

## Automated measurement of ommatidia in the compound eyes of beetles

Bartosz W. Schramm, Agnieszka Gudowska, Filip Kapustka, Anna Maria Labecka, Marcin Czarnoleski, and Jan Kozłowski  
*Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland*

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The size of the ommatidia that compose the insect compound eye is linked to visual capacity, physiological performance, and cell size. Therefore, rapid and reliable methods for measuring ommatidia can advance research on insect ecology and evolution. We developed an automated method to measure ommatidia in nail polish imprints of the eyes of three Carabidae beetle species using the widely available, free software ImageJ. Our automated method was equivalent to a traditional manual method in terms of accuracy but had the advantage of being 70 times faster. We provide access to our algorithm, which can be used to investigate biological phenomena ranging from the functional architecture of the compound eye to the cellular basis of the evolution of body size and metabolic rates.

The compound eye of an insect consists of subunits called ommatidia, and the size of the ommatidia is linked to visual capacity and organismal performance (1). These relationships most likely explain why the size of the ommatidia correlates with the lifestyle (2), body size (3), and standard metabolic rate of the organism (4). The ommatidia within a single eye vary in size (5), and they differ genetically between individuals (6) and species (3). Additionally, the size of the ommatidia changes plastically in response to developmental conditions (7). Moreover, because ommatidia develop from a defined number of cells (8), and the sizes of different cell types

are usually correlated (3,9), the size of the ommatidia approximates the average cell size in other tissues (4).

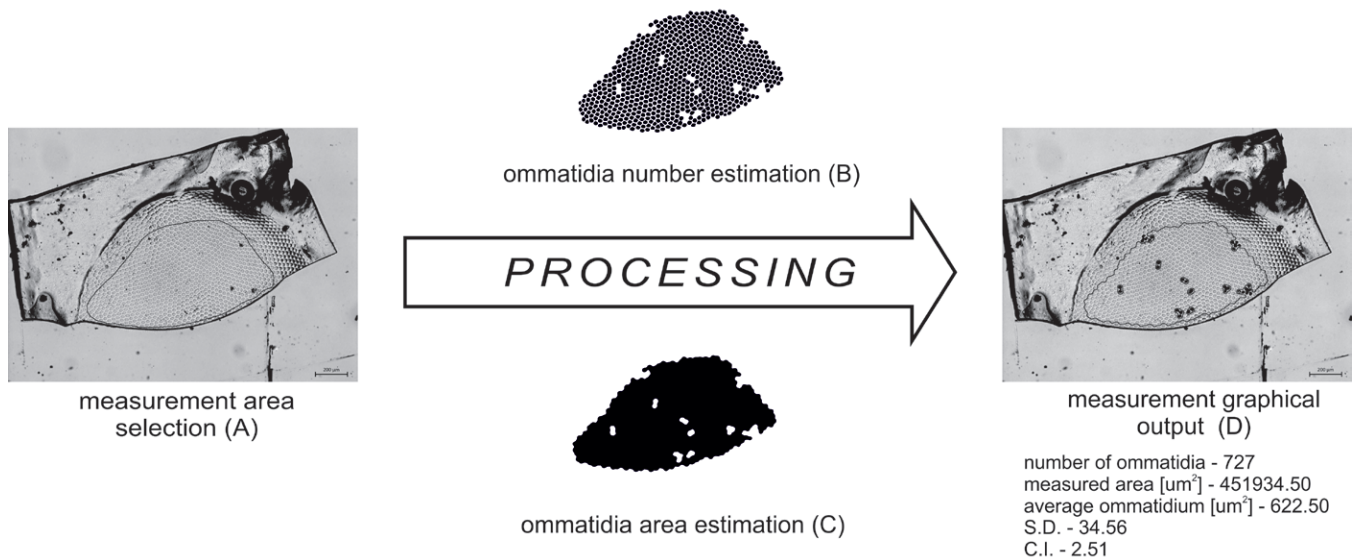
Different methods are available for measuring ommatidia, but none allow for the rapid measurement of large numbers of ommatidia in a simple and inexpensive manner. This limitation hinders the performance of large-scale experimental and comparative studies. Methods based on light microscopy and an eyepiece micrometer or manual imaging software (3) are inexpensive but tedious to perform. Electron microscopy is much more accurate and precise but is also costly and time-consuming (4).

We have developed a method that maximizes throughput and accuracy while minimizing cost. This method involves the preparation of eye imprints with nail polish (a technique that has been used successfully in previous studies) (10), taking digital images of the imprints under a light microscope, and using the free software ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>) to analyze the images in an automated fashion. We used this method to measure the size of ommatidia in three species of Carabidae beetles (Supplementary Material) and compared the accuracy, time, and cost associated with this and three alternative methods. Our goal is to provide a protocol that can be applied whenever reliable and rapid measurements are required for studies concerning sexual, regional, interspecific, and intra-specific variations in ommatidium size.

To measure the ommatidia, 10 females per species were euthanized with ethyl acetate (Avantor Performance Materials Poland S.A., Gliwice, Poland). A nail polish imprint of each eye was produced as described by Ribí et al. (10) and mounted on a microscope slide. Digital images of imprints were taken under a light microscope (Supplementary Material) and used to assess the average area of the ommatidia in each beetle. We used four different methods: manual small area (MSA), automatic small area (ASA), automatic large area (ALA), and manual linear (ML). For MSA, we manually defined 3 non-overlapping areas in an image of each eye, with approximately 50 ommatidia per area. The areas were delimited along the edges of the ommatidia and measured in  $\mu\text{m}^2$  with a free-hand selection tool in ImageJ 1.48k. The ommatidia were manually counted, and the average area of an ommatidium was calculated as the total area measured in each beetle divided by the number of ommatidia. This method was used in previous studies (4), and we considered it as a reference method that may be the most reliable among the tested methods. ASA was

### METHOD SUMMARY

We provide an automated method to measure ommatidia in nail polish imprints of the compound eye using the widely available, free software ImageJ. Our automated method was equivalent to a traditional manual approach in terms of accuracy but because it is 70 times faster, it can be used to investigate a wide range of biological phenomena.



**Figure 1. A scheme depicting the automated procedure for ommatidium size measurements.** (A) The area used for the ommatidium measurements is selected based on the image quality. (B) The algorithm manipulates the picture for the automatic counting of ommatidia. (C) The algorithm manipulates the picture for the automatic measurement of area. (D) The output image is generated by the algorithm to provide a visual representation of the measured ommatidia.

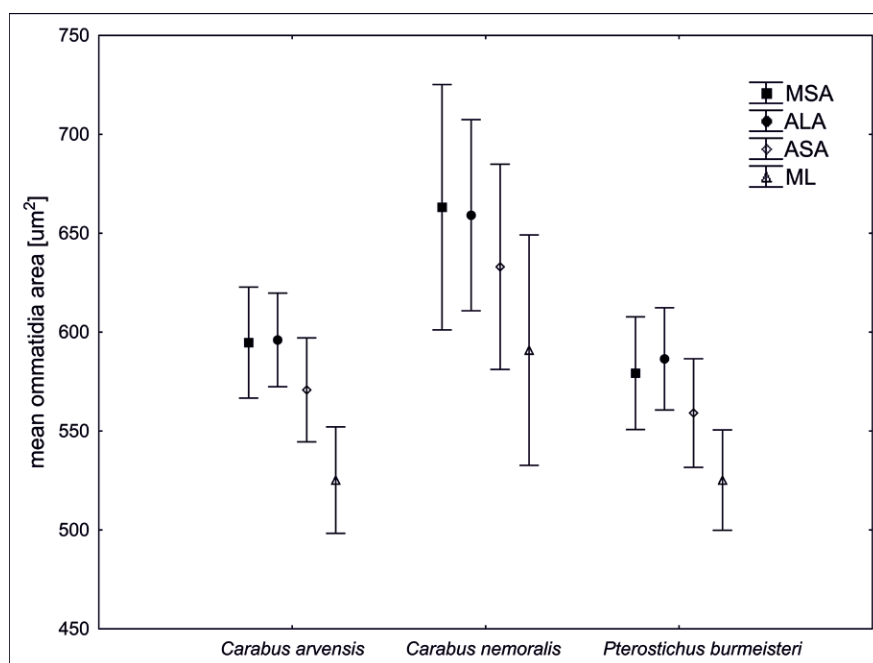
performed using the identical areas used for MSA, but the ommatidium recognition and counting was conducted using our automated algorithm developed for ImageJ. ALA was also performed with our automated algorithm, but using the area that spanned all ommatidia of acceptable quality for image processing (therefore, ALA analyzed more ommatidia than ASA). Finally, ML measured four non-crossing linear transects (in µm), which were drawn manually with ImageJ within the borders outlined in the MSA method. Each transect spanned at least three adjacent ommatidia aligned along a straight line and passed through the central point of each ommatidium (11). Dividing the total length of the transects by the number of ommatidia, we calculated the average diameter of an ommatidium. Then, assuming a circular shape, we calculated the average area of an ommatidium. The time required to measure 1000 ommatidia using each method was recorded.

To perform automatic measurements (ASA and ALA), a macro was designed in ImageJ (see Macro S1 in the Supplementary Material). The algorithm recognizes ommatidium edges based on the difference in brightness between their saturated centers and the bright border areas. After selecting the region of an eye imprint for ommatidium measurement (Figure 1A), digital images were contrasted using an automatic brightness and contrast adjustment

function based on the built-in adjustment tool in ImageJ. Then, a binary transformation using automatic thresholding was performed. The resulting image presented black ommatidium centers separated by white borders (Figure 1B). To exclude non-ommatidium objects and superimposed ommatidia, a small number of ommatidia was measured manually in five individuals from each species (note that this step calibrates the method to the size range of the ommatidia in the studied animals). Based on these measurements, objects smaller than 30% or larger than 130% of the manually estimated average area (655 µm<sup>2</sup>) were automatically excluded by the software as non-ommatidium artifacts. These objects are shown in Figure 1, B and C as white insertions. The automated procedure counted the remaining black ommatidium centers. Then, the white borders of the ommatidia were removed (Figure 1C) because ommatidia adjoin their neighbors, and the area of the black surface was measured by the software. Based on the number of ommatidia determined in Figure 1B, the average area of a single ommatidium was calculated. A graphical presentation of the results was generated to check for abnormalities; the excluded ommatidia were marked in black, and a line delimited the measurement area (Figure 1D).

The methods we tested produced different estimates of the average area

of the ommatidia ( $F_{3,81} = 162.23$ ;  $P < 0.001$ ) (Figure 2). Additionally, differences between species ( $F_{2,6} = 6.16$ ;  $P = 0.006$ ) and individual beetles ( $F_{27,81} = 34.02$ ;  $P < 0.001$ ) explained significant portions of the variance in ommatidium size. The interaction between method and species was non-significant ( $F_{6,81} = 0.57$ ;  $P = 0.752$ ), indicating that our conclusions regarding the methods do not depend on the species tested. Importantly, the estimations of ommatidium size using the automatic large area method (ALA) and the manual reference method (MSA) were not different (Tukey's test,  $P = 0.99$ ). The estimations based on the remaining 2 methods (ASA and ML) differed from each other ( $P < 0.001$ ), and both methods produced lower measurements than either MSA or ALA ( $P < 0.001$ ). The laboriousness of methods differed greatly. The ALA method was 2 times faster than ASA, 20 times faster than ML, and 70 times faster than the reference MSA method. Overall, our results indicate that all of the tested methods provided reasonable estimates of the area of the ommatidia. Only the fully automated method (ALA) was as accurate as our reference approach (MSA), and it was also the most rapid. We suggest that this method may be an efficient tool for future large-scale studies that require extensive measurements of ommatidia with high accuracy. Although we have not directly tested this method in all possible appli-



**Figure 2.** The average area of an ommatidium ( $\pm 95\%$  confidence interval) estimated for three species of beetles (10 females per species) using different methods. The estimation of ommatidium size did not differ between the manual small area (MSA) and the automatic large area (ALA) methods, but estimations based on the automatic small area (ASA) and manual linear (ML) measurements were significantly lower than either the MSA or the ALA estimation.

cations, it can be easily adopted to study patterns in the size of ommatidia within a single compound eye as well as to investigate individual and species differences.

When developing ALA, we considered throughput and accuracy as our main goals. Therefore, the potential sources of error should be highlighted. Deformations resulting from the preparation of the eye imprint can affect the estimation of ommatidium size. Thus, we suggest omitting the eye margin, which might be the region that is most affected by this process. Next, improper adjustments of the brightness, contrast, and binarization levels in ImageJ may result in the exclusion of some ommatidia from the analysis due to incorrect separation or imposition. We tested the effects of different values of these parameter, but the default values proposed by ImageJ and by Guimond and Miura (IJ\_BCauto-Macro: The 2nd release, <http://dx.doi.org/10.5281/zenodo.12249>) yielded satisfactory results. In our experience, only substantial deviations from the default values had visible effects.

Each ommatidium in its digital form has a spherical cornea lens and an adjoining edge. In the automated procedure, these features are converted into black areas

with white rims of a fixed width around the center (Figure 1B). These rims are included in the ommatidium area measurement (Figure 1C). An error may occur if the ratio of the edge to the measured area is high. To minimize this effect, we suggest analyzing large non-fragmented areas that contain a small fraction of excluded portions and choosing comparable areas in all studied specimens. The MSA method should minimize the aforementioned errors, but it introduces subjectivity and is time-consuming.

Overall, low-cost and automated methods will allow future studies to investigate the size of ommatidia on an unprecedented scale. The mass measurements of ommatidia will advance our understanding of the ecology of insects and the evolutionary significance of cell size.

## Author contributions

B.W.S., F.K., A.G., A.M.L., M.C., and J.K. conceived and designed the method. B.W.S., F.K., A.G., and A.M.L. performed the measurements and created the macro. B.W.S., M.C., F.K., and J.K. analyzed the data. B.S., M.C., J.K., F.K., A.G., and A.M.L. drafted and worked on the manuscript.

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## Competing interests

The authors declare no competing interests.

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Address correspondence to Bartosz W. Schramm, Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland. E-mail: bartosz.schramm@gmail.com

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