SHORT COMMUNICATION

A Randomised Approach for Enumerating Migrated Cells in a Transwell Migration Assay

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ABSTRACT

The transwell migration assay is commonly used for assessing cell migration. It involves the enumeration of cells that have migrated across a pore-containing membrane. We describe a randomised approach to quantifying migrated cells and compare it to a conventional full cell count. We used ATP as a chemoattractant and automatic cell quantification performed on all fields (Full count; FC) or 10 randomly selected fields (Randomised count; RC). The two methods were compared by evaluating standard deviations (SD), coefficient of variation (CV) and using the Bland-Altman analysis. The dispersion of data is higher with the RC approach (3.77-6.66% CV for control; 3.89-4.48% CV for ATP-treated wells) compared to FC (0.27-0.46% CV for control; 0.05-0.09% CV for ATP-treated wells), but are acceptable considering that the number of migrated cells are in the thousands. Both methods verified that an ATP migration assay for BV2 microglia was established, demonstrating that the RC approach is reliable and comparable to a full count.

Keywords: Migration, Transwell apparatus, Microglia, Randomised count

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INTRODUCTION

Cell migration is an important event in development (1), inflammatory responses (2) and metastases of cancers (3). It is a highly regulated process that involves chemotaxis, homing, diapedesis and tissue digestion by matrix metalloproteinases (4). The migration of cells can be observed in vitro by various techniques including the scratch or wound healing assay, Boyden chamber assay, and invasion assay (5, 6). The Boyden chamber assay, also known as the chemotaxis chamber assay or transwell migration assay, consists of a chamber with two compartments (7). Cells are placed on the top compartment that contains a membrane with pores. Chemoattractant is placed in the lower compartment and cells are allowed to migrate towards the lower compartment. The cells are then fixed, stained and quantified (7).

In our laboratory, we utilise the transwell migration assay to evaluate the migration of microglia (8). Microglia are tissue-specific macrophages that mediate inflammation within the brain and spinal cord. In healthy tissue, microglia actively traverse the CNS parenchyma, monitoring changes in homeostasis (9). When there is tissue injury, they migrate towards areas of tissue damage (10), including sites of infection (11) and tumours (12) as a response towards tissue damage. The migration of microglia therefore serves as an indicator of their activation, along with upregulation of activation markers and production of inflammatory mediators such as prostaglandins, leukotrienes, reactive oxygen species, nitric oxide and proinflammatory cytokines (13-15). Here, we compare an approach of quantifying microglia in 10 random microscope fields compared to a conventional full analysis of cell number for a transwell migration assay.

MATERIALS AND METHODS

BV2 microglia culture

The BV2 immortalised murine microglia cell line was maintained in Dulbecco Modified Eagle Medium (DMEM; Gibco, USA Cat. No. 12100-046) supplemented with 5% foetal bovine serum (FBS; Gibco, USA Cat. No. 10270-098), 100 U/ml penicillin (Gibco, USA Cat. No. 15140-122), 100 µg/ml streptomycin (Gibco, USA Cat. No. 15140-122), 1 ml/L gentamicin (Gibco, USA Cat. No. 15710-064) and 250 µg/ml fungizone (Gibco, USA Cat. No. 15140-122), 1X non-essential amino acids (NEAA; Gibco, USA Cat. No. 11140-050) and 1.5g/l sodium bicarbonate in a 95% humidified atmosphere containing 5% CO2 at 37°C. Cells were harvested upon reaching 80% to 90% confluency by incubating with 0.25% trypsin in 1mM EDTA for 5 minutes at 37°C.

Transwell migration assay

BV2 microglia (1 x 105 cells in 500µl of serum-free culture media) were seeded onto polycarbonate cell-culture inserts with pore size of 8 microns (BD Falcon Cat No. 353097) in 24-well plates. ATP (250µM prepared in 500µl serum-free media; Sigma-Aldrich, Cat. No. 34369-07-8) was added to the bottom of the well. Serum-free media without ATP was used as the negative control. After 6 hours of incubation, inserts were carefully removed from the plate, fixed using 2% paraformaldehyde (PFA) for 1 hour followed by permeabilisation of the cells with 0.01% Triton-X for 1 hour. Cells were then stained with crystal violet (Merck, Cat. No. 101408) for 10 minutes. The transwell inserts were rinsed using phosphate buffer solution (PBS) in between fixation, permeabilisation and staining steps. BV2 microglia cells that did not migrate across the membrane were swabbed carefully by using a cotton bud dipped into PBS. The transwell inserts were then cut with care using a sterile blade and mounted using DPX Cytoseal™ 60 (Thermo Scientific, 23244256) onto a slide to be viewed under phase-contrast microscopy (Olympus CK30). Slides were stored at 4°C.

Quantification of migrated BV2 cells

Images were taken with 40X magnification and the entire transwell membrane was imaged with a total of 20 microscope fields. For Full counts (FC), cells in all 20 fields were automatically quantified using the ImageJ® software. To increase accuracy of the automated counts, contrast and threshold level adjustments were made till majority of cells were marked for quantification (Fig. 1). For Randomised counts (RC), 10 random fields were selected out of the total of 20 fields for automatic quantification, as aided by an online randomiser. The cells in the 10 fields were then subjected to the automated count and multiplied by two to juxtapose the RC count to FC.

Statistical analysis

Counts were performed thrice on each well, with randomisation performed each time for RC. The migration assay was repeated twice as independent experiments. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, CA, USA). Concordance of RC with FC counts were determined with the Bland-Altman test.

RESULTS AND DISCUSSION

The transwell migration assay is a commonly used assay to assess cell migration. Here, we describe a randomised count approach to replace the conventional full count analysis of migrated cells in a transwell migration assay for a more time-saving option. For the migration assay, ATP at a concentration of 250µM was used as the chemoattractant and migration of BV2 microglia was assessed at 6 hours. The negative control consisted of serum-free media as serum itself has chemoattractive properties (manuscript in preparation).

The steps for performing the counts are as follows: viewing and delineating the transwell membrane into 20 microscope fields, followed by performing an automated cell count using the ImageJ® software. Automated counts for both randomised counts and full counts were adjusted to improve contrast and threshold levels to prevent under or overestimation of migrated cells. The difference between the full count and random count method is that the full count method enumerates all 20 fields while the random count method enumerates 10 randomly selected fields. The cell number obtained with the RC method was then multiplied by two to allow comparisons to FC. Therefore the assumption is that the distribution of cells in the sampled 10 fields mirror that of the entire transwell membrane.

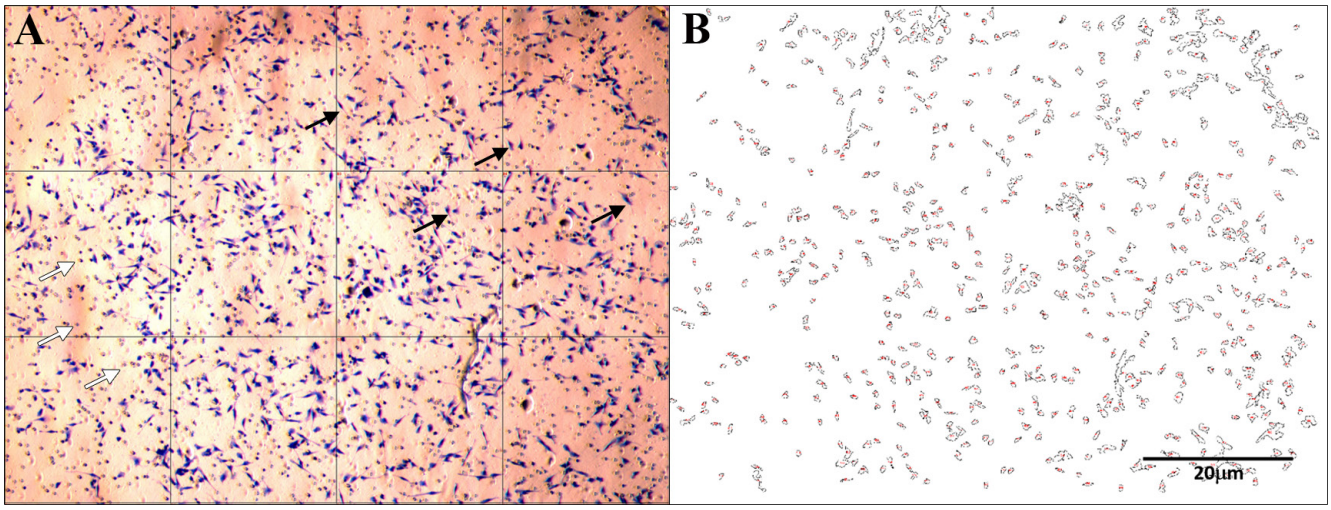

Figure 1: Quantification of migrated BV2 microglia. (A) Representative photomicrograph of one microscope field of the transwell membrane. Black arrows depict migrated BV2 microglia and white arrows depict pores on the transwell membrane. (B) Automated enumeration output of the photomicrograph from (A) by the ImageJ® software. Each red dot represents a cell count.

Comparison of counts

All cell counts were performed thrice on the same transwell using both the Full count (FC) and Randomised count (RC) approach to compare the two different methods. The counts were performed with an interval of one day to prevent familiarity or bias by the researcher. Table I shows the mean differences in cell counts between the FC and RC approach for control transwells and ATP-induced migration. Replicate 1 and Replicate 2 are counts performed on 2 independent experiments.

The coefficient of variation (CV) was first evaluated for all counts. CV is the ratio of the standard deviation (SD) to the mean, therefore, for instance, the SD for RC of control wells is 6.66% of its mean (Table I). For Replicate 1, the CV of FC and RC for control wells were 0.27% and 6.66% respectively and 0.09% and 4.48% for ATP wells. This demonstrates that the data for RC is more variable than FC. Replicate 2 also exhibited more variable data for the RC counts, with CV of 3.77% and 3.89% for control and ATP wells respectively compared

Table I: Mean counts and related statistics of migrated cells for the Full count (FC) and Randomised count (RC) methods

	Full count (FC)		Randomised count (RC)		95% CI	
	Mean (SD)	CV (%)	Mean (SD)	CV (%)	from	to
Control						
Replicate 1	1284 (3.5)	0.27	1387 (92.4)	6.66	-5.27	20.39
Replicate 2	1422 (6.6)	0.46	1459 (54.9)	3.77	-5.57	10.56
ATP						
Replicate 1	5389 (4.7)	0.09	5700 (255.4)	4.48	-3.30	14.39
Replicate 2	3795 (2.1)	0.05	4063 (157.9)	3.89	-0.73	14.24

SD = standard deviation; CV = coefficient of variation; 95% CI = 95% confidence intervals

to 0.46% and 0.05% for the FC. Concordance of the two approaches for the counts were assessed through a Bland-Altman test (16). The 95% confidence interval (CI) of the two approaches are considered by us to be acceptable as it is not large enough to render the data unreliable. For instance, the CI for control wells of Replicate 1 is from -5.27 to 20.39. For cell counts that average 1387 ± 92.4, this limit of agreement is not large enough to be of any significance.

ATP induces BV2 microglia migration

Based on these two counting appraoches, both methods revealed that ATP significantly increases BV2 microglia migration by 4.1-4.2 fold and 2.7-2.8 fold for each independent experiment (Fig. 2) demonstrates the data when plotted as a model of the ATP migration assay. Both the FC and RC values demonstrate that an ATP migration assay for BV2 microglia was established, with ATP significantly increasing microglia migration by 4.1-4.2 fold increase for Replicate 1 and 2.7-2.8 fold increase for Replicate 2 compared to control ($p<.05$).

Determining the reliability of two different methods for the same measure is an important and necessary step and both design of the experiment and appropriate statistical analysis must be taken into account. Here, the same transwell membrane for each treatment (control and ATP) was counted thrice with both counting methods, with a one-day interval to limit familiarity and bias. Although correlation analyses such as the Pearson correlation coefficient and Spearman's rank correlation are typically used to determine congruence of data, it is a statistical analysis that studies the relationship of one measurement with another, not its differences (2). When

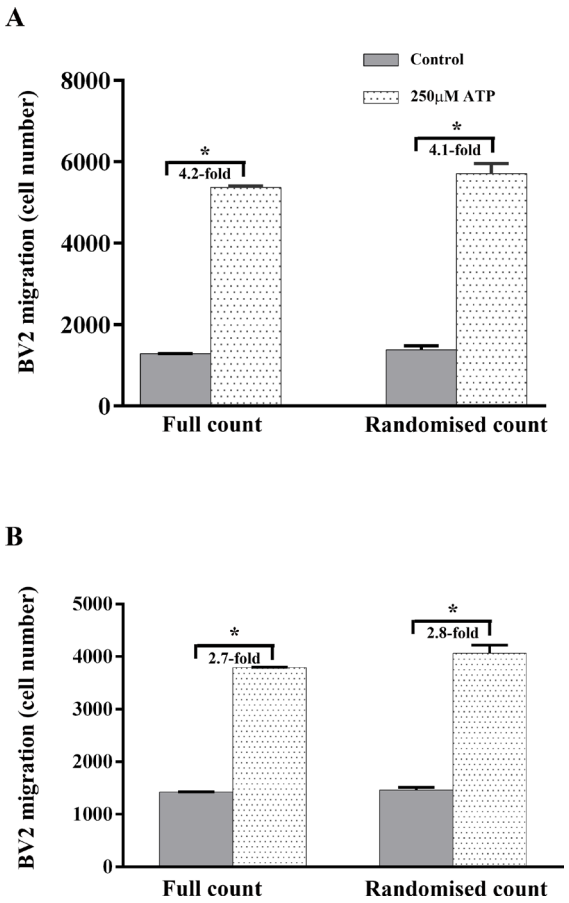

Figure 2: ATP-induced migration of BV2 microglia as determined by the Full count and Randomised count methods. Migration was assessed at 6 hours by automated enumeration of the number of BV2 microglia stained with crystal violet on the converse side of transwell culture inserts. Control consists of wells with serum-free media. A and B are data from two independent experiments respectively. Data is mean ± SD of triplicate counts. Mann-Whitney test; *$p<.05$.

assessing the comparability between methods, the Bland-Altman analysis is more suitable (16). This analysis is based on the quantification of the agreement between two measurements by studying the mean differences and constructing limits of agreement (16). Therefore this was the chosen analysis and the data acquired by the RC method was demonstrated to be reliable. The higher variation for RC owes to the fact that each time a count is performed, 10 different random fields are selected. As the distribution of cells is not entirely even (see Fig. 1), this accounts for the variation. Whereas for full counts, any variation recorded would be only due to contrast and threshold level adjustments during the automated counts. It is important to note that the Bland-Altman analysis does not define whether these limits are acceptable, as these must be determined based on biological considerations. For cell counts ranging from 1,284 to 5,389 (depending on the treatment), the limits of agreement that we report here are considerably narrow. The two counts do not offer information that are conflicted with each other, and both demonstrate that ATP significantly increases BV2 cell migration by 4.1-4.2 fold.

CONCLUSION

The randomised approach to quantifying migrated cells in a transwell assay described here can replace a full count of migrated cells and is both a time and labour-saving improvement to the technique.

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