

## RESEARCH ARTICLE

# Improvement of cell counting method for Neubauer counting chamber

Minghao Zhang<sup>1</sup>  | Lingui Gu<sup>2</sup> | Peihua Zheng<sup>2</sup> | Zhixin Chen<sup>3</sup> | Xinqi Dou<sup>4</sup> | Qizhong Qin<sup>1</sup> | Xiaozhong Cai<sup>1</sup>

<sup>1</sup>Lab Teaching and Management Center, Chongqing Medical University, Chongqing, China

<sup>2</sup>College of Pediatrics, Chongqing Medical University, Chongqing, China

<sup>3</sup>First College of Clinical Medicine, Chongqing Medical University, Chongqing, China

<sup>4</sup>College of Public Health, Chongqing Medical University, Chongqing, China

## Correspondence

Qizhong Qin and Xiaozhong Cai, Lab Teaching and Management Center, Chongqing Medical University, No.1, Yixueyuan Road, Chongqing 400016, China. Emails: qqizhong@sina.com (QQ); 346360049@qq.com (XC)

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## Abstract

**Background:** We compared the cell counting accuracy of the conventional method and the improved method by using Neubauer counting chamber.

**Methods:** In the improved method, all the border cells were counted and then divided by two; while, in the conventional method, only border cells on the two boundaries (top and left) were counted.

**Results:** About 55.814% of the samples showed more accurate results by improved counting method, about 38.372% had more accurate results by conventional counting method, and about 5.814% were counted with similar counting error by both methods. The improved method had significantly smaller counting error than conventional method ( $P < .05$ ). The distribution ratio of the border cells was an independent factor for counting accuracy ( $P < .05$ ).

**Conclusion:** Together, the improved counting method can reduce the counting error of the Neubauer counting chamber to some extent, assess the distributing uniformity of border cells, and help to eliminate the samples with large differences in distribution.

## KEYWORDS

border cells, cell counting method, distributing uniformity, errors, Neubauer counting chamber

## 1 | INTRODUCTION

Accurate counting of body fluid cells contributes to the diagnosis and treatment of diseases.<sup>1,2</sup> According to the guidelines of the College of American Pathologists, the current method for counting body fluid cells is manual counting or automated cell counting.<sup>3</sup> Both manual counting and automated cell counting have their own advantages and disadvantages. Manual counting relies mainly on microscopes and counting chambers, and the counting principle is scientific. However, manual counting is labor and time-consuming with poor repeatability.<sup>1,2,4,5</sup> In recent years, manual counting has gradually been replaced by automated cell counting.<sup>6</sup> Automated cell

counting is fast and efficient. However, there are also some disadvantages. For example, the result is not accurate after a long-time use of machine and instrument calibration should be performed.<sup>7,8</sup> According to the report of Yang et al, the use of automatic blood cell counters did not effectively identify cell morphology, which may lead to missed diagnosis.<sup>9</sup> Therefore, compared with the automatic cell counter, the measurement principle of manual counting is more scientific, and it is often used for instrument calibration in clinical practice. In order to improve the accuracy of cell counting, counting chambers are constantly being innovated in practice. Douglas et al<sup>10</sup> reported that there was a large distribution error in the low depth counting cell because of the fluid dynamics. However, this

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phenomenon does not exist in the counting chamber with a 0.1 mm counting cell. Due to the siphon effect, the low depth counting cell may result in a low counting result.<sup>10,11</sup> At present, the universal counting cell depth of the Neubauer counting chamber is 0.1 mm.

Neubauer counting chamber is widely used to count cells in body fluid, and its result is generally considered to be "gold standard." Despite this, there are many shortcomings in the Neubauer counting chamber that need further improvement.<sup>11-13</sup> For example, when counting with a Neubauer counting chamber, there are usually a lot of border cells on the four outer lines of each counting square. The conventional method only counts cells on one side of the upper and the lower boundaries and one side of the left and the right boundaries. The sum of cells on the two outer lines is used as the total number of cells on border.<sup>14</sup> However, the premise of such counting is that the cells on the outer lines are evenly distributed; otherwise, it will cause a large shift in the counting result.

Here, we improved the conventional method by counting the cell number on all the outer lines, which was further divided by two to serve as the valid border cells. While, for those distributed inside each counting square, the conventional counting method was used. The counting accuracy and error of the conventional method and the improved method were compared.

## 2 | MATERIAL AND METHODS

### 2.1 | Sample collection

From June 2015 to March 2017, 416 fresh EDTA-2K anticoagulant blood samples were collected from individuals underwent physical examination at the University-Town Hospital of Chongqing Medical University. All individuals signed informed consent. The study was approved by the medical Ethics Board of the Chongqing Health Economics Association. After counting analysis, the average number of RBCs in all blood samples was  $(4.39 \pm 0.53) \times 10^{12}/L$ , the maximum value of RBCs was  $6.05 \times 10^{12}/L$ , and the minimum value of RBCs was  $3.24 \times 10^{12}/L$ .

### 2.2 | Counting instruments and reagents

The standard value of red blood cells (RBCs) for each blood sample was detected by a KX-21 automatic blood cell analyzer (SYSMES, KOBE, Japan) or an XN-1000i automatic blood cell analyzer (SYSMES, KOBE, Japan). In order to reduce the error, the KX-21 and the XN-1000i blood cell analyzer were calibrated before use, and their maximum error was <5%.

Improved Neubauer counting chamber (Shanghai Qijing Biochemical Instrument Co., Ltd.) was used. The depth of counting cell is 0.1 mm, and the counting area is 0.3 mm<sup>2</sup>. The four corners and the central medium square of the central large square were used for RBC counting. Cells were counted under the microscope (Ningbo Haoyu Instrument Co., Ltd.). Hemoglobin pipette (Shandong Osset Medical Devices Co., Ltd.) was used for sample preparation. The homemade RBCs dilution solution was 3.13% (m/v) tri-sodium citrate.

### 2.3 | Sample preparation and cell counting

Sample preparation was done by an experienced technician. In order to reduce the errors caused by subjective central tendency, 46 blood samples with relatively lower RBCs content (around  $3.24-4.21 \times 10^{12}/L$ ) and 60 samples with relatively higher RBCs content (around  $4.41-5.16 \times 10^{12}/L$ ) were selected. The samples with lower RBCs content were diluted with normal saline at 3:1 ratio. Meanwhile, the samples with higher RBCs content were concentrated after centrifugation (ie, about one-sixth of the plasma was discarded after centrifugation). Before the manual counting, the investigators were told that the concentration of some samples was adjusted and thus the results of the samples may differ greatly from the normal range of RBCs. Cell counting of 416 samples was performed by four people in accordance with the National Clinical Laboratory Procedures (the third Edition)<sup>14</sup> using the improved Neubauer counting chamber. The samples were added to the counting chamber with a hemoglobin pipette, and cell counting was performed after three minutes. In order to avoid errors caused by the destruction of RBCs, the time interval between the instrument counting and manual counting of each blood sample was controlled within half an hour.

Counting principle: The cells in each medium square were counted in the order of "S." The improved method was different from the conventional method in the counting of the border cells of each medium square. According to the conventional counting principle, cells on the top and the left boundaries are counted, whereas, cells on the bottom and the right boundaries are not counted. While the improved method is to count the total cell number on the four outer lines and then divide it by two.

The cell number was calculated as follows:

Conventional method: Number of RBCs (/L) = [Number of RBCs in five squares (excluding the border cells) + Number of RBCs in the upper and left sides of five squares]  $\times 5 \times 10 \times 201 \times 10^6$  (/L);

Improved method: Number of RBCs (/L) = [Number of RBCs in five squares (excluding the border cells) + 1/2 (Total number of RBCs distributed on the four sides of five squares)]  $\times 5 \times 10 \times 201 \times 10^6$  (/L).

### 2.4 | Definitions

The error between the conventional method and standard value was shown as  $E_c$ , while the error between the improved method and standard value was shown as  $E_i$ . When the sample with  $E_c$  and  $E_i$  was both <10%, the sample was considered valid. In all the valid samples, the samples with  $E_c$  and  $E_i$  both <2% were classified as "low error samples." The samples were classified as "medium error samples" when both  $E_c$  and  $E_i$  <5%, with at least one of them no <2%. The remaining valid samples were classified as "high error samples." During statistical analysis, the "low error samples" and the "medium error samples" were further grouped into "medium-low error samples"; and, the "medium error samples" and the "high error samples" were further grouped into

“medium-high error samples.” Compared with the standard values, the results of the two manual counting methods were defined as “both big,” “inconsistent,” and “both small.”

There were two reference values for the distributing uniformity of the border cells, which were the absolute difference (A) and the ratio (R). They were calculated according to the following formula:

$$A (\geq 0) = |(\text{Number of RBCs in the upper and left sides of five squares}) - (\text{Number of RBCs in the lower and right sides of five squares})|.$$

When the (Number of RBCs in the upper and left sides of five squares) was more than (Number of RBCs in the lower and right sides of five squares),

$$R = (\text{Number of RBCs in the upper and left sides of five squares}) / (\text{Number of RBCs in the lower and right sides of five squares}).$$

Or else,  $R = (\text{Number of RBCs in the lower and right sides of five squares}) / (\text{Number of RBCs in the upper and left sides of five squares}).$

## 2.5 | Statistical analysis

Data were statistically analyzed by SPSS 17.0 software (SPSS Inc.). The nonparametric independent sample *t* test was used for comparison. The correlation analysis was performed by using Spearman's test. The chi-square test was used to analyze whether the two counting methods have independent effect on the counting error. Binary logistic regression and receiver operating characteristic (ROC) curves were used to determine the correlation between the distributing uniformity of the border cells and the counting error. The difference was statistically significant if  $P < .05$ .

## 3 | RESULTS

### 3.1 | The counting results

Of all the 416 blood samples, 158 samples were excluded due to large errors, and 258 samples were valid. Compared with the standard values obtained by the automatic analyzer, the counting results of the two manual methods revealed that the count value was higher than their standard values in 111 samples, and lower in 108 samples, and not consistent in 39 samples. The average number of RBCs in each blood sample distributed on the upper left and lower right border lines was about  $70.42 \pm 22.62$  cells/L and  $70.44 \pm 22.65$  cells/L, respectively, and the difference between these two was not significant ( $P > .05$ ).

Additionally, there was no significant difference among the average number of RBCs calculated by the conventional method, improved method, and the automatic blood cell analyzer ( $P > .05$ ).

### 3.2 | The frequency analysis of lower error between the improved counting method and the conventional counting method

The frequency analysis and chi-square test of the error were performed on the counting results of the two methods (Table 1). The results showed that in all valid samples, the improved counting method had a significant advantage over the conventional method in controlling the number of error specimens ( $P < .05$ ).

### 3.3 | Significant differences in the mean $E_c/E_i$ were shown in the improved counting method

Nonparametric independent sample *t* test analysis for difference of the mean  $E_c$  and  $E_i$  were shown in Table 2. In all the valid samples and the low/high error samples, the error between the improved method and the standard value was significantly smaller than that between the conventional method and the standard value ( $P < .05$ ).

### 3.4 | Correlation analysis between the two manual counting value and standard values

A nonparametric correlation test was performed on the results of the two counting methods and the standard values (Table 3). In the four groups of samples, the counting results of the two methods had a very high positive correlation with the standard values ( $r > .9$ ,  $P < .001$ ). In addition, the correlation between the counting results of the improved methods and the standard values was higher than the correlation between the conventional method counting results and the standard values, indicating that the improved counting method is more reliable.

### 3.5 | Significant differences in the ratio of the distributing uniformity of the border cells were shown in different sample groups

Nonparametric independent sample *t* test was performed on the absolute difference and ratio of the distributing uniformity of the border cells in high, medium and low error sample groups (Table 4). The results showed that there was a significant difference in the ratio of

**TABLE 1** Frequency comparison of small count error in each RBC sample under two manual counting methods

Groups	n (%)	The conventional method, n (%)	The improved method, n (%)	Same error, n (%)	$\chi^2$	P
The valid samples	258 (100)	99 (38.372)	144 (55.814)	15 (5.814)	3.931	.047
Low error samples	90 (100)	34 (37.778)	50 (55.556)	6 (6.667)	1.434	.231
Medium error samples	99 (100)	39 (39.394)	52 (52.525)	8 (8.081)	0.862	.353
High error samples	69 (100)	26 (37.681)	42 (60.870)	1 (1.449)	1.853	.173

**TABLE 2** The average value of the difference between the count result and the standard value of each group of samples by using the two counting methods (mean  $\pm$  SD,  $\times 10^{12}/L$ )

Groups	The conventional method	The improved method	Z	P
The valid samples	0.149 $\pm$ 0.13	0.140 $\pm$ 0.13	3.649	<.001
Low error samples	0.038 $\pm$ 0.025	0.030 $\pm$ 0.023	2.414	.016
Medium error samples	0.129 $\pm$ 0.052	0.121 $\pm$ 0.053	1.921	.055
High error samples	0.324 $\pm$ 0.108	0.310 $\pm$ 0.113	2.039	.041

the distributing uniformity of the border cells among the high, medium, and low error samples ( $P < .01$ ).

### 3.6 | Error controlling analysis by four binary logistic regression models

The binary logistic regression of the error value of the counting results was conducted on the standard value of the sample RBCs count, the distributing uniformity of the border cells, and the relationship between the counting results of the two methods and the standard values (Table 5). The distributing uniformity of the border cells in Model I and Model III was represented by the absolute difference, while that in Model II and Model IV was represented by its ratio. The binary values of the counting results in Model I and Model II respectively referred to the "medium and low error samples" and the "high error samples," while the binary errors of the counting results in Model III and Model IV referred to the "low error samples"

**TABLE 3** Correlation analysis between the count results and the standard values of the two groups of samples under the two counting methods

Groups	The conventional method (r, P)	The improved method (r, P)
The valid samples	.963, <.001	.966, <.001
Low error samples	.995, <.001	.998, <.001
Medium error samples	.974, <.001	.977, <.001
High error samples	.917, <.001	.919, <.001

**TABLE 4** Comparison of the absolute difference and the ratio of the distributing uniformity of the border cells (mean  $\pm$  SD)

	Medium error samples	Medium error samples	High error samples	$\chi^2$	P
The absolute difference of the distributing uniformity of the border cells	5.678 $\pm$ 4.321	6.899 $\pm$ 7.108	9.508 $\pm$ 13.116	3.058	.217
The ratio of the distributing uniformity of the border cells	1.091 $\pm$ 0.091	1.117 $\pm$ 0.14	1.179 $\pm$ 0.215	11.418	.003*

\* $P < .01$

and the "medium-high error samples." Our results revealed that the distribution ratio or absolute difference of the border cells could be used as independent risk factors affecting the counting accuracy (Model I:  $P < .05$ , Model II:  $P < .01$ , Model III:  $P < .05$ , Model IV:  $P < .05$ ).

### 3.7 | ROC curve analysis of the ratio or the absolute difference of the distributing uniformity of the border cells for predicting the counting error

In order to distinguish between the "low error samples" and the "medium-high error samples," the ROC curves were plotted using the absolute difference and ratio of the distributing uniformity of the border cells. As shown in Figure 1, the AUC for "border cells" of the ROC curve of absolute difference in the uniformity of distribution was 0.539 [95% CI (0.467-0.611),  $P > .05$ ]. The critical value was 4.5, the predictive sensitivity was 54.8% and specificity was 47.8%. The AUC for "border cells" of the ratio of the distributing uniformity was 0.574 [95% CI (0.503-0.645),  $P < .05$ ]. The critical value was 1.112, and the predictive sensitivity and specificity were 41.7% and 71.1%, respectively. Therefore, the ratio but not the absolute difference of the distributing uniformity of the border cells was able to better distinguish the "low error samples" and the "medium-high error samples."

In order to distinguish between the "low-medium error samples" and the "high error samples," the ROC curves were plotted using the absolute difference and ratio of the distributing uniformity of the border cells (Figure 2). The AUC for border cells of the ROC curve of absolute difference in the uniformity of distribution was 0.57 [95% CI (0.490-0.649),  $P > .05$ ]. The critical value was 7.5. The predictive sensitivity was 43.5%, and specificity was 68.3%. The AUC for border cells of the ratio of the distribution uniformity was 0.636 [95% CI (0.559-0.712),  $P < .01$ ]. The critical value was 1.098, and the predictive sensitivity and specificity were 56.5% and 64.6%, respectively. Therefore, the ratio of the distributing uniformity of the border cells was much better than the absolute difference for distinguishing the "medium-low error samples" and the "high error samples."

## 4 | DISCUSSION

Our study revealed that in all valid samples, the average of the counting error of the improved counting method was significantly

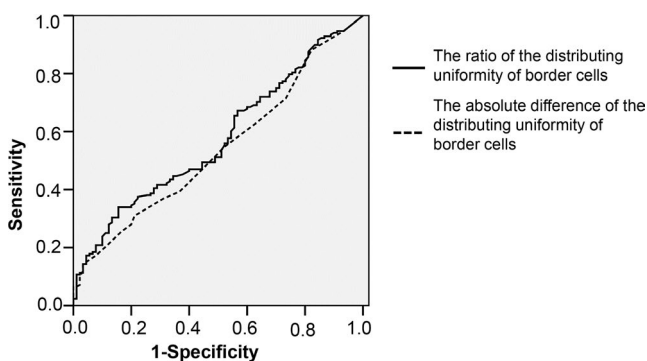
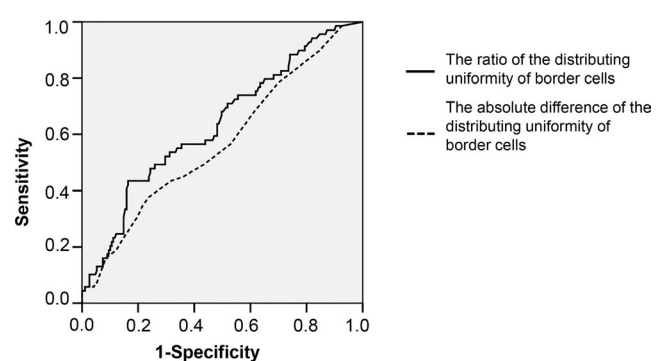
**TABLE 5** Binary logistic regression analysis was performed on the counting error according to the standard value of RBCs number, the distributing uniformity of the border cells, and relationship between the two method and standard value

	Model I			Model II			Model III			Model IV		
	B	Wald	P	B	Wald	P	B	Wald	P	B	Wald	P
The standard value of RBCs number	-0.049	0.072	.789	0.067	0.131	.717	0.005	0.001	.977	0.109	0.395	.53
The absolute difference of the distributing uniformity of the border cells	0.042	5.935	.015	/	/	/	0.045	3.91	.048	/	/	/
The ratio of the distributing uniformity of the border cells	/	/	/	2.984	9.21	.002	/	/	/	3.268	6.302	.012
The relationship between the counting results of the two methods and the standard value	0.238	2.265	.132	0.217	1.877	.171	0.038	0.07	.791	0.017	0.014	.906
constant	-1.589	3.231	.072	-5.128	11.119	.001	0.233	0.083	.774	-3.526	4.003	.045

smaller than that of the conventional counting method. And similar result was also shown in the low error samples and the high error samples. Additionally, in all the valid samples, the results of the two counting methods were significantly correlated with the standard values. Moreover, the correlation between the counting results of each group using the improved counting method and the standard value was better than the conventional counting method. It is suggested that the improved counting method is more reliable, which is consistent with the report of Zhang et al.<sup>15</sup> Meanwhile, our study also found that among all the valid samples, 55.814% of the samples had more accurate results by using the improved method, 38.372% of the samples showed more accurate results by using the conventional method, and 5.814% of the errors were the same by using the two counting methods. This difference was statistically significant. However, this significance was not reported by Zhang et al.<sup>15</sup> Moreover, our study innovatively introduced the probability of “the distributing uniformity of the border cells” and used its absolute difference and ratio as reference. When the absolute difference was close to zero or R close to one, it was considered as an ideal distributing uniformity of the border cells. Moreover, we found that absolute

difference and ratio of the distributing uniformity of the border cells could be used as independent risk factors affecting the counting accuracy. According to the ROC curve analysis, the ratio of the distributing uniformity of the border cells was more effective than the absolute difference to predict the counting error.

The counting error of the Neubauer counting chamber includes the operation error and the inherent error. Operation errors come from the unreasonable sampling, the improper use of equipment, the inaccurate dilution factor, and cell identification errors. The error caused by inaccuracies such as counting chambers, coverslips, and hemoglobin pipettes is called instrument error. The distribution error refers to the error caused by the uneven distribution of cells in the counting chamber. Instrument error and distribution error are collectively referred to as inherent errors. Operating error and instrument error can generally be avoided by improving the experimenter's technical proficiency and standard operating procedures, but cell distribution errors are difficult to eliminate. To overcome the above problems, clinical laboratory personnel are constantly developing new methods.<sup>16</sup> In order to cope with the possible distribution error, our group carefully analyzed the principle of “Poisson distribution”<sup>17</sup> and filtered the sample with large

**FIGURE 1** The ROC curve analysis of the absolute difference and ratio of the distributing uniformity of the border cells, and the “low error sample” and the “medium-high error sample” were analyzed**FIGURE 2** The ROC curve analysis of the absolute difference and ratio of the distributing uniformity of the border cells, and the “low-medium error sample” and the “high error sample” were analyzed

distribution error by calculating the distributing uniformity of the border cells. During the experiment, we strictly controlled the operation error. For example, the blood cell transportation process may be affected by many factors. To avoid errors caused by the sample, we used EDTA-2K anticoagulant sample to protect red blood cells from agglutination or hemolysis.<sup>18,19</sup> Another study reported that a properly positioned coverslip should have several iridescence lines visible where the coverslip is attached to the counting chamber and should not be easily dislodged.<sup>11</sup> In this study, we also carefully examined the thickness of the coverslip to ensure its well match with the counting chamber.

There are several limitations in this study. For example, although our improved method had a significant effect on controlling the count error, the improvement was not big. Additionally, any changes and updates to analytical methods should be based on simplified operations and quality improvement.<sup>11</sup> Our improved counting method increases the counting of border cells, which would lead to more time costs. Further studies are warranted.

In conclusion, compared with the conventional method, the improved counting method reduces the counting error of the Neubauer counting chamber to some extent. Meanwhile, the improved counting method improves the counting methods of border cells, which can evaluate the distributing uniformity of samples and help eliminate the samples with large distribution errors in time.

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## CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Minghao Zhang  <https://orcid.org/0000-0002-0240-2244>

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