Electronic determination of size and number in isolated unfixed adipocyte populations[1]

Bradley J. Maroni,[2] Robert Haesemeyer, Lois K. Wilson, and Mario DiGirolamo

Divisions of Nephrology and Endocrinology, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322

Abstract Adipose tissue cellularity and metabolism are traditionally expressed in terms of mean cell size and number. The need for a simple method allowing rapid determination of cell size and number of freshly isolated, unfixed adipocyte preparations led us to compare estimates of cell size determined by the established method of optical sizing to a proposed method of electronic cell sizing and counting. In collagenase-isolated, unfixed adipocytes whose mean diameters ranged from ~40 to 65 μm (obtained from healthy rats weighing 100–360 g) the electronic method provided estimates of the mean cell diameter and size distribution that did not differ from the optical sizing technique. Estimates of mean cell diameter and cell number by the electronic method were rapid and reproducible (coefficients of variation 0.5 and 3.8%, respectively) and a <20 sec delay until sample analysis, after mixing of the adipocyte suspension, did not alter these estimates. Electronic determination of cell size and number, using freshly isolated, unfixed rat adipocyte populations (mean cell diameter ≤60 μm), is rapid and reliable. It will be particularly useful for studies of hormone binding and transport processes where it may be necessary to tightly control cell density.—**Maroni, B. J., R. Haesemeyer, L. K. Wilson, and M. DiGirolamo.** Electronic determination of size and number in isolated unfixed adipocyte populations. *J. Lipid Res.* 1990. **31:** 1703–1709.

Supplementary key words cell diameter • size distribution • Coulter Counter[R]

Several methods have been described for estimating adipocyte size and number, each with its particular advantages and disadvantages (1, 2). A simple method allowing rapid determination of cell size and number in freshly isolated, unfixed adipocytes would be particularly useful for studies of transport and hormone binding, since the fat cell concentration is known to influence their metabolism during metabolic incubations and must be carefully controlled between experiments.

We therefore compared estimates of cell size determined by an established method of optical sizing (3) to a proposed method of electronic sizing and cell counting. The results were indistinguishable by the two methods and indicate that, within certain limits, the electronic sizing of unfixed cells may offer the advantage of rapidity and reproducibility without major pitfalls due to cell lysis or other technical problems.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 100–360 g (Harlan Sprague-Dawley, Inc., Prattville, AL) were used in all studies. Animals were housed in a temperature-controlled room with a 12-h light/dark cycle; they were fed standard rat chow (Lab Chows 5001; Purina Mills, Inc., St. Louis, MO) and water ad libitum for at least 5 days before being studied. Experiments were performed between 900 and 1500 h.

Materials

Collagenase A (0.26 U/mg) was obtained from Boehringer Mannheim Biochemical (Indianapolis, IN); bovine serum albumin (fraction V) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Adipocyte isolation

Adipocytes were isolated by the method of Rodbell (4) and all procedures were performed using polypropylene tubes. Rats were anesthetized with 50 mg pentobarbital sodium/kg (ip) and the epididymal fat pads were removed via a midline abdominal incision. The fat pads were minced, gassed for 15 sec (O_2–CO_2, 95:5%), and incubated at 37°C in a shaking water bath (Model G76, New Brunswick Scientific Co., Edison, NJ) for 45 min in Krebs-Henseleit bicarbonate buffer (KRB), pH 7.4, containing 1.5 mg/ml collagenase, 5 mM dextrose, and 4 g/dl albumin. Subsequently, adipocytes were gently filtered through ~200-μm diameter mesh silk, washed three times in KRB buffer, and adjusted by visual inspection to a "lipocrit" of ~10–20%.

[1]Presented in part in New Orleans at the annual meeting of the Southern Section, American Federation for Clinical Research, 17–19 January 1990.

[2]To whom correspondence should be addressed at: Renal Division, Emory Univeristy Hospital, 1364 Clifton Road, N.E., Atlanta, GA 30322.

Cell sizing

Adipocyte sizing was determined in two ways: *1*) a novel method utilizing electronic cell sizing of unfixed freshly isolated fat cells and *2*) the established technique of microscopic sizing of freshly isolated stained fat cells (3).

Electronic sizing

Cells size and number were determined in a standardized manner using a Coulter Multisizer™ (Coulter Electronics, Inc., Hialeah, FL) connected to an Epson LX-80 printer (Beckman Instruments, Inc.). An orifice tube containing a 280-μm aperture was used for all experiments and the Multisizer™ was calibrated with latex particles of known diameter. Cell analysis was performed in the "siphon mode" (2 ml volume) and results were displayed graphically in terms of linear diameter (x-axis) and cell number (y-axis). The left and right cursors set the limits of the analysis to exclude particles less than 22.03 μm or greater than 176.3 μm diameter with the x-axis divided into 64 consecutive channels, 2.75 μm in diameter (**Fig. 1**). The exclusion of cells less than 22 μm was chosen to approximate the lower limits of the techniques of Hirsch and Gallian (5) and of DiGirolamo, Mendlinger, and Fertig (3). After each Coulter "sizing," a graphic printout was saved for data analysis. The printout graphically displays a frequency distribution (y-axis) of the cell population by cell diameter (x-axis), as well as the number of cells within each channel and the cumulative number of cells counted. The mean cell diameter was determined by multiplying the mid-point of each channel by the number of cells in that channel; a weighted average was then calculated.

For cell sizing and counting, the cell suspension was removed from the shaking water bath (37°C), gently mixed by inverting three times, and 50 μl of the original cell suspension was added to a 20-ml plastic Accuvette™ (Coulter Electronics, Inc.) containing 10 ml of a isotonic electrolyte solution (Isoton II™; Coulter Electronics, Inc.). After quickly attaching the Accuvette™ cap, the cell dilution was mixed by gently inverting the suspension three times and immediately placed on the sampling stand for analysis. To insure uniformity, the sampling stand was adjusted so that the orifice tube was immersed in the diluted cell suspension and just contacted the bottom of the Accuvette™; the aperture was thereby situated ~1 cm above the base.

After a preliminary determination of the cell count on the diluted sample, the lipocrit of the original cell suspension was adjusted to obtain ~6 × 10^3 cell counts in the diluted sample. This cell concentration was empirically chosen based on Coulter's™ recommendation that the best compromise for measuring the size distribution with

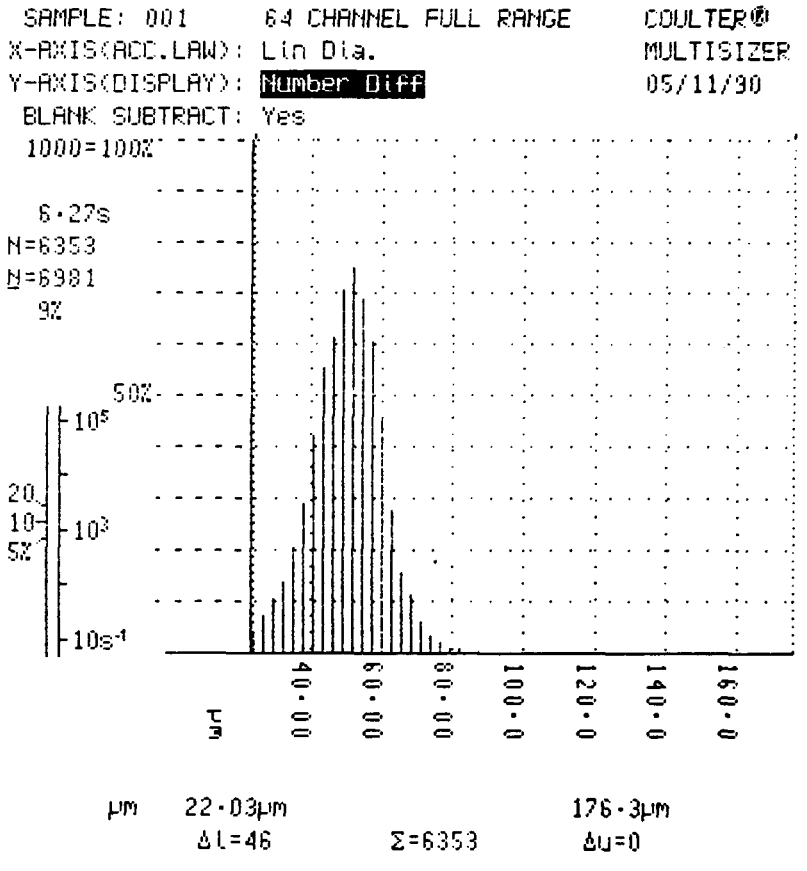

Fig. 1. Graphic printout of a representative cell population displaying a frequency distribution (y-axis) of the cell population by cell diameter (x-axis), and the cumulative (N) and coincidence corrected ($\overline{N}$) cell counts.

both accuracy and speed was to maintain the coincidence error of the total counts between 5 and 10% of the total counts. Measurements of cell number and diameter were then performed in rapid sequence on triplicate 50-μl aliquots from the original cell suspension as described.

Experiments designed to examine the influence of time delay on the estimates of cell number and mean cell diameter were performed as described with the following modification. Fifty μl of the original cell suspension was added to an Accuvette™ containing 10 ml of Isoton™, capped, gently inverted three times, and placed on the Coulter sampling stand. A timer preset to the indicated time (5–40 sec) was then activated with sampling initiated when the alarm sounded.

Optical sizing

The optical sizing method of DiGirolamo et al. was utilized (3). In brief, after the appropriate lipocrit was determined, and immediately prior to the electronic sizing, 50 μl of the original cell suspension was added to 2 ml of KRB media containing 1–2 drops of methylene blue (1%, w/v). The cells were incubated for 5–10 min at 37°C, and then an aliquot was transferred to a microscope slide containing three wells; cover slides were placed over the cells and the diameters of 300 adipocytes were measured. The circular wells (1–2 mm deep by 1 × 1 cm wide) were prepared by expressing silicon gel from a 20 ml plastic syringe, while microscope and cover slides were siliconized by prior immersion in Dri-Cote™ (Fisher Scientific Co., Pittsburgh, PA). A frequency distribution of cell diameters was obtained and the mean cell diameter was determined by multiplying the midpoint of each class interval by the number of cells; a weighted average was then calculated (3).

Statistics

Data are presented as means ± SD. The variability of repeated measures of cell size and number was assessed by determining the coefficient of variation. Comparisons between the electronic and microscopic sizing techniques were assessed with the Student's *t* test. The influence of animal weight and delaying sample analysis on the estimates of cell size and number were determined using two-way ANOVA (Systat, Inc., Evanston, IL).

RESULTS

A prerequisite of any method for determining the mean cell diameter of a cell population is measurement reproducibility. Illustrated in **Table 1** are the electronically determined triplicate mean cell diameter estimates and their averages determined on adipocytes isolated from 16 rats of various weights. Over a range of mean cell diameters from 41 to 63 μm, the coefficient of variation (CV) of replicate measures obtained from adipocytes of a given animal averaged 0.5% (range 0–1.8%).

Since adipocytes will rise to the surface of the suspending solution as a function of cell size and time, it is conceivable that the mean cell diameter could be underesti-

TABLE 1. Reproducibility of electronic mean cell diameter determination

Rat weight	Replicates 1	2	3	Mean Diam. ± SD	CV
g		*μm*		*μm*	*%*
116	42.1	42.0	41.8	42.0 ± 0.1	0.2
118	44.0	43.2	43.9	43.7 ± 0.4	0.9
131	42.7	42.7	42.9	42.8 ± 0.1	0.2
136	41.3	41.2	41.4	41.3 ± 0.1	0.2
204	46.2	45.7	45.5	45.8 ± 0.4	0.9
210	51.0	49.6	49.8	50.1 ± 0.0	0.0
216	51.7	51.5	51.8	51.7 ± 0.1	0.2
227	54.5	54.5	54.4	54.4 ± 0.1	0.2
300	57.1	57.0	56.8	57.0 ± 0.0	0.0
301	55.0	54.4	54.2	54.5 ± 0.4	0.7
304	53.0	52.7	53.0	52.9 ± 0.2	0.4
309	54.1	54.1	54.2	54.1 ± 0.1	0.2
350	59.7	58.9	58.6	59.0 ± 0.6	1.0
355	60.8	60.2	60.8	60.6 ± 0.3	0.5
358	61.9	62.8	62.6	62.4 ± 0.4	0.6
360	62.7	61.4	60.6	61.6 ± 1.1	1.8
					Mean = 0.5 ± 0.5

Illustrated are the triplicate determinations of the mean cell diameter determined from aliquots of the original cell suspension (see Methods), their respective means ± standard deviations (SD), and coefficients of variation (CV) for the adipocyte populations isolated from individual rats weighing ~116–360 g.

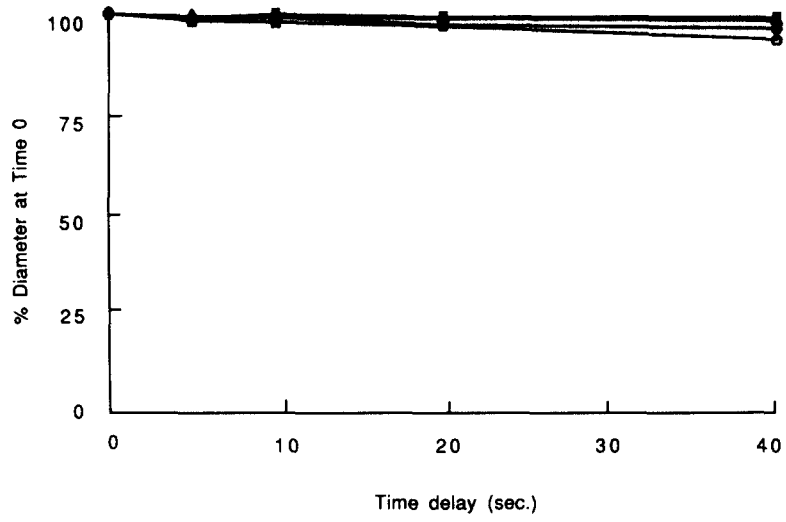

Fig. 2. The influence of delaying sample analysis by 5–40 sec on the mean cell diameter estimated by electronic sizing (□, ~100 g (n = 2); Δ, ~300 g (n = 4); ◇, ~200 g (n = 4), ⌂, ~360 g (n = 4)).

mated as the time interval between mixing the cell suspension and analysis increased. The effect of delaying sample analysis by 5–40 sec, on the mean adipocyte diameter is illustrated in **Fig. 2**. ANOVA revealed that adipocyte mean cell diameter increased with increasing animal weight ($P<0.0001$), but over the range of mean cell diameters examined, a delay in sample analysis of ≤40 sec did not significantly alter our estimates of mean cell diameter, (e.g., after a 40-sec delay the average value was 96.1% of that at time 0).

The reproducibility of the optical sizing technique for determining mean cell diameter was evaluated by comparing measurements by two individuals experienced with this technique. Inter-observer agreement was excellent and no significant difference was noted between the two observer estimates (data not shown). The results obtained by one observer using the optical sizing method were then compared with those determined by the electronic counting technique (**Table 2**). There was no significant difference in mean cell diameter obtained by either method, although in rats weighing ~360 g there was a tendency for the mean cell diameter to be slightly smaller and the size distribution to be somewhat broader with the electronic counter.

Because a rapid and reproducible method for estimating cell number of freshly isolated adipocytes would by useful, we examined the reproducibility of the electronic technique for determining cell number. Presented

TABLE 2. Comparison of optical sizing and electronic counting techniques for determining mean cell diameter

Rat Weight	Optical Sizing (mean diam. ± SD)	Electronic Counting (mean diam. ± SD)	Δ
g	*μm*	*μm*	
116	42.8 ± 9.9	42.0 ± 9.8	+0.8
118	42.1 ± 9.7	43.7 ± 9.5	−1.6
131	40.5 ± 10.9	42.8 ± 10.5	−2.2
136	39.9 ± 11.6	41.3 ± 11.1	−1.4
204	46.4 ± 10.8	45.8 ± 12.1	+0.6
210	48.5 ± 11.7	50.1 ± 11.4	−1.7
216	51.9 ± 10.6	51.7 ± 10.0	+0.2
227	56.0 ± 11.0	54.4 ± 11.5	+1.6
300	57.7 ± 11.1	57.0 ± 13.3	+0.7
301	56.3 ± 11.0	54.5 ± 13.5	+1.8
304	54.1 ± 10.2	52.9 ± 10.1	+1.2
309	55.5 ± 11.9	54.1 ± 13.4	+1.4
350	60.4 ± 10.6	59.0 ± 13.1	+1.3
355	66.6 ± 13.6	60.6 ± 14.3	+6.0
358	67.7 ± 12.9	62.4 ± 16.6	+5.2
360	64.8 ± 11.4	61.6 ± 15.5	+3.2
			mean = 1.1 ± 2.3
			P = NS

Estimates of the mean cell diameter of size distributions from adipocyte populations of rats weighing 116–360 g.

TABLE 3. Reproducibility of cell count using electronic counter

Rat weight	Replicates 1	2	3	Mean ± SD	CV	Cells/ml ($\times 10^3$)
g					%	
116	5168	5098	4976	5,081 ± 97	2.0	510.61 ± 9.77
118	5741	5573	5852	5,722 ± 141	2.0	575.06 ± 14.12
131	6138	6398	6181	6,239 ± 139	2.0	627.02 ± 14.01
136	4612	4433	4657	4,567 ± 119	3.0	459.02 ± 11.91
204	5180	5512	4573	5,088 ± 476	9.0	511.38 ± 47.85
210	5893	5815	5658	5,789 ± 120	2.0	581.76 ± 12.03
216	5568	5508	5854	5,643 ± 185	3.0	567.16 ± 18.58
227	5551	5370	5382	5,434 ± 101	2.0	546.15 ± 10.17
300	5736	5738	5558	5,677 ± 103	2.0	570.57 ± 10.39
301	5744	5480	5330	5,518 ± 210	4.0	554.56 ± 21.07
304	5735	5332	5448	5,505 ± 208	4.0	553.25 ± 20.85
309	6225	6634	6187	6,349 ± 248	4.0	523.40 ± 20.30
355	4073	3897	3772	3,914 ± 151	4.0	393.36 ± 15.20
358	5262	5089	4742	5,031 ± 265	5.0	505.62 ± 26.61
360	5599	4940	4669	5,069 ± 478	9.0	509.47 ± 48.07
					mean = 3.8%	

Illustrated are the triplate determinations of cell count determined from aliquots of the original cell suspension (see Methods), their respective means ± SD and coefficients of variation (CV) for adipocyte populations isolated from individual rats weighing 116–360 g. Column 7 represents the cell concentration per ml of the original cell suspension derived from the mean of the triplicate dilutions (dilution factor 1:100.5; i.e., 50 μl of original cell suspension added to 10 ml of Isoton™ of which 2 ml are counted).

in **Table 3** are the individual values and means of the triplicate determinations of adipocyte cell number; reproducibility was quite good as reflected by the 3.8% CV of replicate cell counts (range 2–9%). By calculating the dilution factor one readily obtains the cell concentration present in the original cell suspension (Table 3, column 7).

Since estimates of cell number may be affected by adipocytes floating to the surface of the suspension, we examined the influence of delaying sample analysis by 5–40 sec on the estimates of cell number (**Fig. 3**). ANOVA revealed a significant influence of time delay on the cell count determinations only when the delay was ≥20 sec. There was a nonsignificant trend for the cell count to decline more rapidly as a function of time in cell populations isolated from heavier animals ($P<0.08$). Hence, under the described conditions, adipocyte populations whose mean diameters range from ~40 to 63 μm, remain homogeneous and migrate slowly to the surface of the suspension, independent of actual cell size.

DISCUSSION

Four general methods have been advocated for estimating adipocyte size and number, each with its advantages or disadvantages (1, 2, 6): *1*) DNA determination of

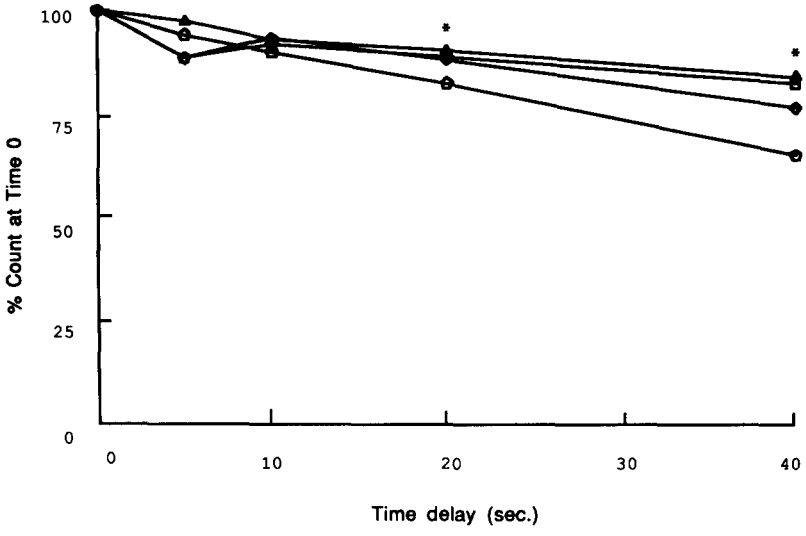

Fig. 3. The influence of delaying sample analysis by 5–40 sec on the electronically determined cell counts (□, ~100 g (n = 2); Δ, ~200 g (n = 4); ◇, ~300 g (n = 4); ⌂, ~360 g (n = 4); *$P \leq 0.0001$; 20 and 40 sec. vs time 0 by two-way ANOVA).

adipocytes in situ or separated from the stromal component of adipose tissue; *2*) microscopic examination of thin sections of fixed adipose tissue or isolated fixed adipocytes; *3*) microscopic sizing of freshly isolated fat cells following collagenase digestion of adipose tissue; and *4*) electronic determination of cell size and number of adipocytes isolated from adipose tissue previously fixed with osmium tetroxide.

It is generally accepted that quantitation of DNA is not an accurate basis for determination of fat cell number in intact adipose tissue or isolated adipocyte suspensions due to contamination by the stromal-vascular component (1, 2). Furthermore, this method provides no information regarding the size distribution of the cell population.

Microscopic examination of fixed histological sections of intact adipose tissue is inexpensive and represents the best method for minimizing the influence of cell loss during morphologic determinations of intact adipose tissue (1, 7, 8). Limitations include the need for a correction factor when thin tissue sections are utilized and the practical difficulty of comparing metabolic data obtained from different samples (1, 9, 10). Furthermore, when a large number of samples need processing, it may become tedious, involving considerable eye strain.

Direct microscopic determination of the diameter distribution of collagenase-isolated stained adipocytes is the third method advocated and served as the reference for validation of the presently proposed method (3). Its strengths include the modest equipment cost and the capacity to determine diameters quickly and accurately. Disadvantages predominantly reflect the potential problem with cell lysis with isolated adipocytes (1, 2), although the addition of adenosine to the collagenase solution and incubation media has been reported to virtually eliminate this problem (11). In addition, microscopic sizing requires practice and may be tedious when multiple samples need processing.

One of the most widely utilized techniques for studying adipocyte cellularity and morphology is the method originally described by Hirsch and Gallian (5) and subsequently extended by others (8, 12, 13). As initially described, small fragments of adipose tissue were fixed for 24–72 h in osmium tetroxide, separated from the tissue stroma by filtration through a 250-μm filter and isolated on a 25-μm filter, resuspended in saline, and counted electronically. With method III, the average cell size (μg lipid/cell) was calculated by dividing the lipid weight of a comparable sample by the electronically determined cell number (5). Alternatively, by repeated sampling and excluding cells of increasing size, one could obtain a frequency distribution of cell size as well as number (method IV). Further modifications of this technique include electronic counting of osmium-fixed adipocytes separated into different size ranges by filtration through nylon filters of successively smaller diameter, thereby providing a frequency distribution by size (12); use of an electronic particle counter capable of segregating cells into distinct size intervals greatly simplified the process while simultaneously reducing the amount of adipose tissue necessary (13).

A major advantage of this latter technique is that electronic counting is automated, providing a direct, objective measure of the size distribution and cell number. Disadvantages include the expense of the electronic counter and osmium tetroxide and the latter's toxicity, as well as the long processing time (2, 10). Finally, some (5), but not all investigators (8, 13) have suggested that osmium may cause cell swelling thereby overestimating true adipocyte size.

Our proposed method for electronic determination of cell size and number represents an extension of the method initially proposed by Hirsch and Gallian (5). It incorporates the strength of the electronic sizing and cell counting with the capability for immediate analysis using freshly isolated unfixed cells. Electronic counting allows rapid quantitation of a large number of cells, thereby minimizing sampling error. Direct electronic determinations are completely objective with cell volume of the individual particles determined by volume displacement (1). A frequency distribution of cell diameter, volume, or surface area can then be derived (Fig. 1). An additional advantage of the Coulter Multisizer^{TM} is that mean cell volume and surface area are calculated from the individual cell data rather than being derived from the mean cell diameter. This is of practical benefit since the latter method is valid only if diameter is distributed normally (2, 14), an assumption that may at times be erroneous (15, 16).

In summary, the described electronic method represents a simple, rapid, and reproducible technique for estimating cell size and number of freshly isolated rat adipocytes. Its accuracy for determining the mean cell diameter and size distribution was externally validated with the established optical sizing method. Cell number of an adipocyte suspension can be rapidly and reproducibly determined with an acceptable degree of accuracy. Even a moderate delay (<20 sec) until sample analysis did not significantly alter the estimates of mean cell size or number (Figs. 2, 3).

It should be recognized that electronic sizing and counting of freshly isolated fat cells has been validated only using adipocyte populations with mean diameter ranging from ~ 40 to 65 μm. Its utility for other species, with adipocytes whose mean diameters lie outside this range or for investigating the metabolism of very small fat cells <22 μm (16), would require additional validation.

We thank Cathy Keeler and Janelle Feathers for their expert editorial assistance. This study was supported in part by National Institutes of Health grants DK-39326 and DK-37175.

Manuscript received 26 February 1990 and in revised form 21 May 1990.

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