Protocol for acquisition and analysis of Oxymap T1 oximetry images
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Current version

- This protocol will be updated when needed and the date changed. The current version is from **November 21, 2013**
- This protocol is for general oximetry measurements and works for many studies but not all. Other protocols have to be written for special cases.
- The protocol is written for the use of Oxymap T1 oximeter and Oxymap Analyzer software, version 2.4. (revision 6813).
- This protocol is divided two main parts; **part A for image acquisition**, **part B for image analysis**. Parts A and B are divided into “background” and “procedure”. The **Appendix** at the end of the document provides further explanations. See also the **References**.
- Video tutorials, explaining the main ingredients of the analysis are available at the **Retinal oximetry** Youtube channel.
- Questions? Suggestions? Write to **sveinnha@hi.is**

Version history

- **April 3 2013**: “Original” version of this document (draft). This was built on previous more informal notes.
- **October 9 2013**: Slight updates of document without changes to the protocol itself (figures updated and minor changes made to the text).
- **October 22 2013**:
  - 20°/35° images of the macula made optional.
  - Analysis never continued over vessel crossings, even if the smaller daughter branch is narrower than 6 pixels in diameter (which was the only exception before).
  - Other changes to the text and layout do not affect the protocol itself.
- **November 21 2013**:
  - Recommendations for focus adjustment (eye piece) omitted.
A. Image acquisition

Image acquisition - background

The purpose of this part of the protocol is to help the researcher obtain standardised, good quality oximetry images.

Image alignment (angle of gaze) has been shown to affect the results of oximetry measurements\(^1\). Figure 1 illustrates an example of this.

![Figure 1](image1.png)

**Figure 1.** An example of the effect of image alignment on oximetry results. In figure 1A, the optic nerve head is at the centre of the image. In figure 1B, the subject looks more downwards, which means that the retinal vasculature shifts upwards in the image frame. According to a recent study\(^1\), the measured oxygen saturation in the same venules is on average 1.9 percentage points higher in images, which have the optic disc displaced upwards as in figure B (compared to A). The corresponding difference for arterioles is 1.3 percentage points.

The effects of image alignment, shown in figure 1, mean that the quality of the data will be increased if images with similar alignment are compared.

Image quality can also affect the oximetry results. Experience indicates that poor contrast / hazy or grainy images and shadows tend to produce lower saturation values than would be seen in better quality images of the same eye. Poor contrast may for example be caused by cataract, poor dilation of the pupil or poor alignment of the fundus camera. Glare and poor focus are less common sources of problems but should be minimised nonetheless. Image quality is difficult to define and studies on the effect of image quality on oximetry results are on-going. Later updates of this protocol may address the issue of image quality differently.

Figures 2-4 show examples of different image quality.
Figure 2. Good quality image.

Figure 3. Image with poor contrast. A grey haze covers the image.
The physiological state of subject and his/her eye can potentially influence the measurements. Below are some potential factors:
- Various medications and the time from when they were last taken.
- Dark / light adaptation.²
- Dry eye / wet eye to the extent that image quality is affected.
- Blood pressure / exercise (for example walking stairs) immediately prior to measurement³.
- Caffeine?
- Smoking?

**Image acquisition - procedure**

**Preparation of the subject (1-3):**

1. The subject should not have performed strenuous activity for at least one hour before measurement. Allow the subject to sit and relax for at least 15 minutes before the measurement is made (use the same ambient light for all subjects). The time may be used for explaining the study and for taking the subject’s history as well as for waiting for dilating drops to function.
2. Dilate pupils adequately. Use the same dilating agent in the same amount whenever possible. When possible, register the type of dilating drops, the amount applied and the time
of application. Register the approximate pupil size at the time of oximetry (use reference circles).

3. Register medical history (diseases, medication, smoking history). Also register approximate time from last intake of caffeine and nicotine.

Room and camera preparation (4-6):

4. Perform all measurements under the same ambient light conditions, in a well-ventilated room. It is recommended that ambient light is kept to a minimum since this makes it easier to obtain good quality images.

5. Following are the standard settings of the fundus camera for oximetry. Only change these if necessary. Register all settings:
   a. Filter wheel should be set at the filter for retinal oximetry (F2 or T setting).
   b. Aiming light setting: Lowest possible setting for good quality imaging.
   c. Flash setting: 50Ws (see Appendix).
   d. Small aperture setting ON (increases depth of field and therefore reduces focus problems).
   e. Large pupil setting ON (use small pupil setting when needed but register this every time).
   f. Make sure that the eyepiece is correctly adjusted for focus.

6. Refer to the Topcon fundus camera manual (and other material) for information on how to take good quality fundus images. Learn, for example, how to use the alignment aid (green dots) and split lines for focus assistance (option only available in Topcon Type IA).

Imaging (7-9):

7. If both eyes are imaged, complete imaging of one eye before starting with the other eye.

8. Take images in the following order (see figures 5-7, note that this has been simplified from a previous protocol):
   a. 50° setting. The optic disc centred.
   b. Image a repeated.
   c. Other images, depending on the particular study. For example a 20° or 35° image of the macula.

9. Write in the Comments field (for individual images or the session as a whole) if the quality of the images is in doubt. Note for example if image acquisition was difficult due to poor cooperation, poor dilation or cataract (etc.).
Figure 5. Image a in the protocol. Optic disc in the centre. 50° image.

Figure 6. Image b in the protocol (image a repeated). 50° image.

Figure 7. Image c in the protocol. An optional image with the macula centred. 20° image.
Note: Repeat imaging until quality is as good as possible (obtain a good quality image in position a and then move to b etc.). It is of course possible to use other image sequences but it is preferable to use always the same sequence for data that needs to be compared. This is explained in the Appendix.

Note: The Oxymap Analyzer software is optimised for use with images taken with the 50° setting. There is little experience yet with images taken with the 20° setting.
B. The image analysis protocol

A video tutorial, explaining the main ingredients of the image analysis are available at the Retinal oximetry Youtube channel.

Image analysis protocol - background

The purpose of this part of the protocol is to help the researcher analyse oximetry images in a standardised manner so that meaningful comparisons can be made between datasets.

The end result of this analysis protocol is mean oxygen saturation in retinal arterioles and venules, weighted by the vessel diameter in the power of four (to approximate weighing with blood flow – see calculation of mean at the end of this section).

Image analysis protocol - procedure

This protocol describes the analysis of 50° images with the optic disc in the centre. At the moment, no protocol has been written for other images.

1. Randomise the order of subjects before analysis. During the course of the analysis of, for example, images from 100 subjects, the person, who analyses the images, may inadvertently make subtle changes in the analysis procedure. If, for example, all the younger people are analysed first and all the older people last, this could create a bias.

2. Image quality:
   a. Check all notes made at the time of image acquisition.
   b. Adjust brightness and contrast in the Oxymap Analyzer program to be better able to evaluate image quality (see figure 8).

![Figure 8](image.png)

*Figure 8*. The brightness and contrast settings can be accessed by clicking the icon in the lower left corner in the program.

   c. Evaluate if the image is of sufficient quality. Observe the following characteristics of the image (most easily observed by removing colour map overlay (F9) and toggling F2/F3):
      i. Contrast (is there haze in the image / is it grainy?).
      ii. Shadows, if present.
exclusion criteria:

iii. Focus.
iv. “Glare” (increased brightness in specific area(s)).
v. Other abnormalities.

Exclude images from analysis if they are of poor quality. Write “nb” (nota bene), followed by an explanation in Notes in the analysis table if the quality is marginal for some reason (measurement included with a comment). The “nb” facilitates searching in Excel files later. The researcher may decide to test the data with and / or without data of marginal quality but should maintain consistency in all comparisons.

d. In this version of the protocol, there are no firm guidelines for excluding images due to poor image quality. It may be feasible to collect reference images to better standardise which images are excluded due to poor quality. A sequence of reference images may also be used to evaluate if image quality is similar in any two groups that are being studied and compared.

3. For each eye, choose the first good quality 50° image in the series, where the optic disc is in the centre and measure this.

4. The aim of the analysis is to measure (almost) all blood entering and leaving with the optic nerve. Therefore, all vessels (above minimum diameter) around the optic disc are measured. Criteria for choosing vessel segments for measurement:

a. Width should be 6 pixels or more. 6-8 pixels may be rather narrow and it may be more reliable to use only vessels with diameter of 8 pixels or more. Even if the 6-8 pixels vessels are included in the measurements, the user can select to exclude any measurements he / she wants during the analysis of the data in Excel (or other spreadsheet programs). Minimum valid vessel width can be adjusted by opening Tools -> Preferences.

b. Using the circle tool, draw two circles, concentric with the optic disc, with diameters 1.5 times and 3 times the optic disc diameter (see figure 11, below). All measurements should be made within the area between these circles.

c. Exclude vessel branching (the branching itself and a few extra pixels on each side). This can be done with the exclusion tool and a convenient setting for this tool is 15 pixels. Exclude also vessel crossings. See figure 9.

![Figure 9. Excluded branching point (lower arrow) and excluded crossing of vessels (upper arrow).](image)

d. Measurement points can be visualised by pressing F7 (when selection is active). Exclude vessel segments if the background points are affected by extremes in brightness (undetected nearby vessels, laser scars, haemorrhages etc.). If the program moves the background points from such extremes, check if the background...
points are in a retinal area with similar background as is (or should be) close to the vessel being measured. See figure 10.

**Figure 10.** Background vessel points (small red dots) on a haemorrhage. This is the view when F7 has been pressed and F9 to remove the pseudocolour for the saturation. The points on the haemorrhage are encircled with blue. The segment between the two blue bars should be excluded, mostly due to the haemorrhage but also due to the close by vessel branching. (The blue colour is added to this image for clarification and is not from the Oxymap Analyzer).

e. Start as close to the inner circle as is allowed according to the above and measure either up to the next branching (figure 11) or up to the larger circle if there is no branching (figure 12). See also item f for minimum length.

**Figure 11.** A vessel segment (white borders, marked with arrow) is chosen from the inner circle and up to the first branching. The immediate vicinity of the vessel branching is excluded (white circles).
f. The minimum length of a vessel segment should be 50 pixels. If there are less than 50 pixels from the start (at the inner circle) to a branching of the vessel, then start the analysis from the vessel branching and measure the daughter branches, provided that they reach the minimum length of 50 pixels (see figure 13 below).

Do not combine measurements on both sides of vessel branching.
Figure 13. The chosen vessel segment (red arrow) on the figure is too short (<50 pixels after the immediate vicinity of the branching has been excluded). The analysis should therefore be started on the other side of the vessel branching and both daughter branches should be measured as separate vessels up to the outer circle (white arrows).

5. Documentation of the measurements in the analysis table and order of measurements:
   a. In notes write od or os for right or left eye.
   b. Divide the retina into superotemporal (st), superonasal (sn), inferonasal (in) and inferotemporal (it) quadrants. In notes, each vessel will be labelled st/sn/in/it according to the quadrant it belongs to. If it is not clear, which quadrant the vessel supplies or drains write s, i, t, n as appropriate.
   c. Start measuring the st arteriole, which is lowest in that part of the image. Label this as a1 in Notes in the analysis table. Then choose the arteriole above (a2) and continue around the optic disc (clockwise for OD and counter-clockwise for OS). Repeat for veins (v1, v2...).
   d. If the measurement is, for some reason, thought to be unreliable, write nb (nota bene) and explain why the measurement is questionable (this makes it easy to find suspicious data later with the Find command in Excel).
   e. Example of notation in Notes in the analysis table: od a1 st

6. Name and label the analysis file carefully so that it can be easily seen what was analysed, by whom and when.

7. In the Excel files, where the measurements are gathered, write the no. of the version of OxyMap Analyzer used and the parameters from the program’s Preferences menu (a, b, c, d and minimum valid vessel width).
Calculation of means

Mean oxygen saturation has previously been calculated by simply averaging measurements from all arterioles or all venules in an image (over a certain diameter). This does not take into account the different blood flow in these vessels. In an attempt to partially correct for this, the means can be weighted with the fourth power of the diameter. The fourth power is used because blood flow is related to diameter in the fourth power. If, for example, eight venules were measured in an eye and each has measured saturation $S_i$ and diameter $d_i$, the mean saturation becomes:

$$\text{Mean oxygen saturation} = \frac{S_1 * d_1^4 + S_2 * d_2^4 + S_3 * d_3^4 + S_4 * d_4^4 + S_5 * d_5^4 + S_6 * d_6^4 + S_7 * d_7^4 + S_8 * d_8^4}{d_1^4 + d_2^4 + d_3^4 + d_4^4 + d_5^4 + d_6^4 + d_7^4 + d_8^4}$$

**Note:** The benefit of weighing with diameter in the fourth power is that the mean saturation should be closer to the actual mean saturation in the retinal circulation. There is some risk that the added complexity of the calculation means that the result will be less stable, i.e. that the variance will increase. According to recent testing, the increase in variability is not great, see table.

### Table.
Standard deviation of ten repeated measurements of one healthy eye.

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<th>All major retinal vessels (simple mean)</th>
<th>All major vessels, weighted with (diameter)$^4$</th>
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<tr>
<td><strong>Arterioles</strong></td>
<td>0.30</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>Venules</strong></td>
<td>1.29</td>
<td>1.94</td>
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Further studies on the effect of weighing with diameter in the fourth power are on-going. Examining un-weighted averages and comparing them to weighted averages is still a good idea. Other possible methods include choosing only the four main vessel pairs and calculating the simple average. The rule could for example be to choose the widest arteriole and widest venule in each quadrant.
C. Appendix

Standardisation in general – with or without evidence of the effect of a particular parameter

This protocol attempts to improve the quality of the data and the validity of analyses and comparisons. Some of the parameters, which are standardised here, have been shown to affect the results, while there is only suspicion that other factors might influence the results. We know for example that image alignment (angle of gaze) can have an effect while the effect of the flash intensity is not well known. Nevertheless, it is generally a good idea to standardise as many procedures and parameters as possible in a scientific study. This usually decreases the chance of confounding effects. If care is taken when measuring and analysing two groups of subjects in the same way, it is more likely that the differences found are really due to the factor of interest (for example a disease) rather than due to differences in methodology.

Justification for image sequence

According to this protocol, two good quality 50° images with the optic nerve in the centre should be obtained and then there is an option of capturing a macular image at 20°or 35°. More images are captured if some of the three images are not successfully captured in the first try. The first two images are taken in the same fashion, i.e. with the optic disc in the centre. The repetition provides the opportunity to check repeatability (consistency) of the data. Furthermore, possible effects of flash exposure could be elucidated by comparing images from the beginning of the sequence to images later in the sequence (see effect of light / dark below).

The effect of light / dark, including flash

In a study comparing oxygen saturation during darkness and during normal office light, the difference in saturation was about 3-5 percentage points on average. This study used an oximeter, which was based on a non-mydriatic fundus camera and the subjects were healthy. The current Oxymap T1 oximeter uses a mydriatic fundus camera. Therefore, all measurements are taken after some light adaptation has occurred. If the level of light adaptation varies, it is unlikely that this will have as large effect as the shift from total darkness to office light. In other words, it is likely that the effects of slight changes in light adaptation of the retina on oxygen saturation are less than 3-5 percentage points. Nonetheless, it is advised to standardise light exposure.

Similarly, exposure to the flashes in the fundus camera (and time between images) should also be standardised as possible. In a recent study, images number two and five in an image sequence gave very similar oxygen saturation, indicating the flashes number two, three and four did not have large influence on measurement number five.

Another study looked at the influence of flash intensity on oximetry measurements and found that increased flash intensity led to increased measured oxygen saturation, particularly outside certain limits of flash intensity. This should clearly be regarded as an artefact. It should be noted that this study was performed with a different oximeter (from Imedos Systems), which uses different wavelengths. Nevertheless the possibility exists that flash intensity influences the results of Oxymap T1. While this potential cause of error is investigated, it is recommended that flash
setting 50W is used for all subjects and that care is taken that images of groups being compared have similar brightness.

**Calibration parameters**

(See also the appendix of the Oxymap T1 instruction manual). The Oxymap Analyzer software uses a set of four parameters to calculate oxygen saturation from optical density ratios and vessel diameters. The parameters can be seen under Tools -> Preferences. This set of parameters was found with calibration¹, using data from Icelandic healthy individuals. The calibration should in general work with most datasets but can be changed if another (local) calibration is performed. It is important to keep note of the calibration parameters used in each study and to compare only values acquired with the same calibration parameters. Only change the calibration parameters if you have good reason to do so, i.e. if you have performed a thorough calibration yourself. In general, it is advisable that each laboratory has its own normative data and this may be used for revising the calibration if the calibration appears to be different from the default. Information on how to perform your own calibration can be requested from Oxymap or from sveinnha@hi.is.
D. References