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# Method Comparison of In Vitro Wound Area Measurements

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**Abstract:** Wound area is a primary outcome measure in wound healing studies. This method comparison study evaluates differences of wound area measurements of a newly developed image analysis method based on wound edge contour to an existing method based on contrast tolerance. Digital images of 64 wounds were taken immediately after wounding matured in vitro 3D organotypic tissues with a biopsy punch. Wound area measurements were calculated using each image analysis method and then normalized. The method comparison study evaluates the difference of each paired measurements for all 64 wound areas. Measurement differences are demonstrated and evaluated in normalized data boxplots, scatter plots with a line of equality, data histogram and Normal probability plots, and a Bland-Altman plot of paired measure difference against mean. The measured wound areas using the tolerance method have large variability in comparison to the contour method measures. The tolerance method measures often underestimate and overestimate what is assumed to be an approximately repeatable initial wound size. Skewness in comparison plots are due to the ‘fat tails’ introduced by the variability of measurements of the tolerance method. In contrast, the contour method results in larger wound area measurements on average at a statistically significant level of difference. The relatively less variable range of contour method measurements suggest this method has more potential to agree with the ‘true’ wound area. Future work to improve the method are proposed for application of image analysis methods to distinguish true wound area and measurement error in time for wound healing treatment-control experiments.

**Keywords:** Bland-Altman plot, Image analysis, In vitro, Measurement error, Method comparison, Planimetry, Reproducibility study, Wound area, Wound assay, Wound healing

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## 1 Introduction

Wound area is a primary measurement in wound healing treatment studies. Previously, we introduced systems to obtain digital images of wounds and an image analysis method to calculate wound area [3]. The method was developed in agar and based on contrast tolerance, where a dark interior was selected and then the algorithm detected a ‘contrast’ edge (defined by a pre-selected tolerance). However, application of this method in the experimental system yielded results with variable agreement in repeated measurements.

We now introduce a newly developed image analysis method to calculate wound area developed using images from the experimental biological system. The basis of the method is to define the wound edge contour and then to calculate the enclosed wound area. As the old method was developed in agar and the new method was developed in the biological system, we expect measure disagreements.

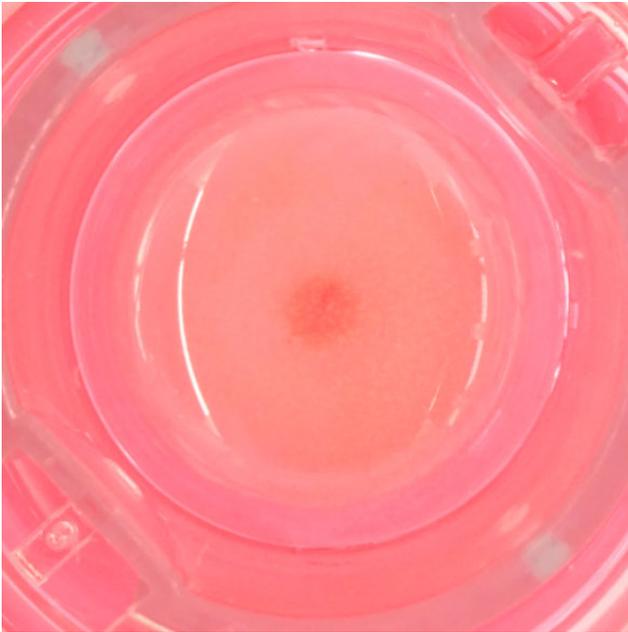
Differences in measurements obtained from two different methods on the same study population will be explored in this paper. Inherent errors for both methods from different sources are discussed. Thus, we conduct a method comparison study of the established tolerance method and the newly developed contour method and evaluate which method is more suitable for our purposes.

## 2 Methods

The contour method is introduced to calculate wound area in vitro. The method is based on calculating pixel area within a user-defined contour. The contour method algorithm consists of: 1) defining the wound edge as a closed and continuous contour, 2) overlaying a pixel-wise grid on the contour, and 3) calculating the pixel-wise area enclosed by the contour.

When the digital image of the wound is imported into MATLAB, the user represents the wound edge as a traced contour. The contour is digitally represented as 2 vectors of coordinate points. To ensure a closed contour, the algorithm linearly joins the first and last coordinate points.

The algorithm then ensures pixel-wise continuous x- and y- values along the entire contour. This is done by finding and adding (as necessary) to the vector all x- and y- coordinates



**Fig. 1:** Digital image of an in vitro 3D organotypic tissue wounded with a biopsy punch. The wound is the center darker circle surrounded by intact tissue. The tissue is in a cup with a semipermeable membrane bottom to allow transfer with the cell culture media (pink liquid). The bottom of the cup is the bright pink circle and the cup top is grey, which meets the cup flanges (upper right and lower left corners) to suspend the cup in the tray well. The image is taken through the tray cover.

along the entire contour. Hence, the wound edge is defined by a closed and continuous contour.

Wound area is defined as the pixel area within the contour. The pixel counting algorithm scans the contour coordinate vector vertically and horizontally to obtain all  $x$ -values and the minimum and maximum  $y$ -coordinate counterparts. This is also repeated in the second direction for all  $y$ -values. Thus, the entire wound area is overlaid with a pixel-wise grid. Finally, the enclosed wound area is defined as the intersections of a continuous pixel-wise grid within a continuous contour.

The study population included 64 samples of in vitro 3D organotypic tissues. All samples were wounded with a biopsy punch. Images were obtained using a digital camera. Wound area was measured for all 64 wound images using both the tolerance method and contour method. Thus, the data set had 64 paired wound area measures. Figure 1 shows an example of a single wound sample.

All wound area results were normalized because optical zoom may vary between images. To normalize all images, 2 points were selected that are shared on all images. The coordinates of these points were used to determine a diameter, then used to calculate the area of a circle, which was the divisor for

all calculated wound areas. Thus, all data presented are normalized wound area measures.

A method comparison study was conducted, according to Bartlett and Frost [2]. A boxplot, scatter plot, Bland-Altman plot, and histogram and Normal probability plot are provided to evaluate differences in wound area measurements obtained from the existing tolerance method to the new contour method. Finally, each method was evaluated as appropriate for our wound healing treatment research.

A boxplot is provided to compare normalized wound area measurements for each method by group. A scatter plot (contour method measurements against tolerance method measurements) is shown to visualize the agreement of the data in comparison to the diagonal line of equality. The Bland-Altman plot is presented to plot the difference of the measures by the two methods (contour method measure - tolerance method measure) against the mean of the measurements [1].

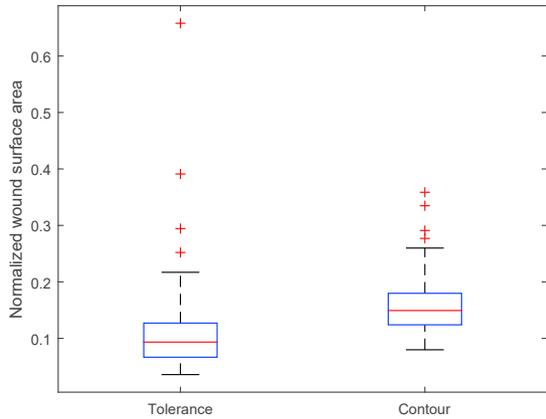
Bias between methods was tested by using a one-sample  $t$ -test of the mean differences. This tests the hypothesis if the true mean of the paired differences is zero. Uniformity for variability in measurements was tested by comparing the standard deviation of the paired differences of the lower median of the means to the that of the higher median of the means. A log transform was taken to improve the variability such that the Bland-Altman plot paired differences are approximately uniform in standard deviation across the range of means. The resulting log transform is shown as a histogram and Normal probability plot of the resulting data distribution.

### 3 Results

Wound images in 64 3D organotypic tissues were taken immediately after wounding with a biopsy punch. Wound area was calculated using both methods for all wounds and then normalized. A method comparison study was then conducted on paired wound area measurements of 64 wound samples.

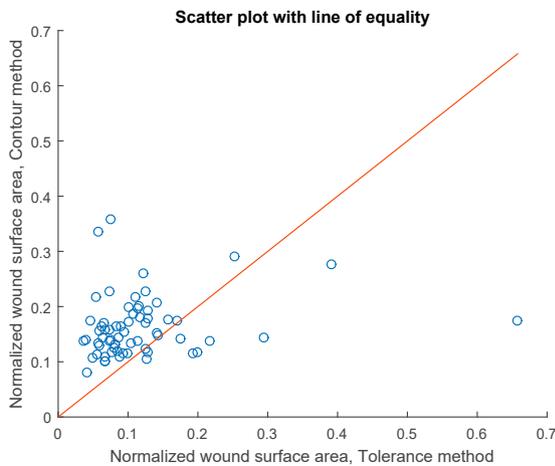
Figure 2 shows boxplots of the normalized wound area measurements using the tolerance method and contour method by groups. User feedback of the tolerance method overlays the resultant area on the wound image, where wound areas are often underestimated or overestimated. This can be observed in the boxplots as the large range of output values. Alternatively, the median value of the contour method is larger than the tolerance method, while the value range is more compact.

The scatter plot of normalized wound area of the contour method is plotted against the normalized wound area of the tolerance method in Figure 3. The majority of the data points lie above the line of equality. Thus, on average, the contour



**Fig. 2:** Boxplot of normalized wound area by group (tolerance method and contour method).

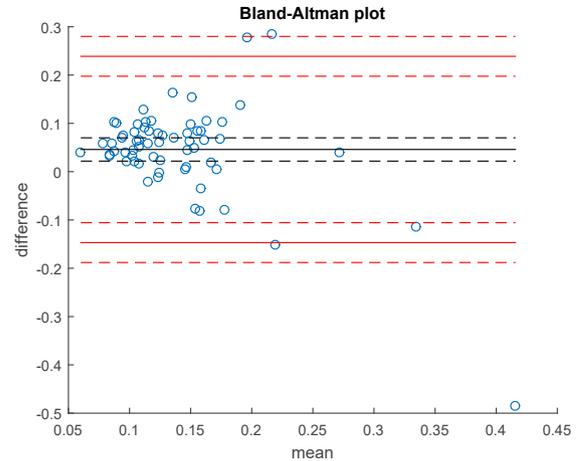
method yields larger measures of wound area as compared to the tolerance method.



**Fig. 3:** Scatter plot of normalized wound area measures (contour method against tolerance method) with the line of equality.

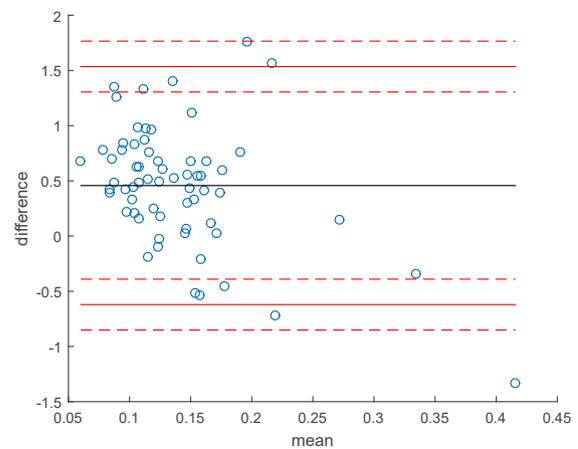
The Bland-Altman plot (Figure 4) is the difference of the wound areas (contour method measure - tolerance method measure) against means for 64 wounds. The solid line is the mean of the paired differences, its distance from 0 provides an estimate of the bias between the two methods. The statistical significance test measures if, on average, a method underestimated or overestimated measurements relative to the other. The paired t-test yields  $p = 4.04E-4$ , so we reject the null hypothesis that the true mean of the differences is 0. The measures of the methods do not agree.

In Figure 4, an increase in the standard deviation of the paired differences with increasing mean can be observed. This



**Fig. 4:** Bland-Altman plot of normalized wound area difference (contour method - tolerance method) against mean. The black line is the mean of the paired differences. The red lines are limits of agreement. 95% confidence interval are dotted lines.

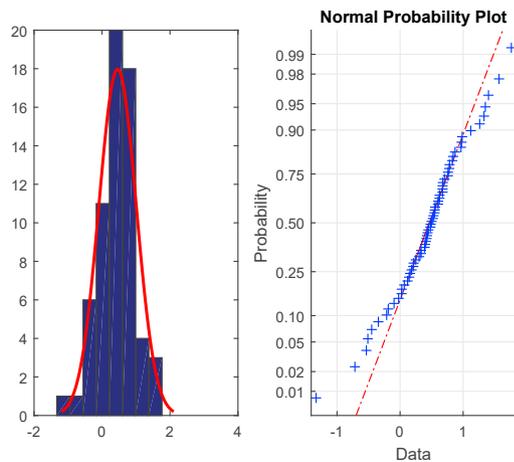
means the limits of agreement may be too wide for smaller measures and too narrow for larger measures. The data is log transformed to reduce the variability in order to have approximately uniform standard deviation across the range of means. The transformed is more uniform variability, as indicated in the log transformed Bland-Altman plot provided in Figure 5.



**Fig. 5:** Bland-Altman plot of transformed normalized wound area difference (log(contour method/tolerance method)) against mean.

A histogram and Normal distribution plot of the log-transformed data was examined in Figure 6. The log transformation yields approximately uniform standard deviation across the range of means (Figure 5) and approximately Normally distributed paired differences. As shown in the Normal probability plot, much of the transformed data lie along the

Normal line. Each tail (20% of the data) is skewed from the Normal line, thus the distribution is 'fat tailed'.



**Fig. 6:** Histogram and Normal probability plot of the logarithm of paired differences ( $\log(\text{contour method}) - \log(\text{tolerance method})$ ).

## 4 Discussion

A method comparison study is typically conducted to explore agreement between method measurements, assuming the existing method is error free and represents a 'true' measured value. However, in our case, the inherent limitations of the existing tolerance method should be addressed. As the tolerance method was developed in agar and based on contrast tolerance, the application of the tolerance method in the biological samples (Figure 1) may have different ranges of contrast that limit accurate wound edge detection. Comparisons are often skewed by the tolerance method, where the 'fat tailed' distribution are likely underestimations and overestimations of wound area. In contrast the relatively lower variation in the contour method measurements may more accurately reflect the initial wound area. Assuming wounding is approximately repeatable, variations in the tolerance method measurements are likely due to differences in image contrast between the changing image conditions (low reproducibility).

In this case, the method comparison study design is applied to examine disagreement between method measurements. Disagreement between method measurements is seen in all plots. Specifically, 'overestimation' of bias of wound area measured by the contour method relative to the tolerance method is seen in all plots and is statistically significant.

The contour method also introduces potential operator bias when manually tracing a contour, which is not addressed

in this work. Evaluation of operator bias is similar to a method comparison study, as both are reproducibility study designs. In one design, the changing condition is the analysis method, while another changing condition is the operator.

Importantly, this method comparison study does not yet evaluate the contour method in relation to the 'true' wound area. A repeatability study should be conducted. In particular, reliability should be examined to compare variation in the 'true' wound area in comparison to measurement error. Ultimately, the variation in the initial wound size (subject variability) and differences in measures by each method suggest the contour method has greater potential for application in our wound healing treatment research.

## 5 Conclusion

This work presents the ongoing development of our image analysis methods for in vitro wound area measurement. A method comparison study highlights differences in 64 in vitro wound area measurements obtained by different methods. The contour method demonstrates less variability than the tolerance method, which may have greater accuracy to the 'true' wound area. Future work includes evaluation of the contour method bias as well as measure error for improvement. Application of the developed method should be reliable enough to distinguish variation in the wound system and measurement error to resolve true differences in wound area closure in time for ongoing wound healing treatment-control experiments.

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