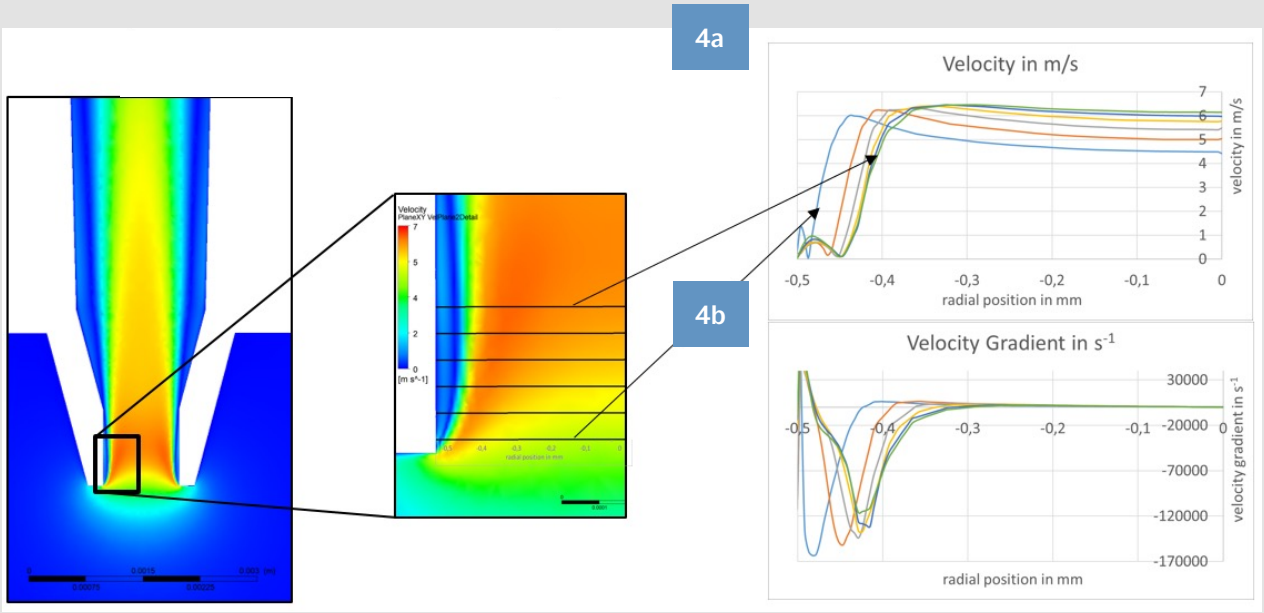


Fig. 4: Computer simulation of effective flow velocity and the velocity gradient during liquid aspiration.
Simulated system: Multipette E3x set to maximum speed and equipped with a 10 mL Combitips advanced tip.
a) Flow velocity.
b) Velocity gradient.

Armed with an understanding that the shear stress stimulus is only connected to the tip's orifice and thus very short, the next question arises: Are the conditions the same for aspiration and for dispensing? In laboratory tests, we cannot observe the influencing factors of aspiration and dispensing separately. In a computer simulation, however, we can. Focusing on the flow inside the tip and neglecting the effects of free jet flow and dispensing against a wall, we found the aspirating process of a liquid to be more critical for functional units than the dispensing process. This is due to the fact that liquid is forced to move around the corner into the tip, which is something that does not occur during dispensing. Fig. 5a shows the aspiration condition at the orifice of a 10 mL Combitips advanced tip with the instrument set to maximum speed. Fig. 5b shows the same setting for dispensing: The velocity gradient is negligible and thus the shear stress is low.

5a **Aspiration**

5b **Dispensing**

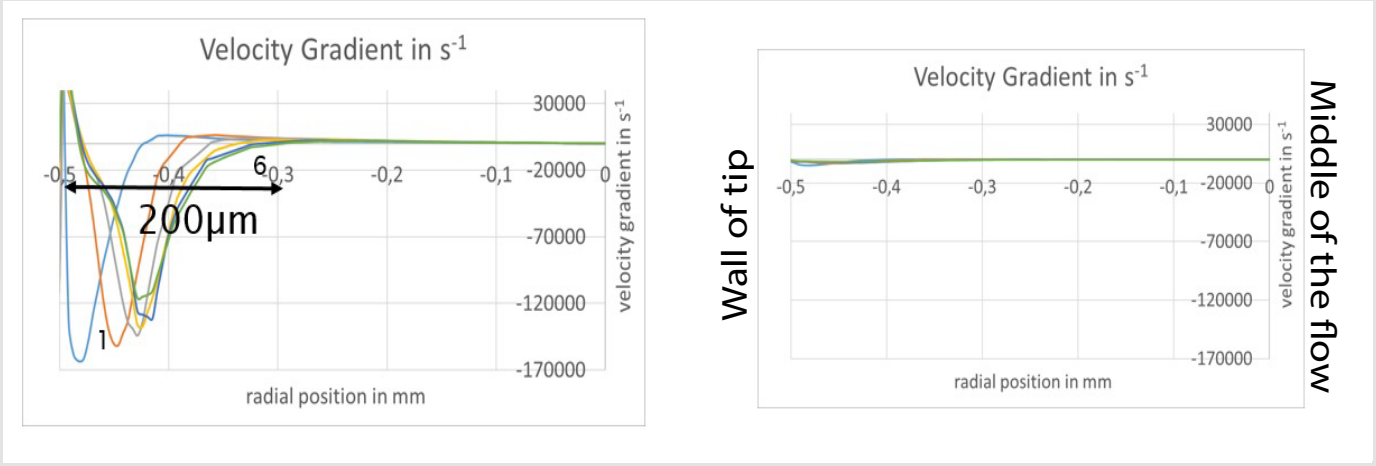


Fig. 5: Comparison of the velocity gradients between aspiration and dispensing of Multipette E3x at maximum speed with Combitips advanced 10 mL.
a) Aspiration
b) Dispensing.

These results indicate that in laboratory practice it is better to use higher speed settings for dispensing than for aspirating. An application example is cell seeding where a comparatively fast transfer of cell cultures is needed.

But another interesting detail emerges from the velocity gradient graph in fig. 5a: the width of the velocity gradient. HEK 293 cells have a size of approximately 20 µm. As shown in fig. 5a, the velocity gradient has a width of approximately 200 µm. This means the cells are small enough to be fully captured and spin around instead of being torn apart between the wall (zero flow velocity) and the middle flow (high flow velocity).

Influence of the bore size

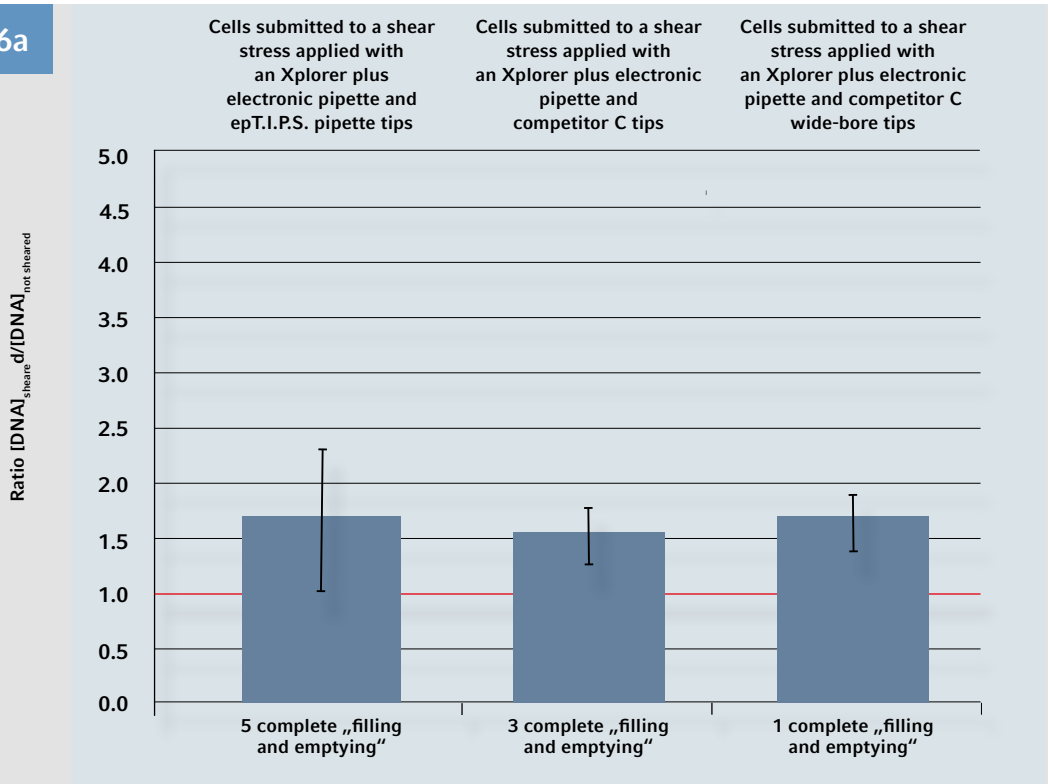
As already mentioned, flow velocity is calculated from the volume flow and the orifice diameter. To define the influence of the bore size, we performed experiments with an electronic air-cushion pipette and pipette controllers that were each equipped with tips having different bore sizes. Competitor tips were included in the experiments to increase the variety of orifice diameters. For the pipette tips, we included manufacturers with standard and wide-bore variants. Their measured orifice diameters are provided in table 5.

Tab. 5: Orifice diameters of the various manufacturer tips and serological pipettes used in the experiment

Manufacturer	Volume (mL)	Standard or wide bore	Orifice diameter (mm)
epT.I.P.S. 1 mL pipette tips	1	Standard	0.83
Competitor C	1	Standard	0.68
Competitor E	1	Standard	0.76
Compeitor C	1	Wide bore	1.89
Competitor E	1	Wide bore	1.45
Eppendorf Serological Pipets	5	Standard	2.17
Competitor F	5	Standard	1.54
Competitor G	5	Standard	1.56
Eppendorf Serolocical Pipets	10	Standard	2.37
Competitor F	10	Standard	1.46
Competitor G	10	Standard	1.45

For the test with HEK 293 cells, the same experimental design was used as for the Multipette E3x tests: Cells were shear stressed by using Eppendorf Xplorer plus or the pipette controller's maximum speeds. Five successive filling and emptying cycles of the respective tip were completed and cells were grown without serum after shearing. The shear stress result was determined by DNA release directly after the shearing event as well as 2 d later by cell proliferation. As shown in fig. 6a and 6b for Eppendorf and competitor C with 1,000 µL tips at maximum speed, we found a small general influence on DNA release and cell proliferation. However, we found no difference between the tips used.

6a



6b

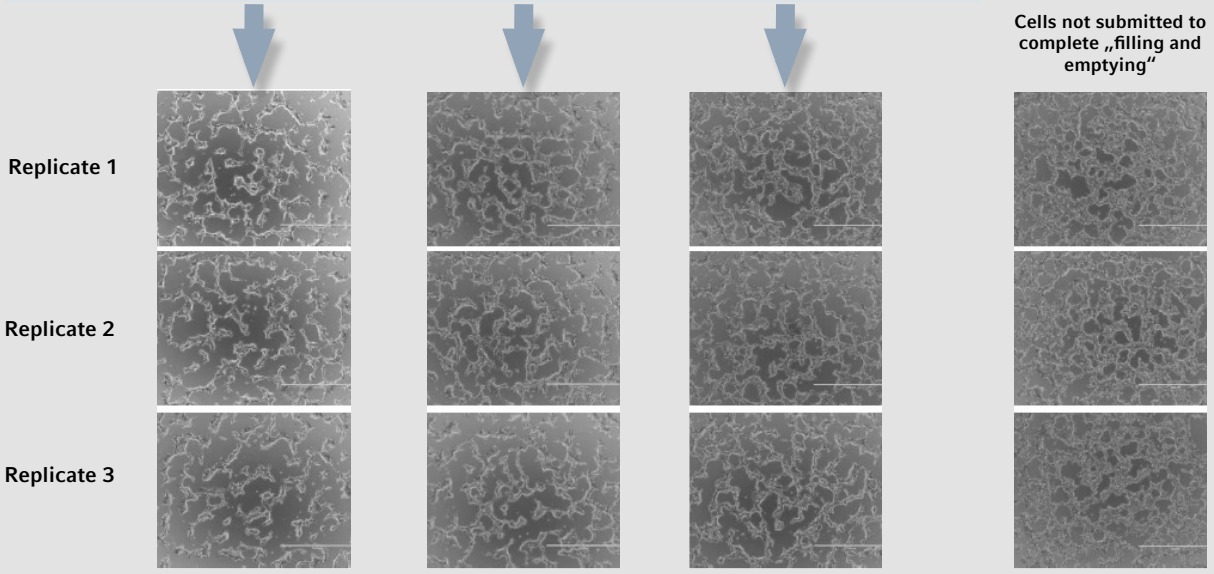
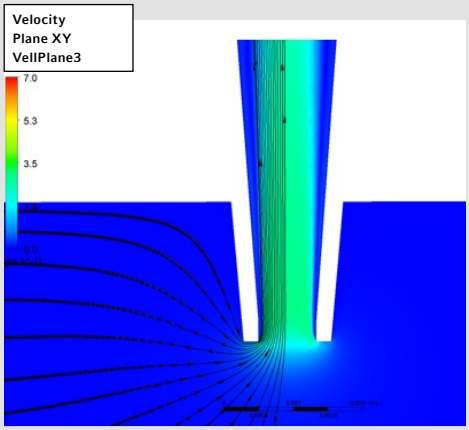


Fig. 6: Standard versus wide-bore pipette tips: No difference in the response of HEK 293 cells after shearing with 1 mL standard and wide-bore pipette tips. Shearing was provoked by completing five successive filling and emptying cycles of the HEK 293 cells using the respective tips and setting the instrument to its highest piston speed. The shearing design was chosen to provoke shear stress. It does not reflect normal laboratory handling.
a) DNA release into the supernatant of the medium directly after the shearing experiment (ratio of sheared to unsheared).
b) Proliferation of the HEK 293 cells after 2 d of growth in medium without serum. Each condition was performed in triplicate (magnification: 4 x).

Running the experiment using three other competitors' tips led to comparable results (data not shown): DNA release slightly increased and cell proliferation decreased, but no differences between standard or wide-bore tips emerged. The computer simulation of the epT.I.P.S. yielded simliar results as with the Combitips advanced: The greatest flow velocity and shear stress were found during aspiration where the liquid entered the tip (fig. 7a). Behind this, the flow velocities and shear stress were low.

7a



7b

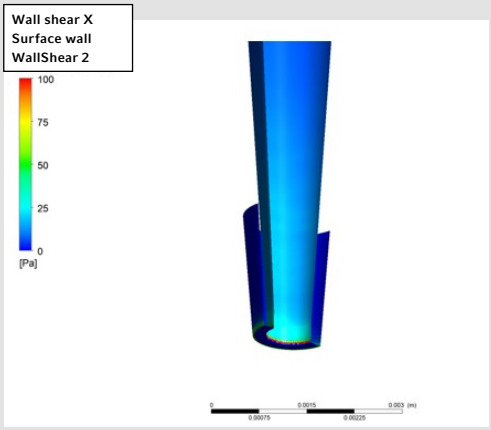


Fig. 7: Computer simulation of flow velocity and shear stress during aspiration with epT.I.P.S. 1000 µL and Xplorer plus pipette with maximum speed setting.
a) Flow velocity.
b) Wall shear stress.

One interesting finding of this experiment was the fact that none of the wide-bore tips improved results, which has led to this conclusion: The slight increase of DNA release and the proliferation effect was not driven by the liquid handling system, but rather the handling itself – with its five successive pipetting steps. The experiment further showed that, as long as flow velocity remains low enough, a larger bore diameter has no further positive influence on cells. From this we were able to conclude that even the highest Eppendorf Xplorer plus piston speed setting enabled safe handling of the HEK 293 cells. Please note that this information cannot be generalized to other electronic pipettes as other manufacturers' electronic pipettes offer different piston speed ranges.

A different picture was found when repeating the HEK 293 cell experiment with serological pipettes. Here we faced the fact that volume flow was higher. The competitors' 5 mL and 10 mL serological pipettes have a smaller orifice diameter than Eppendorf Serological Pipets (table 5). As shown in fig. 8a and 8b, we found a clear difference between the serological pipettes from Eppendorf and those from competitors. While proliferation was slightly reduced due to the "process stress" of repetitive liquid movement, no cells lysed when using the Eppendorf Serological Pipets. Instead, with competitors' serological pipettes, cells lysed and proliferation clearly decreased. It was not possible to calculate an exact flow velocity with this dispensing system as it had user-specific speeds that were not reproducibly adjustable. However, transferring the outcome of the results we received using the different Combitips advanced sizes, we were able to conclude for the serological pipette that the volume flow was simply too high for the competitors' bore sizes. The flow velocity at the narrowest point increased too much and with it the shear stress. Here, the Eppendorf Serological Pipets performed better – even on a competitor pipette controller.

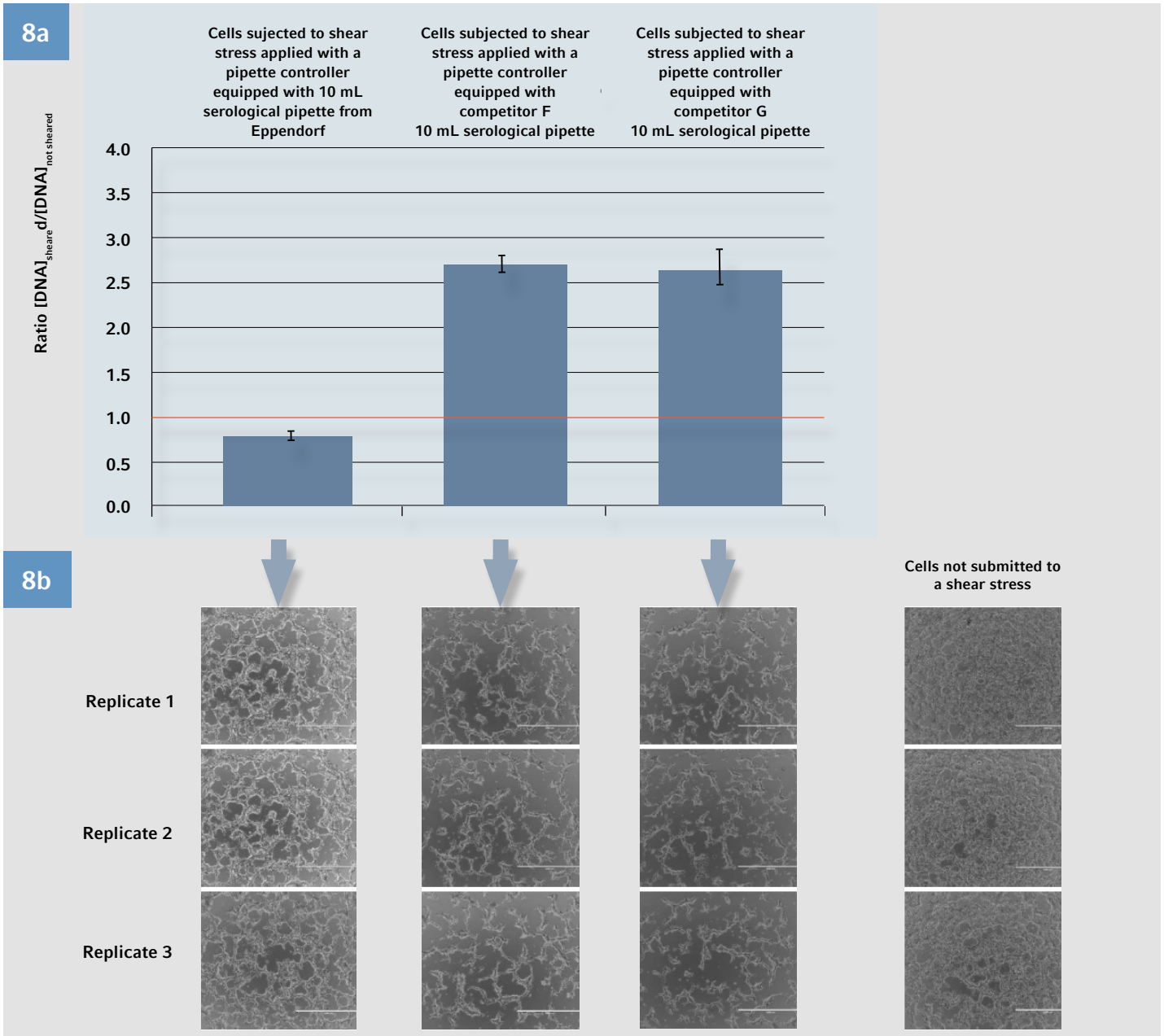


Fig. 8: Smaller orifices of serological pipettes increase shear stress: Response of HEK 293 cells after shearing stimulus with different consumables.
a) DNA release into the supernatant of medium directly after shearing experiment (ratio of sheared to unsheared).
The < 1 result of the ratio with the Eppendorf serological pipette is based on normal fluctuation as the difference with the control was not significant.
b) Proliferation of the HEK 293 cells after 2 d growth in medium without serum. Shearing was provoked by completing five successive filling and emptying steps of the HEK 293 cells using the respective tips and setting the instrument to its highest speed. Each condition was performed in triplicate (magnification: 4x).

The improved results using Eppendorf Serological Pipets were also demonstrated using the more sensitive iPSC cells. Although we did not find an influence on immunostaining (data not shown), the cell clumps were smaller after applying the competitors' serological pipettes, as shown in fig. 9.

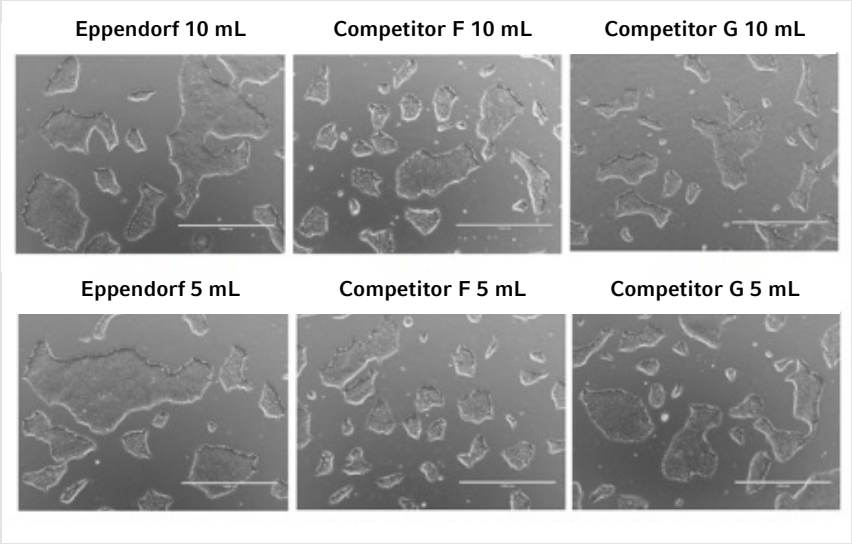


Fig. 9: Orifice diameter influences clump size: hiPSCs morphology 2 d (D2) after passage P5 according to the consumable used to pipette the cell suspension (magnification: 40x).

As mentioned before, proliferation is an unspecific indicator for second-level cell responses. To get a more differentiated picture about the cells' responses to pipetting and to apply a more sensitive test system, we conducted experiments with the more sensitive iPSCs and analyzed their proliferation and immunostaining following pipetting. The liquid handling systems used were the Eppendorf Xplorer plus pipette with competitor E's standard and wide-bore tips as well as a pipette controller with the 5 mL serological pipette from Eppendorf. The standard 1,000 µL tip of competitor E was chosen on purpose for this experiment as its orifice diameter is smaller than 1,000 µL tips from Eppendorf.

The ability of cells to efficiently adhere and proliferate can be affected by a range of parameters, including clump size after passage and intensity of mechanical force used to break up cell clumps. Indeed, clumps should be as uniform as possible. If clumps are too large, increased differentiation may be observed within the resulting colonies. Very small clumps may induce cellular stress and poor attachment. Moreover, clumps are fragile and should not be submitted to excessive shear stress.

Drawing on the greater sensitivity of iPSCs, we used lower velocities in these experiments: Eppendorf Xplorer plus with speed level 5 and serological pipettes with slow speed. Fig. 10a shows that the impact of the liquid handling system and consumable used to perform the clump passaging was not drastic enough to obviously affect the iPSCs adhesion and short-term proliferation. Indeed, whatever the consumable, cells presented the typical morphology of hiPSCs and grew efficiently until requiring a new passage 3 d after seeding. By using a serological pipette as well as air-cushion pipette tips (standard and wide bore), an iPSC culture was successfully maintained during five successive passages with no increasing spontaneous differentiation. Nevertheless, some issues did emerge with the standard tips: They showed more cell debris after the first seeding (P0D2) and smaller colonies (P2D2-P4D2). These minor issues were not observed when the serological pipette or wide-bore tips were used to pass the iPSCs.

To confirm the pluripotency status of iPSCs cultured using different liquid handling systems and consumables, immunofluorescence staining was used to assess the expression of two nuclear markers (OCT4 and SOX2) and two cell surface markers (SSEA-4 and TRA-1-60). These markers are specific to iPSC physiology and are required to maintain these cells in an undifferentiated state. OCT4 and SOX2 are transcription factors highly expressed in undifferentiated iPSCs while SSEA-4 and TRA-1-60 are surface proteins specific for human iPSCs. The staining was performed on cells expanded during five passages. Besides the specific staining, cells were also counterstained with a standard nuclear marker (DAPI).

As shown in fig. 10b, hiPSCs exhibited a high expression level of two nuclear transcription factors (SOX2 and OCT4) and one specific surface protein (SSEA-4) regardless of the liquid handling system and consumable used during expansion. The fourth marker evaluated (TRA-1-60) was also highly expressed in cells passed using a serological pipette (at low speed) and a wide-bore pipette tip (at Eppendorf Xplorer plus medium speed). By contrast, in cells cultured

using the standard tips (at Eppendorf Xplorer plus medium speed), the staining of this specific marker had significantly decreased, including a direct impact on cells. As TRA-1-60 antigenic sites (found on pluripotent stem cells) are lost when these cells start to differentiate, this result represents an initial sign that cells are starting to be impacted by the use of a standard pipette tip. The loss of TRA-1-60 is particularly obvious when viewing the merged images of fig. 10c.

The staining profile obtained with cells cultured by using standard tips was significantly different. In contrast, profiles obtained with cells cultured by using the 5 mL serological pipette from Eppendorf or Xplorer plus electronic pipette with competitor E wide-bore tips are obviously similar. These results hint that in specific cases, a consumable with a larger orifice is a good option as the flow velocity and shear stress are further reduced.

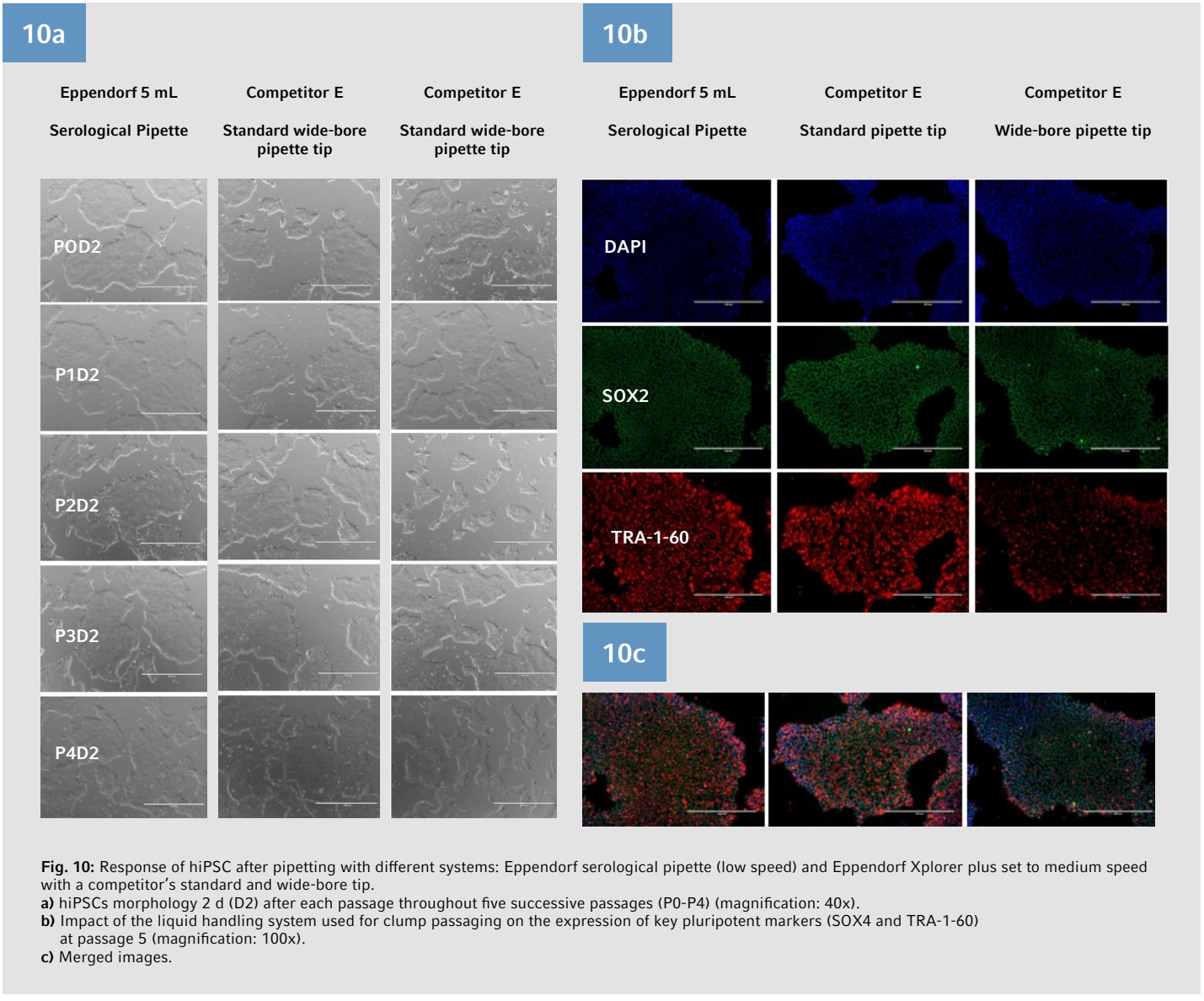


Fig. 10: Response of hiPSC after pipetting with different systems: Eppendorf serological pipette (low speed) and Eppendorf Xplorer plus set to medium speed with a competitor's standard and wide-bore tip.
a) hiPSCs morphology 2 d (D2) after each passage throughout five successive passages (P0-P4) (magnification: 40x).
b) Impact of the liquid handling system used for clump passaging on the expression of key pluripotent markers (SOX4 and TRA-1-60) at passage 5 (magnification: 100x).
c) Merged images.

Within this context it should be mentioned that the correlation between the orifice diameter and the flow velocity is quadratic, not linear. This aspect is important to consider because orifice diameters may vary between tips with the same volume variant – for example, standard or filter tips or tips from different manufacturers. Switching to a different supplier can also lead to a change in flow velocities and thus different shear forces. For example, the 8 % smaller diameter of competitor E's pipette tips used in the iPSC experiments results in a 16 % higher theoretical flow velocity compared with the epT.I.P.S.

The results of this study show the importance of understanding that the tip and the pipette form one system. This is because the tip defines the orifice diameter and the instrument defines the volume flow. Both together define the flow velocity that influences shear stress. Where, in the case of HEK 293 cells, no improvement using wide-bore tips was found, the iPSC reacted positively to the wide-bore variants 5 mL serological pipette from Eppendorf and 1 mL pipette tip. This indicates the need for wide-bore tips also depends on the sensitivity of the functional unit. However, the results for the iPSC have been confirmed by Agashi et al. [29] who found a direct relationship between a needle's bore diameter respective to the increase in flow rate and the response of primary murine mesenchymal stem cells (mMSCs).

For the two liquid handling systems we examined through computer simulation for this study (Multipette/Combitips advanced, Eppendorf Xplorer plus/epT.I.P.S.), we found shear stress to be proportional to the effective (CFD) flow velocity as well as to the theoretical flow velocity (calculated on the basis of orifice diameter and volume flow). A simple rule for laboratory practice can be derived from this: The higher the flow velocity, the greater the shear force. For this reason, flow velocity may be a more practical value to work with than shear stress. Calculating shear stress is difficult as it should not be estimated for liquid handling systems using Hagen-Poiseuille's equation.

Bearing this in mind, users who pipette functional units such as cells should pay attention first and foremost to the piston speed they use for their work. Electronic devices offer an important advantage over mechanical systems: They can dispense very slowly and reproducibly. Brindley et al. [8] mentioned that sources of bioprocess forces are highly variable among operators. Experience has shown that users usually move the pistons of mechanical pipetting systems too fast. In contrast, slow speeds can be set with electronic devices. This ensures the permissible shear stresses for certain functional units are not exceeded as well as prevents differing flow velocities among different users. This is of particular interest when working in teams. If team members use mechanical dispensing systems and apply different piston speeds, they apply different shear stresses that may result in different assay results. Programming functions support these users because pipetting speeds, once defined, can be saved and automatically applied when running the respective program at a later point.

Conclusion

We found it possible to generate shearing conditions during liquid handling with unfavorable conditions for HEK 293 cells. However, the conditions applied were very harsh with maximum piston speed, five successive filling and emptying cycles, and cells grown in medium without serum.
When handling functional units such as cells in the laboratory, one should consider that shear stress is influenced by a triad of factors: Physical factors (for example, flow velocity), biological factors (for example, cell size or age), and experimental factors (for example, duration of shearing, treatment of cells like freezing or thawing, and growth in medium without serum). For this reason, the transfer of "critical limits" for tolerable shear stress from the literature is only possible to a very limited extent. Instead, users need to define the shear stress limits specifically for their specific cell type and treatment. From our experiments, we were, however, able to derive the following recommendations for the daily laboratory routine:

> Liquid handling devices are constructed to support a large number of applications. Not every speed setting from the **wide range of piston speeds** may be useful for transferring functional units. Make sure to apply a speed level suitable to the chosen tip and soft enough for the functional unit to be transferred.

> Understand the liquid handling device and its tip as a **system** as both elements influence the flow velocity at the narrowest point: The tip's size and orifice diameter and the instrument's piston speed. It does not help to cut pipette tips when using a mechanical pipette with a fast piston movement.

> **Literature values** for "critical limits" can **only be transferred to a very limited extent** to liquid handling systems. The reported systems and their conditions are too different. For example, a plate-and-cone viscometer does not envision the conditions of the liquid's entry into the pipette tip, and the shearing stimulus duration is much longer than with liquid handling systems.

> Liquid handling systems that can be used with **different tip sizes** create very different, tip-specific volume flows and thus very different flow velocities. Make sure to apply suitable speed levels when switching between tips. This also applies when changing from one tip manufacturer to another as bore sizes differ.

> **Flow velocity is proportional to the shear stress** that is created. Since the effective shear stress is difficult to calculate, we recommend applying flow velocity as a measure for laboratories because it is easy to calculate. Note that we demonstrated that the Hagen-Poiseuille's equation is not suitable for describing shear stress in liquid handling systems. To support our users, flow velocity values for different Eppendorf electronic instrument-tip combinations are provided in the appendix (see tables 7 and 8).

> With liquid handling systems, the **duration of the shear stress stimulus** is very short. It predominantly occurs at the tip's orifice. Our results indicate that a short but high shear stress is tolerable as long as the threshold for the membrane capacity is not exceeded. No influence on HEK 293 cell death or proliferation was observed with one pipetting step but high flow velocity. However, we have not determined more specific factors like gene expression.

> **Reducing pipetting steps** avoids processing stress. When resuspending cells, check how many pipetting cycles are actually needed for a particular cell culture. One pipetting step may already be sufficient [25].

> **Aspiration is more critical than dispensing**. When needing to work faster, increase the piston movement of the dispensing step, not the aspiration step. In addition, the tip should not touch the ground as a narrow gap between the tip and wall or bottom creates unnecessary shear stress.

> When transferring functional units, **use electronic liquid handling instruments**. These instruments offer very slow piston speeds, which the user typically does not reach with mechanical systems. And they allow reproducible speed settings. Instead of leaving it to the user, the variable "differing influence on analysis by shear stress" can be removed from the equation when electronic systems are, for example, used for passaging, cell seeding or resuspension.

> **Wide-bore tips** are not always needed. Our results show that with the Eppendorf Xplorer plus, wide-bore tips did not improve the number of lysed cells nor the proliferation of HEK 293 cells. However, if sensitive functional units, such as iPSC, are to be transferred using wide-bore tips can be advantageous.

> Eppendorf electronic liquid handling systems offer flow velocities that are slow enough to minimize **shear stress to below 1 N/m²** (see table 6). This makes them a very good option for gently handling functional units.

When it comes to transferring very sensitive functional units, one should make sure to choose liquid handling system that offer slow enough volume flows. This is not standard with, for example, all manufacturers' electronic pipettes and should be mentioned before purchase. In this study, we used Eppendorf liquid handling systems to create extreme testing conditions by setting the systems' speed levels to maximum, repeating the pipetting five times, and growing cells without serum. This was done on purpose to study the creation of shear stress in liquid handling systems and derive the recommendations outlined above. However, as shown in table 6, these systems can also be set to very slow flow velocities. Considering the very short duration of exposure to shear stress, it is obvious that the very low shear stimulus associated with Eppendorf liquid handling systems set to minimum speed levels is negligible.

Tab. 6: Smallest adjustable effective flow velocities and shear stress of the liquid handling systems examined in this study

Liquid handling system	Effective flow velocity (m/s)	Shear stress (N/m²)
Multipette E3/E3x with Combitips advanced 10 mL	0.32	1.8
Multipette E3/E3x with Combitips advanced 5 mL	0.27	0.87
Multipette E3/E3x with Combitips advanced 2.5 mL	0.15	0.27
Eppendorf Xplorer plus pipette with epT.I.P.S. 1 mL	0.23	0.97

Our results show that the instrument's speed level and the tip's orifice alone are not the right measure – it is simply not enough to consider the volume flow or to use a wide-bore or bigger tip. Only flow velocity, a factor calculated on the basis of the orifice diameter and the instrument's volume flow, derives a value that can be applied as a measure to estimate the situation of functional units. To support our users, flow velocity values for different Eppendorf electronic instrument-tip combinations are provided in the appendix.

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Appendix

As the effective wall shear stress is difficult to estimate in the laboratory routine, the flow velocity, which was found to be proportional to the wall shear stress in the tested systems, may be taken as an approximative value for the load of the functional units by pipetting. In any case, the flow velocity (m/s) is a better indicator than the volume flow (mL/s) being stated in the instrument's operating manual as the latter does not consider the tip's orifice diameter. This underlines the importance to understand that the instrument and the tip build a system – it is insufficient to consider only the tip's bore or the instrument's volume flow.

The application of the flow velocity offers two advantages over the volume flow: 1) the narrowest point of the system becomes considered and 2) different instrument types or different tips used with the same instrument can be compared. Table 7 and 8 state the theoretical flow velocities of Xplorer / Xplorer plus pipette and Multipette E3 / E3x for the different instrument - tip combinations. Please note that this information is not transferrable to other manufacturer instruments or tips. For further information please refer to chapter "results and discussion".

Tab. 7: ep Dualfilter T.I.P.S.® pipette tips with Xplorer and Xplorer plus pipettes at different speed settings
Theoretical flow velocities of different epDualtilter T.I.P.S. pipette tips with Xplorer and Xplorer plus at different speed settings

	Theoretical flow velocity (m/s) with ep Dualfilter T.I.P.S.								
Speed level	0.1 - 10 µL M	2 - 100 µL	2 - 200 µL	20 - 300 µL	50 - 1,000 µL	50 - 1,250 µL L	0.5 - 2.5 mL	0.1 - 5 mL	1 - 10 mL
1	0.008	0.039	0.059	0.088	0.106	0.193	0.157	0.195	0.294
2	0.012	0.059	0.088	0.133	0.159	0.289	0.235	0.292	0.441
3	0.025	0.118	0.177	0.265	0.318	0.578	0.314	0.390	0.588
4	0.035	0.168	0.253	0.379	0.455	0.825	0.392	0.487	0.735
5	0.045	0.214	0.322	0.482	0.579	1.050	0.523	0.650	0.980
6	0.061	0.294	0.442	0.663	0.796	1.444	0.589	0.731	1.102
7	0.082	0.392	0.589	0.884	1.061	1.925	0.673	0.835	1.260
8	0.109	0.523	0.786	1.179	1.415	2.310	0.724	0.899	1.357

Tab. 8: Theoretical flow velocities of different Combitips advanced dispenser tips with Multipette E3 and E3x at different speed settings

Theoretical flow velocity (m/s) with Comitips advanced									
Speed level	0.1 mL	0.2 mL	0.5 mL	1 mL	2.5 mL	5 mL	10 mL	25 mL	50 mL
1	0.013	0.030	0.042	0.043	0.083	0.166	0.212	0.180	0.186
2	0.030	0.060	0.085	0.087	0.124	0.249	0.318	0.325	0.279
3	0.045	0.091	0.127	0.130	0.199	0.398	0.509	0.541	0.478
4	0.072	0.145	0.204	0.208	0.332	0.663	0.849	0.928	0.837
5	0.151	0.302	0.424	0.433	0.663	1.326	1.698	1.547	1.395
6	0.181	0.362	0.509	0.520	0.829	1.658	2.122	1.911	1.674
7	0.226	0.453	0.637	0.650	1.105	2.210	2.829	2.499	2.093
8	0.302	0.604	0.849	1.866	1.658	3.316	4.244	3.609	2.790

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