Numerical measurement of viable and non-viable adipocytes and other cellular components in aspirated fat tissue

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Abstract

Background: A reliable method to assay viability and number of adipocytes and other cellular components in adipose tissue remains to be established.

Methods: We assessed cell viability and number obtained from 1 g suctioned adipose tissue and respective layers (the top, middle, and bottom layers) before and after digestion and centrifugation, using cell staining with Hoechst 33342 and propidium iodide (PI) and the XTT and glycerol-3-phosphate dehydrogenase (GPDH) assays (n=10). The correlation between the number of prepared cells (adipocytes, adipose stromal cells [ASCs], and white blood cells [WBCs]) and the resulting values from the XTT and GPDH assays was also examined (n=5). The cell composition of the stromal vascular fraction isolated from the same adipose tissue was determined by multicolor flow cytometry (n=5).

Results: Hoechst 33342 and PI staining allowed distinguishing of viable adipocytes from lipid droplets, dead adipocytes, and cells other than adipocytes. We obtained 6.9 $\times 10^5$ non-ruptured adipocytes from 1 g suctioned adipose tissue; 30% of the original adipocytes appeared to have been ruptured. Both the XTT and GPDH assays provided good correlations between the number of viable adipocytes and resulting values, but only the GPDH assay was strictly specific for adipocytes. The ratio of ASCs to adipocytes was found to be much larger than previously described.

Conclusions: Single use or a combination of the viability assays used in this study can appropriately determine the number of adipocytes and other cells, although it remains difficult to assess original cells directly without tissue dissociation.

Introduction

Adipose tissue has been widely studied in basic research, especially in relation to obesity and metabolic diseases such as diabetes.[1] In the clinical setting, autologous fat grafting has long been an effective technique for soft tissue augmentation. Furthermore, multipotent stem cells have been identified in adipose tissue, resulting in a new field in adipose biology.[2-7] In spite of the intense interest in adipose tissue, however, its cellular composition—either excised or aspirated—remains unknown. Although cell counting with cell staining,[8-13] colorimetric assays (the XTT and/or MTT assays),[9,14-17] and the glycerol-3-phosphate dehydrogenase (GPDH) assay[9,10,18] have been used in previous studies to analyze cell number and viability in adipose tissue, a reliable method has not yet been established. Specifically, the number of mature adipocytes is difficult to measure mainly because of their fragility and the presence of adjacent progenitors.

Trypan blue staining is a well established procedure for detecting viable cells. In adipocytes, however, this technique is difficult because of the scant cytoplasm of mature adipocytes.[12] Other staining methods should be used to distinguish viable adipocytes, and differentiating adipocytes and pure lipid droplets is also necessary.

A colorimetric assay uses tetrazolium salt (XTT and/or MTT), which can be reduced by active mitochondrial dehydrogenase in viable cells, yielding a highly colored formazan product.[19] This assay provides a good correlation between the number of viable cells and the resulting formazan absorbance values, but it is not specific for adipocytes. In addition, because adipose tissue contains not only adipocytes but also blood-derived cells, endothelial cells, and adipose-derived stem cells (ASCs), these other cells could influence the total absorbance values.

In the GPDH assay, GPDH activity is determined by measuring the decrease in NADH concentration.[18] This assay has the advantage of adipocyte specificity, but extracellular GPDH from damaged adipocytes is measured together with intracellular GPDH from viable adipocytes, affecting the accuracy of viability analysis. Furthermore, a correlation between the number of viable adipocytes and the resulting values of GPDH activity has not been reported.

Thus, how many adipocytes and other cellular components exist in the adipose tissue remains unclear.[8,10,13] In this study, to seek a reliable method for evaluating adipose tissue, we assessed the viability and number of cellular components obtained before and after collagenase digestion of suctioned adipose tissue using cell staining, the XTT assay, and the GPDH assay.

Materials and methods

Tissue sampling and processing

After obtaining informed consent using an institutional review board-approved protocol, we acquired adipose tissue from 10 healthy female donors undergoing liposuction of the abdomen or thighs. We used a cannula with a 2.5-mm inner diameter and a conventional liposuction machine. From each donor, five sample tubes were prepared. We carefully collected the most homogeneous samples possible, making an effort not to include debris or clumps. The sample was divided by weighing 1 g for each specimen. One sample of the five was directly subjected to the XTT assay, and another sample to the GPDH assay. The other three samples were processed by digestion with collagenase and centrifugation; each sample was mixed with 2 ml of 0.075% collagenase (Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline (PBS), and incubated at 37 °C for 30 minutes. The digestion was terminated with 2 ml of PBS containing 10% fetal bovine serum (FBS), and the digested adipose tissue was centrifuged at 430 ×*g* for 5 minutes. After centrifugation, the top, middle, and bottom layers were respectively assessed by a cell-staining morphometric assay, the XTT assay, and the GPDH assay (Fig. 1). To avoid waiting time, we performed these assays simultaneously. We also performed each assay as immediately as possible.

Cell-staining morphometric assay

Respective layers after digestion and centrifugation were resuspended in PBS to a total of 5 ml. First, to distinguish adipocytes from floating lipid droplets released from broken adipocytes, cells were stained with Nile Red (AdipoRed^{TM}) (Cambrex, Walkersville, MD) and Hoechst 33342 (Dojindo, Kumamoto, Japan). Cells stained with both Nile Red and Hoechst 33342 were defined as adipocytes, while cells stained only with Hoechst 33342 but negative for Nile Red were defined as non-adipocytes. Particles stained only with Nile Red were defined as floating lipid droplets.

Next, to distinguish viable cells from dead cells, cells were stained with Hoechst 33342 and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO). This staining distinguished four types of nucleated cells: viable adipocytes (positive for Hoechst 33342, negative for PI, and including lipid); dead adipocytes (positive for PI and including lipid); viable cells other than adipocytes (positive for Hoechst 33342, negative for PI, and not including lipid); and dead cells other than adipocytes (positive for PI and not including lipid). Erythrocytes, which are not stained with the nuclear staining, were not counted in this study. Samples were placed on a hematocytometer under a microscope. At least three fields (×100) were randomly photographed and the number of cells in each group counted. Then, the total cell number obtained from 1 g suctioned adipose tissue was calculated.

The XTT assay

A Cell Proliferation Kit II (XTT) (Roche Diagnostics, Indianapolis, IN) was used according to the manufacturer's instructions. In brief, 1 g of each sample was suspended in PBS containing 10% FBS to a total of 5 ml. An XTT-labeling reagent and electron-coupling reagent were mixed at a ratio of 50:1, and 2.5 ml of this reagent mixture was added to the sample, followed by incubation in a 6-well plate for 6 hours at 37 °C. After the incubation, 150 µl of the solution was transferred to a 96-well plate, and the absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 650 nm. The baseline absorbance of the negative control, PBS containing 10% FBS and no cells, was subtracted from each result.

To examine any correlation between the number of viable cells and the resulting values from the XTT assay for each cell type, several concentrations of adipocytes, white blood cells (WBCs), and ASCs were prepared for assessment by the XTT assay. Adipocytes were separated from adipose tissue by digestion and centrifugation as described above, and viable adipocytes in the top layer were used. WBCs were obtained from the peripheral blood of five healthy donors. ASCs were separated from adipose tissue as described previously[6] and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. ASCs at passage 1 or 2 were used. For each cell type, samples of 1, 2, 5, and 10×10^4 viable cells/ml were prepared; the cell number was determined by cell staining with Hoechst 33342 and PI for adipocytes, and it was determined by cell counter (NucleoCounter, Chemometec, Allerod, Denmark) for WBCs and ASCs.

The GPDH assay

A GPDH Assay Kit (Cell Garage, Tokyo, Japan) was used according to the manufacturer's instructions. In brief, 1 g sample (adipose tissue or respective layers after digestion and centrifugation) was mixed with 0.25 M sucrose solution to a total of 5 ml and homogenized. The mixture was then centrifuged at 430 $\times g$ for 5 minutes. A 1-ml aliquot of the aqueous layer was taken and again centrifuged at 17700 $\times g$ for 5 minutes. The supernatant after the second centrifugation was diluted 10 times with an enzyme-extracting reagent, and the optical absorption was measured at 340 nm for 10 minutes on a 96-well plate after addition of twice the volume of a substrate reagent. GPDH activity was calculated based on the following formula: GPDH activity (U/ml) $= \Delta OD \times 0.482 \times 10$ (ΔOD: change in optical density per minute).

To examine correlation between the number of viable cells and the resulting values of GPDH activity in each cell type, we assessed several concentrations of adipocytes, WBCs, and ASCs using the GPDH assay, following the design used for the XTT assay.

Flow cytometry

To determine the presence of non-adipocytes, we used multicolor flow cytometric analysis to examine cells in the bottom layer, the stromal vascular fraction (SVF), for surface marker expression. The following monoclonal antibodies conjugated to fluorochromes were used: anti-CD31-PE, anti-CD34-PE-Cy7, and anti-CD45-FITC (BD Biosciences, San Jose, CA). Cells were analyzed with a LSR II (BD Biosciences), and cell composition percentages were determined according to surface marker expression profiles.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons of multiple groups were made using one-way ANOVA with Bonferroni's multiple *t*-test. Values of $P<0.05$ were considered statistically significant.

Results

Cell staining

After digestion and centrifugation, many pure lipid droplets, not cells, were included together with cells in the top layer (Fig. 2). The top layer contained 4.4 (± 0.66) × 10^5 viable adipocytes; 2.5 (± 0.28) × 10^5 dead adipocytes; 1.5 (± 0.23) × 10^5 viable cells other than adipocytes; and 7.5 (± 0.72) × 10^5 dead cells other than adipocytes, which were distinguished by cell staining with Hoechst 33342 and PI (Fig. 3). The middle layer contained no cells. The bottom layer contained 10.0 (± 2.8)× 10^5 viable cells other than adipocytes and 2.3 (± 0.34) × 10^5 dead cells other than adipocytes (Table 1).

The XTT assay

Adipose tissue before digestion resulted in the highest and similar to the sum of three layers after digestion. The top layer produced a much higher value than that of the bottom layer, although the number of viable cells directly counted by cell staining was higher in the bottom layer (Fig. 4).

Good correlations between the number of prepared viable cells and the XTT values were respectively obtained in adipocytes, WBCs, and ASCs. Adipocytes showed higher absorbance than WBCs and ASCs, especially at a high concentration, differences that were statistically significant (Fig. 5). Thus, the results suggest that the value derived from the XTT assay would not be proportional to the total viable cell number when different cell types are mixed.

The GPDH assay

Adipose tissue before digestion and centrifugation showed the highest value. After digestion and centrifugation, the value of the top layer, which was regarded as intracellular GPDH from floating non-ruptured adipocytes, was more than twofold that of the middle layer, which was regarded as extracellular GPDH released from ruptured adipocytes. The bottom layer, which contained no adipocytes, showed a low value (Fig. 6).

The GPDH assay provided a good correlation between the number of prepared viable adipocytes and the resulting values, while WBCs and ASCs showed no GPDH activity even at a high concentration (Fig. 7).

Flow cytometry

Flow cytometric analysis revealed that the SVF contained CD45-positive cells, corresponding to blood-derived cells at 37 ± 5.2%; ASCs ($CD45^-$, $CD31^-$, $CD34^+$) at 37 ± 4.0%; and endothelial cells ($CD45^-$, $CD31^+$, $CD34^+$) at 15 ± 4.9% (Fig. 8; Table 2).

Discussion

In the present study, suctioned fat samples obtained from the abdomen or thighs were used. Although regional (donor site) difference in adipose cell components may exist, a previous study reported that no statistical differences in adipose tissue viability were demonstrated among abdominal fat, thigh fat, flank fat, or knee fat, based on the XTT assay.[14] In our study, no differences in adipose stromal cell yield were observed between the samples from the abdomen and the thigh (data not shown).

In staining and counting of adipocytes, distinguishing them from pure lipid droplets is necessary. In our study, many lipid droplets derived from ruptured adipocytes were observed together with adipocytes, as shown in Figures 2 and 3. Lipid droplets the same size as adipocytes (50 to 120 μm) cannot be distinguished from adipocytes without nuclear staining. In previous studies,[8,10,13] trypan blue staining was performed, but it cannot distinguish pure lipid droplets from viable adipocytes because because it does not stain either of them well. The previous studies[8,10,13] reported adipocyte numbers (2.5–90 million/ml) which were much larger than those in our result (0.7 million/ml), and the difference may arise from the confusion of lipid droplets with adipocytes. Nuclear staining with Hoechst 33342 only is not appropriate for a viability assay, because it also stains the nuclei of dead cells. We combined PI, which stained only the nuclei of nonviable cells, with Hoechst 33342 staining, and with this combination, we distinguished four types of cells: viable adipocytes, dead adipocytes, viable cells other than adipocytes, and dead cells other than adipocytes. A combination of fluorescein diacetate and PI may be another option.[12]

We found that many non-adipocyte cells were present in the top layer after collagenase digestion and centrifugation, although the viable cell ratio was much lower than that of the bottom layer. Cells, especially adherent cells, may have been trapped in the floating layer and not released by centrifugation. A previous study using ceiling culture showed that cells tightly attached to mature adipocytes could generate fibroblastic cell populations with multiple differentiation potential,[20] and the cells were regarded as ASCs. Thus, the viable non-adipocyte cells detected in the top layer may contain ASCs as well as WBCs, although more than 80% of the viable non-adipocyte cells were dead in the current study.

Among colorimetric assays of cell viability, the XTT and MTT assays have been widely used. We first assessed the viability of adipose tissue using both the XTT and MTT assays, but the MTT assay did not give reproducible results (data not shown) unlike the XTT assay. In the MTT assay, the resulting colored formazan product is insoluble and requires a dissolving procedure,[19] which may impair reproducibility, especially when using adipose tissue.

We also found that the resulting values of the XTT assay differed among cell types. This difference is likely the reason for the higher value from the XTT assay in the top layer compared to the bottom layer; the results from the cell-staining assays made it clear that the bottom layer contained a much larger number of viable cells. Adipocytes exhibited much higher absorbance in the XTT assay compared to WBCs and ASCs. This difference may arise because of the increase in mitochondrial capacity (number of mitochondrion profiles, concentration of numerous mitochondrial proteins, and oxygen consumption rate) during adipogenesis.[21] Some authors have reported in previous studies[14,16] that the XTT assay is well suited to assessing adipose tissue viability; however, it is not specific for adipocytes, and careful interpretation of results is needed when contamination by other cell types is present. Our results demonstrate that the bottom layer, which contains no adipocytes, had a substantial XTT value. Given this finding, it is possible that in previous studies, vascular stromal fraction cells such as white blood cells, adipose stromal cells, and vascular endothelial cells were mistakenly counted as adipocytes.

GPDH is normally intracellular, and extracellular GPDH is considered proportional to the amount of adipocyte destruction.[9,18] Therefore, examining total GPDH in adipose tissue is not appropriate for viability assessment. To gain a full understanding of the results of previous studies, we would have to confirm whether the reported GPDH value was intracellular or extracellular or a mixture of both. In our study, the GPDH activity of the top layer was regarded as intracellular GPDH from non-ruptured adipocytes, although this layer appeared to contain both viable and dead (but not ruptured) adipocytes. These latter adipocytes may die because of the effects of collagenase, although they did not ruptured and the intracellular contents were not released into the middle layer. The GPDH activity of the middle layer indicated that approximately 30% of total adipocytes were ruptured. The rate of this extracellular GPDH can change, depending on adipose tissue sampling methods[22] and the digestion procedure.[18] The GPDH assay is superior to the XTT assay because of its strict specificity for adipocytes, which the current findings support. Although the previous studies[9,10,18] appropriately used the GPDH assay, a control curve using premeasured adipocytes or a combination of direct cell counting is needed to evaluate the absolute number of adipocytes.

From 1 g suctioned adipose tissue, we obtained 6.9×10^5 adipocytes (viable:dead = 4.4:2.5) and 2.1×10^6 non-adipocyte cells (viable:dead = 11.5:9.8). Based on the GPDH data for the top and middle layers, we speculate that 1 g suctioned adipose tissue originally contains approximately 1 million adipocytes (6.9×10^5 adipocytes in the top layer and approximately 3×10^5 adipocytes in the middle layer). Although the size of adipocytes may differ according to nutritional status,[23] previous studies[24,25] reported relatively similar adipocyte sizes, ranging from 50–150 µm, with a mean size between 90 and 100 µm regardless of severity of obesity. Assuming that adipocytes are cubes (10^6 µm^3) or spheres (5.23×10^5 µm^3) with a side or diameter of 100 µm, the number of adipocytes in 1 ml adipose tissue would correspond to 0.95 million or 1.81 million under the condition that adipocytes occupy 95% of adipose tissue volume; thus, the theoretical number of adipocytes in the 1ml of adipose tissue is likely to be between the two numbers. This suggestion is supported by the results of a previous study[23] indicating that adipocyte number per 1 g adipose tissue is 1.51 ± 0.47 million (mean ± SD). Because the adipocyte number of non-centrifuged suctioned adipose tissue is likely to be smaller than that of intact adipose tissue,[26] the estimated number in our study (1 million per 1 ml suctioned adipose tissue) seems to be reasonable.

Multicolor flow cytometry revealed that 37% of non-adipocyte cells in SVF (the bottom layer) are ASCs, a ratio compatible with our previous findings.[5] Assuming that the non-adipocyte cell composition in the top layer is similar to that of the bottom layer, we can speculate that the number of ASCs in 1 g suctioned adipose tissue is about half that of adipocytes. Considering also that the number of ASCs in suctioned adipose tissue is significantly smaller than in intact adipose tissue,[6] the ASC number in intact adipose tissue may be similar to that of adipocytes. In addition, it may be much larger than that described in a previous histological study,[27] which reported percentages of adipocytes and stromal cells in the adipose tissue as 89–98% and 2–10%, respectively. In a histological assessment of the adipose tissue, a single adipocyte 100 µm in size, for example, could appear in 12 serial sections (8 µm thick; 96 µm in all), but each section contains different ASCs; thus, a single adipocyte could be repeatedly counted by mistake, and a careful evaluation of the specimens is necessary for counting cell numbers. Therefore, thicker sections, as thick as 200 µm, combined with an appropriate calculation formula such as that used in a previous study[25] may contribute to avoiding mistakes in cell counting.

In conclusion, single use or a combination of the viability assays employed in this study can accurately determine the number of viable adipocytes and other cells, although other assays like the ATP assay[28] and glucose transport experiment[29] should be further investigated for applications to adipose tissue. The major limitation of the current study is that the assays are performed only after enzyme digestion and centrifugation, which partly destroys adipocytes and other cells. Thus, directly assessing the original adipocytes and other cellular components without tissue dissociation is difficult. A three-dimensional measurement that can allow analysis without tissue dissociation should be developed for a more accurate anatomical evaluation of adipose tissue.

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Figure legends

Fig. 1. A schematic diagram of tissue sampling and processing. One sample was directly subjected to the XTT assay, and another sample to the GPDH assay. The other three samples were processed by digestion with collagenase and centrifugation. After centrifugation, the top, middle, and bottom layers were respectively assessed by a cell-staining morphometric assay, the XTT assay, and the GPDH assay.

Fig. 2. Cell staining with Nile Red and Hoechst 33342. Bright field (above, left), Nile Red (above, right), Hoechst 33342 (below, left), and merge (below, right). A white arrow indicates an adipocyte. A yellow arrow indicates a pure lipid droplet. Scale bar = 100 µm.

Fig. 3. Cell staining with Hoechst 33342 and PI.

(A) Bright field (above, left), PI (above, right), Hoechst 33342 (below, left), and merge (below, right). A white arrow indicates a viable adipocyte. A yellow arrow indicates a dead adipocyte. A white arrowhead indicates a viable cell other than adipocytes. A yellow arrowhead indicates a dead cell other than adipocytes. Scale bar = 100 µm.

(B) Representative views of each layer stained with Hoechst 33342 and PI. In the top layer, four types of cells (viable adipocytes, dead adipocytes, viable cells other than adipocytes, and dead cells other than adipocytes) as well as pure lipid droplets were observed. No cells were detected in the middle layer, while non-adipocytes, viable or dead, were seen in the bottom layer. Scale bar = 100 µm.

Fig. 4. Results of the XTT assay for 1 g suctioned adipose tissue and respective layers after digestion and centrifugation (n = 10). Adipose tissue before digestion resulted in the highest value, which is similar to the sum of three layers after digestion. Statistical significance was detected between any combinations of groups. Values are mean ± SEM.

Fig. 5. Correlation between the number of prepared viable cells and the resulting values of the XTT assay (n = 5). Good correlations were respectively obtained in adipocytes, WBCs, and ASCs. Values are mean ± SEM.

Fig. 6. Results of the GPDH assay for 1 g adipose tissue and respective layers after digestion and centrifugation (n = 10). Adipose tissue before digestion and centrifugation showed the highest value. The bottom layer, which contained no adipocytes, showed a low value. Statistical significance was detected between any combinations of groups. Values are mean ± SEM.

Fig. 7. Correlation between the number of prepared viable cells and the resulting values of the GPDH assay (n = 5). The GPDH assay provided a good correlation in adipocytes. WBCs and ASCs showed no GPDH activity even at a high concentration. Values are mean ± SEM.

Fig. 8. Multicolor flow cytometric analysis of cells in the bottom layer. $CD45^+$ cells were regarded as blood-derived cells. $CD45^-$ cells were regarded as adipose-derived cells and were processed to the next analysis. $CD45^-CD31^-CD34^+$ cells were regarded as ASCs, while $CD45^-CD31^+CD34^+$ cells were regarded as endothelial cells.

Table 1.

Number of cells obtained from 1 g adipose tissue ($\times 10^5$)

	Adipocytes, viable	Adipocytes, dead	Other cells, viable	Other cells, dead
Top layer	4.4 ± 0.66	2.5 ± 0.28	1.5 ± 0.23	7.5 ± 0.72
Middle layer	0	0	0	0
Bottom layer	0	0	10.0 ± 2.8	2.3 ± 0.34

Data are expressed as mean ± SEM (n = 10).

Table 2.

Cell composition in the bottom layer

	Blood-derived cells	Adipose-derived cells		
		ASCs	Endothelial cells	Other cells
CD45	+	−	−	−
CD31		−	+	−
CD34		+	+	−
Percentage in all cells	36.8 ± 5.2	62.8 ± 5.2		
Percentage in adipose-derived cells		60.7 ± 8.5	21.4 ± 5.8	17.9 ± 3.8
Calculated percentage in all cells	36.8 ± 5.2	37.4 ± 4.0	14.6 ± 4.9	11.2 ± 3.1

+: positive for the cellsurface marker.

−: negative for the cell surface marker.

Data are expressed as mean ± SEM (n = 5).

Figure 1

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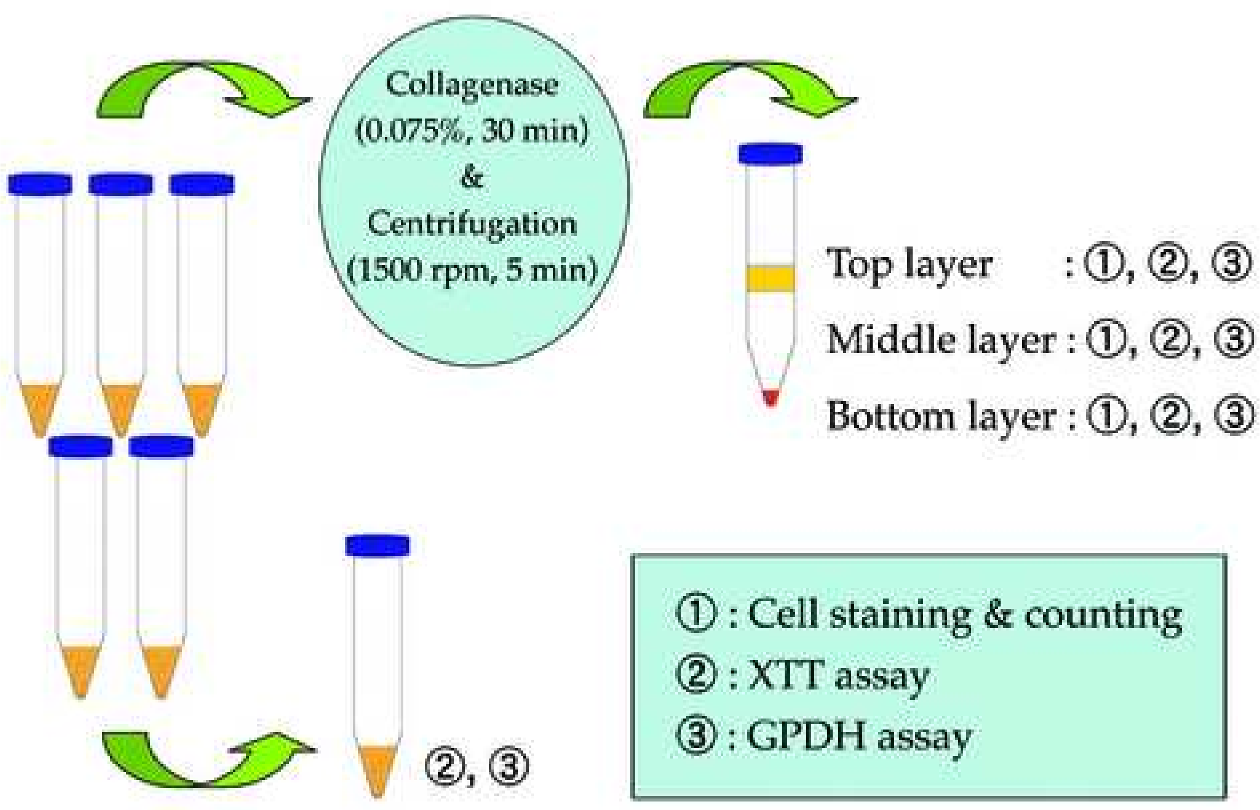

Figure 2
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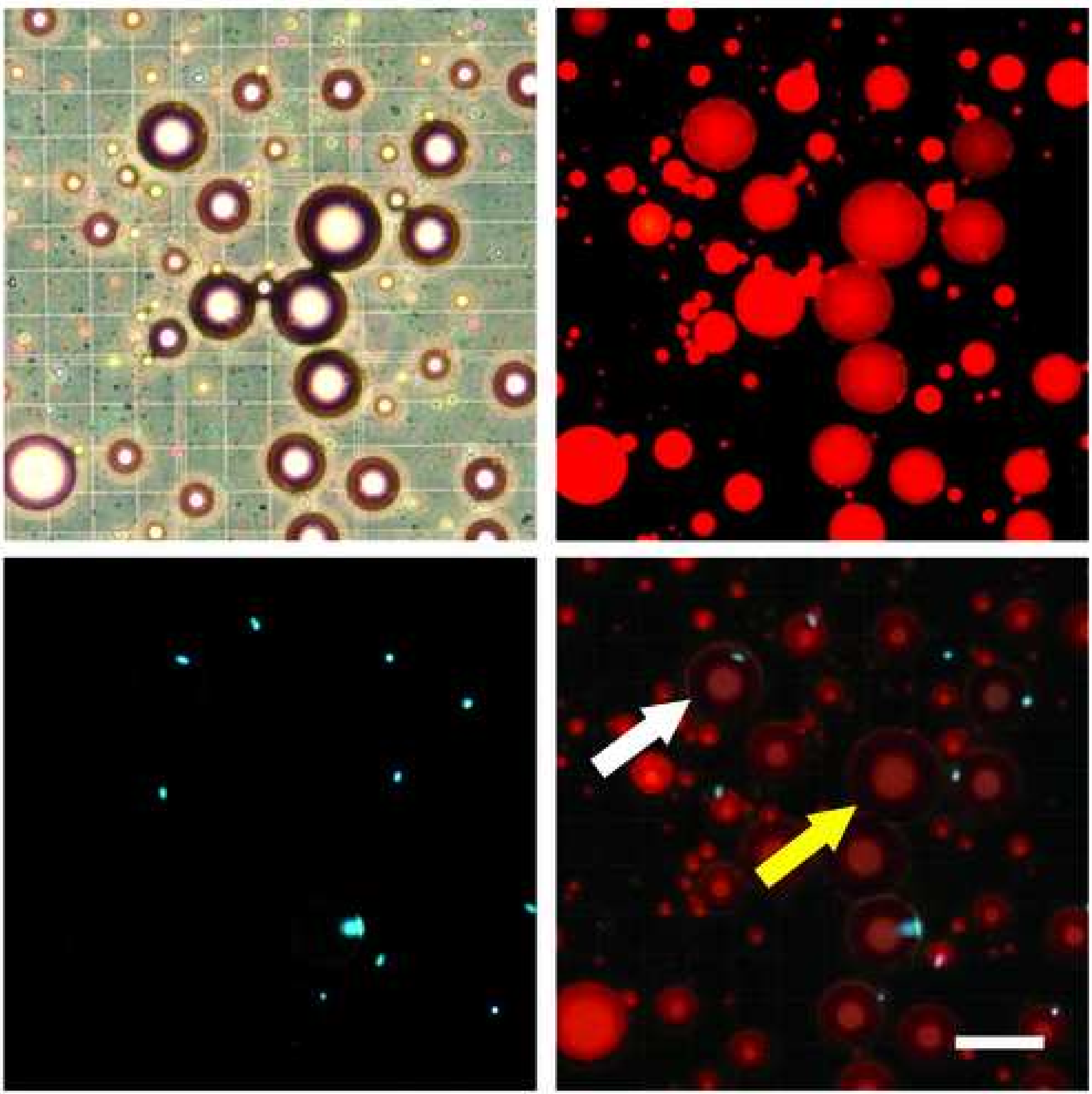

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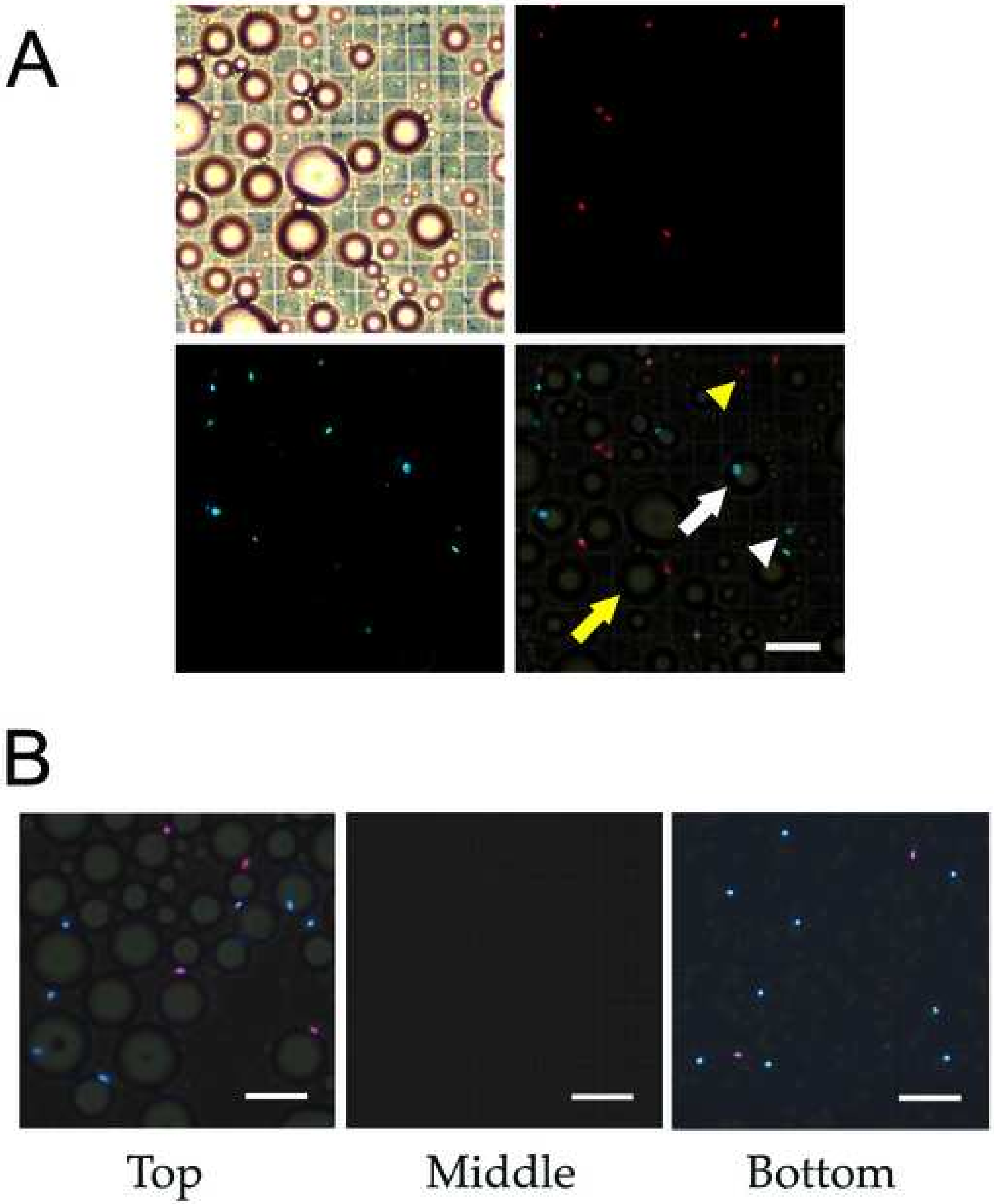

Figure 4
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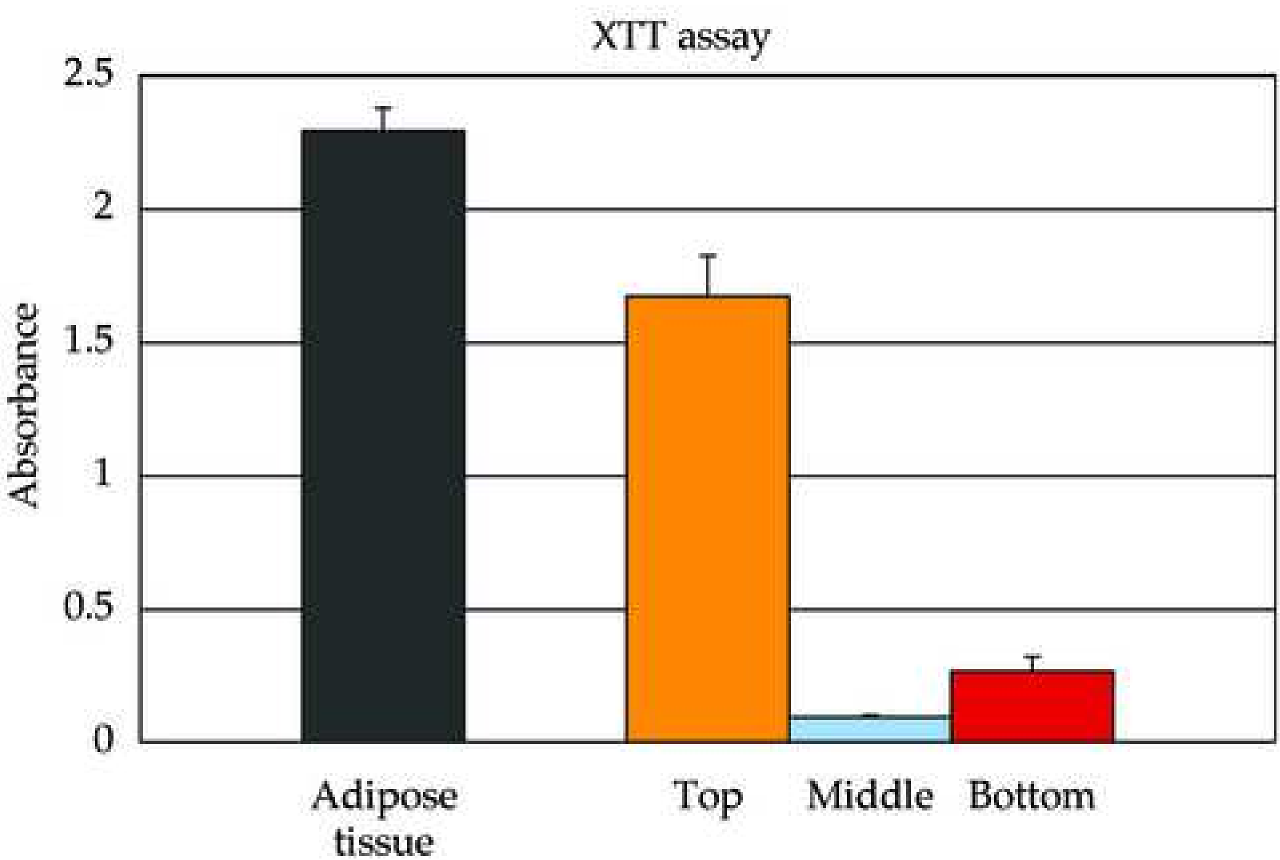

Figure 5

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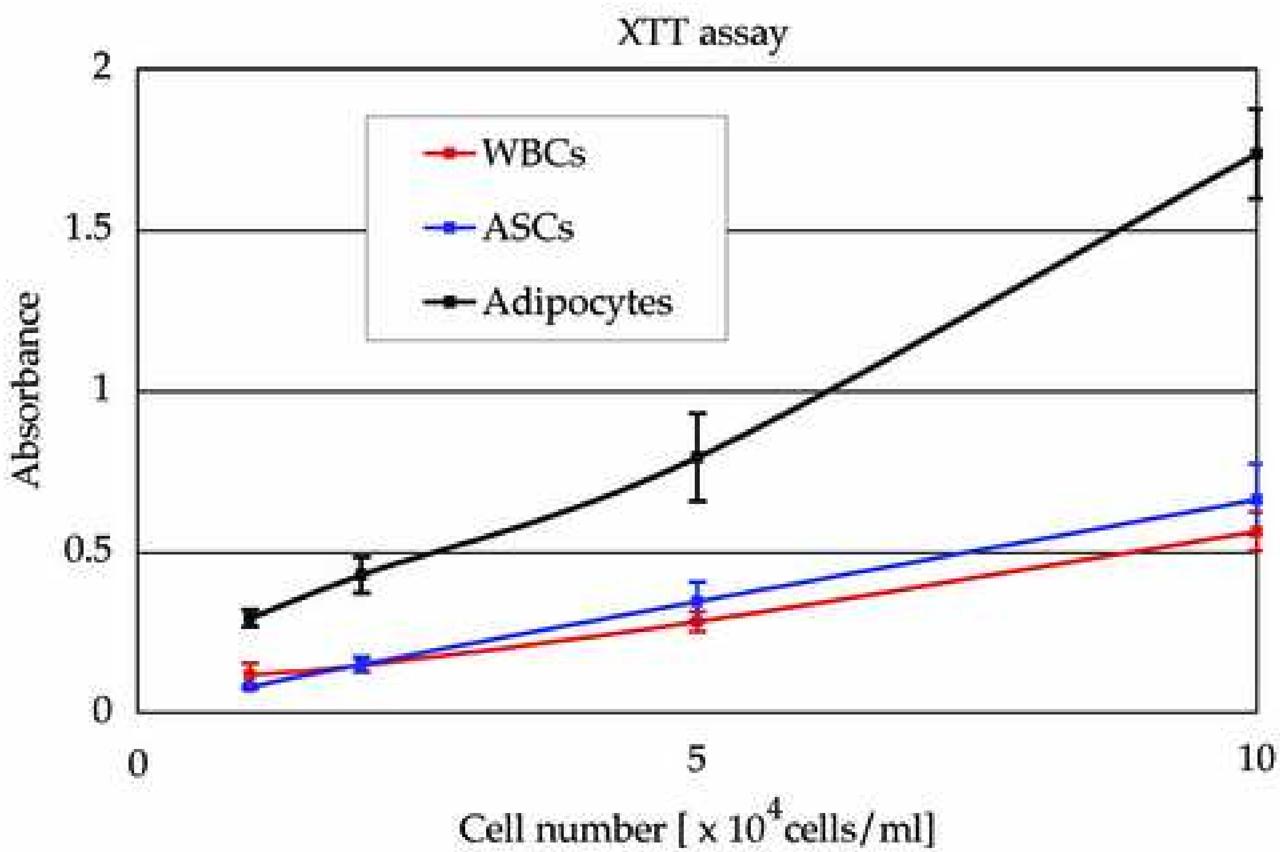

Figure 6

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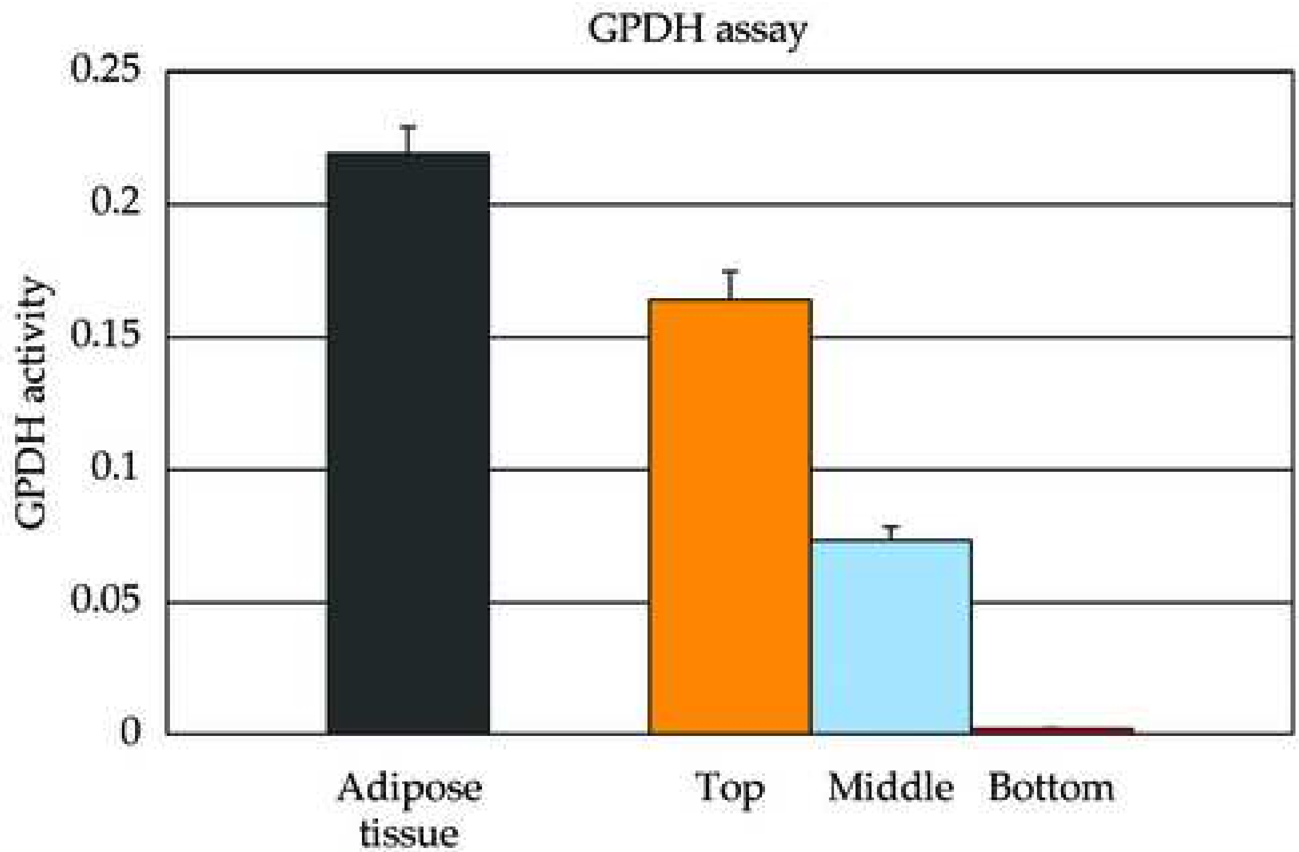

Figure 7

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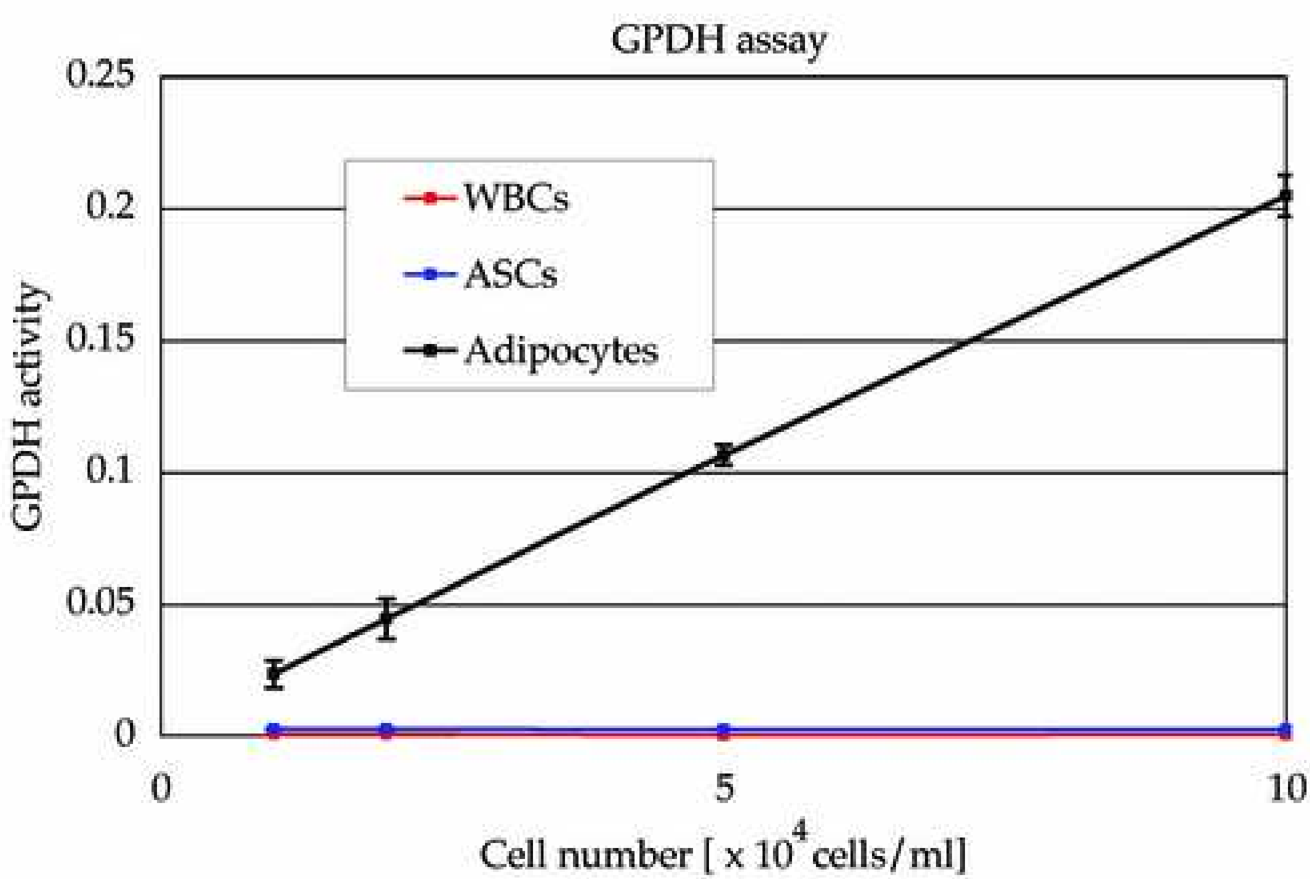

Figure 8

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