SPOTS v2

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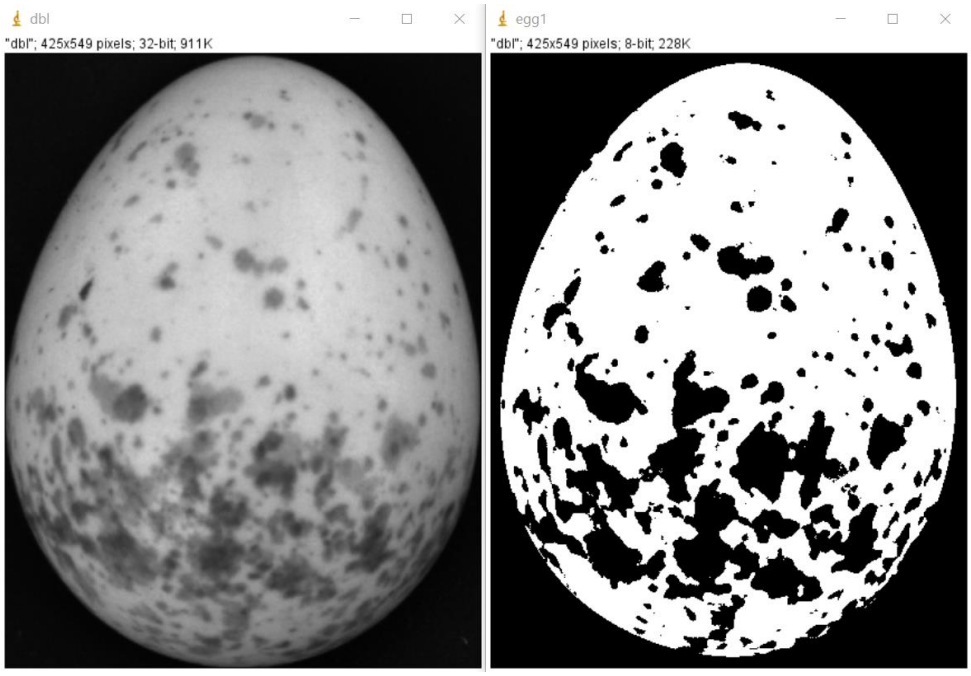
If you have any questions or found a bug in the code, please, feel free to contact me.

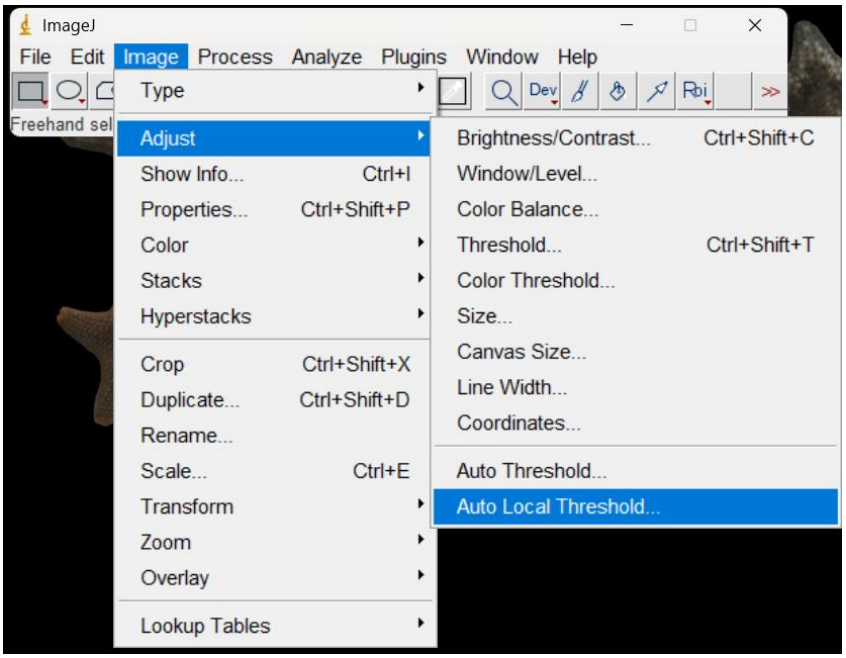
INSTALLATION

These scripts require installation of ImageJ (Schneider et al., 2012) version 1.54. If you use older version of ImageJ, please, contact me. Further, the scripts work together with MICA Toolbox plugin (Troscianko & Stevens 2015, here you can download MICA: http://www.empiricalimaging.com/). I tested the scripts with MICA's version 2.1.

To use the scripts directly from ImageJ, you should place the whole unzipped folder in ImageJ/plugins folder. Then you can access them through plugins -> SPOTS_v2 in the menu bar.



Before starting to use scripts, make sure that "Auto Local Threshold" option is available in your version of ImageJ. When you select in the menu bar: Image -> Adjust, „Auto Local Threshold" should be somewhere in the list among the other options, see Figure below.



If it is not there, then download the script "Auto_Threshold.jar" from https://imagej.net/plugins/auto-local-threshold and put it in ImageJ -> plugins -> jars folder.

DESCRIPTION

The scripts use normalised digital images (.mspec files) created with the MICA Toolbox software (Troscianko and Stevens, 2015). For more information on how to create an mspec file, please visit: http://www.empiricalimaging.com/knowledge-base/ . Apart from normalising images, a scale bar and eggs should be selected (that is - added to ROI manager) for scripts to work correctly.

The main advantage of the SPOTS v2 package is that it measures different aspects of eggshell patterning and allows to measure brightness and colour of spots and eggshell background separately. Spots are detected from double cone channel when images are transformed to a visual model of a bird. If no visual model is selected, then green channel is used, as it match the spectral sensitivity of avian double cones (Spottiswoode and Stevens, 2010). Detection of spots uses auto local threshold function in ImageJ with Phansalkar method (Phansalkar et al., 2011). In contrast to global thresholding, local thresholding calculates the threshold for each pixel using not the whole image, but neighbouring pixels that lie within a radius around the focal pixel. Such an approach allows to cope with high variability in pixels values that occur in small spatial scales (Phansalkar et al. 2011). You can customise the value for radius as spots on eggshells highly differ between species. It is good to find a good trade of between small and large radius values. Smaller values are sensitive to small changes in pixel intensities and can detect even very little spots, but in the case of larger blotches even slightly brightened areas can be incorrectly classified as background. On the other hand, larger radius values will classify all pixels within big spots as spots, but are less sensitive for small spots. Therefore, if eggs of the studied species are covered with large blotches that have some internal variation in coloration, it makes sense to use larger radius values and if your species lay eggs with small and uniform in coloration spots, it's better to use small radius values. However, it is especially tricky in the case of eggs that have both large and ununiform blotches and small speckles. In that case, one have to find a golden trade-off, between accurate detection of small and large spots. In my work on red-backed shrikes (whose eggs belong to the third category), I use a radius value between 20 and 50.

Second version of the spots package have two major changes.

First, because of their shape it is quite difficult to take a picture of an egg that would be evenly illuminated – especially in fieldwork conditions. As a consequence, edges of eggs that are visible in the picture are very often darker – not because they are darker in reality, but only because less light reflects from them and reach camera sensor (Gómez and Liñán-Cembrano, 2017). Following (Gómez et al., 2018) I used Gaussian blur to correct for shaded edges of eggs. You can customise the scale of Gaussian blur – it should be similar to the scale of the eggs (that is lower values for species that lay small eggs and larger for species laying large eggs). I used the scale of 128 or 181 px for red-backed shrikes. It is important to underline here, that the more care one takes at the stage of taking pictures: accurately aligning eggs and grey standards, taking photos in as uniform light conditions as possible and diffusing light, the easier it will be later at the image analysis stage and the more accurate the measurements will be. If a picture was taken with no care, no post processing can help.

Second, in the recent version, images can be converted to a selected visual model. This way you can model how eggs look from the ecologically relevant receiver's perspective. It is possible to prepare a custom visual model for your own camera set using X-Rite ColorChecker and "Generate Cone Mapping Model From Chart" function in MICA Toolbox (unfortunately only in visible light) – please visit http://www.empiricalimaging.com/knowledge-base/ for a detailed instruction. Most scripts work with both pictures taken only in visible light and with pictures taken in visible and UV light. Therefore, it is possible to model eggs appearance form birds perspective. As far as we know, many avian species are tetrachromats. Missing information in the UV band is for sure a simplification, but if you photographed eggs only in visible light it is still possible to model eggs appearance from birds' perspective using trichromatic models, for example Bluetit LMS model for UVS species and Peafowl LMS for VS species (see Ödeen and Håstad, 2013 to learn which species are UVS and which VS). There are two separate functions to calculate colour of spots and background, depending on whether your pictures are only in visible light ("spots colour trichromats") or in visible and UV light ("spots colour tetrachromats").

Spots are detected from avian double cones channel and if a visual model was selected, then spots and background coloration is measured in terms of quantum catches for long-, medium- and short-sensitive cones, (and UV- or V- sensitive cones for "spots colour tetrachromats" script). Further, for "spots colour trichromats" function, it is possible to calculate X and Y coordinates in the trichromatic colour space. If a visual model is selected, X coordinate tells us where a specific colour lies in a red-green opponency and Y coordinate – in blue-yellow opponency. Saturation is distance from the achromatic centre of the colour space.

I cannot guarantee that spots detection will be accurate, especially in the case when photographs were not taken in diffused light. As I already mentioned, because of their shape, eggs are difficult objects to measure and their edges are often shaded what makes spots detection much more difficult. In the current version of the program, I introduced a correction of uneven illumination using Gaussian blur (following Gómez et al. 2018) and moreover, you can shrink selection around the egg, to avoid measuring very dark edges (or colour of the supporter that an egg lies on – if selection around the egg wasn't very accurate). But – I will repeat it again – always the first and very important step will be to make effort to take the best pictures you can take, for example using a sheet of PTFE to diffuse light, and not photographing in very variable light conditions, etc. It will be also a good practice to select a subset of highly variable eggs from your set and check spots detection using "preview spotsDetection" function to work out the best settings and make sure there are no surprises (for example distinct shades or light bouncing off the shell that makes pixels overexposed). You can also save images of eggs with detected spots using "percent spots" function.

Here is a brief description of all functions (more detailed information are below):

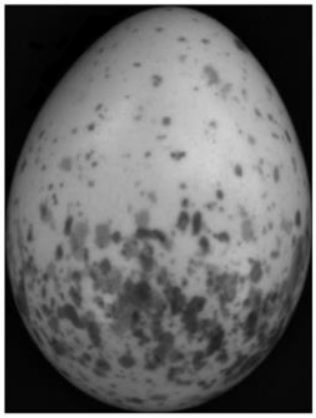
- **preview spotsDetection**: it allows to preview the performance of the spots detection with different settings to work out the best settings for your set of photos
- **check overexposition**: it checks what percent of an egg's area in pixels is overexposed, separately in every channel. It is best to avoid measuring overexposed pixels, so if there are some, try to use a picture with shorter exposition.
- **percent spots**: counts percent of an egg's surface that is covered with spots
- **spots size**: measures average size of spots (in pixels)
- **pattern dispersion**: measures dispersion of patterning along the egg
- **spots colour trichromats**: measures brightness and colour of spots and background separately. If a visual model is selected, then luminance and colour in terms of quantum catches is calculated (in linear, log or hiperbolic scale). Additionally, you can calculate coordinates in trichromatic colour space. These are X, Y coordinates and saturation in the Maxwell triangle if no visual model was selected and red-green and blue-yellow opponency (and saturation) in Receptor Noise Limited models (Vorobyev and Osorio, 1998) for a selected visual model. Finally, it calculates achromatic and chromatic contrast between spots and background. It works only with pictures taken in visible light (three channels: red, green and blue – no UV) or with images transformed to trichromatic models.
- **spots colour tetrachromats**: measures brightness and colour of spots and background separately. If a visual model is selected, then luminance and colour in terms of quantum catches is calculated (only in linear scale). It also calculates achromatic contrast between spots and background. It works only with tetrachromatic models. It works only with pictures taken in visible and UV light (five channels: red, green, blue, UVB and UVR) or with images transformed to tetrachromatic models.

All images should be scaled uniformly for spots measurements to be comparable across photographs. First use plugins -> micaToolbox -> Image Analysis -> Batch Scale Bar Calculation to calculate minimum px/mm factor in the whole folder of images. You should use this value or round it down (never up! – see details in the User Guide to MICA Toolbox, http://www.empiricalimaging.com/). For example if minimum px/mm is 27.78 you can use 27.78, or round it down for example to 27.5, to 27, to 25 and so on (but do not round up to 28!). You will need this value in most of functions - just provide it next to "Scale images (px/mm)" in the window with settings that will pop up when you select one of scripts and the code will do the rest.

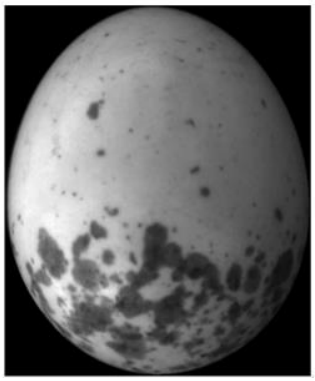
Detailed description of all scripts

preview spotsDetection

This is a supplementary script that allows to check how detection of spots works on different eggs and using different settings. Hopefully, it will be helpful at working out the best settings specifically for your own dataset. I recommend selecting a subset of pictures with highly variable eggs and testing different settings to check what works best in your specific case. If you select a visual model, spots are detected from double cones channel and if you do not select any visual model, then green channel is used for detection.

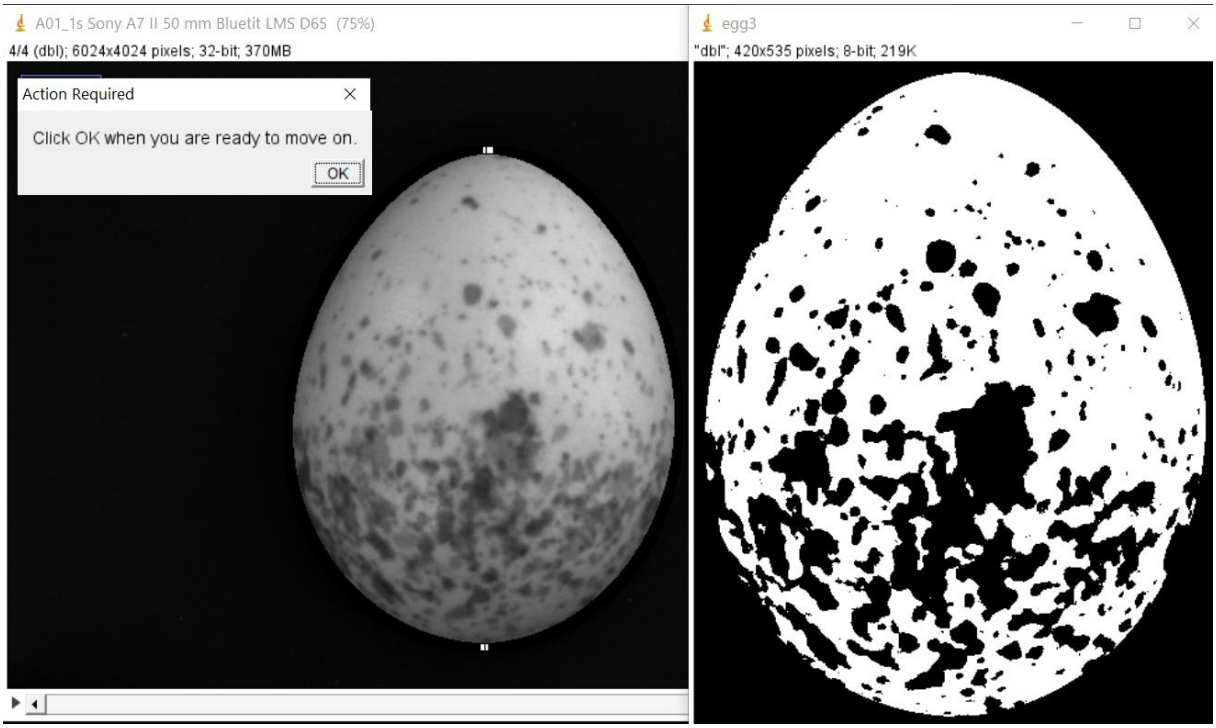


Egg with accurately detected spots:



Egg with some artefacts:

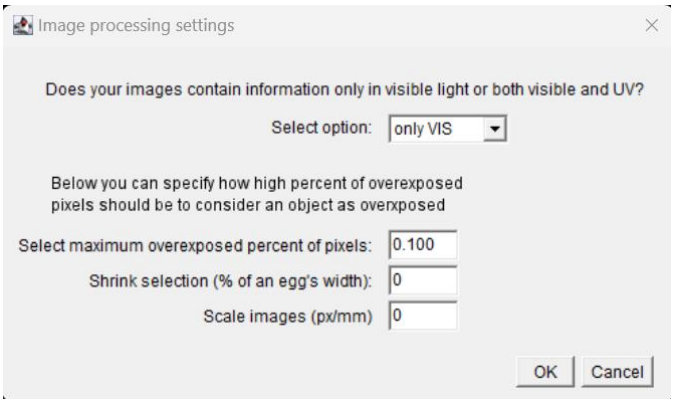
Select in the Menu Bar: Plugins -> SPOTS_v2 -> preview spotsDetection. First you will be asked to select an mspec file that will be loaded. Next you will be asked if you would like to scale your image. If you prefer not to scale image, then type “0”. Then you will be asked if you would like to convert image to a selected visual model – if you select “Yes”, in the next step you can select visual model. Finally you will be asked to select the egg that you would like to preview. Then a window with settings will pop up. There you can specify radius value for thresholding, scale of Gaussian blur and how much the selection will be shrank. Feel free to play a bit with different settings. When you click “OK” a window with a binary image of egg will appear. At this moment you can zoom in, zoom out and move between windows. When you are ready to move on, click “OK” button in the “Action Required” window. The script works in a loop – after previewing one egg, you can select the same or another egg from the loaded image, load another image or exit.


A01_1s Sony A7 II 50 mm Bluetit LMS D65 (75%)
4/4 (dbl); 6024x4024 pixels; 32-bit; 370MB
Action Required
Click OK when you are ready to move on.
OK
egg3
"dbl"; 420x535 pixels; 8-bit; 219K

check overexposition

This is a supplementary script that allows to check if no eggs are overexposed (pixel values above 100). It can often be the case if light was not diffused when taking photographs. It is quite problematic, because pixels values can't go higher than 100, which means the information is lost for overexposed pixels and their values are not true. Using this script you can specify maximum percent of overexposed pixels and this should help to decide whether you can use this picture for analyses or not.

Select in the Menu Bar: Plugins -> SPOTS_v2 -> check overexposition. Next select a folder, where are saved all your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear. There, you can specify if your photos are only in visible light or on both visible and UV light. Select maximum overexposed percent of pixels on egg for the script to treat an egg as overexposed. Small values should be used, for example 0.1. You can also scale all images and decrease selection around the egg (percent of an egg width).



After clicking "OK", analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with "Processing finished" information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named "Results_overexposed_temp.csv") is saved in the folder with pictures, so in the case script aborted, you won't lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren't analysed yet. If you would like to keep the table with temporary results, please, change the file's name – when script will be run again, original temporary file will be overwritten.

In the file with results there are following columns:

- Label: ID of an egg constructed as name of mspec file + name of ROI;
- Pixel_area: area of the egg selection (in pixels)
- R_pixel_overexposed – number of overexposed pixels in the red channel
- R_percent_overexposed – percent of overexposed pixels in the red channel
- R_is_overexposed – is the egg overexposed in the red channel?
- G_pixel_overexposed – number of overexposed pixels in the green channel

- G_percent_overexposed – percent of overexposed pixels in the green channel G_is_overexposed – is the egg overexposed in the green channel?
- B_pixel_overexposed – number of overexposed pixels in the blue channel
- B_percent_overexposed – percent of overexposed pixels in the blue channel
- B_is_overexposed – is the egg overexposed in the blue channel?

If your images are in both visible and UV light, then additionally there will be:

- UVB_pixel_overexposed – number of overexposed pixels in the UVB channel
- UVB_percent_overexposed – percent of overexposed pixels in the UVB channel
- UVB_is_overexposed – is the egg overexposed in the UVB channel?
- UVR_pixel_overexposed – number of overexposed pixels in the UVR channel
- UVR_percent_overexposed – percent of overexposed pixels in the UVR channel
- UVR_is_overexposed – is the egg overexposed in the UVR channel?

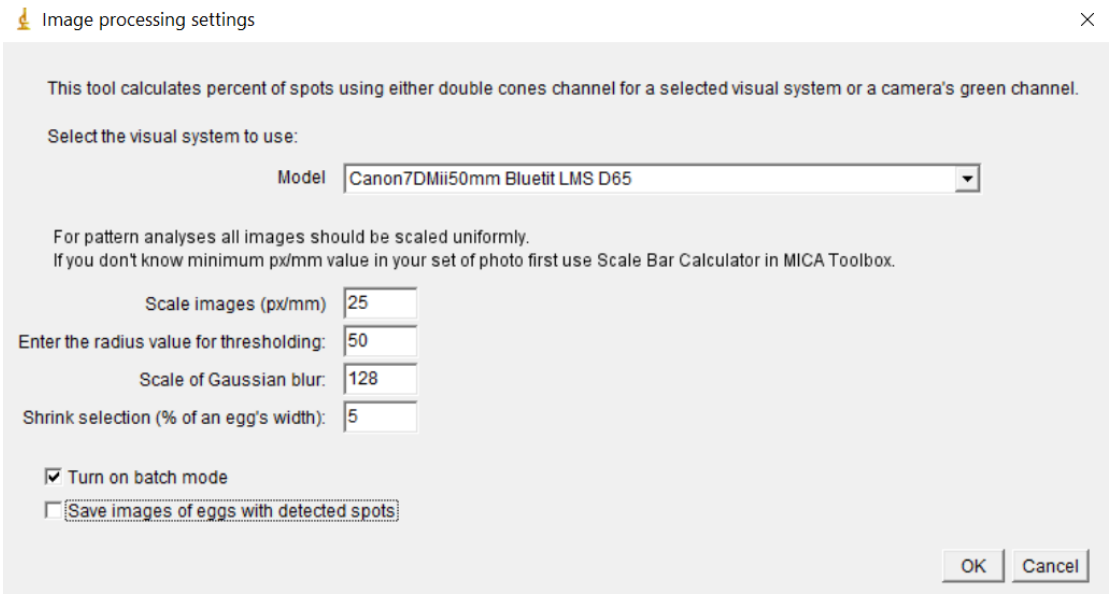
Using „…_is_overexposed" columns, it is pretty straightforward to filter eggs that are overexposed (they will have a value "yes" in some of these columns). If that is the case, try to check a picture with shorter exposition (shorter integration time) or lower EV step and check again if this solved the problem with overexposition.

It is a good practice to check if there are any overexposed eggs before starting further analyses. It saves a lot of time and frustration.

percent spots

Select in the Menu Bar: Plugins -> SPOTS_v2 -> percent spots. Next select a folder, where are saved all your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear.

- **Model**: If you select “None”, normalised images will be used instead and spots will be detected from a camera’s green channel. If you select a visual model, images will be first converted into cone catch values for the model and spots will be detected using double cones channel.
- **Scale images (px/mm)**: minimum px/mm factor in your set of photographs. It has to be first measured for the whole set using Batch Scale Bar Calculator in MICA Toolbox. If you would like to round it, then round down, never up!
- **Radius value for thresholding**: scale for calculating threshold when detecting spots. Something between 20 and 50 should work well.
- **Scale of Gaussian blur**: Gaussian blur corrects for shaded edges, scale should be similar (in pixels) to the scale of an egg.
- **Shrink selection**: it is worth to slightly decrease selection around the egg, to avoid measuring the background that egg lies upon. While MICA Toolbox fits egg shape very well, there is always a possibility that some thin areas of the background will be within selection. Further, some eggs just have a strange shape and it is difficult to fit the selection. The value that you type here is a percent of an egg’s width, e.g. 2 will decrease selection of each egg by 2% of its width.
- **Turn on batch mode**: if you turn it on, spots detection will be processing in the background. It does not affect results. However, I noticed that turning it on makes analyses faster.
- **Save images of eggs with detected spots**: if selected, binary jpg images of eggs with detected spots (black spots on white background) will be saved in a selected directory.



If you select “save images of eggs with detected spots” option, you will be additionally asked to choose a directory where images will be saved (it’s good to save them in a separate folder).

After clicking “OK”, analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with “Processing finished” information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named “Results_percentSpots_temp.csv”) is saved in the folder with pictures, so in the case script aborted, you won’t lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren’t analysed yet. If you would like to keep the table with temporary results, please, change the file’s name – when script will be run again, original temporary file will be overwritten.

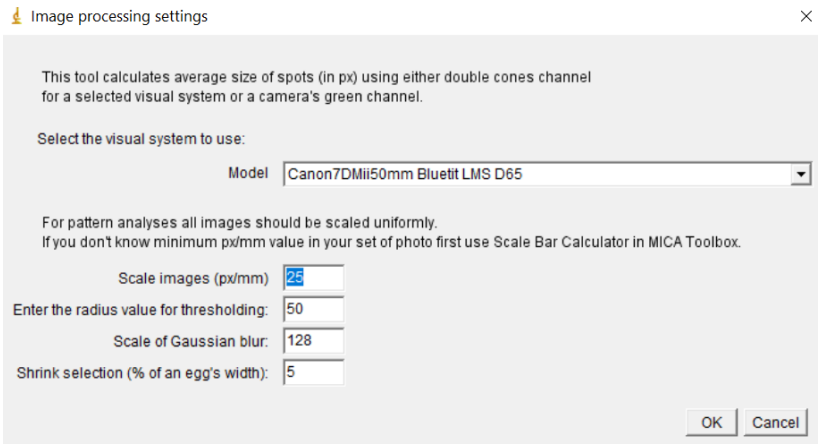
In the file with results there are following columns:

- Label: ID of an egg constructed as name of mspec file + name of ROI;
- Area: area of the egg (in pixels);
- Background: number of pixels that were classified as egg’s background coloration;
- Spots: number of pixels that were classified as spots;
- Percent_spots: percent of egg surface that is covered with spots.

spots size

Select in the Menu Bar: Plugins -> SPOTS_v2 -> spots size. Next select a folder, where are saved all your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear.

- **Model**: If you select “None”, normalised images will be used instead and spots will be detected from a camera’s green channel. If you select a visual model, images will be first converted into cone catch values for the model and spots will be detected using double cones channel.
- **Scale images (px/mm)**: minimum px/mm factor in your set of photographs. It has to be first measured for the whole set using Batch Scale Bar Calculator in MICA Toolbox. If you would like to round it, then round down, never up!
- **Radius value for thresholding**: scale for calculating threshold when detecting spots. Something between 20 and 50 should work well.
- **Scale of Gaussian blur**: Gaussian blur corrects for shaded edges, scale should be similar (in pixels) to the scale of an egg.
- **Shrink selection**: it is worth to slightly decrease selection around the egg, to avoid measuring the background that egg lies upon. While MICA Toolbox fits egg shape very well, there is always a possibility that some thin areas of the background will be within selection. Further, some eggs just have a strange shape and it is difficult to fit the selection. The value that you type here is a percent of an egg’s width, e.g. 2 will decrease selection of each egg by 2% of its width.



After clicking “OK”, analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with “Processing finished” information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named “Results_spotsSize_temp.csv”) is saved in the folder with pictures, so in the case script aborted, you won’t lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren’t analysed yet. If you would like to keep the table with temporary results, please, change the file’s name – when script will be run again, original temporary file will be overwritten.

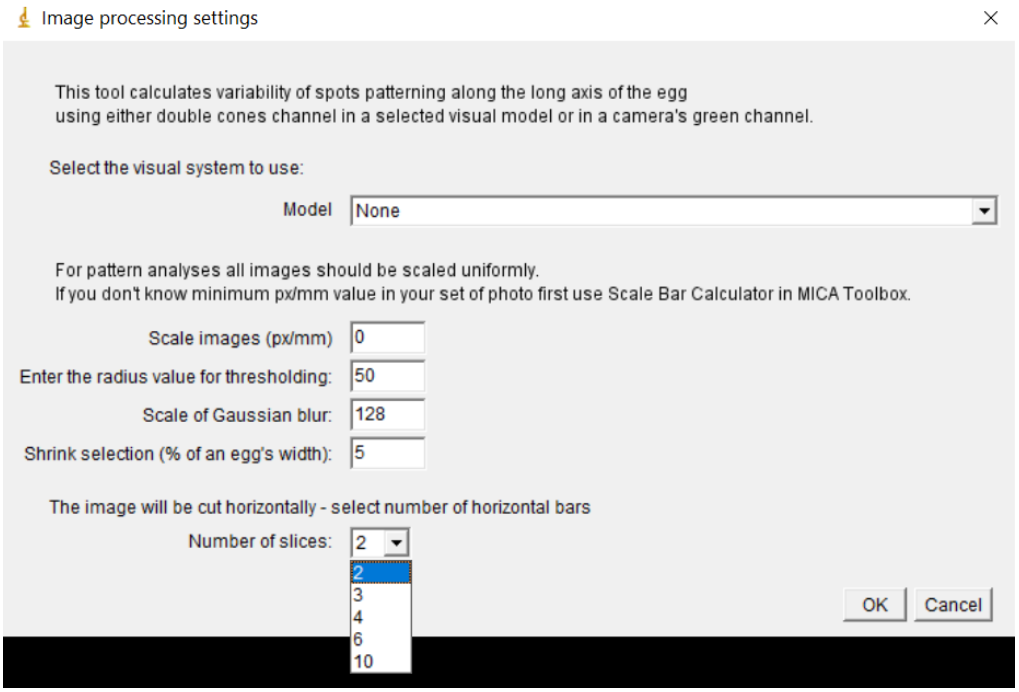
In the file with results there are following columns:

- Label: ID of an egg constructed as name of mspec file + name of ROI;
- avg_spot_size: average size of spots on egg (in pixels)

pattern dispersion

Select in the Menu Bar: Plugins -> SPOTS_v2 -> pattern dispersion. Next select a folder, where are all saved your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear.

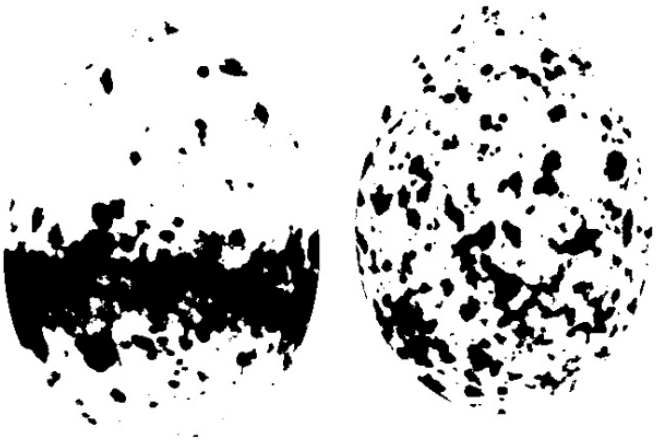
- **Model**: If you select “None”, normalised images will be used instead and spots will be detected from a camera’s green channel. If you select a visual model, images will be first converted into cone catch values for the model and spots will be detected using double cones channel.
- **Scale images (px/mm)**: minimum px/mm factor in your set of photographs. It has to be first measured for the whole set using Batch Scale Bar Calculator in MICA Toolbox. If you would like to round it, then round down, never up!
- **Radius value for thresholding**: scale for calculating threshold when detecting spots. Something between 20 and 50 should work well.
- **Scale of Gaussian blur**: Gaussian blur corrects for shaded edges, scale should be similar (in pixels) to the scale of an egg.
- **Shrink selection**: it is worth to slightly decrease selection around the egg, to avoid measuring the background that egg lies upon. While MICA Toolbox fits egg shape very well, there is always a possibility that some thin areas of the background will be within selection. Further, some eggs just have a strange shape and it is difficult to fit the selection. The value that you type here is a percent of an egg’s width, e.g. 2 will decrease selection of each egg by 2% of its width.
- **Number of slices**: number of horizontal bars that eggs will be cut into. You can select one of following values: 2, 3, 4, 6, 10. When selecting 2, egg will be cut into two halves and percent of area covered by spots in both halves will be compared. When selecting 10, egg will be cut into 10 slices and percent of area covered by spots will be compared for all of them. And so on.



After clicking “OK”, analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with “Processing finished” information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named “Results_patternDispersion_temp.csv”) is saved in the folder with pictures, so in the case script aborted, you won’t lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren’t analysed yet. If you would like to keep the table with temporary results, please, change the file’s name – when script will be run again, original temporary file will be overwritten.

In the file with results there are following columns:

- Label: ID of an egg constructed as name of mspec file + name of ROI;
- Mean: average percent of area covered with spots for all slices
- SD: standard deviation of average percent of area covered with spots for all slices
- CV: coefficient of variation, SD divided by mean and multiplied by 100. It tells us how dispersed spots are along the long axis of the egg. Lower values indicate eggs that have spots evenly dispersed across the whole surface. Higher values suggest that spots are concentrated in one part, for example they can create a corona ring or concentrate at one end.



CV = 126.77 CV = 46.96

spots colour trichromatic

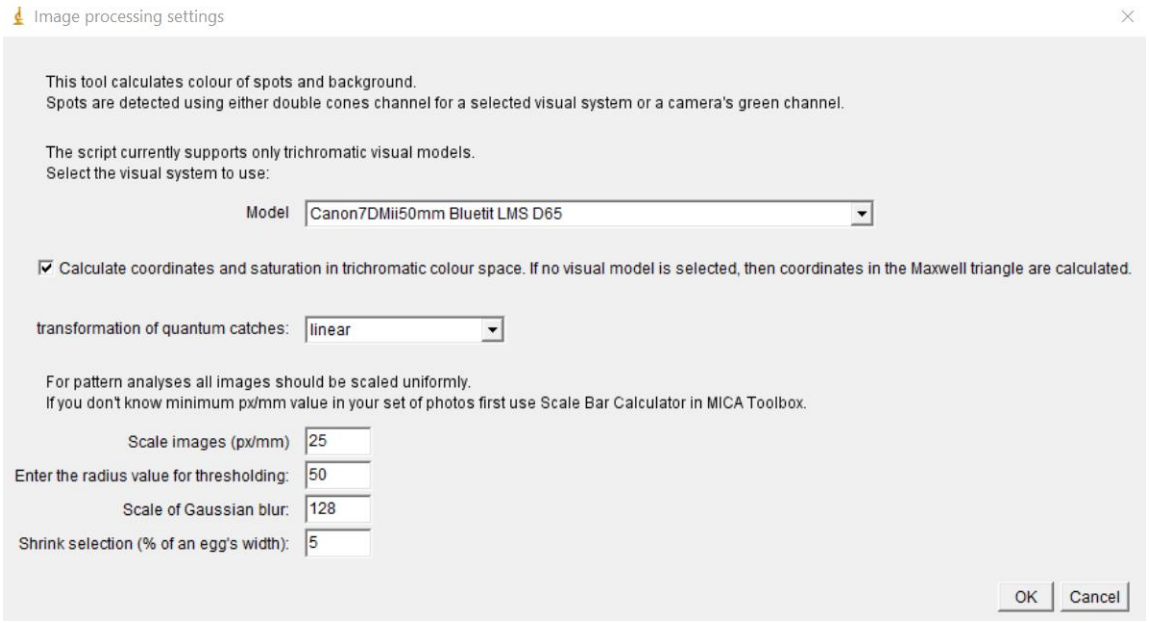
It works only with pictures taken in visible light (three channels: red, green and blue – no UV) or with images transformed to trichromatic models, e.g. Bluetit LMS D65 or Peafowl LMS D65 models.

Select in the Menu Bar: Plugins -> SPOTS_v2 -> spots colour trichromatic. Next select a folder, where are saved all your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear.

- **Model**: If you select "None", normalised images will be used instead and spots will be detected from a camera's green channel. Spots and egg's background brightness will be calculated as sum of pixel values in red, green and blue channels, and colours as proportional values in red, green and blue channels (e.g. red / brightness) and achromatic contrast will be calculated as absolute difference between brightness of spots and background. If you select a visual model, images will be first converted into cone catch values for the model and spots will be detected using double cones channel. Spots and background brightness will be calculated as values in double cone channel (so we can call it luminance in that case) and colour will be calculated as quantum catch values for each cone type. Achromatic contrast will be calculated as absolute value of the difference between logarithm of luminance of spots and logarithm of luminance of background divided by Weber fraction (assumed to be 0.05), following equation 7 in (Siddiqi et al., 2004).
- **Calculate coordinates and saturation in trichromatic colour space**: if selected, the script will calculate X and Y coordinates in trichromatic colour space and saturation (a distance of a point from achromatic centre). If no visual model was selected, these will be coordinates in the Maxwell triangle that is an objective trichromatic colour space that does no assume any specific visual system (Renoult et al. 2017). To compute coordinates in the Maxwell triangle, I used equations A2, A4 and A4 from (Kelber et al., 2003). If a visual model was selected, X coordinate can be interpreted as red-green opponency and Y coordinate as blue-yellow opponency in log-version of Receptor Noise Limited models (Vorobyev and Osorio, 1998) and saturation as a distance of a point from achromatic centre. To compute coordinates using RNL models, I used equations B5 and B6 from (Kelber et al., 2003). Chromatic contrast between spots and background in both cases is calculated as Euclidian distance between two points using equation B7 from (Kelber et al., 2003).
- **Transformation of quantum catches**: If a visual model is selected, photoreceptor signals can be linearly related to quantum catches or transformed to a logarithmic or hyperbolic scale. Here, I used equations A1.5, A1.6 and A1.7 from (Renoult et al., 2017).
- **Scale images (px/mm)**: minimum px/mm factor in your set of photographs. It has to be first measured for the whole set using Batch Scale Bar Calculator in MICA Toolbox. If you would like to round it, then round down, never up!
- **Radius value for thresholding**: scale for calculating threshold when detecting spots. Something between 20 and 50 should work well.
- **Scale of Gaussian blur**: Gaussian blur corrects for shaded edges, scale should be similar (in pixels) to the scale of an egg.

- **Shrink selection**: it is worth to slightly decrease selection around the egg, to avoid measuring the background that egg lies upon. While MICA Toolbox fits egg shape very well, there is always a possibility that some thin areas of the background will be within selection. Further, some eggs just have a strange shape and it is difficult to fit the selection. The value that you type here is a percent of an egg's width, e.g. 2 will decrease selection of each egg by 2% of its width.

If a visual model was selected, in the next step you will be asked to select Weber fractions. If you selected a Bluetit LMS model, then select "Bluetit LMS 0.05" Weber fraction. If you selected Peafowl LMS model, then select "Peafowl LMS 0.05". Pay attention, because "Bluetit 0.05" and "Bluetit LMS 0.05" are two very different things! To learn more about Weber fractions, please visit: http://www.empiricalimaging.com/knowledge-base/cone-ratios/.



After clicking "OK", analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with "Processing finished" information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named "Results_spotsColour_temp.csv") is saved in the folder with pictures, so in the case script aborted, you won't lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren't analysed yet. If you would like to keep the table with temporary results, please, change the file's name – when script will be run again, original temporary file will be overwritten.

Depending on whether you selected a visual model or not and whether you selected "Calculate coordinates…" option, results will look slightly different.

- Label: ID of an egg constructed as name of mspec file + name of ROI – this column will be always.

Columns in results file with no visual model:

- spots_brightness – brightness of spots calculated as R + G + B for spots
- spots_Rchroma – how much spots are reflecting in red channel, calculated as R / brightness
- spots_Gchroma – how much spots are reflecting in green channel, calculated as G / brightness
- spots_Bchroma – how much spots are reflecting in blue channel, calculated as B / brightness
- bg_brightness – brightness of egg's background, calculated as R + G + B for background
- bg_Rchroma – how much background is reflecting in red channel, calculated as R / brightness
- bg_Gchroma – how much background is reflecting in green channel, calculated as G / brightness
- bg_Bchroma – how much background is reflecting in blue channel, calculated as B / brightness
- dL – achromatic contrast between spots and background, calculated as absolute difference between brightness of spots and brightness of background.

Additionally, if you selected "calculate coordinates…" option, there will be:

- spots_X – X coordinate in the Maxwell triangle for spots (higher values mean more red colour, lower – more green)
- spots_Y – Y coordinate in the Maxwell triangle for spots (higher values mean more blue colour, lower – more yellow)
- spots_Sat – saturation of spots (distance from achromatic centre)
- bg_X – X coordinate in the Maxwell triangle for background (higher values mean more red colour, lower – more green)
- bg_Y – Y coordinate in the Maxwell triangle for background (higher values mean more blue colour, lower – more yellow)
- bg_Sat – saturation of background (distance from achromatic centre)
- dS – chromatic contrast between spots and background, calculated as Euclidian distance between the point for spots and the point for background in the chromatic space.

Columns in results file when a visual model was selected:

- spots_lum – luminance of spots calculated as quantum catch of double cones for spots
- spots_lw – how much long-sensitive cones are stimulated
- spots_mw – how much medium-sensitive cones are stimulated
- spots_sw – how much short-sensitive cones are stimulated
- bg_lum – luminance of background calculated as quantum catch of double cones for background
- bg_lw – how much long-sensitive cones are stimulated
- bg_mw – how much medium-sensitive cones are stimulated
- bg_sw – how much short-sensitive cones are stimulated

- dL – achromatic contrast between spots and background, calculated as dL = | (log(bg_lum) – log(spots_lum)) / 0.05 | following equation 7 in Siddiqi et al. (2004).

Additionally, if you selected “calculate coordinates…” option, there will be:

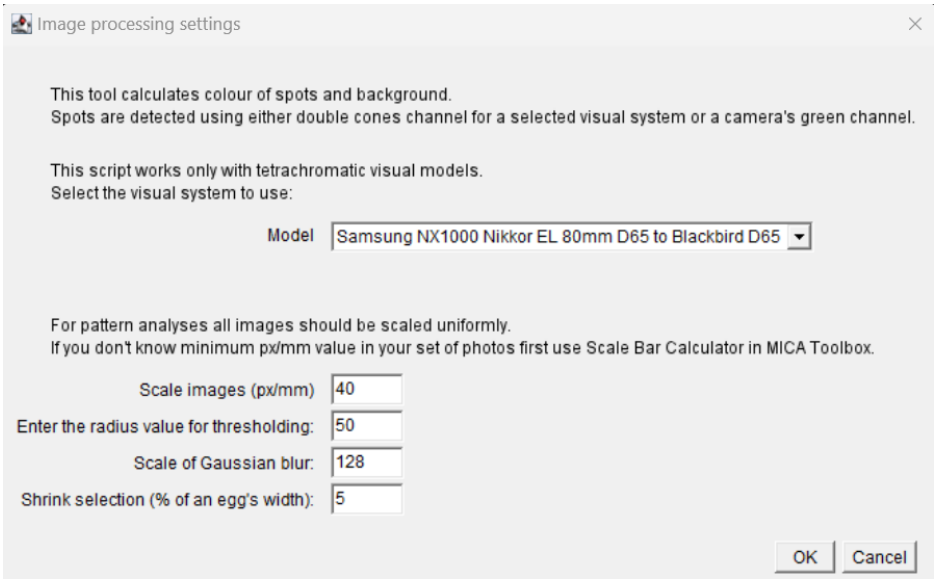
- spots_X – red-green opponency in RNL models (higher values mean more red colour, values around zero grey colour and the more below zero, the more green)
- spots_Y – blue-yellow opponency in RNL models (higher values mean more blue colour, values around zero grey colour and the more below zero, the more yellow)
- spots_Sat – saturation of spots (distance from achromatic centre)
- bg_X – red-green opponency in RNL models (higher values mean more red colour, values around zero grey colour and the more below zero, the more green)
- bg_Y – blue-yellow opponency in RNL models (higher values mean more blue colour, values around zero grey colour and the more below zero, the more yellow)
- bg_Sat – saturation of background (distance from achromatic centre)
- dS – chromatic contrast between spots and background, calculated as Euclidian distance between the point for spots and the point for background in the chromatic space.

spots colour tetrachromatic

It works only with pictures taken in both visible and UV light (five channels: red, green, blue, UVB and UVR) or with images transformed to tetrachromatic models.

Select in the Menu Bar: Plugins -> SPOTS_v2 -> spots colour tetrachromatic. Next select a folder, where are saved all your normalised images that you would like to measure (RAW files, together with mspecs and zip folders). After that a window with settings will appear.

- **Model**: If you select "None", normalised images will be used instead and spots will be detected from a camera's green channel. Spots and egg's background brightness will be calculated as sum of pixel values in all channels, and colours as proportional values (e.g. Rchroma = red / brightness, UVchroma = (UVB + UVR) / brightness). Achromatic contrast will be calculated as absolute difference between brightness of spots and background. If you select a visual model, images will be first converted into cone catch values for the model and spots will be detected using double cones channel. Spots and background brightness will be calculated as values in double cone channel (so we can call it luminance in that case) and colour will be calculated as quantum catch values for each cone type. Achromatic contrast will be calculated as absolute value of the difference between logarithm of luminance of spots and logarithm of luminance of background divided by Weber fraction (assumed to be 0.05), following equation 7 in (Siddiqi et al., 2004).
- **Scale images (px/mm)**: minimum px/mm factor in your set of photographs. It has to be first measured for the whole set using Batch Scale Bar Calculator in MICA Toolbox. If you would like to round it, then round down, never up!
- **Radius value for thresholding**: scale for calculating threshold when detecting spots. Something between 20 and 50 should work well.
- **Scale of Gaussian blur**: Gaussian blur corrects for shaded edges, scale should be similar (in pixels) to the scale of an egg.
- **Shrink selection**: it is worth to slightly decrease selection around the egg, to avoid measuring the background that egg lies upon. While MICA Toolbox fits egg shape very well, there is always a possibility that some thin areas of the background will be within selection. Further, some eggs just have a strange shape and it is difficult to fit the selection. The value that you type here is a percent of an egg's width, e.g. 2 will decrease selection of each egg by 2% of its width.



After clicking “OK”, analyses will launch. In that time it is better not to use computer to avoid interfering with analyses. When measurements will be finished a window with “Processing finished” information will pop up and then you will be asked to select directory where you would like to save the results and to provide name for the file (all results are saved as csv files). During analyses, a temporary table with results (named “Results_spotsColour_temp.csv”) is saved in the folder with pictures, so in the case script aborted, you won’t lose all results. In this file you can check at which moment the script stopped working and re-run analyses only on the files that weren’t analysed yet. If you would like to keep the table with temporary results, please, change the file’s name – when script will be run again, original temporary file will be overwritten.

Depending on whether you selected a visual model or not, results will look slightly different.

- Label: ID of an egg constructed as name of mspec file + name of ROI – this column will be always.

Columns in results file with no visual model:

- spots_brightness – brightness of spots calculated as R + G + B + UVB + UVR for spots
- spots_Rchroma – how much spots are reflecting in red channel, calculated as R / brightness
- spots_Gchroma – how much spots are reflecting green channel, calculated as G / brightness
- spots_Bchroma – how much spots are reflecting in blue channel, calculated as B / brightness
- spots_UVchroma – how much spots are reflecting in UV channels, calculated as (UVB + UVR) / brightness
- bg_brightness – brightness of egg’s background, calculated as R + G + B + UVB + UVR for background
- bg_Rchoma – how much background is reflecting in red channel, calculated as R / brightness

- bg_Gchroma – how much background is reflecting in green channel, calculated as G / brightness
- bg_Bchroma – how much background is reflecting in blue channel, calculated as B / brightness
- bg_UVchroma – how much background is reflecting in UV channels, calculated as (UVB + UVR) / brightness
- dL – achromatic contrast between spots and background, calculated as absolute difference between brightness of spots and brightness of background.

Columns in results file when a visual model was selected:

- spots_lum – luminance of spots calculated as quantum catch of double cones for spots
- spots_lw – how much long-sensitive cones are stimulated
- spots_mw – how much medium-sensitive cones are stimulated
- spots_sw – how much short-sensitive cones are stimulated
- spots_uv – how much very short-sensitive cones (UVS or VS) are stimulated
- bg_lum – luminance of background calculated as quantum catch of double cones for background
- bg_lw – how much long-sensitive cones are stimulated
- bg_mw – how much medium-sensitive cones are stimulated
- bg_sw – how much short-sensitive cones are stimulated
- bg_uv – how much very short-sensitive cones (UVS or VS) are stimulated
- dL – achromatic contrast between spots and background, calculated as dL = | (log(bg_lum) – log(spots_lum)) / 0.05 | following equation 7 in Siddiqi et al. (2004).

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