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Heterogeneity of Calcium Compartmentation: Electron Probe Analysis of Renal Tubules

Ann LeFurgey, Peter Ingram†, and Lazaro J. Mandel
Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710 and †Research Triangle Institute, Research Triangle Park, North Carolina, 27709

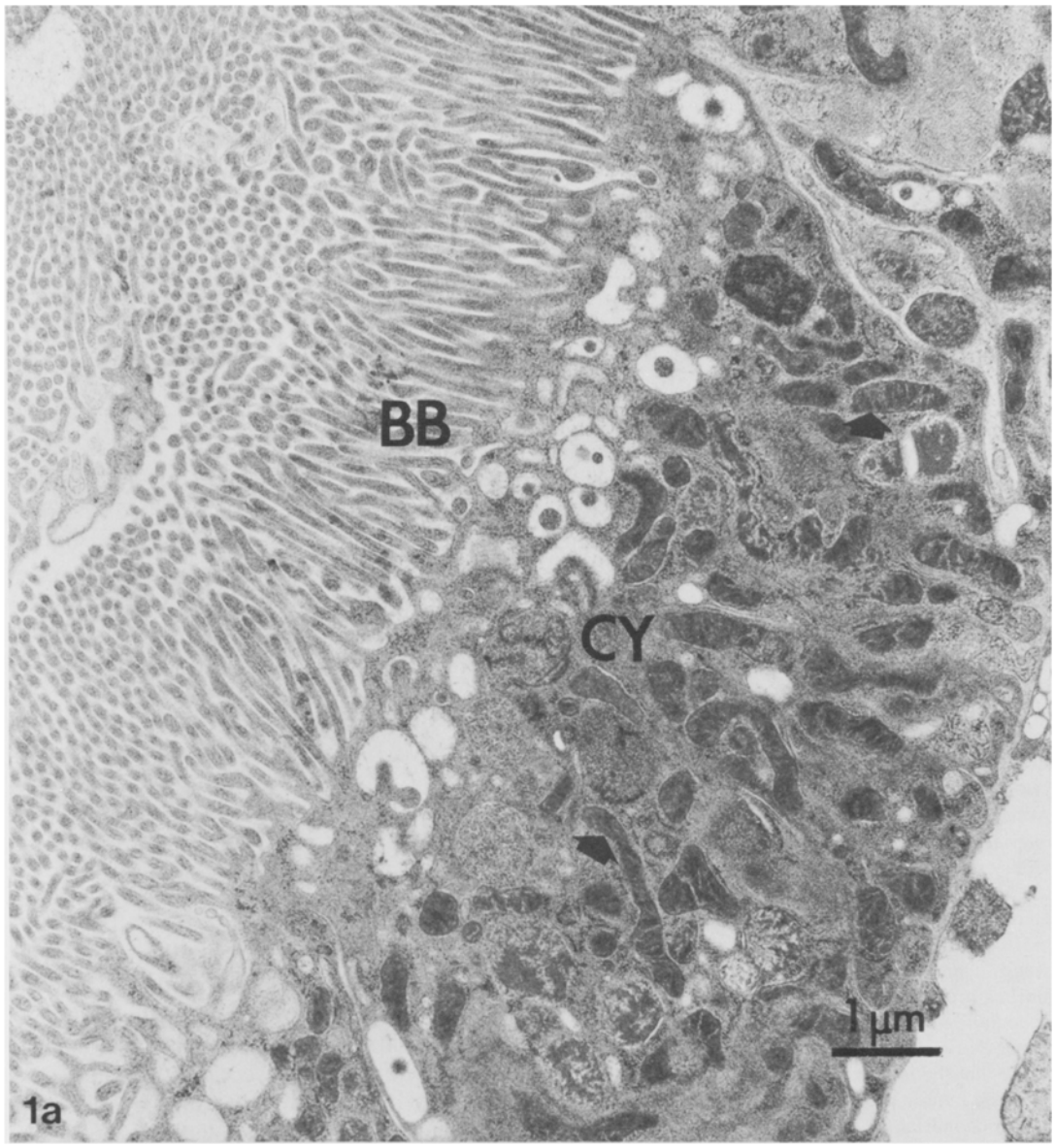
Summary. The objective of this study has been to determine the intracellular localization of calcium in cryofixed, cryosectioned suspensions of kidney proximal tubules using quantitative electron probe X-ray microanalysis. Two populations of cells have been identified: 1) "Viable" cells, representing the majority of cells probed, are defined by their relatively normal K/Na concentration ratio of ~4 : 1. Their measured Ca content is 4.1 ± 1.4 (SEM) mmol/kg dry wt in the cytoplasm and 3.1 ± 1.1 mmol/kg dry wt in the mitochondria, or an average cell calcium content of ~3.8 mmol/kg dry wt. 2) "Nonviable" cells, defined by the presence of dense inclusions in their mitochondria and a K/Na concentration ratio of ~1. The Ca content is 15 ± 2 mmol/kg dry wt in the cytoplasm and 685 ± 139 mmol/kg dry wt in the mitochondria of such cells. Assuming 25 to 30% of the cell volume is mitochondrial, the overall calcium content of such nonviable cells is ~210 mmol/kg dry wt. The presence of these inclusions in 4 to 5% of the cells would account for the average total Ca content measured in perchloric acid extracts of isolated proximal tubule suspensions (≈ 18 nmol/mg protein or 12.6 mmol/kg dry wt). Whole kidney tissues display a large variability in total Ca content (4.5 to 18 nmol/mg protein, or 3.4 to 13.5 mmol/kg dry wt), which could be accounted for by inclusions in 0 to 4% of the cells. The electron probe X-ray microanalysis (EPXMA) data conclusively demonstrate that the *in situ* mitochondrial Ca content of viable cells from the kidney proximal tubule is low and support the idea that mitochondrial Ca may regulate dehydrogenase activity but probably does not normally control cytosolic free Ca.

Key Words calcium · kidney proximal tubule · electron probe X-ray microanalysis · mitochondria · cytoplasmic calcium

Introduction

Two mutually exclusive roles have been attributed to mitochondrial Ca transport which are critically dependent on the mitochondrial Ca content. Studies of Ca^{2+}-sensitive mitochondrial dehydrogenase enzymes demonstrate that their activity is controlled with a half-maximal Ca^{2+} concentration of about 1 μM [6, 8]. This intramitochondrial concentration would be achieved with a mitochondrial Ca content of 1 to 2 nmol/mg protein [9]. On the other hand, isolated mitochondria containing 5 nmol Ca/mg protein or higher are capable of buffering the extramitochondrial (cytoplasmic) Ca^{2+} level to about 0.3 to 0.8 μM [4, 20]. In order to distinguish between these two possible roles for mitochondrial Ca, it is extremely important to accurately measure mitochondrial Ca content *in situ* in each tissue under consideration. Somlyo and coworkers [5, 28] using electron probe X-ray microanalysis (EPXMA) have measured mitochondrial contents of about 1 to 2 nmol/mg protein (0.8 to 1.6 mmol/kg dry wt) in both liver and smooth muscle cells. On the other hand, values of 10 to 20 nmol Ca/mg protein were found in liver [12, 18] and kidney [1, 35] mitochondria by other investigators using other methods.

The present study was designed to reconcile these differences between mitochondrial Ca contents measured by EPXMA and other methods. To this end, we measured the intracellular localization of calcium in cryofixed, cryosectioned suspensions of kidney proximal tubules using quantitative EPXMA. Results show a sharp heterogeneity between two cellular populations. The majority of cells display normal plasma membrane transport and morphology, and an average mitochondrial Ca content of 3.1 mmol/kg dry wt (4.3 nmol/mg protein). The other population consists of a small percentage of injured cells within the preparation, many of which contain mitochondria with large concentrations of Ca, precipitated in association with phosphate. While this phenomenon has been noted before [8, 30], this is the first time that such a heterogeneity has been quantitated by direct correlation with other physiological techniques. These results show that the normal mitochondrial Ca content in the kidney is sufficiently low for control of dehydrogenase activity [6, 8] and probably too low for the control of cytoplasmic-free Ca concentration [4, 20]. Furthermore, the heterogeneity in Ca distribu-



tion presents serious problems for the interpretation of results of Ca flux experiments using populations of cells or intracellular organelles. This result has far reaching implications for the understanding of the role of Ca in normal cell function and in the progression of cell injury, especially in the kidney.

Materials and Methods

Tubule fragments are isolated from the rabbit kidney by methods developed in our laboratory [2, 26]. They have open lumens, actively transport ions, and the individual cells are characterized by a well-developed apical brush border and a high proportion of mitochondria. The tubules are incubated in a shaker bath at a concentration of 4 to 5 mg protein/ml for 20 min at 37°C in a solution containing (mM): 115 NaCl, 25 $NaHCO_3$, 2 NaH_2PO_4, 1 $CaCl_2$, 5 KCl, 1 $MgSO_4$, 5 glucose, 4 lactate, 1 alanine, 5 malate, and 2 butyrate. The suspension is continuously gassed with 95% O_2/5% CO_2 to maintain a pH of 7.4. In some experiments, ouabain (10^{-4} M) is added to the suspension after this 20-min period and the incubation continued for another 5 min. For X-ray microanalysis, a 0.5-ml sample is taken from the suspension and rapidly centrifuged in a microfuge (about 3 sec), the supernatant is aspirated, a sample is taken from the loose pellet and placed on a wooden stub which is then rapidly frozen without cryoprotectants by immersion in liquid nitrogen-cooled liquid propane. The frozen tubule suspension is then processed in one of two ways: 1) by freeze substitution with osmium tetroxide in acetone [33] for

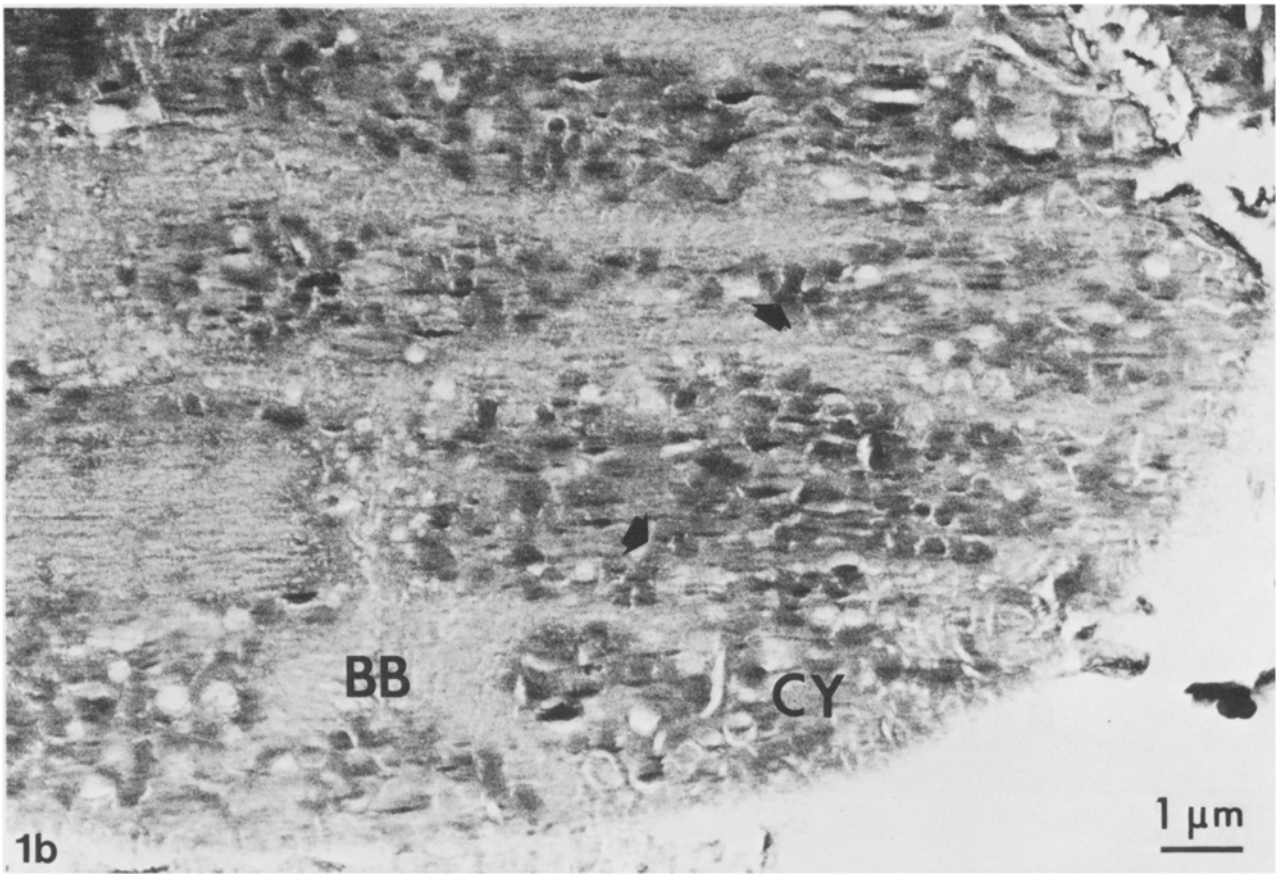


Fig. 1. (*a*) (facing page) Transmission electron micrograph of proximal tubule preparation fixed by quick freezing in liquid nitrogen-cooled liquid propane and freeze-substituted in OsO_4 in acetone [33] prior to epoxy embedding and routine sectioning. No cryoprotectants were used in freezing. *BB*, brush border; *CY*, cytoplasm; arrows, mitochondria. (*b*) Transmission electron micrograph of proximal tubule preparation quick frozen by plunging into liquid nitrogen-cooled liquid propane and cryosectioned at −130° prior to freeze-drying for 48 hr in a vacuum evaporator at 10^{-3} Torr. No cryoprotectants or stains were used in the preparation. *BB*, brush border, *CY*, cytoplasm; arrows, mitochondria. (Micrograph 1*b* taken by B.L. Craig.)

examination by transmission electron microscopy (TEM) of the quality of quick freezing and 2) by cryosectioning at −130°C, freeze-drying and examination by TEM, scanning transmission electron microscopy and energy dispersive X-ray microanalysis.

Results

EPXMA Measurement of Intracellular Calcium

Figure 1(*a*) illustrates the appearance of a proximal tubule following freeze substitution. The brush border and mitochondria of the cells are well preserved with little ice crystal damage evident. Figure 1(*b*) shows a typical cryosection used for X-ray microanalysis. Mitochondria and cytoplasmic regions are clearly identifiable in the absence of heavy metal stains or fixatives, thus allowing the electron beam to be positioned on individual structures for microanalysis.

Figure 2(*a*) and (*b*) show the type of EPXMA spectra obtained from the cytoplasm and mitochondria of the great majority of cells. The averaged elemental contents obtained from a number of such spectra are displayed in Table 1. The K/Na ratio of between 3 and 4 : 1 obtained in these tubules is the value expected for viable cells, based on measurements using other methods [27]. The Ca content was obtained by deconvolution of the overlapping K Kβ peak from the Ca Kα peak using the peak centroid shift correction method of Kitazawa et al. [13]. Calculated values for the Ca content were 4.1 ± 1.4 mmol/kg dry wt for cytoplasm and 3.1 ± 1.1 mmol/kg dry wt for mitochondria.

Results for other elements, e.g. Na, K, in viable

Table 1. Elemental contents in cytoplasm and mitochondria of kidney proximal tubules[a]

	Elemental content (mmol/kg dry wt)							*n*
	Na	Mg	P	S	Cl	K	Ca	
A. Viable cells								
Cytoplasm	125 ± 12	27 ± 4	356 ± 18	124 ± 8	141 ± 11	348 ± 23	4.1 ± 1.4	23
Mitochondria	95 ± 7	27 ± 3	349 ± 22	158 ± 6	131 ± 12	349 ± 22	3.1 ± 1.1	23
B. Nonviable cells								
Cytoplasm	261 ± 16	26 ± 5	288 ± 16	147 ± 14	70 ± 10	206 ± 11	15 ± 2	9
Mitochondria	154 ± 29	37 ± 16	612 ± 85	112 ± 17	187 ± 23	124 ± 16	685 ± 139	10

[a] *n* = number of 500-sec raster probes obtained from each region. The tubules were pre-incubated at 37°C for 20 min as described in the text. Values are mean ± SEM.

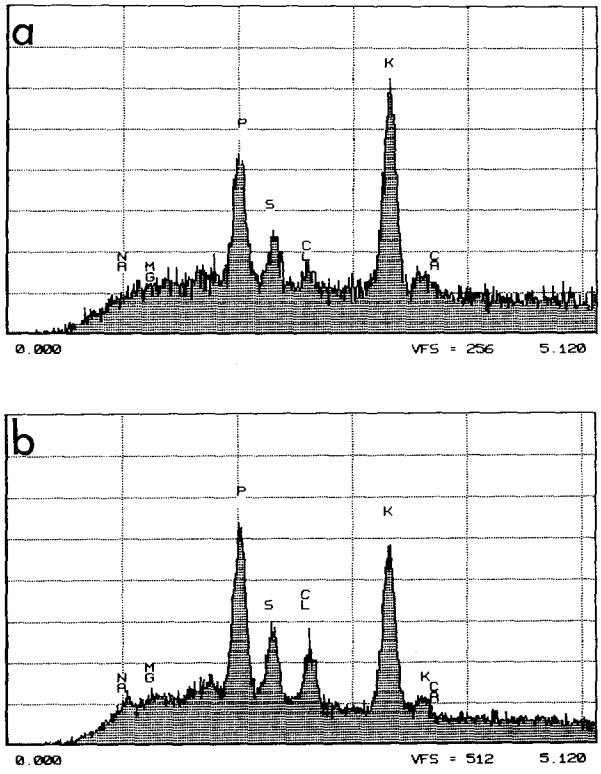


Fig. 2. Representative energy dispersive X-ray spectra from freeze-dried cryosections of kidney proximal tubule cells. (*a*) Cytoplasm; (*b*) mitochondria. All analyses were performed for 500 sec at −120°C in the raster mode at magnifications of 15,000 to 200,000, with specimen tilt, 30°; beam current, ~1 nanoamp; and accelerating voltage, 80 kV, in a scanning transmission electron microscope (JEM 100CX-II TEMSCAN) equipped with a 30-mm² Si(Li) energy dispersive X-ray detector and microcomputer system (Tracor Northern 5500). Standards were prepared according to the methods of Shuman, Somlyo and colleagues [25] and quantitation performed using the Hall continuum normalization method [7] with the peak centroid shift correction of Kitazawa et al. [13]

cells are in agreement with values published by Beck et al. [3] and Sauberman et al. [22, 23], using similar methodology. These findings also concur with qualitative and semi-quantitative values published much earlier by Trump and colleagues [34] and Kriz and co-workers [15]. Assuming that the dry weight is 25 to 30% of the wet weight [24, 29], it is possible to calculate Na and K concentrations of about 35 to 40 and 110 to 130 mM, respectively. These values are in close agreement to those obtained by other methods in rabbit proximal tubules [27].

As a test for the accuracy of the K deconvolution method for calculation of Ca content, another series of experiments was performed in which ouabain (10^{-4} M) was added to the tubule suspension for 5 min at 37°C prior to cryofixation. Ouabain, a cardiac glycoside widely used in experimental and clinical applications, inhibits Na, K-ATPase at the cell membrane and thus alters the cytoplasmic ion (Na,K) content of target cells. In the proximal tubule, ouabain rapidly ($t_{1/2}$ = 2 min) causes a decrease in intracellular K [16, 26], but does not change ultrastructure, intracellular total or free Ca for up to 30 min [17, 19]. This maneuver is akin to rubidium substitution used in smooth muscle cells to obtain an improvement in calcium measurements [14]. After 5 min of ouabain, cytoplasmic Na increased to 471 ± 12 mmol/kg dry wt, cytoplasmic K decreased to 26 ± 2 mmol/kg dry wt, while mitochondrial Ca content remained unchanged at 3.8 ± 1.0 mmol/kg dry wt (n = 11 for these experiments).

Calcium Distribution among Cells

A subpopulation of cells can be identified in the control tubule suspension: injured or "nonviable" cells, which are defined by dense inclusions in their mitochondria and a K/Na concentration ratio of about 1:1 (Table 1B). The average Ca content of the mitochondria in "nonviable" cells was obtained by taking X-ray spectra from small rasters which included only the dense inclusions and their concomitant mitochondrial matrix space. The average value for mitochondrial calcium content in these cells is 685 ± 139 mmol/kg dry wt. Assuming 30% of the cell volume to be mitochondrial [10, 21], the

Ca values obtained in Table 1 indicate that the viable cells have an average Ca content of 3.8 mmol/kg dry wt which, using the conversion factor of 0.7 mg protein/mg dry wt [2], corresponds to 5.4 nmol/mg protein. This value is only 20 to 30% of the total Ca usually measured in the suspension by atomic absorption spectrophotometry [17, 19]. A simple calculation (Table 2) shows that the presence of inclusions in 4 to 5% of the "nonviable" cells would account for the remaining 70 to 80% of the total Ca content measured over the whole cell population. These microprobe data suggest that heterogeneity of calcium content exists not only among intracellular compartments of the same cell but also among cells within the same suspension.

As a further illustration of the validity of the comparison of probe measurements to bulk content measurements, we have also taken rasters which are large enough to include within their borders intracellular contents of one or more cells but exclude extracellular material. These regions were chosen at random and some contained mitochondrial inclusions. There was a high variability in Ca ion content ranging from <1 (not measurable) to about 70 mmol/kg dry wt; however, the average value is 23 ± 7 mmol/kg dry wt which is within the range encountered by the bulk measurement.

Table 2. Average calcium content of the total cell population calculated with a variable percentage of nonviable cells.[a]

% Nonviable cells	Average Ca content of total cell population	
	(mmol/kg dry wt)	(nmol/mg protein)
1	5.9	8.4
2	7.9	11.3
3	10.0	14.3
4	12.0	17.2
5	14.1	20.1
6	16.2	23.1
7	18.2	26.0
8	20.3	29.0
9	22.4	31.9
10	24.4	34.9

[a]The average calcium content of each viable (3.8 mmol Ca/kg dry wt) or nonviable (210 mmol Ca/kg dry wt) cell was calculated using the values for mitochondrial and cytoplasmic calcium from Table 1 and assuming mitochondria to be 30% of cell volume [10, 21]. If mitochondria are assumed to be 25% of cell volume, the final values for the average calcium content of the total cell population range from 5.6 to 21.7 mmol/kg dry wt (8 to 31 nmol/mg protein) for 1 to 10% of nonviable cells. All these calculations assume that the Ca content in the nucleus is the same as in the cytoplasm, as obtained in preliminary measurements (*data not shown*).

Discussion

Kidney Cell Mitochondrial Calcium is Low

The present results using EPXMA allow the reconciliation of conflicting values obtained by other techniques for kidney cell mitochondrial calcium content. We find that in viable cells of the kidney proximal tubules, the mitochondrial calcium content is low, about 3 mmol/kg dry wt. These data are consistent with results obtained by EPXMA in smooth and striated muscle [5, 29], liver [28], and retinal rods [32] showing that mitochondrial calcium is about 1 to 3 mmol/kg dry wt. Our results in renal epithelial cells, as well as those in other nonmuscle and muscle cell types, support the notion that mitochondrial Ca may regulate dehydrogenase activity [6, 8, 9], while lending less credence to the suggestion that mitochondria regulate cytosolic free Ca [4, 20].

Calcium Distribution is Heterogeneous

We have also demonstrated the occurrence of both "viable" and "nonviable" cells in the tubule preparations, which can be distinguished on the basis of morphological (presence or absence of mitochondrial inclusions) and content (K/Na, 4:1 *vs.* 1:1) criteria. The occurrence of an abnormal, heavily Ca-loaded population has been previously observed with EPXMA measurements in isolated mitochondria [31], in cell cultures [11], and in freshly dissected muscle fibers [29]. In the current experiments, the population of cells which are nonviable may: 1) reflect the natural proportion of cells in the kidney *in situ* which are in some phase of degradation or turnover, and/or 2) may represent cells damaged during one or more steps of preparation of the tubule suspension. The first possibility is supported by the large variability encountered in the bulk Ca content (4.5 to 18 nmol/mg protein) of the kidney cortex *in vivo* [17]. However, additional damage may also occur during tubule isolation. The presence of such a sharp heterogeneity in Ca content emphasizes the need for caution in interpreting the results from other techniques such as Ca flux studies or measurement of bulk samples of cell suspensions or isolated organelles, which only yield an average value for the total cell population.

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