Critical location of cell viability loss during the cell injection process in hepatocyte transplantation using a rectangular microchannel model

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Abstract
A sufficient number of functional live hepatocytes delivered to a recipient is necessary for cell therapy. Preventing cell viability loss during the cell injection process is important to improve the clinical outcomes of hepatocyte transplantation. The critical location of cell viability loss is important to identify the causal relationship between the viability loss and cell injection process. In this study, the critical location of cell viability loss was determined experimentally in a rectangular microchannel by microscopic high-speed camera observations. Live hepatocyte distributions were investigated upstream and downstream, and measured on three planes, top, center, and bottom, under horizontally or vertically supplied conditions of the syringe orientation. Sedimented and uniform dispersion conditions of the live hepatocyte distribution at upstream of the microchannel were classified according to observations at horizontal and vertical syringe orientations, respectively. Higher hepatocyte viability loss was found under the sedimented condition. The results suggested that the critical location of hepatocyte viability loss was on the bottom plane of the microchannel. Furthermore, physical causes of the hepatocyte viability loss were found by micro-scale observations of the cell velocity and diameter during the cell injection process. This information may contribute to development of a guideline for the cell injection process to improve hepatocyte transplantation.

Keywords: Hepatocyte, Viability loss, Accidental cell death, Rectangular microchannel, Cell transplantation

1. Introduction

Cell transplantation is a promising alternative therapy to treat end-stage diseases of an organ. The shortage of functional donor organs for transplantation has initiated a cell transplantation trend. The fundamental therapeutic concept of cell transplantation is the replacement of dysfunctional cells by functional live cells to treat a disease (Monahan et al., 1991). Cell transplantations for disease therapy in preclinical and clinical trials include bone marrow (Thomas, 2005), neurons (Huang et al., 2010), islets (Shapiro et al., 2006), and hepatocytes (Enosawa et al., 2014).

Hepatocyte transplantation can cure inborn error diseases, biliary atresia, cirrhosis, fulminant hepatic failure, and viral hepatitis (cirrhosis) (Gramignoli et al., 2015). The benefits of hepatocyte transplantation are that cells can be received by multiple recipients through simple administration using cryopreserved cells, and the cells can be repeatedly transplanted (Forbes et al., 2015). Hepatocyte transplantation also reduces costs compared with organ transplantation (Forbes et al., 2015; Strom et al., 2006). Transplantation of hepatocytes is a promising treatment for liver diseases because of various administration choices and cost considerations. However, its translation to clinical practice has some problems.
Some of the problems in hepatocyte transplantation are a limited supply of donor livers, isolation of high-quality cells, viability loss upon cryopreservation, low cell engraftment and proliferation, and allograft rejection (Forbes et al., 2015). Important considerations for hepatocyte transplantation are its long-term efficacy (Dhawan et al., 2010) and therapeutic benefits (Forbes et al., 2015). As a basic quality indicator of cells, the viability of hepatocytes is measured after cell isolation or before cell injection (Soltys et al., 2010).

Viability measurement post-injection for purposes other than cell transplantation has been reported by some research groups. Studies have reported the effects of cell injection by a needle or cannula on the viabilities of embryonic rat brains cells (Torres et al., 2015), fibroblasts (Amer et al., 2015), mesenchymal cells (Agashi et al., 2009; Heng et al., 2009; Walker et al., 2010; Mamidi et al., 2012; Amer et al., 2016), neural stem cells (Rossetti et al., 2015) and hepatocytes (Meyburg et al., 2009a). Improvement of cell injection is desirable to increase the quantities of functional live cells delivered to the target organ, and mechanistic investigation of the phenomena may facilitate development of the cell injection process.

Hepatocyte apoptosis (Fisher et al., 2004), anoikis (Smets et al., 2002), necrosis (Carini et al., 1999), and necroptosis might occur in cell transplantation. However, mechanically induced accidental cell death (Galluzzi et al., 2015), which is considered as the primary cause of hepatocyte viability loss during cell injection for transplantation, is not clearly defined. Knowledge of mechanically induced accidental cell death is necessary for improvement of the cell injection process because the duration of the event is faster than programmed cell death such as apoptosis, anoikis, necrosis, and necroptosis. Identification of mechanically induced accidental cell death is necessary to achieve safe cell injection conditions.

Safe clinical conditions for hepatocyte transplantation have been determined empirically by cell injection experiments using various catheter sizes and flow rates (Meyburg et al., 2009a). Safe cell injection conditions for clinical application are suggested to be a flow rate of less than 2 mL/minute using a catheter of 4.2 F (Meyburg et al., 2009). However, wall shear stress in the 4.2 F catheter at a flow rate of 2 mL/minute is higher than that in 5 and 6.6 F catheters at the same flow rate with respective values of 1.3, 0.5, and 0.3 Pa (Sufiandi et al., 2015).

Shear-stress-induced hepatocyte viability loss has not yet been evaluated, especially in terms of a mechanical cause during cell injection. The cell injection problem of sedimentation in the syringe is a clinical obstacle for cell delivery in transplantation. In practice, gently shaking and rotating the syringe are usually undertaken to prevent sedimentation (Meyburg et al., 2009b). Therefore, observations of the effects of sedimentation and shear stress on cell viability loss may further our understanding of the phenomena.

The effect of shear stress and sedimentation have been investigated at macro-scale using a rectangular microchannel model (Sufiandi et al., 2015). This results were found, the vertical syringe orientation reduced viability loss during hepatocyte delivery and removed the sedimentation effect in the syringe. Avoiding sedimentation can prevent a hepatocyte from experiencing high shear stress located near the wall of the delivery line. However, the process of hepatocyte viability loss at the near wall of a microchannel has not been verified experimentally.

This study identified the critical location of hepatocyte viability loss. It was determined whether the hepatocyte viability loss was caused by shear stress on a cell in suspension or near the wall of the delivery line. Therefore, this study determined the critical location of live hepatocyte loss by comparing the live cell distribution at the upstream and downstream in a microchannel. The distribution difference in the microchannel identified the cell loss location, which is preliminary information to understand the processes of viability loss in the delivery line during hepatocyte injection.

2. Materials and methods

Hepatocyte injection was observed in a microchannel to determine the critical location of hepatocyte viability loss on an axis perpendicular to the direction of fluid suspension. A suspension flow rate of 0.4 mL/minute (1.3 Pa) was chosen based on the same wall shear stress of the safe clinical injection condition (Meyburg et al., 2009a, Sufiandi et al., 2015) and a significant difference of viability loss (Sufiandi et al., 2015) in the two supply methods of horizontal and vertical syringe orientations. The viability loss difference might be caused by shear stress on a cell, which depends on the cell location in the microchannel and the distribution of live hepatocytes. Live hepatocytes were assessed by the impermeable fluorescent dye propidium iodide, which indicates integrity of the cell membrane, and observed under an inverted microscope by a high-speed camera to identify the live hepatocyte distribution. Live hepatocyte distributions while passing through the microchannel at the upstream and downstream positions were measured on three planes, top, bottom, and center, as shown
in Fig. 1, and compared to determine the critical location of live hepatocyte loss.

Fig. 1 Experimental setup and observation area in the microchannel. The delivery line consisted of the inlet tube (0 to 1), microchannel (1 to 2), and outlet tube (2 to 3). The inlet tube was connected to a syringe and the microchannel. A sample-collecting port was an outlet tube connected at the downstream of the microchannel.

2.1. Hepatocyte preparation
Nine-week-old male Sprague-Dawley rats (specific pathogen free) were purchased from Sankyo Labo Service., Co. Inc. (Tokyo, Japan). Animals provided with standard rat chow and water ad libitum were used for hepatocyte isolation.

Hepatocyte isolation was performed according to the method of Seglen (Seglen, 1976). The isolation procedure for hepatocytes (Hsu et al., 2013; Sufiandi et al., 2015; Yasuda et al., 2015) was performed as follows. A rat was anesthetized with 1.0 % isoflurane (Mylan Inc., Canonsburg, PA, USA). The blood in the liver was flushed out by perfusion of calcium and magnesium-free Hanks Balanced Salt Solution (14175-095, Life Technologies, Carlsbad, CA, USA) through a cannula inserted into the portal vein. Then, connective tissues in the liver were digested by perfusion of 100 mL of a 1 mg/mL collagenase solution (Wako Pure Chemical Industries, Tokyo, Japan) at 38°C for 10 minutes. The whole liver was dispersed in 50 mL Williams Medium E (12551-032; Gibco, Life Technologies, Carlsbad, CA, USA). The hepatocyte suspension was sequentially filtered through sterile gauze (071116A, Suzuran Sanitary Goods Co., Ltd., Nagoya, Japan) and a 100 µm nylon mesh (SN-91-1194, Sansyo Co., Ltd., Tokyo, Japan). Then, the cells were isolated by centrifugation.

The filtered hepatocyte suspension was washed three times by centrifugation for 1 minute at 60 g and 4°C (Heraeus Labofuge 400R, Thermo Fisher Scientific Inc., MA, USA). Then, the hepatocyte suspension in Williams Medium E was diluted to a concentration of 1×10⁶ cells/mL. The viability and concentration of hepatocytes were determined by the 0.04% trypan blue (15250-061, Gibco, Life Technologies, Carlsbad, CA, USA) exclusion method using a hemocytometer (Digital Bio Technology Co. Ltd., Seoul, Korea). The hepatocyte suspension was stored in a cold box in the vertical orientation to prevent prolonged cell contact with the container wall. Before experiments, the hepatocyte suspension was stained with propidium iodide (P3566, Life Technologies, Carlsbad, CA, USA) at a concentration of 20 µL for 1×10⁶ cells at room temperature in the range of 20°C to 26°C in the dark for 30 minutes. The hepatocyte suspension was stored in a cold box during experiments at a temperature range from 4°C to 10°C.

Animal experiments followed the guidelines of the care and use of laboratory animals of the National Research Institute for Child Health and Development. Experimental procedures were approved by the Institutional Animal Ethics Committee of the National Center for Child Health and Development (Tokyo, Japan) (Reference No. 2000-001).

2.2. Determination of the hepatocyte distribution
Hepatocyte suspensions were supplied to the microchannel using a syringe under two orientation conditions. One was a supply condition with a horizontal orientation of the syringe plunger perpendicular to the gravitational and cell
sedimentation direction (Fig. 1). The other was a vertical orientation of the syringe plunger with gravitational and cell sedimentation moving in the same direction (Fig. 1). The syringe outlet was connected using a tube to the upstream of the microchannel for micro-scale observations.

The conditions of hepatocyte suspension passing through a catheter was simulated using the microchannel, and the live cell distribution in the microchannel was observed under a microscope. The various microchannel lengths, heights, and sizes in this study were chosen to match the viewable area of the observation stage in the microscope and objective lens travel height. The microchannel with dimensions of 50 mm × 2 mm × 0.2 mm (Vitrotubes Rectangle Capillary Tubing Borosilicate Glass; Vitrocom, Mountain Lakes, NJ, USA) was connected to a tube with a length and internal diameter of 0.2 and 0.8 mm, respectively (Tygon E-3603, ACF00001, Saint Gobain, Tokyo, Japan), at both sides. The edges of the tube and microchannel were covered with a silicone seal of a photo-curing type adhesive composition. The inlet side of the tube was connected to a syringe (1 mL; Terumo Syringe, Tokyo, Japan) and driven by a syringe pump (Fusion 200 Classic Syringe Pump; Chemyx, Inc., Stafford, TX, USA) at a flow rate of 0.4 mL/minute. The flow rate was chosen according to a previous significant difference of viability loss in a macro-scale observation condition between vertical and horizontal syringe orientations with 1.3 Pa wall shear stress (Sufiandi et al., 2015). The microchannel and tube assembly defined as the delivery line, consisted of the inlet tube (0 to 1), microchannel (1 to 2), and outlet tube (2 to 3). The inlet tube was connected to a syringe and the microchannel. A sample-collecting port was an outlet tube connected at the downstream of the microchannel. The assembly was used to simulate a catheter with clinical wall shear stress of the 4.2 F catheter at a flow rate of 2 mL/minute (Meyburg et al., 2009a). Observations in the microchannel were performed under the inverted microscope and recorded by the high-speed camera.

The microchannel was installed horizontally on the observation stage of the inverted microscope (Nikon Eclipse TE2000-U) with 40x objective lens magnification (CFI Plan Fluor ELWD 40XC). The focal travel range of the microscope objective lens on the z-axis was calibrated by visually checking the inside of the microchannel using 1 µm particles (R0100, Thermo Fisher Scientific Inc., USA) suspended in pure water at a concentration of 1×10^6 particles/mL. The measurement plane depth (Meinhart et al., 2000) of the microscope was observed in the median range of 49 µm (35 µm to 65 µm) on the focal plane, depending on the diameter of hepatocytes. The measurement depth nearly covered the required range of measurement planes, which is the same as a 1/3 microchannel height of 67 µm. A live cell was distinguished from a dead cell using the propidium iodide exclusion method.

The live hepatocyte distribution in the microchannel was determined by the propidium iodide exclusion method using a G-2A filter (excitation filter, 510560 nm; dichroic mirror, 575 nm; barrier filter, 610 nm). The light sources were a 100 W mercury lamp (Nikon LH-M100CB-1) and 100 W halogen lamp. The hepatocyte conditions were distinguished by adjusting the brightfield light source intensity to maintain the visibility of fluorescent propidium iodide (Fig. 2). Images of the hepatocyte condition were recorded simultaneously through the microscope front port by a high-speed camera (NAC Memrecam GX-1 Monochrome 2 GB) at a 1024 × 464 pixel resolution, 666 µs shutter speed, and 1000 fps frame rate. Each data set contained 493 frames with a 1 ms interval. The numbers of unstained live hepatocytes and stained dead hepatocytes in each data set were manually counted by observation of the image sequences in ImageJ software (National Institutes of Health, Bethesda, MD, USA). Observations were performed on three measurement planes: bottom, center, and top.

The live hepatocyte distributions in the two syringe orientations were measured in the microchannel under the inverted microscope and recorded by the high-speed camera. The live hepatocyte distribution was measured using the non-dimensional position of cells on the z-axis, \( z/h = -3/8, 0, \) and 3/8, at the upstream (1) and downstream (2) position as shown in Fig. 1. The position of \( z/h = -3/8, 0, \) and 3/8 was denoted as the planes (p) of the bottom (b), center (c), and top (t). Recorded measurement data on the top, center, and bottom planes were then processed for data presentation.

### 2.3. Calculations and analysis of data

The number of live hepatocytes \( n_L \) was measured in suspension based on the principle that live cells possess intact cell membranes that exclude certain dyes (Strober, 2001). Trypan blue and propidium iodide dyes were used to assess the numbers of dead hepatocytes \( n_D \) in a suspension by a hemocytometer or microchannel, respectively. The numbers of live \( n_L \) and dead \( n_D \) hepatocytes were calculated in the observation locations. The observation locations \( m \) at (0), (1), (2), and (3) for the inlet side of the tube, upstream of the microchannel, downstream of the microchannel, and outlet side of the tube, respectively, are shown in Fig. 1. Cell viabilities at the tube inlet (0) and outlet (3) were measured in hepatocyte suspensions of pre- and post-injection samples using the hemocytometer. The cell viabilities during movement in the microchannel were measured in hepatocyte suspensions using the high-speed camera at the observation locations \( m \) of...
Fig. 2  Cell observation in the microchannel using a high speed camera. Live hepatocytes (A); dead hepatocyte (B).

upstream (1) and downstream (2). The hepatocytes were observed on the three measurement planes $p$ of the bottom ($b$), center ($c$), and top ($t$) as denoted by $n_{L,m,p}$ for the number of live hepatocytes and $n_{D,m,p}$ for the number of dead hepatocytes. Therefore, the number of live cells at location $m$ for the microchannel measurement defined in Eq. (1)

$$n_{Lm} = \sum n_{L,m,p} = n_{Lm,b} + n_{Lm,c} + n_{Lm,t}$$ (1)

and the number of dead cells at location $m$ for the microchannel measurement defined in Eq. (2).

$$n_{Dm} = \sum n_{D,m,p} = n_{Dm,b} + n_{Dm,c} + n_{Dm,t}$$ (2)

The $n_L$ and $n_D$ were then used for calculation of the following parameters.

The number of live hepatocytes $n_L$ is presented as a percentage ratio to their initial condition with the aim to independently observe the parameter because there was a tendency for the disappearance of dead cells (Yasuda et al., 2015), which influenced the calculation of cell viability. The ratio of live hepatocytes is defined as the ratio of $n_L$ at a particular location of $m$ to $n_L$ at their initial condition $m(i)$ and shown as a percentage (Eq. (3)). For measurements using the hemocytometer, their initial condition $m(i)$ was at (0). For measurements in the microchannel, their initial condition was at (1).

$$R_{Lm} = \frac{n_{Lm}}{n_{Lm(i)}} \times 100$$ (3)

Dead hepatocytes were also calculated using the same ratio of Eq. (4).

$$R_{Dm} = \frac{n_{Dm}}{n_{Dm(i)}} \times 100$$ (4)

The live hepatocyte distribution was also calculated to observe their distribution in the microchannel.

The live hepatocyte distribution was used as initial data to determine the location of cell viability loss on the three observation planes in the microchannel. The live hepatocyte distribution in the microchannel at $m$ on measurement plane $p$ is defined as the ratio of $n_{L,m,p}$ to the number of live hepatocytes upstream $n_{L1}$. The values are presented as a percentage ratio to the total live hepatocytes measured upstream. Therefore, the live and dead hepatocyte distributions were calculated by the formula defined in Eq. (5) and Eq. (6).

$$R_{Lm,p} = \frac{n_{L,m,p}}{n_{L1}} \times 100$$ (5)

$$R_{Dm,p} = \frac{n_{D,m,p}}{n_{D1}} \times 100$$ (6)

$R_{m,1}$ was used for calculation of the live hepatocyte difference.

Viability is used to determine the number of viable cells in a cell suspension (Strober et al., 2001). The viability of hepatocytes at location $m$ is defined as the ratio of $n_{Lm}$ to the total number of cells in a suspension at location $m$ and shown as a percentage (Eq. (7)).

$$V_m = \frac{n_{Lm}}{n_{Lm} + n_{Dm}} \times 100$$ (7)
Viability data were then used to calculate cell viability loss.

The cell viability loss was calculated as the difference between the viabilities in the initial and post-injection conditions. The viability loss in the delivery line $\Delta V_D$ was calculated as the difference between the viability measured in the outlet (3) and inlet (0) of the delivery line as defined in Eq. (8).

$$\Delta V_D = V_3 - V_0$$  \hspace{1cm} (8)

Conversely, the viability loss in the microchannel $\Delta V_C$ was calculated as the difference between viabilities measured downstream (2) and upstream (1) as defined in Eq. (9).

$$\Delta V_C = V_2 - V_1$$  \hspace{1cm} (9)

The live $\Delta R_{LD}$ or dead hepatocyte difference $\Delta R_{DD}$ in the delivery line was calculated as the difference between the ratios of live or dead hepatocytes at the outlet $R_3$ and inlet $R_0$ as defined in Eq. (10) and Eq. (11), respectively.

$$\Delta R_{LD} = R_{L3} - R_{L0}$$  \hspace{1cm} (10)

$$\Delta R_{DD} = R_{D3} - R_{D0}$$  \hspace{1cm} (11)

The live $\Delta R_{LC}$ or dead hepatocyte difference $\Delta R_{DC}$ in the microchannel was calculated as the difference between the ratios of live or dead hepatocytes downstream $R_2$ and upstream $R_1$ as defined in Eq. (12) and Eq. (13), respectively.

$$\Delta R_{LC} = R_{L2} - R_{L1}$$  \hspace{1cm} (12)

$$\Delta R_{DC} = R_{D2} - R_{D1}$$  \hspace{1cm} (13)

Positive or negative values indicated a hepatocyte surplus or loss, respectively, between measured and initial locations.

The differences of the live hepatocyte distribution in the microchannel on each measurement plane $\Delta R_{L,p}$ were calculated as the differences between the live hepatocyte distribution downstream $R_{L2,p}$ and upstream $R_{L1,p}$ as defined in Eq. (14).

$$\Delta R_{L,p} = R_{L2,p} - R_{L1,p}$$  \hspace{1cm} (14)

The differences of the dead hepatocyte distribution in the microchannel on each measurement plane $\Delta R_{D,p}$ were calculated as the differences between the live hepatocyte distribution downstream $R_{D2,p}$ and upstream $R_{D1,p}$ as defined in Eq. (14).

$$\Delta R_{D,p} = R_{D2,p} - R_{D1,p}$$  \hspace{1cm} (15)

Data are presented as mean values with error bars or a box indicating the standard deviation or standard error, respectively. In graphs without a box, error bars indicate the standard error of the mean. The Students t-test was used to compare differences between the measurements. P-values of $<0.05$ and $<0.005$ are marked with a single and double asterisk, respectively.

The live hepatocyte distribution in the microchannel downstream (2) on each plane $R_{L2,p}$ was modeled by calculating the cell trajectory movement that starts at the upstream (1) location of the top $R_{L1,c}$, center $R_{L1,c}$, and bottom $R_{L1,b}$ planes. The number of live hepatocytes observed experimentally at the upstream was used as the input value for the calculation model to determine the live hepatocyte distribution downstream in the microchannel. The location of cells at a particular time was dependent on the trajectory movement at position $x$ and $z$ calculated by the formulas in Eq. (16) and Eq. (17), respectively, as the product of velocity on the horizontal axis $u_x$ from sedimentation (Eq. (19)) (Happel and Brenner, 1973).

$$x(\gamma) = x(0) + u_x \cdot \gamma$$  \hspace{1cm} (16)

$$z(\gamma) = z(0) + u_z \cdot \gamma$$  \hspace{1cm} (17)

$$u_x = -\frac{1}{2\mu} \cdot \frac{\Delta P}{\Delta x} \cdot \left( \left( \frac{1}{2} \right)^2 - \gamma^2 \right)$$  \hspace{1cm} (18)

$$u_z = \frac{d^2 \cdot (\rho - \rho_0) \cdot g}{18\mu}$$  \hspace{1cm} (19)
To calculate the velocity profile in Eq. (18), the pressure difference between upstream and downstream was calculated by the formula

$$\Delta P = \frac{l}{d_h} \cdot \frac{\bar{u}^2 \rho_0}{2}$$

with $\lambda = 64/Re$, and microchannel length as $\Delta x = 50 \times 10^{-3}$ m. The hydraulic diameter of a rectangular microchannel $d_h = 4w h / (w + h)$, and Reynolds number $Re = (\rho_0 \cdot \bar{u} \cdot d_h) / \mu$. Average velocity was calculated by the formula $\bar{u} = Q / A$, and the cross-sectional area of the microchannel by the formula $A = w \cdot h$.

Parameters used for calculation, such as the flow rate of the syringe pump, were $Q = 6.7 \times 10^{-9} \text{ m}^3/\text{s}$ (0.4 mL/minute), width of the microchannel $w = 2 \times 10^{-3}$ m, and height of the microchannel $h = 0.2 \times 10^{-3}$ m. Other parameters for calculations referred to prior measurements in a macro-scale investigation (Sufiandi et al., 2015), such as viscosity of Williams Medium E, $\mu = 1.329 \times 10^{-3}$ Pa.s measured at 10°C. Measured median diameters of hepatocytes, $d = 18.4 \mu$m (12.425.0 $\mu$m) (Sufiandi et al., 2015). The density of hepatocytes $\rho = 1100$ to 1130 kg/m$^3$ (Sakiska, 1982). The density of Williams Medium E, $\rho_0 = 1007$ kg/m$^3$ was measured using a density meter (DA-130, Kyoto Electronics Manufacturing, Tokyo, Japan) and gravitational acceleration, $g = 9.8$ m/s$^2$.

The calculations employed a low density of hepatocytes (Sakiska, 1982). The calculations were performed on spreadsheet software using LibreOffice Calc (The Document Foundation, Berlin, Germany). The live hepatocyte numbers inputted in the model of experiment results at the upstream were then grouped based on their $z$-axis location for each cell size at the downstream position. Live hepatocyte distributions at downstream, which are $R_{L2,b}$, $R_{L2,c}$, and $R_{L2,t}$, were compared between experimental and calculated results to analyze the location of hepatocyte viability loss and predict the cause of hepatocyte viability loss in the microchannel.

The predicted location of viability loss was on the bottom plane. The live hepatocyte difference indicated viability loss when the live hepatocyte distribution at the downstream $R_{L2,b}$ was less than that at the upstream $R_{L1,b}$. The live hepatocyte difference between upstream and downstream was represented by the value of $AR_{L,b}$. Therefore, to understand relationships between live hepatocytes that passed upstream on the bottom plane $R_{L1,b}$ and their viability loss on the same plane $AR_{L,b}$, both parameters were presented respectively by scatter graphs. The influence of passing on the bottom plane on the viability loss of live hepatocytes was identified by calculating the Pearson correlation coefficient between $R_{L1,b}$ and $AR_{L,b}$.

### 2.4. Cell velocity and diameter measurements

Individual live cell velocities were measured to determine the cell location during movement on the bottom plane of the microchannel because a cell follows the flow in the velocity profile. The cell velocity was calculated by the movement of cells measured on the bottom plane of the microchannel upstream (1) and downstream (2) using Fiji (ImageJ for living science). The cell was tracked manually frame-by-frame using Plug-in M Track J to measure their velocity. The measurements were then grouped into three categories of (A) live cells on the bottom plane of the microchannel, (B) live cells floating on the bottom plane of the microchannel, and (C) floating dead cells on the bottom plane of the microchannel. The number of cells tracked for each category was 15. Each tracked cell diameter was calculated by measuring their shape and area using ImageJ software with the assumption their form was spherical.

### 3. Results and Discussion

#### 3.1. Cell viability loss

Cell viability losses in the delivery line and microchannel measured on horizontal and vertical syringe orientations are shown in Fig. 3. The cell viability loss of the vertical syringe orientation was lower than that of the horizontal syringe orientation(Fig. 3). This trend was found in both microchannel and delivery line measurements. The delivery line consists of the inlet tube, outlet tube, and microchannel. The same decreasing trend indicated that observations in the microchannel represented the condition in the delivery line.

Cell viability loss is a common parameter used in clinical practice, and cell viability is dependent on the ratio of dead and total cells in the measured condition as shown in Eq. (7). Conversely, the live hepatocyte difference is calculated from the ratio of live hepatocytes downstream and upstream. The live hepatocyte calculation was not influenced by the number of dead cells (Eq. (3)) or the condition of cell lysis (Fig. 4). Therefore, the live hepatocyte losses in the microchannel and delivery line were measured to show the similarity of results and reflect the actual number of live hepatocytes lost.
Cell viability loss ∆V (%)
−25
−20
−15
−10
−5
0
Syringe Orientation
H
V
H V
Delivery line (∆VD)
microChannel (∆VC)

Fig. 3 Cell viability loss in the delivery line and microchannel.
H and V indicate horizontal and vertical syringe orientations, respectively. n=18

Fig. 4 Cell morphology observation in the hemocytometer.
Live hepatocytes (A), dead hepatocytes (B), cells beginning to lyse (C), and cell debris (D).

3.2. Live hepatocyte loss

Differences in the numbers of live and dead hepatocytes in the delivery line and microchannel measured at horizontal and vertical syringe orientations are shown in Fig. 5. About half of the live hepatocytes were lost during cell injection (Fig. 5). Ideally, when dead cells are not destroyed, the number of live hepatocytes lost and the dead hepatocyte surplus might be similar. However, the difference between live hepatocyte loss and dead hepatocyte surplus was significant, indicating that dead hepatocytes were destroyed as shown in the delivery line and microchannel at horizontal and vertical syringe orientations (Fig. 5).

Fig. 5 Live hepatocytes (L) and dead hepatocytes (D) in the delivery line (D) and microchannel (C). H and V indicate horizontal and vertical syringe orientations, respectively. Negative and positive values indicate loss and surplus of hepatocytes. n=18

3.3. Live hepatocyte distribution in the microchannel

The characteristics of the live hepatocyte distribution were classified based on the differences of live hepatocyte distributions at $R_{L1,b}$ and $R_{L1,c}$ compared with the baseline $R_{L1,t}$. The grouped distribution characteristics were used to determine the location of hepatocyte viability loss by comparing them between downstream and upstream of each group at the three measurement planes of the bottom (b), center (c), and top (t).
3.3.1. Sedimented condition

The live hepatocyte distribution $R_{L_{m,p}}$ in the sedimented condition is shown in Fig. 6. The live hepatocyte distribution $R_{L_{1,p}}$ at the upstream position in the horizontal syringe orientation produced the sedimented condition (Fig. 6A). The distributions at the downstream on the bottom plane decreased after live hepatocytes had passed through the microchannel (Fig. 6B). The differences in the live hepatocyte distribution indicated that the location of the highest loss was the bottom plane of $z/h = -3/8$ (Fig. 6C). Therefore, the location of hepatocyte viability loss might be at the bottom plane of the microchannel.

3.3.2. Uniform dispersion condition

The live hepatocyte distribution $R_{L_{m,p}}$ in the uniform dispersion condition is shown in Fig. 7. The live hepatocyte distribution $R_{L_{1,p}}$ at the vertical syringe orientation produced the uniform dispersion condition (Fig. 7A). The distributions at the downstream were increased on the bottom plane and decreased on the center plane after live hepatocytes had passed through the microchannel (Fig. 7B and Fig. 7C). The decreased live hepatocytes on the center plane might be caused by sedimentation as indicated by the live hepatocyte surplus at the bottom plane.

A decrease in the number of live hepatocytes in the sedimented condition was found on the bottom plane. However, a decrease in the number of live hepatocytes in the uniform dispersion condition was found on the center plane. The contradiction in the location of the decrease in live hepatocyte numbers between the sedimented and uniform dispersion conditions indicated the need for confirmation of the correct location of hepatocyte viability loss. A live hepatocyte distribution model utilizing cell sedimentation during flow in the microchannel describe in the following to confirm the live hepatocyte decrease at center plane in uniform dispersion condition was caused by cell sedimentation.

3.4. Live hepatocyte distribution model

The distribution difference between downstream and upstream of the sedimented condition (Fig. 6B and 6A) and uniform dispersion condition (Fig. 7B and 7A) was examined by the cell distribution model. Cell trajectories of live hepatocytes in the microchannel were calculated in two axes (Fig. 8). The number of live hepatocytes inputted in the model for sedimented and uniform dispersion conditions were the experiment results at upstream of Fig. 6A and Fig. 7A, respectively. Then, the number of live hepatocytes at each layer of the model were grouped based on their $z$-axis location for each cell size at the downstream position.
Fig. 8 Model of the live hepatocyte distribution in the microchannel: Trajectories. The trajectory of live hepatocytes with maximum (25 μm), median (18.4 μm), and minimum (12.4 μm) diameters are denoted by dashed, solid, and dotted lines respectively.

Cell distributions at the downstream were compared between the model and experiments. The calculation range of the live hepatocyte distribution model was covered or near the experimental results for the measurement planes of \( R_{L2,r} \) and \( R_{L2,c} \). The modeled results quantified the number of live hepatocytes on the center plane of the uniform dispersion condition (Fig. 7C), and the decrease in the number of live hepatocyte on the center plane was confirmed as the settling result of live hepatocytes from the center to bottom planes. The cell trajectory model showed that a large difference between experimental and modeled results had occurred on the bottom plane of the microchannel for both sedimented (Fig. 9A) and uniform dispersion conditions (Fig. 9B). Therefore, the decrease in the number of live hepatocytes on the center plane of the uniform dispersion condition was the result of cell sedimentation, and the location of the hepatocyte viability loss was on the bottom plane of the microchannel. The phenomena of hepatocyte viability loss on the bottom plane of the microchannel was then analyzed in more detail by comparing the parameters of \( R_{L1,b} \) with \( \Delta R_{L,b} \).

Fig. 9 Model of the live hepatocyte distribution in the microchannel: Model and experimental results. (A) Sedimented condition of the live hepatocyte distribution downstream. (B) Uniform dispersion of the live hepatocyte distribution downstream. • denotes experimental results with errors bar as the standard error of the mean. ☐ denotes model results with error bars as the range of calculation.

3.5. Uniform dispersion of dead hepatocytes

The dead hepatocyte distributions \( R_{Dm,p} \) in sedimented and uniform dispersion conditions are shown in Fig. 10 and Fig. 11, respectively. The dead hepatocyte distribution in the microchannel at horizontal (Fig. 10A) and vertical (Fig. 11A) syringe orientations produced a uniform dispersion condition. The results indicated that the dead hepatocyte distribution was not influenced by sedimentation. The differences in the dead hepatocyte distribution indicated that the location of the highest loss was the bottom plane of \( z/h = -3/8 \). Based on the dead hepatocyte location, critical live hepatocyte loss and cell lysis were at the bottom plane of the microchannel.

3.6. The critical location of live hepatocyte loss

The distribution of \( R_{L1,b} \) and \( \Delta R_{L,b} \) (Fig. 12) indicated decreasing relationships between the live hepatocytes distributed on the bottom plane of the microchannel and their distribution difference on the same plane. The uniform dispersion condition resulted in a surplus of live hepatocytes. The result was confirmed a settling process of live hepatocytes from the center plane to the bottom plane. Five data points of the uniform dispersion condition were under the 0 % line.
and resulted in the loss of the live hepatocyte distribution, indicating that hepatocyte viability loss might increase upon a distribution increase of live hepatocytes on the bottom plane of the microchannel. The sedimented condition resulted in a loss of the live hepatocytes distribution, and confirmed that the critical location of hepatocyte viability loss was on the bottom plane of the microchannel. Three data points of the sedimented condition were over the 0 % line, resulting in a surplus of live hepatocytes. Based on the high-speed camera images of these three data points, the phenomena might be caused by floating cell movement on the bottom plane of the microchannel. The floating cell movement on the bottom plane of the microchannel might be related to their form. Most surviving cells were in a clustered form as observed at the downstream position. The cell cluster form might have prevented the cells from contacting the bottom plane of the microchannel because their cross-sectional area is larger than a single cell, which may influence their sedimentation velocity. The distribution of $R_{L1,b}$ and $\Delta R_{L,b}$ indicated that the increased number of live hepatocytes on the bottom plane of the microchannel may decrease downstream. Changes in the live hepatocyte size were observed during their motion on the bottom plane of the microchannel by measuring their diameters and velocities.

### 3.7. Live cell size and velocity change

Live hepatocyte velocities (I) and their respective diameters (II) were measured to observe changes in cell size during their motion on the bottom plane of the microchannel (Fig. 13). Category A is live hepatocyte movement on the bottom plane of the microchannel. Cell locations were confirmed by the calculated Poiseuille velocity profile. The velocity at location $29.2\pm3.5$ µm of the cell diameter at upstream as shown in Fig. 13 II-A was the same as the measured velocity at the upstream position of $9.8\pm1.8$ mm/s as shown in Fig. 13 I-A. The result of category A indicated that cells rotate or slide on the bottom plane of the microchannel. Floating cells in category B (Fig. 13 II-B) and C (Fig. 13 II-C) were indicated by velocities higher than 10 mm/s. The result of the hepatocyte viability loss process might be related to the cell size change on the bottom plane of the microchannel, which was verified as the critical location of cell viability loss. Live hepatocytes in category A had a decrease in their size after passing through the microchannel (Fig. 13 II-A), and their size difference between upstream and downstream was significant. The decrease in the size of live hepatocytes might be related to the regulatory volume...
Fig. 12  **Live hepatocytes upstream and their change downstream.**

$R_{L,b}$ is the live hepatocyte distribution upstream on the bottom plane of the microchannel. $\Delta R_{L,b}$ is the live hepatocyte difference on the bottom plane of the microchannel with negative or positive values indicating a decrease or increase in the number of live hepatocytes, respectively. $n=36$ (18 for each condition)

Fig. 13  **Cell velocities and sizes on the bottom plane of the microchannel.**

I and II indicate cell velocities and equivalent cell diameters, respectively. AC indicate live hepatocyte movement on the bottom plane of the microchannel, and live and dead hepatocyte movement above the bottom plane of the microchannel, respectively. U and D indicate upstream and downstream positions of observation, respectively. $n=90$ (15 pairs for each condition)
decrease mechanism to maintain their steady state condition (Okada et al., 2001). The relationship between movement on the bottom plane of the microchannel and the regulatory volume decrease may explain the process of hepatocyte viability loss.

The process of hepatocyte viability loss is described as follows. First, along with the cell injection process, live hepatocytes settle on the bottom plane of the microchannel because their density is higher than that of the suspension medium (Fig. 8). Upon settling, live hepatocytes may contact the bottom plane of the microchannel. The settled cells then move on the bottom plane of the microchannel in the category A condition. The category A condition is cell movement that contacts the bottom plane of the microchannel (Fig. 13 II-A) as indicated by the location of the cells in the velocity profile (Fig. 13 I-A). The cell movement along the bottom plane of the microchannel with a particular velocity is the product of the force applied to the cell by the suspension fluid flow parallel to the x-axis direction. Upon the condition of cells contacting the solid surface, shear force is applied to the cells. The shear force may damage the cell membrane as indicated by the live hepatocyte loss on the bottom plane of the microchannel (Fig. 6C, Fig. 9A and Fig. 9B). In addition, an increase of dead cells on the bottom plane was confirmed as shown in Fig. 10C and Fig. 11C. The decrease and increase in cell size may be an effect of shear force along their motion on the bottom plane of the microchannel and may be related to the process of hepatocyte viability loss.

The regulatory processes of the cell volume in apoptosis, necrosis (Okada et al., 2001), and cell anoikis (Smets et al., 2002) occur within minutes. However, the decrease in cell size on the bottom plane of the microchannel occurred in about 5 seconds as calculated from the microchannel length of 50 mm and the measured velocity of 9.8 mm/second (Fig. 13 I-A). The difference in the cell response time, which is within seconds during mechanical load and within minutes for cell death processes of apoptosis or necrosis, indicates the importance of mechanical factors to explain the viability loss mechanism during cell injection.

4. Conclusion

This study has confirmed that the critical location of hepatocyte viability loss is at the bottom plane of the microchannel and introduced a physical explanation for cell viability loss during cell injection. The cell viability loss process might be induced by cell contact along movement on the solid surface. In addition, the study has characterized two live hepatocyte distributions in the horizontal and vertical syringe orientations, which produced sedimeted and uniform dispersion conditions, respectively. Taken together, maintaining a uniform distribution during cell injection is preferable because it distributes cells along the center, which can increase the delivered number of live hepatocytes and prevent a cell from contacting a solid surface. The hepatocyte viability loss is proportional to the number of live hepatocytes distributed on the bottom plane of the delivery line. Therefore, preventing the sedimented condition and preventing a cell from contacting a solid surface in the cell injection process is an engineering challenge to enhance cell delivery in hepatocyte transplantation.

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References

Agashi, K., Chau, D. Y. S. and Shakesheff, K. M., The effect of delivery via narrow-bore needles on mesenchymal cells, Regenerative medicine, Vol. 4, No. 1 (2009), pp.49–64, DOI:10.2217/17460751.4.1.49
Amer, M. H., White, L. J. and Shakesheff, K. M., The effect of injection using narrow-bore needles on mammalian cells: Administration and formulation considerations for cell therapies, Journal of Pharmacy and Pharmacology, Vol. 67, No. 5 (2015), pp.640–650, DOI:10.1111/jphp.12362
Amer, M. H., Rose, F. R., White L. J. and Shakesheff, K. M., A detailed assessment of varying ejection rate on delivery efficiency of mesenchymal stem cells using narrow-bore needles, Stem Cells Translational Medicine, Vol. 5, No. 3 (2016), pp.366–378, DOI:10.5966/sctm.2015-0208
Carini, R., Autelli, R., Bellomo, G. and Albano, E., Alterations of Cell Volume Regulation in the Development of Hepatocyte Necrosis, Experimental Cell Research, Vol. 248, No. 1 (1999), pp.280–293, DOI:10.1006/excr.1999.4408
