Uncertainty determination of a microvolume characterized through confocal microscope technique

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Abstract: Standardized measurements protocols are widely used and necessary in order to ensure a reliable and repetitive data. This work introduces a new procedure to measure the volume of any irregular micro-hole on a rough surface. A filtering and fitting model is proposed for calculating the void volume of the cavity. Experimental point cloud is acquired by optically measuring the micro-hole with a confocal microscope. RANSAC algorithm is used to establish the reference plane, while the limits of the cavity and the volume are determined by a proposed algorithm. The mentioned model is validated by measuring six real samples. In addition, the corresponding uncertainty is estimated by using Monte Carlo method.

Keywords: Microvolume, Confocal microscope, Uncertainty, Monte Carlo method.

1. Introduction

The contrast and verification of many scientific achievement rely on scientific metrology, which is responsible for not only gathering global measurements, but also for the establishment of adequate measuring protocols [1,2]. Thus, comparison of several experiments could be carried out if those measurements have been performed under standardized procedure.

In the last decades, the development and miniaturization of the technology has led to instruments, systems, and devices in the micro and nanometric scales. This technological advance is accompanied by the need of measurement tools adapted to those scales. In the case of micro-holes, several techniques are currently available. Those based on the direct contact measurement are widely used to determine the diameter and depth of a micro hole. Acoustic, electric, or optical fibre probes for instance, are reported in the literature. However, the samples could be damaged due to physical contact, high accuracy is only achieved in the millimetre scale, and not all of them ensure depth measurements. Therefore, those could be a poor option for volume and roughness evaluation [3-5]. By contrast, optical microscopy characterization techniques provide an image (data set) information based on a non-contact measurement. Even so, the acquired data given by a microscopy must be properly processed to ensure a truthful volume estimation. This is why most of optical characterization techniques are combined with computer-based image processing systems [6].

Despite the existence of many techniques such as the ones above mentioned, the lack of a normalized protocol for measuring irregular micro-cavities avoids the comparison of results obtain from different
micro-spots or different instruments. The only regulation regarding to volume determination (ISO 4787) is centred on macro-volumes as those used for laboratory glasses [7].

It is possible to define a standard protocol to process the matrix of data points, which could be obtained by measuring the micro-cavity with any optical microscopy technique, determine the volume and the related uncertainty. In this sense, there are many data processing methods that could be apply. For instance, Weizheng defines a method using tetrahedrons that are adjusted to the data cloud provided by pictures from different positions [8]. Other protocol reported in the literature is the one proposed by Siswantoro, that adapts the Monte Carlo method for area calculation to volume determination and it is also based on microscope images [9]. As a drawback, it is difficult to distinguish between the data point belonging to the micro-cavity and any data from the surrounding areas with those methods.

The procedure described in this work is based on data points measured with confocal microscopy. For the volume measurement and corresponding uncertainty estimation, it has been fundamental to define a cut-off level in Z axis (reference plane), Probability Density Function (PDF), and the number of interactions of the mathematical model [10,11].

Volume measurement of small, not regular cavities on a rough surface faces two basic problems:

- The surface roughness and porosity hinder telling apart those points belonging to the microcavity.
- A reference plane is needed for measuring the volume. However, selecting that plane is difficult due to the surface unevenness.

The methodology proposed in this work solve the previous problems. Morphological characterization of the cavity using a confocal microscope provides a point cloud, which must be analysed and filtered. The RANSAC algorithm is used establish the reference plane. Cavity volume is measured with a proper algorithm and the associated uncertainty is assessed using Monte Carlo method.

This novel procedure aims to stablish a standard regulation for micro-volume determination by analysing data points of the cavity acquired from any optical instrument.

2. Methodology and Experimental Procedure

2.1. Confocal Microscope

Morphological measurements of micro-cavities have been performed using the Leica DCM 3D confocal microscope installed in Laboratorio de Investigación de Materiales de Interés Tecnológico (LIMIT) at Escuela Técnica Superior de Ingeniería y Diseño Industrial de la Universidad Politécnica de Madrid (ETSIDI – UPM). A coaxial 460 nm LED lightning source and a 20X N Plan L microscope objective have been used.

The cavity is nearly place in the centre of the rectangular scanning area. The in-depth optical measurement performed by the microscope provides a 3D \((x_i, y_i, z_i)\) data matrix. Figure 1 shows the set of samples used where the irregular micro-holes and original roughness of the surfaces can be appreciated. In this work, 2D \((x_i, y_i)\) matrix comprise a set of data points with a constant pitch of 1.66 \(\mu m\) both in the X and Y axes. Thus, a 3D \((x_i, y_i, z_i)\) data matrix consisting of around 6 million points is obtained. The visualization of the point cloud and the comparison of the results from different experiments have been done using CloudCompare software [12].

2.2. Numerical Method

Once the micro-cavity has been characterized with the confocal microscope, it is necessary to tell apart surface and cavity and to clearly identify which points belong to the cavity to measure its volume.

Firstly, the upper limit of the cavity must be determined. The exact Z position of the surface is not straightforward due to surface roughness or irregularities.
2.2.1. RANSAC algorithm. A RANSAC (Random Sample Consensus) algorithm was selected because it allows fitting data including points with large differences respect to the mean. RANSAC allows to reach a compromise between the random selection of points and the fitting of those points to a user defined model. This widely used algorithm has been implemented in MATLAB R2019b within the function pcfitplane to fit a point cloud to a plane. The fundamental parameters used are as follow: an horizontal reference plane is defined in order to provide to the algorithm as a first approach, d parameter is also defined (maximum distance between the reference plane and the point cloud), \(v_{\text{ref}}\) (unit vector perpendicular to the plane), \(v_{\text{ran}}\) (unit vector perpendicular to the plane determined by RANSAC), \(\Upsilon\) (largest angle between \(v_{\text{ref}}\) and \(v_{\text{ran}}\) the unit vector perpendicular to the fitted plane determined by the algorithm), and C (confidence interval). The actual value used in the simulations are shown in table 1.

| \(d\) [\(\mu\text{m}\)] | \(v_{\text{ref}}\) | \(\Upsilon\) [\(^\circ\)] | C [%] |
|----------------------|----------------|----------------|-------|
| 1                    | (0,0,1)        | 10             | 95    |
| 10                   | (0,0,1)        | 10             | 99    |

In general, the vector obtained after applying RANSAC algorithm, \(v_{\text{ran}}\), is different than \(v_{\text{ref}}\), the difference is given by an angle \(\theta\). Therefore, a rotation must be applied to correct any deviation. The objective is to determine a matrix \(B\), equation (6), comprising the positions of the point cloud but rotated respect the original matrix \(A\). Vectors \(v_{\text{ran}}\) and \(v_{\text{ref}}\) define a plane. The rotation is made through a rotation matrix \(G\) (equation (1)), where \(v_{\text{ran}} \cdot v_{\text{ref}} = \cos \theta\) (i.e. \(\theta\) is the angle between vectors), and \(|v_{\text{ran}} \times v_{\text{ref}}| = |\sin \theta|\). The new reference system is defined by unit vectors \((u,v,w)\) in equation (2) to (4). \(F\) is the change-of-basis matrix, equation (5), that provide the rotation of \(A\) to obtain \(B\).
\[
G = \begin{pmatrix}
\mathbf{v}_\text{ran} \cdot \mathbf{v}_\text{ref} & -|\mathbf{v}_\text{ran} \times \mathbf{v}_\text{ref}| & 0 \\
|\mathbf{v}_\text{ran} \times \mathbf{v}_\text{ref}| & \mathbf{v}_\text{ran} \cdot \mathbf{v}_\text{ref} & 0 \\
0 & 0 & 1
\end{pmatrix}
\]

(1)

\[
u = \mathbf{v}_\text{ran}
\]

(2)

\[
\mathbf{v} = \frac{\mathbf{v}_\text{ref} - (\mathbf{v}_\text{ran} \cdot \mathbf{v}_\text{ref}) \mathbf{v}_\text{ran}}{|\mathbf{v}_\text{ref} - (\mathbf{v}_\text{ran} \cdot \mathbf{v}_\text{ref}) \mathbf{v}_\text{ran}|}
\]

(3)

\[
w = \mathbf{v}_\text{ref} \times \mathbf{v}_\text{ran}
\]

(4)

\[
F = (\mathbf{u}, \mathbf{v}, \mathbf{w})^{-1}
\]

(5)

\[
B = F^{-1}GFA
\]

(6)

Once the reference plane is properly defined, it is possible to make a first selection of which points belong to the hole. Initially, any point with a height below the reference plane, which means Z values lower than 0, is considered to be part of the hole (figure 2).

Figure 2. Classification of the point cloud according to RANSAC algorithm (purple plane).

2.2.2. Filtering of the cavity points. Then, it is necessary to determine the limits of the hole near to the reference plane. Filtering starts determining the lowest point in the data cloud. From this point, the data row is scanned in the X axis and those points with height lower than the reference plane are identified. Repeating the process for each row yields a point cloud that represents the cavity in the X axis (figure 3 left). The same procedure can be implemented in the Y axis, with similar results (figure 3 right). Due to the algorithm, several lines of point are wrongly identified as part of the cavity. Those false positives are discriminated by combining both X and Y axis point clouds and applying an OR superposition filter, in which the points of boundary of the cavity are identified if they are present in the X-axis filtering, the Y-axis filtering or in both. Alternatively, an AND addition filter has been also studied, in which the points of the boundary are those belonging to both X-axis filtering and Y-axis filtering.
Figure 3. On the left: filtering with data points along X axis. On the right: filtering with data points along Y axis.

Figure 4. On the left: filtering by OR overlapping. On the right: filtering by AND intersection.

Choosing between OR and AND filters would depend on the appearance of secondary microcavities around the main hole. If so, OR filter yields better results. Table 2 shows the filters used in the case of the images tested.

Table 2. OR and AND filter used in several microcavities.

|   | 1  | 2  | 3  | 4  | 5  | 6  |
|---|---|---|---|---|---|---|
| OR | OR | OR | OR | AND | OR | OR |

2.2.3. Volume measurement and uncertainty estimation. The volume of the microcavity is measured as the sum of the infinitesimal volumes \( \Delta X \Delta Y \Delta Z \), for each point \((x_i, y_i, z_i)\) in the filtered point cloud, in which \( \Delta X \Delta Y \) are constant. The uncertainty is estimated using the procedure shown in supplement 1 of GUM [13]. The confocal microscope has previously been calibrated [11] and the Z coordinate has normal distribution with mean value of 0 \( \mu m \) and standard deviation 0.1\( \mu m \), \( N(0,0.1) \).

2.2.4. Assessment of the model. The measuring procedure has been validated by means of determining the volume of a computer-generated 3D gaussian cavity, equation (7), which is like the real cavities to be experimentally measured. The generated point cloud has the same X and Y axis distribution and approximately the same number of points (2240x2340).

\[
Z_i = f(X_i, Y_i) = -A \cdot \exp \left( -\frac{x_i^2}{2\sigma_x^2} + \frac{y_i^2}{2\sigma_y^2} \right) \tag{7}
\]

Theoretical volume is calculated using equation 8, where \( A = 902.90 \mu m \) \( \sigma_x = \sigma_y = 226.07 \mu m \):

\[
V = 2\pi A \sigma_x \sigma_y = 2.8994 \cdot 10^8[\mu m^3] \tag{8}
\]
Table 3 shows the results of applying the algorithm to measure the Gaussian volume. According to those results, a confidence in the RANSAC algorithm of 95% and 99% is selected.

Table 3. Validation of the algorithm for measuring an ideal Gaussian volume. From left to right: number of iterations, confidence percentage, volume, standard deviation, confidence interval at 95%.

| Nº Iter. | Confidence RANSAC [%] | V [µm$^3$] (x 10$^8$) | u(V) [µm$^3$] (x 10$^8$) | Interval at 95% [µm$^3$] (x 10$^8$) |
|----------|------------------------|------------------------|------------------------|---------------------------------|
| 15000    | 50                     | 2.8932                 | 0.0260                 | (2.8244, 2.9067)                |
| 26000    | 80                     | 2.8965                 | 0.0166                 | (2.8606, 2.9041)                |
| 220000   | 90                     | 2.8970                 | 0.0143                 | (2.8685, 2.9038)                |
| 214000   | 95                     | 2.8983                 | 0.0092                 | (2.8903, 2.9006)                |
| 186000   | 99                     | 2.8989                 | 0.0053                 | (2.8975, 2.8996)                |

3. Results and discussion

Once the method has been validated against a perfect gaussian cavity, it has been used for measuring six real samples with different surface rugosities.

In every sample, the uncertainty has been calculated using the protocol stablished by the Supplement 1 to the GUM [13]. The effect of the RANSAC confidence and $d$ parameter on the results has been studied. Table 4 shows the fitting results and figure 5 shows the histograms for two different samples.

Table 4. Results obtained for 6 samples.

| Item | Nº Iter. | Confidence RANSAC [%] | $d$ RANSAC [µm] | V [µm$^3$] (x 10$^8$) | u(V) [µm$^3$] (x 10$^8$) | Interval at 95% [µm$^3$] (x 10$^8$) |
|------|----------|------------------------|----------------|------------------------|------------------------|---------------------------------|
| 1    | 8560     | 95                     | 10             | 8.275                  | 0.207                  | (7.900, 8.673)                  |
| 1    | 8610     | 99                     | 10             | 8.260                  | 0.170                  | (7.954, 8.588)                  |
| 2    | 9980     | 95                     | 10             | 13.839                 | 0.360                  | (13.158, 14.515)                |
| 2    | 6630     | 99                     | 10             | 13.880                 | 0.317                  | (13.274, 14.475)                |
| 3    | 8630     | 95                     | 10             | 16.234                 | 0.322                  | (15.606, 16.835)                |
| 3    | 5760     | 99                     | 10             | 16.236                 | 0.275                  | (15.709, 16.764)                |
| 4    | 7500     | 95                     | 10             | 18.096                 | 0.338                  | (17.405, 18.684)                |
| 4    | 3940     | 99                     | 10             | 18.136                 | 0.304                  | (17.448, 18.641)                |
| 5    | 25480    | 95                     | 10             | 3.354                  | 0.273                  | (2.887, 3.881)                  |
| 5    | 17540    | 99                     | 10             | 3.357                  | 0.245                  | (2.909, 3.805)                  |
| 6    | 147950   | 95                     | 10             | 2.878                  | 0.107                  | (2.650, 3.094)                  |
| 6    | 19190    | 95                     | 1              | 2.895                  | 0.023                  | (2.851, 2.938)                  |
| 6    | 19730    | 99                     | 1              | 2.894                  | 0.020                  | (2.855, 2.943)                  |

The method is quite sensitive to the position of the reference plane, because small variations in position lead to large differences in the volume calculates. Thus, the correct selection of the RANSAC parameter is an issue when calculating the uncertainty. The RANSAC confidence and $d$ parameter have been set to 95% and 99% and 10 µm respectively. In order to keep computational complexity low, the number of iterations were limited, especially when using a 99% confidence. This limitation is according to the section 7.2 of the cited supplement [13].

Low surface rugosity eases searching the reference plane using RANSAC, because it is possible to achieve a larger number of iterations with the same calculation complexity. In the case of the flattest sample (sample 6), several values for $d$ parameter have been tested and the results using a value of $d$ of 10 µm and 1 µm were compared. When using $d = 1$ µm the variation in the measured volume is only a
0.6% but the uncertainty was decreased by 80%. On the other hand, sample 5, which has the largest rugosity, yields the worst fitting results and the largest relative uncertainty.

![Histograms from sample 1 and 6 with different confidence and d parameter.](image)

**Figure 5.** Histograms from sample 1 and 6 with different confidence and d parameter.

### 4. Conclusions

In this work, a new procedure for measuring the volume of microcavities and the corresponding uncertainty from confocal microscopy images (according to the protocol described in the Supplement 1 to the GUM) is proposed. The reference plane, which is the critical element for performing such measurement, has been found using RANSAC algorithm.

The computer complexity was high, so a relatively small number of iterations have been done. Reaching the objective of $10^6$ iterations demands increasing the computational speed by filtering the data before the algorithm is applied. Those data clearly not belonging to the cavity could be eliminated without losing information and thus the reference plane could be determined more straightforward.
Another way of improving the method is by knowing the surface of the sample, because parameter \( d \) is related to the mean distance between points in the surface of the sample, i.e., its surface rugosity. Therefore, if the sample rugosity were previously known, it would be easier to select a proper parameter \( d \) which would yield in smaller relative uncertainties.

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**References**

[1] Kind D and Lübbig H 2003 Metrology - The present meaning of a historical term *Metrologia* **40** (5) pp 255–257

[2] Brown R J C 2021 Measuring measurement – What is metrology and why does it matter? *Meas. J. Int. Meas. Confed.* **168** p 108408

[3] Elfurjani S, Ko J and Jun M B G 2016 Micro-scale hole profile measurement using rotating wire probe and acoustic emission contact detection *Meas. J. Int. Meas. Confed.* **89** pp 215–222

[4] Kim B, Masuzawa T and Bourouina T 1999 The vibroscanning method for the measurement of micro-hole profiles *Meas. Sci. Technol.* **10** (8) pp 697–705

[5] Murakami H, Katsuki A, Onikura H, Sajima T, Kawagoishi N and Kondo E 2010 Development of a system for measuring micro hole accuracy using an optical fiber probe *J. Adv. Mech. Des. Syst. Manuf.* **4** (5) pp 995–1004

[6] Jordan H J, Wegner M and Tiziani H 1998 Highly accurate non-contact characterization of engineering surfaces using confocal microscopy *Meas. Sci. Technol.* **9** (7) pp 1142–1151

[7] Asociación Española de Normalización y Certificación UNE-EN ISO 4787:2011: Vidrio para laboratorio. Instrumentos Volumétricos. Métodos para el Ensayo de la Capacidad y su Uso 2010 (ISO 4787:2010)

[8] Zhang W, Wu X, Qiu Z and He Y 2016 A novel method for measuring the volume and surface area of egg *J. Food Eng.* **170** pp 160–169

[9] Siswantoro J, Prabuwono A S and Abdullah A 2014 Volume measurement algorithm for food product with irregular shape using computer vision based on Monte Carlo method *J. ICT Res. Appl.* **8** (1) pp 1–17

[10] Mihai Ricean F 2019 Caracterización de microvolúmenes *Universidad Politécnica de Madrid*

[11] Wang C, Caja J, Gómez E and Maresca P 2019 Procedure for Calibrating the Z-axis of a Confocal Microscope: Application for the Evaluation of Structured Surfaces *Sensors* **19** (3) p 527

[12] CloudCompare (http://cloudcompare.org/) accessed 06 March 2021

[13] J C for G in M (JCGM) 2008 101:2008 Evaluation of Measurement Data—Supplement 1 to the ‘Guide to the Expression of Uncertainty in Measurement’—Propagation of Distributions Using a Monte Carlo Method