

A quick, reliable, and automated method for fat cell sizing

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Abstract Mean diameters of fat cells from abdominal tissues from 31 volunteers were determined by three methods based on fat cell isolation after collagenase digestion and methylene blue staining. The three methods were direct microscopy (Micro), manual measurement of diameters from digital images by using the public domain NIH Image program (Scion), and automated measurement of diameters from digital images using a customized program developed by Biomedical Imaging Resource at Mayo Clinic (AdCount). There was excellent agreement between the methods' measurement of mean abdominal fat cell diameter (concordance correlation coefficient >0.84). The Scion method gave slightly but systematically lower mean abdominal fat cell diameters than did either AdCount or Micro. The AdCount approach produced results that are comparable to those from Micro. Comparison of AdCount and Micro in measuring diameters of fat cells from thigh confirmed the good comparability between the two methods independent of fat depot. **AdCount is very reliable, and the quickest and most objective of the three methods in measuring fat cell diameters from various depots.**—Tchoukalova, Y. D., D. A. Harteneck, R. A. Karwoski, J. Tarara, and M. D. Jensen. A quick, reliable, and automated method for fat cell sizing. *J. Lipid Res.* 2003. 44: 1795–1801.

Supplementary key words fat cell diameter • fat tissue cellularity • adipocyte • collagenase

One of the routine variables of interest for researchers of adipose tissue physiology is adipocyte size, which is commonly measured as fat cell diameter using light microscopy (Micro). From the diameter, mean fat cell volume and lipid content are derived mathematically. This technique requires collagenase digestion of the adipose tissue sample, separating adipocytes by centrifugation, methylene blue staining to identify the nuclei, and subsequent microscopic measurement of the diameter (1). Although inexpensive and faster, this method results in considerable eyestrain when large numbers of samples must be processed and produces no permanent visual record. Attempts to circumvent these inconveniences by taking photomicrographs have been suggested (2). Photomicrographs provide visual records that

can be analyzed at a later time but do not change the time required for the measurement procedure. In this report, we describe two techniques based upon computer analysis of digital images. The first method involved manually measuring fat cell diameters from digital images using NIH Image analysis software (Scion), similar to the photomicrographic method. Because this approach was actually more time-consuming than direct Micro, we developed another technique to further streamline and standardize measurements from digital images. A computer program for automated measurement of fat cell area from digital images and calculation of fat cell diameter, volume, and mass was developed at Mayo Clinic (AdCount). Mean diameters of fat cells obtained from abdominal tissue were determined and compared across three methods: direct Micro, Scion, and AdCount. To evaluate the comparability of AdCount with Micro when the source of fat cells is a different depot, mean diameters of fat cells from thigh were measured additionally by AdCount and Micro. The reliability of AdCount was tested by evaluating the reproducibility of the results when measured by one observer at two different times and by two observers once. Finally, after assessing the relation between the fat cell droplet size and cell maturity of very small fat cells using fluorescence Micro, we reassessed the effects of our choice of lipid droplet size on the estimated mean fat cell diameter.

MATERIALS AND METHODS

Sample preparation

Twenty-six overweight and obese [body mass index (BMI) 27.5–36.8] and 14 lean (BMI 20–27) volunteers, all of whom had given informed written consent, participated in this study. Subcutaneous adipose tissue samples (200–600 mg) from the periumbilical area of 21 overweight and obese and 10 lean volunteers

Abbreviations: AdCount, automated measurement of diameters from digital images using a customized program developed by Biomedical Imaging Resource at Mayo Clinic; Micro, microscopy; Scion, manual measurement of diameters from digital images by using the public domain NIH Image program.

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and from the thigh of four overweight and obese and five lean volunteers were obtained by needle biopsy. Tissue was digested in 1 mg/ml collagenase type II (Sigma C-6885) in HEPES buffer [0.1 M HEPES, 0.12 M NaCl, 0.05 M KCl, 0.005 M glucose, 1.5% w/v BSA, CaCl₂ (pH 7.4)] in a 37°C water bath employing shaking at 115 rotations/min for 20–30 min. The cell suspension thus formed was centrifuged for 5 min at 300 g at room temperature. A 50–150 µl aliquot from the top layer was added to 450 µl of 0.2% methylene blue/HEPES solution for nuclei staining and incubated for 15 min at 37°C in the water bath. Five to 10 µl from the cell suspension were placed in each well of an 8-well glass slide, coverslipped, and measured optically using a Nikon Labophot 2/2A microscope equipped with an eyepiece having a 10 mm scaled reticle at phase contrast and 100× magnification. Next, cells were photographed using a Nikon Coolpix 990 digital camera attached to the microscope. Fat cells of at least 35 µm were measured by all methods.

Measuring of fat cell size using microscope only

The diameters of at least 100 fat cells were defined optically by comparison with the scale from the reticle. The values were not recorded but appointed to groups that differ by 10 µm, creating a histogram with bins of 10 µm. The histograms were used to calculate the mean diameter and standard deviation of the mean diameter and to assess whether the distribution of adipocyte diameters was normal.

Measuring fat cell diameter using Scion

Fat cells from the digital images were analyzed on a PC computer using the public domain image analysis program developed at the US National Institutes of Health (Scion). Briefly, the recorded images were resized to 45% by using Photo Editor software and saved in a TIFF format that is compatible with Scion. The modified images were then imported into the program, and a calibration was performed for each image by drawing a line over the scale that was introduced into the image field prior to digitization. Using the line tool, the diameters of at least 100 adipocytes were manually drawn and individually measured. The output of the measured diameters was transferred to an Excel program that was used for calculations of mean diameters and standard deviation and for creating histograms with 10 µm bins to check the normality of the data distribution.

Measuring fat cell diameter using AdCount

For the purpose of measuring the fat cell size, a stand-alone program designated AdCount was written by the Biomedical Imaging Resource, Mayo Clinic by using the comprehensive visualization workshop image-processing library developed and maintained by the Biomedical Imaging Resource, Mayo Clinic (3). The choice of the parameters to be analyzed was based on the suggestions of DiGirolamo and Fine (4).

Each image is processed with an inhomogeneity correction filter to address the uneven illumination of the microscope field. The original image from the digital camera is imported into the AdCount program and a calibration performed as described for Scion (Fig. 1A). A threshold value is interactively determined to separate the cells from the background. A connected component algorithm is then applied to the image to define potential cells, and the area, diameter, and circularity measurements are computed. Predefined and changeable limits for the diameter and circularity measures further restrict the potential cells that are counted. The circularity measurement is computed as follows: $\text{Circularity} = (P \cdot P/A)/(4 \cdot \text{Pi})$, where P is the perimeter of the potential cell and A is its area. The circularity of a perfect circle is 1.0. The diameter of the circular cells is derived as follows: $\text{Diameter} = 2 \cdot \sqrt{A/\text{Pi}}$. The area equivalent diameters are

shown in Fig. 1B. Cells cut by the borders or with uneven edges resulting in a visual underestimation of the area may then be eliminated manually. After analysis, the program computes and displays a running total of counted cells, a histogram of the diameters of the counted cells, a numbered list of the individual diameters, the mean diameter, standard deviation of the diameters, heterogeneity of the fat cell population (standard deviation/mean), and mean lipid content. The program also delineates the measured cells with different colors and designates to each cell a number that corresponds to the number in the output of the results (Fig. 1C). The last helps in visualization of a cell of interest and its diameter. By clicking either on the cells or on a diameter value in the output, the program simultaneously highlights both. The output of the results is logged to a text file. Images were analyzed until ~300 cells were sized.

Nile Red fluorescent staining

The Nile Red staining of lipid droplets was performed according to a published protocol (5) supplemented with nuclear counterstain. Fat cells were isolated by collagenase digestion, washed in PBS, and fixed in 400 µl of 0.5% paraformaldehyde in PBS for 5 min. Meanwhile, a stock solution of Nile Red (Sigma-Aldrich, St. Louis, MO) prepared in DMSO (1 mg/ml) was diluted 1:100 in PBS (10× stock). Forty-four microliters of the 10× Nile Red stock was added to the fat cell suspension for 5 min. The nuclear staining was carried out for 5 min using a stock solution of Hoechst 33258 (Sigma-Aldrich) in water (10 mg/ml) diluted 1:20 in PBS (100× stock) with an aliquot added to a final concentration of 5 µg/ml Hoechst 33258. The cells were then washed three times with PBS. Five microliters of the fat cells in suspension were put on an 8-well glass slide and coverslipped. Fifty images of small, fluorescently labeled cells were collected immediately. The equipment and methods were as follows: an LSM510 Confocal Microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with an Axiovert 100 M inverted microscope and an LD-Achroplan 40×/0.6na objective lens was used with an argon laser for excitation at 488 nm and emission using a 505–550 nm bandpass filter. The spectra settings may vary over a wide range, resulting in fluorescences of different colors (6). The settings we chose detected green fluorescence and gave a very good delineation of the fat droplets. The diameters of the fluorescent fat droplets of the unilocular cells and of the largest fat droplet in the multilocular cells were measured using an independent program, KS400 image analysis software (Carl Zeiss, Inc.).

Statistical analysis

The populations of fat cells from 31 subcutaneous abdominal and nine femoral fat tissue samples were analyzed by the three methods. By necessity, the direct Micro method assayed a unique population of cells from each sample. The Scion and AdCount methods analyzed the same set of images, but the cells chosen for analysis were not controlled to be identical. The histograms obtained from the direct Micro and the raw data obtained by using both Scion and AdCount were used to calculate the mean fat cell diameter for each sample. The mean fat cell diameters were expressed in micrometers, rounded to a whole number, and then subjected to analysis of method comparisons. Method comparison was performed for each pair of methods from the three possible combinations: Micro versus Scion, AdCount versus Micro, and AdCount versus Scion for the abdominal fat depot. Method comparison for AdCount and Micro only was performed for the femoral fat depot.

For visual assessment of the agreement, the paired readings from two methods were plotted, and a line of identity (7) or a concordance line (8) was drawn through the origin at 45°. The agreement between two methods was quantified with Lin's con-

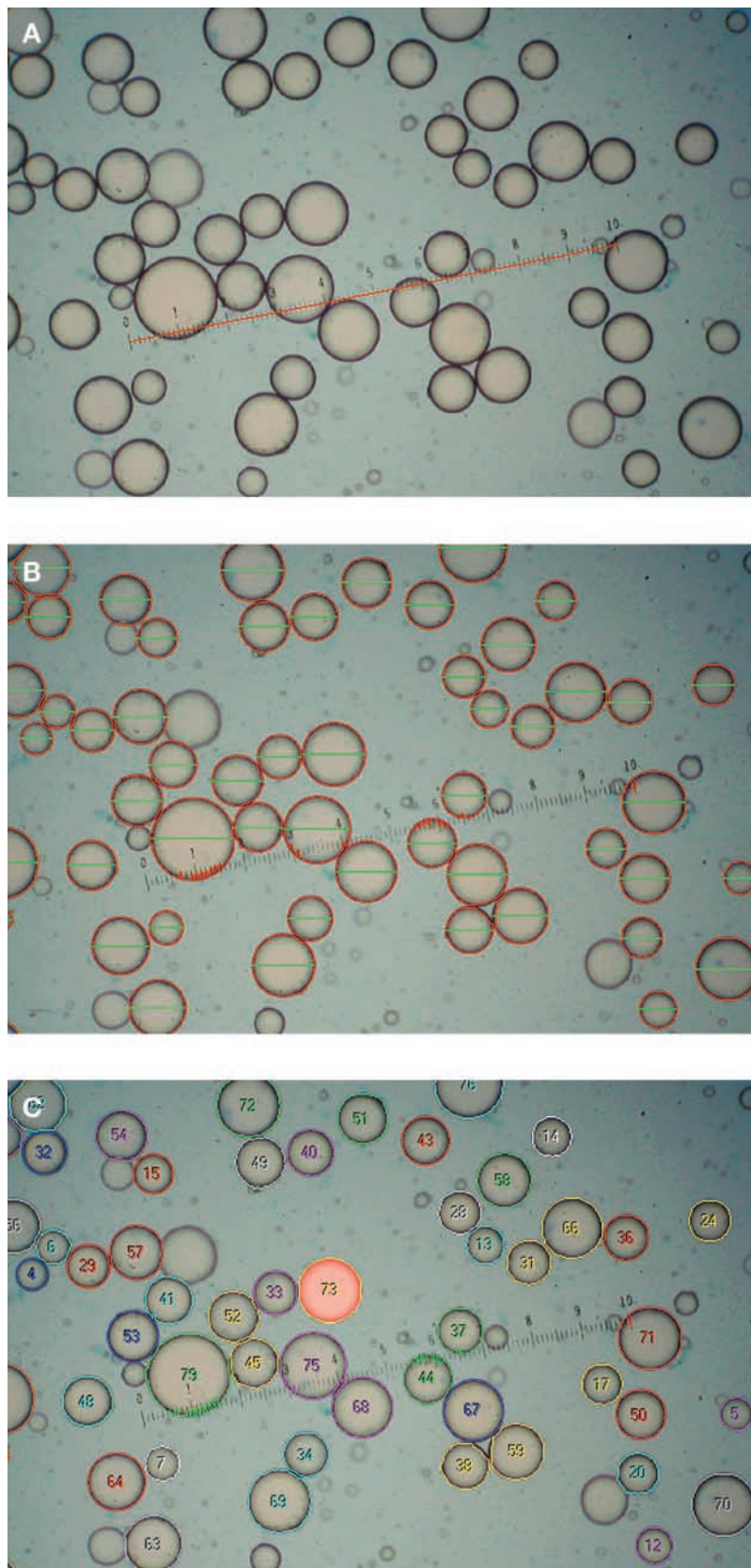


Fig. 1. Stepwise images derived by automated measurement of diameters from digital images using a customized program developed by Biomedical Imaging Resource at Mayo Clinic (AdCount) during analysis: calibration (A), thresholding (B), and marking out the measured cells (C).

cordance correlation coefficient (CCC) (8). Calculations were based on a mathematical formula that contains both a measure of accuracy (how far the best-fit line deviates from the concordance line) and a measure of precision (how far each observation deviates from the best-fit line). The level of agreement was classified as excellent (0.81–1.00), substantial (0.61–0.80), moderate (0.41–0.60), fair (0.21–0.40), slight (0.00–0.20), and poor (<0.00) (9).

Next, methods suggested by Altman and Bland (7) were applied to the data to evaluate the between-methods disagreement and the relative contribution of bias and error. The differences between two methods were plotted against their average for visual assessment of bias and error to spot outliers, and to see whether there was any tendency for the amount of variation to change with the magnitude of the measurements. The mean and the standard deviation of the between-methods differences, estimates of the bias, and error were determined. The hypothesis of zero/no bias was tested by a paired *t*-test.

Separately, the reproducibility of measurements was quantified for only the AdCount method by intraclass correlation coefficient (ICC). One observer measured the diameters of one marked cell from each of 31 abdominal samples at two different times and a second observer measured the diameters of the same cells once. The results from the repeated measurement by one observer were used to calculate the test-retest ICC, and the results from the one-time measurement by the two observers were used to calculate the inter-rater ICC.

RESULTS

Between-method agreement

The identity/concordance line plot showed the diameters of abdominal adipocytes from the same patients by two methods clustered around the line of identity (Fig. 2). All of the between-method comparisons for the abdominal adipocyte diameters had CCCs >0.84, which indi-

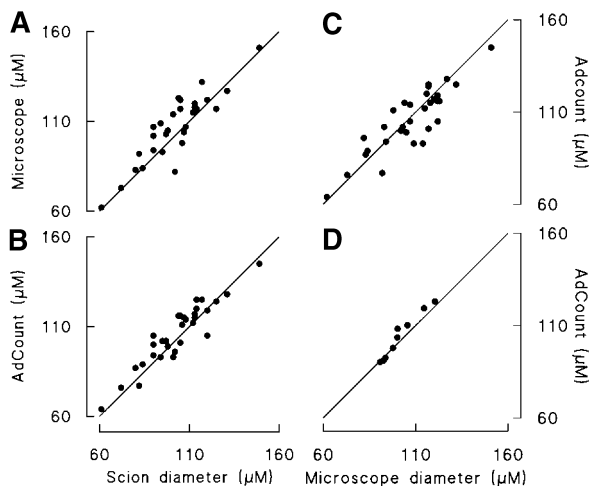


Fig. 2. Agreement between mean diameters (μm) of abdominal adipocytes from 31 volunteers determined by three methods plotted in pairs with an identity line: microscopy (Micro) by manual measurement of diameters from digital images by using the public domain NIH Image program (Scion) (A), AdCount by Scion (B), and AdCount by Micro (C), as well as of femoral adipocytes from nine volunteers determined by AdCount and Micro only (D).

cates an excellent between-methods agreement. For femoral adipocytes, AdCount versus Micro comparison had a CCC of 0.79 (on the border between substantial and excellent categories of agreement).

Between-method disagreement

Between-method differences for each sample plotted against their corresponding mean of the two methods (Fig. 3) spread on both sides of the “0” line. Comparing the patterns of spreading of between-methods differences, it is noticeable that a greater number of AdCount-Scion and Micro-Scion differences are above the “0” line (21:11 and 18:12, respectively), suggesting a tendency for the Scion method to provide smaller abdominal fat cell diameters. The mean and standard deviation of the between-method differences (which represent a quantitative expression of bias and error, respectively, and hence the degree of disagreement) are presented in Table 1. The means of the differences between AdCount or Micro and Scion methods are positive, indicating a tendency for the Scion method to provide smaller mean diameters. Testing of the hypothesis of zero mean/no bias showed that Scion method gives results that are significantly different from the AdCount and from Micro.

Reliability of adipocyte counter method

Both the test-retest ICC and inter-rater ICC were 0.999, showing an excellent reproducibility of the AdCount method, indicating that dual observations or dual observers do not contribute to the variation in the measurements with the AdCount method.

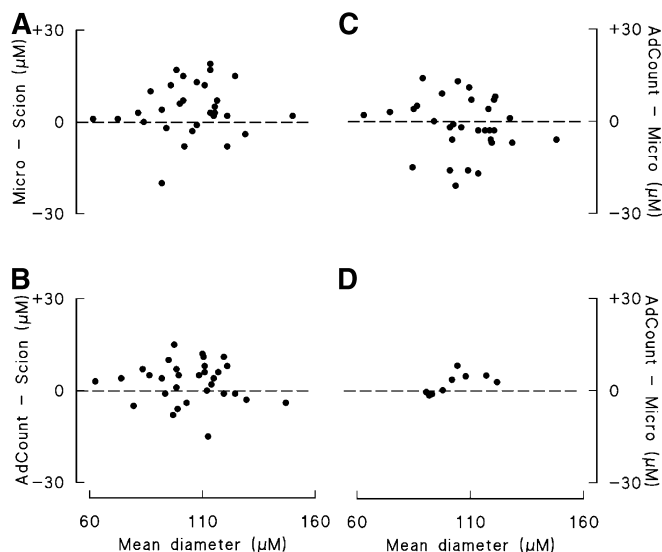


Fig. 3. Disagreement between mean diameters (μm) of abdominal adipocytes from 31 volunteers determined by three methods compared in pairs: Micro versus Scion (A), AdCount versus Scion (B), and AdCount versus Micro (C), as well as of femoral adipocytes from nine volunteers determined by AdCount and Micro only (D). Differences between each pair of methods plotted against their average mean fat cell diameters.

TABLE 1. Between-method differences in the mean diameters of fat cells from two fat tissue depots. Results are given as mean \pm SD

Methods ^a	Abdominal (n = 31)	Thigh (n = 9)
AdCount, Micro	-1.5 \pm 9.0	2.3 \pm 3.3
Micro, Scion	4.3 ^b \pm 8.5	
AdCount, Scion	2.8 ^b \pm 6.6	

The mean diameters of fat cells, isolated after collagenase digestion of fat tissue from the abdomens of 31 volunteers were determined using three methods: direct Micro, Scion, and AdCount. The mean diameters of fat cells, isolated from femoral fat tissue of nine volunteers were determined with Micro and AdCount. Differences between methods, in pairs, were calculated. The mean and standard deviation of the between-method differences were tested for zero/no bias.

^a AdCount, automated measurement of diameters from digital images using a customized program developed by Biomedical Imaging Resource at Mayo Clinic; Micro, microscopy; Scion, manual measurement of diameters from digital images by using the public domain NIH Image program.

^b $P < 0.05$ from the testing of the hypothesis of zero/no bias.

Relation between size of fat cell droplets and maturity stage of very small fat cells

Observing the images from the fluorescent staining for fat droplets with Nile Red and nuclei with Hoechst 22258 prompted us to categorize the small fat cells into three groups. We used as indices of maturation the number of lipid droplets in the cell and/or the disposition of the nuclei and the cytoplasm; representative cells are shown in Fig. 4. The first group comprises cells with multiple fat droplets of sizes within the range of 1–10 μm ; we considered these to be early, immature adipocytes (Fig. 4A). The second group also includes multilocular cells, but with one of the droplets standing out as a dominant droplet. We observed diameters up to 22 μm for the dominant droplet, as in Fig. 4B (late immature fat cells). Cells from these first two groups often did not have a spherical shape. The cytoplasm tended to form protrusions containing the fat droplets that were at a distance from the nuclei, and the nuclei may not have eccentric position. Cells we included in the third group were of two types: those with multiple fat droplets, but with the largest droplet having a diameter in the range of 25–55 μm , as in Fig. 4C (late immature cells), or a single droplet with a diameter greater than 25 μm , as in Fig. 4D (very small mature fat cells). A feature of this third group is that the cells had more-spherical shapes and less cytoplasmic volume unoccupied by the lipid droplet. The nuclei also tended to be more dense and were in close proximity to the fat droplet(s).

Based upon these findings, we reanalyzed seven biopsy samples using AdCount with different lower limits for cell size (25 μm). The new data were compared with an analysis using our original lower limit of 35 μm and with the records of the visual method for the same samples. The same threshold on each picture was used so that the only change was the lower limit of cell size included. For the Micro, AdCount at 35 μm , and AdCount at 25 μm methods, 189 \pm 13 cells, 317 \pm 58 cells, and 319 \pm 58 cells per sample, respectively, were counted. The mean fat cell diameter for these samples was 102 \pm 10 μm , 101 \pm 11 μm , and 102 \pm 12 μm for the Micro, AdCount at 35 μm , and

AdCount at 25 μm , respectively ($P = \text{NS}$ for all between-group comparisons). Thus, selecting a lesser value for the lower limit of fat cell diameter does not significantly change the average fat cell size.

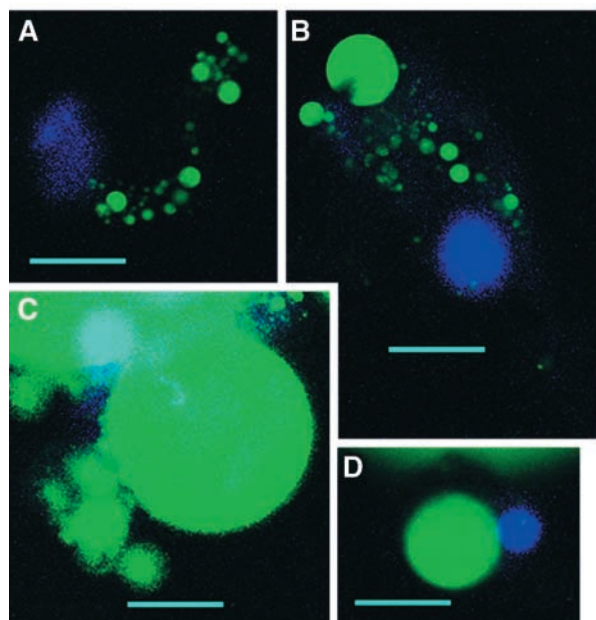
DISCUSSION

Measuring adipocyte diameter using light Micro is an easy, reliable, and inexpensive way to determine fat cell size (1). Unfortunately, it does not allow for a retrievable record, and if more than a few samples need to be processed in one day, it can be a tedious and difficult task. The ready availability of digital photomicrographs and image analysis software suggested to us a means to reduce the time needed for fat cell sizing and to provide a permanent record for later review if necessary. Unfortunately, our original approach using the Scion software actually required more time for analysis. Because of the simple shape characteristics of mature adipocytes, we adapted existing software to automate the sizing process. One advantage of an automated software solution is the ability to size larger numbers of cells from each sample, while at the same time reducing the time needed for the collection of data. These studies showed that the results from the automated software are comparable to the currently accepted optical method.

Another approach to measuring fat cell size uses osmium tetroxide fixation and subsequent automated size fractionation by Coulter counter (10). We did not compare AdCount with this approach because, despite the automation of this procedure (10), it does not seem to be commonly used. This may relate to the long processing time required and the use of the expensive and toxic osmium reagent.

We found that the agreement between methods for abdominal fat cell diameter was excellent ($\text{CCC} = 0.81\text{--}1.00$) for all the two-by-two comparisons. There was a substantial agreement between AdCount and Micro for diameters of fat cells from thigh despite the small sample number (31 for the abdomen vs. nine for the thigh). Our between-method comparisons revealed a bias between Scion and either of the other two methods; consistently smaller mean diameters were noted using Scion (Table 1). Although both Scion and AdCount used the same set of digital images, they actually utilized different populations of cells. Moreover, AdCount measured usually over 300 cells per sample with much less effort and in less time, whereas 100–150 were measured using Scion and required more time than even the direct Micro approach (1.5–2 h per site). Therefore, the mean fat cell diameters we measured by the Scion method might have been less representative for the sample compared with AdCount. If so, measuring more cells would compensate for this problem, but at the expense of an even greater time commitment. We note that the Scion procedure is also more subjective than the AdCount approach in that a certain degree of ‘selection bias’ is inevitable, whereas this occurrence is avoided with AdCount. Finally, the analyst electronically draws the “largest” diame-

Fluorescence



Transmission overlay

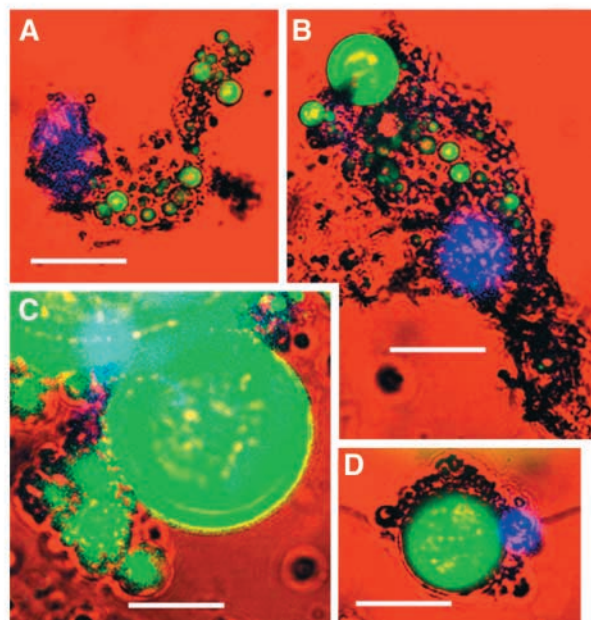


Fig. 4. Representative images of variations in the number and size of fat droplets and their position in relation to nuclei and cytoplasm in very small fat cells; Nile Red fluorescent lipid staining. Fat cells from abdominal subcutaneous fat tissue were isolated by enzymatic digestion, fixed with paraformaldehyde, and stained with Nile Red (green fluorescence) for cytoplasmic fat droplets and with Hoechst 33258 for nuclei. A: A cell full of multiple small droplets with diameters uniformly distributed in the range of 1–10 μm . B: A cell similar to the cells in image A, but including one droplet that is bigger than the rest, with diameter in the range of 10–25 μm . Note the cytoplasmic protrusions and the distance between the nuclei and the fat droplets in the cells in A and B. C: A cell having a large fat droplet with a diameter $\geq 25 \mu\text{m}$ and a few other smaller droplets. D: A cell with a single lipid droplet with diameter $\geq 25 \mu\text{m}$. Note the more rounded shape of the cytoplasm and the close proximity of the more compact nuclei in the cells in C and D. Bar = 25 μm .


ter through the cell center according to the operator's visual judgement while AdCount derives the diameter of each cell from measurement of the cell area. The latter may be a better approach when there is slight variation in circularity of the cells. The good agreement between AdCount and Micro in measuring thigh fat cells further supports the comparability between AdCount and Micro independent of adipose tissue depot.

A potential drawback to the use of digital photographic images is that the field of focus is set, whereas with the manual optical method it is possible to adjust the focus on each cell horizon to perhaps better determine the true cell diameter. The excellent between-methods agreement in diameter measurement of cells from abdomen and thigh and the lack of bias between AdCount and Micro imply that digital image analysis per se does not result in systematic errors when compared with the manual optical method.

When the manual optical microscopic method is used, one can include cells with lipid droplet diameters $\geq 11 \mu\text{m}$, whereas the Coulter counting of osmium fixed cells analyses lipid droplets $\geq 25 \mu\text{m}$. Our study of the morphology of very small fat cells using the Nile Red combined with nuclear staining revealed that cells with multiple small (diameters $< 10 \mu\text{m}$) fat droplets are likely early immature adipocytes. However, other types of cells that may accumulate fat, such as macrophages, may also have droplets of similar size and thus could be mischaracter-

ized unless specific adipocyte markers are used. The relatively equal distribution of the size of these droplets and the distant position of the nuclei of these cells in relation to the fat droplets would make it very difficult to differentiate these early immature fat cells from free fat droplets using the manual optical method. The smallest diameter of an unilocular lipid droplet we detected using the Nile Red-stained sample was $\sim 25 \mu\text{m}$, and that the diameter of the largest droplet in a multilocular small fat cells was $\sim 55 \mu\text{m}$. This suggests that it would be difficult to differentiate immature from mature fat cells when the diameter of the fat droplets is in the range of 25 μm to 55 μm . Therefore, investigators may choose to select the lower end cutoff value for the diameter of fat cells they wish to count, depending on the study objectives. The clear detection of stained nuclei overlaying or in the immediate proximity of the fat droplets was readily and consistently seen for small mature fat cells of at least 35 μm and served as a basis of the choice of 35 μm . The primary object of this study was to compare the methods for measuring fat cell diameters, and any value of the lower end cutoff for the cells measured would not affect the between-method comparisons if the same cutoff has been used for all the methods. The AdCount program is flexible and permits the user to adjust the limits of the diameter included.

In summary, we report the use of digital image analysis using Scion and AdCount to measure average fat cell size.

The approach provides a quick and permanent visual record that can be assessed at a convenient time. Although Scion is available at no cost, the AdCount method is automated and rapid (10–15 min for measuring diameters of 300 fat cells). AdCount eliminates potential bias in selecting cells of particular size and, thus, should minimize subjective error. Adipocyte diameters determined by AdCount were in excellent agreement with those determined by Micro. Therefore, fat cell sizing based on automated determinations utilizing digital images of fat cells should allow measurement of fat cells from a large number of samples in a short period of time. The excellent reproducibility indices, when the same observer has performed two measurements or when two observers have performed one measurement, imply that AdCount can be a reliable method in the hands of any operator. The algorithm and the associated procedure used for segmenting and counting objects described in this paper have many potential applications in biomedical imaging. A generalized version of this counting application is implemented as part of the general-purpose Analyze[®] software system, allowing its direct use with other cell counting applications or with other tasks, such as vessel counting. The cost of implementing and validating this approach in the laboratory would include the purchase of a digital camera and the software, and the technician time for between-method comparisons. In our experience, this cost is definitely worthwhile considering the long-term advantages that it provides. 

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