

Skin autofluorescence for the risk assessment of chronic complications in diabetes: a broad excitation range is sufficient

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Abstract: Skin autofluorescence (AF) is becoming an accepted clinical method for assessing the risk of chronic complications in diabetes mellitus (DM). In this study, the role of the excitation wavelength in the recognition of increased risk of diabetes-related chronic complications was investigated. An Excitation Emission Matrix Scanner (EEMS) was used to perform non-invasive measurements in four age-matched groups of patients with type 1 and type 2 DM, with and without chronic complications, as well as in a control group (N=97 in total). AF was calculated for excitation wavelengths in the range 355 – 405 nm. Mean spectra were assessed per group. AF values in both type 1 and type 2 DM patients with complications were increased compared to the control subjects ($p < 0.01$); this ratio remained practically constant, independent of the excitation wavelength. No emission peaks were distinctive for specific patient groups. We conclude that in these groups, no characteristic fluorophores dictate the use of a specific wavelength or set of wavelengths. The results show the validity of applying a broad excitation wavelength range for risk assessment of chronic complications in diabetes.

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References and links

1. K. König and H. Schneckenburger, "Laser-induced autofluorescence for medical diagnoses," *J. Fluorescence* **4**, 17–40 (1994).
2. N. Ramanujam, *Fluorescence spectroscopy in vivo*, vol. Biomedical Spectroscopy of *Encyclopedia of Analytical Chemistry*, (John Wiley & Sons Ltd, Chichester, 2000) pp. 20–56.
3. M. C. Skala, G. M. Palmer, K. M. Vrotsos, A. Gendron-Fitzpatrick, and N. Ramanujam, "Comparison of a physical model and principal component analysis for the diagnosis of epithelial neoplasias in vivo using diffuse reflectance spectroscopy," *Opt. Express* **15**, 7863–7875 (2007). URL <http://dx.doi.org/10.1364/OE.15.007863>.
4. R. Na, I. M. Stender, M. Henriksen, and H. C. Wulf, "Autofluorescence of human skin is age-related after correction for skin pigmentation and redness," *J. Invest. Dermatol.* **116**, 536–540 (2001). URL <http://dx.doi.org/10.1046/j.1523-1747.2001.01285.x>.

5. H. Chang, J. Qu, P. Yuen, J. Sham, D. Kwong, and W. Wei, "Light-induced autofluorescence spectroscopy for detection of nasopharyngeal carcinoma in vivo," *Appl. Spectrosc.* **56**, 1361–1367 (2002).
6. L. Brancalione, A. J. Durkin, J. H. Tu, G. Menaker, J. D. Fallon, and N. Kollias, "In vivo fluorescence spectroscopy of nonmelanoma skin cancer," *Photochem. Photobiol.* **73**, 178–183 (2001).
7. D. C. G. de Veld, M. Skurichina, M. J. H. Witjes, R. P. W. Duin, H. J. C. M. Sterenborg, and J. L. N. Roodenburg, "Autofluorescence and diffuse reflectance spectroscopy for oral oncology," *Lasers Surg. Med.* **36**, 356–364 (2005). URL <http://dx.doi.org/10.1002/lsm.20122>.
8. D. Goujon, M. Zellweger, A. Radu, P. Grosjean, B.-C. Weber, H. van den Bergh, P. Monnier, and G. Wagnières, "In vivo autofluorescence imaging of early cancers in the human tracheobronchial tree with a spectrally optimized system," *J. Biomed. Opt.* **8**, 17–25 (2003). URL <http://dx.doi.org/10.1117/1.1528594>.
9. M. Hammer, E. Nagel, D. Schweitzer, S. Richter, F. Schweitzer, E. Königsdörffer, and J. Strobel, "[Spectral separation in ocular fundus autofluorescence images in patients suffering from age-related macular degeneration]," *Ophthalmologie* **101**, 1189–1193 (2004). URL <http://dx.doi.org/10.1007/s00347-004-1019-z>.
10. R. Gillies, G. Zonios, R. R. Anderson, and N. Kollias, "Fluorescence excitation spectroscopy provides information about human skin in vivo," *J. Invest. Dermatol.* **115**, 704–707 (2000). URL <http://dx.doi.org/10.1046/j.1523-1747.2000.00091.x>.
11. L. T. Vo, P. Anikijenko, W. J. McLaren, P. M. Delaney, D. H. Barkla, and R. G. King, "Autofluorescence of skin burns detected by fiber-optic confocal imaging: evidence that cool water treatment limits progressive thermal damage in anesthetized hairless mice," *J. Trauma* **51**, 98–104 (2001).
12. J. Sandby-Møller, E. Thieden, P. A. Philipsen, J. Heydenreich, and H. C. Wulf, "Skin autofluorescence as a biological UVR dosimeter," *Photodermatol. Photoimmunol. Photomed.* **20**, 33–40 (2004).
13. V. M. Monnier, V. Vishwanath, K. E. Frank, C. A. Elmets, P. Dauchot, and R. R. Kohn, "Relation between complications of type I diabetes mellitus and collagen-linked fluorescence," *N. Engl. J. Med.* **314**, 403–408 (1986).
14. A. J. Smit and H. L. Lutgers, "The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceuticals to reduce AGE accumulation," *Curr. Med. Chem.* **11**, 2767–2784 (2004).
15. J. W. Baynes and S. R. Thorpe, "Glycoxidation and lipoxidation in atherogenesis," *Free Radic. Biol. Med.* **28**, 1708–1716 (2000).
16. M. Brownlee, "Advanced protein glycosylation in diabetes and aging," *Annu. Rev. Med.* **46**, 223–234 (1995). URL <http://dx.doi.org/10.1146/annurev.med.46.1.223>.
17. T. Miyata, A. Saito, K. Kurokawa, and C. van Ypersele de Strihou, "Advanced glycation and lipoxidation end products: reactive carbonyl compounds-related uraemic toxicity," *Nephrol. Dial. Transplant.* **16 Suppl 4**, 8–11 (2001).
18. R. Ramasamy, S. J. Vannucci, S. S. D. Yan, K. Herold, S. F. Yan, and A. M. Schmidt, "Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation," *Glycobiology* **15**(7), 16R–28R (2005). URL <http://dx.doi.org/10.1093/glycob/cwi053>.
19. J. J. Jager, P. H. N. Oomen, W. J. Sluiter, W. D. Reitsma, A. J. Smit, "Improved reproducibility of the 'large-window' method of assessing transcapillary and interstitial fluorescein diffusion in the skin in healthy subjects and in subjects with insulin-dependent diabetes mellitus," *Int. J. Microcirc. Clin. Exp.* **17**, 150–158 (1997).
20. T. Abiko, A. Abiko, S. Ishiko, M. Takeda, S. Horiuchi, and A. Yoshida, "Relationship between autofluorescence and advanced glycation end products in diabetic lenses," *Exp. Eye Res.* **68**, 361–366 (1999). URL <http://dx.doi.org/10.1006/exer.1998.0615>.
21. E. Hull, M. Ediger, A. Unione, E. Deemer, M. Stroman, and J. Baynes, "Noninvasive, optical detection of diabetes: model studies with porcine skin," *Opt. Express* **12**, 4496–4510 (2004).
22. N. Kollias, G. Zonios, and G. Stamatas, "Fluorescence spectroscopy of skin," *Vib. Spectrosc.* **28**, 17–23 (2002).
23. R. Na, I. M. Stender, and H. C. Wulf, "Can autofluorescence demarcate basal cell carcinoma from normal skin? A comparison with protoporphyrin IX fluorescence," *Acta Derm. Venereol.* **81**, 246–249 (2001).
24. R. Meerwaldt, R. Graaff, P. H. N. Oomen, T. P. Links, J. J. Jager, N. L. Alderson, S. R. Thorpe, J. W. Baynes, R. O. B. Gans, and A. J. Smit, "Simple non-invasive assessment of advanced glycation endproduct accumulation," *Diabetologia* **47**, 1324–1330 (2004). URL <http://dx.doi.org/10.1007/s00125-004-1451-2>.
25. R. Graaff, R. Meerwaldt, H. L. Lutgers, R. Baptist, E. D. de Jong, J. Zijp, T. P. Links, A. J. Smit, and G. Rakhorst, "Instrumentation for the measurement of autofluorescence in the human skin," *Proc. SPIE* **5692**, 111–118 (2005).
26. D. J. Mulder, T. V. D. Water, H. L. Lutgers, R. Graaff, R. O. Gans, F. Zijlstra, and A. J. Smit, "Skin autofluorescence, a novel marker for glycemic and oxidative stress-derived advanced glycation endproducts: an overview of current clinical studies, evidence, and limitations," *Diabetes Technol. Ther.* **8**, 523–535 (2006). URL <http://dx.doi.org/10.1089/dia.2006.8.523>.
27. H. L. Lutgers, R. Graaff, T. P. Links, L. J. Ubink-Veltmaat, H. J. Bilo, R. O. Gans, and A. J. Smit, "Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes," *Diabetes Care* **29**, 2654–2659 (2006). URL <http://dx.doi.org/10.2337/dc05-2173>.
28. R. Meerwaldt, J. W. L. Hartog, R. Graaff, R. J. Huisman, T. P. Links, N. C. den Hollander, S. R. Thorpe, J. W. Baynes, G. Navis, R. O. B. Gans, and A. J. Smit, "Skin autofluorescence, a measure of cumulative metabolic stress and advanced glycation end products, predicts mortality in hemodialysis patients," *J. Am. Soc. Nephrol.*

- 16, 3687–3693 (2005). URL <http://dx.doi.org/10.1681/ASN.2005020144>.
29. D. J. Mulder, P. L. van Haelst, S. Gross, K. de Leeuw, J. Bijzet, R. Graaff, R. O. Gans, F. Zijlstra, A. J. Smit, "Skin autofluorescence is elevated in patients with stable coronary artery disease and is associated with serum levels of neopterin and the soluble receptor for advanced glycation end products," *Atherosclerosis* **197**, 217–223 (2008). URL <http://dx.doi.org/10.1016/j.atherosclerosis.2007.03.027>.
30. J. Blaauw, A. J. Smit, M. G. van Pampus, J. J. van Doormaal, J. G. Aarnoudse, G. Rakhorst, and R. Graaff, "Skin autofluorescence, a marker of advanced glycation end products and oxidative stress, is increased in recently preeclamptic women," *Am. J. Obstet. Gynecol.* **195**, 717–722 (2006). URL <http://dx.doi.org/10.1016/j.ajog.2006.06.086>.
31. K. de Leeuw, R. Graaff, R. de Vries, R. P. Dullaart, A. J. Smit, C. G. Kallenberg, and M. Bijl, "Accumulation of advanced glycation endproducts in patients with systemic lupus erythematosus," *Rheumatology (Oxford)* **46**, 1551–1556 (2007). URL <http://dx.doi.org/10.1093/rheumatology/kem215>.
32. N. den Hollander, D. Mulder, R. Graaff, S. Thorpe, J. Baynes, G. Smit, and A. Smit, "Advanced Glycation end products and the absence of premature atherosclerosis in glycogen storage disease Ia," *J. Inherit. Metab. Dis.* **30**, 916–923 (2007). URL <http://dx.doi.org/10.1007/s10545-007-0507-0>.
33. E. G. Gerrits, H. L. Lutgers, N. Kleefstra, R. Graaff, K. H. Groenier, A. J. Smit, R. O. Gans, and H. J. Bilo, "Skin autofluorescence: a tool to identify type 2 diabetic patients at risk for developing microvascular complications," *Diabetes Care* **31**, 517–521 (2008). URL <http://dx.doi.org/10.2337/dc07-1755>.
34. R. Meervaldt, H. L. Lutgers, T. P. Links, R. Graaff, J. W. Baynes, R. O. B. Gans, and A. J. Smit, "Skin autofluorescence is a strong predictor of cardiac mortality in diabetes," *Diabetes Care* **30**, 107–112 (2007). URL <http://dx.doi.org/10.2337/dc06-1391>.
35. K. A. Destrampe and G. M. Hieftje, "New instrumentation for use in excitation-emission fluorescence-polarization measurements," *Appl. Spectrosc.* **47**, 1548–1554 (1993).
36. S. J. Hart and R. D. JiJi, "Light emitting diode excitation emission matrix fluorescence spectroscopy," *Analyst* **127**, 1693–1699 (2002). URL <http://dx.doi.org/10.1039/b207660h>.
37. J. W. Tunnell, A. E. Desjardins, L. Galindo, I. Georgakoudi, S. A. McGee, J. Mirkovic, M. G. Müller, J. Nazemi, F. T. Nguyen, A. Wax, Q. Zhang, R. R. Dasari and M. S. Feld, "Instrumentation for multi-modal spectroscopic diagnosis of epithelial dysplasia," *Technol. Cancer Res. Treat.* **2**, 505–514 (2003).
38. A. R. Young, "Chromophores in human skin," *Phys. Med. Biol.* **42**, 789–802 (1997).
39. N. Ahmed, P. J. Thornalley, "Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and intrinsic fluorescence," *Biochem. J.* **364**, 15–24 (2002).
40. D. C. G. D. Veld, M. J. H. Witjes, H. J. C. M. Sterenborg, and J. L. N. Roodenburg, "The status of in vivo autofluorescence spectroscopy and imaging for oral oncology," *Oral Oncol.* **41**, 117–131 (2005). URL <http://dx.doi.org/10.1016/j.oraloncology.2004.07.007>.
41. R. Na, I.-M. Stender, L. Ma, and H. C. Wulf, "Autofluorescence spectrum of skin: component bands and body site variations," *Skin Res. Technol.* **6**, 112–117 (2000).
42. V. M. Monnier, O. Bautista, D. Kenny, D. R. Sell, J. Fogarty, W. Dahms, P. A. Cleary, J. Lachin, and S. Genuth, "Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA1c as markers of diabetic complications. DCCT Skin Collagen Ancillary Study Group. Diabetes Control and Complications Trial," *Diabetes* **48**, 870–880 (1999).
43. S. Genuth, W. Sun, P. Cleary, D. R. Sell, W. Dahms, J. Malone, W. Sivitz, V. M. Monnier, and D. C. C. T. S. C. A. S. Group, "Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes," *Diabetes* **54**, 3103–3111 (2005).

1. Introduction

Autofluorescence of human tissue upon excitation with UV-A light, is caused by endogenous fluorophores including collagen, elastin, NADH, tryptophan and porphyrins [1, 2]. Application of tissue autofluorescence in vivo has been used previously to detect local disorders such as cancer [3–8], age-related macular degeneration [9], psoriasis [10], and skin burns [11] and the effects of chronic ultra violet exposure [12].

Moreover, determination of skin autofluorescence from biopsies has been used in studies on systemic diseases such as diabetes mellitus (DM) [13]. Advanced glycation endproducts (AGEs) that cross-link collagen, are the main source of collagen linked fluorescence (CLF) in skin biopsies and are classically determined by fluorescence at 440 nm upon excitation at

370 nm [13]. AGEs are products of glycemic and oxidative stress, and their formation is increased in pathological conditions like DM, renal disease, and also in atherosclerosis [14–18]. Tissue-AGE determination is usually performed in skin biopsies.

Several investigators have studied skin or lens autofluorescence non-invasively [19–23]. We developed an Autofluorescence Reader (AFR) for non-invasive measurement of skin autofluorescence (*AF*) [24–27]. With this AFR, a prototype of the current AGE Reader (DiagnOptics, The Netherlands), the skin on the volar side of the forearm was illuminated with light in an excitation range of 350 – 420 nm (maximum intensity at 370 nm), and emission in the range 420 – 600 nm was measured with a spectrometer. With these measurements, a strong correlation was not only found with CLF from dermal skin biopsies, but also with skin AGEs (pentosidine, N^ε-(carboxymethyl)lysine, N^ε-(carboxyethyl)lysine) from the same skin biopsies from DM patients, renal failure patients and healthy persons. These biopsies were taken from the same site as where the autofluorescence was measured [24, 28]. In later studies, *AF* has been studied in a range of other conditions such as atherosclerosis, preeclampsia, systemic lupus erythematosus (SLE), and glycogen storage disease (GSD) Ia. [29–32] Moreover, *AF* has been reported to be an independent predictor of macrovascular and microvascular complications [33] and of mortality in hemodialysis and DM patients [28, 34].

The spectrum of the UV-A light source as used in the AFR and in the AGE Reader is shown in Fig. 2 (continuous line). As shown above, this broad and fixed excitation range already provides important clinical information. However, it is not known which excitation wavelengths provide this information. For the investigation of the influence of excitation wavelength within the current excitation range, Excitation-Emission Matrices (EEMs) [35–37] can be used. An instrument was developed with a similar setup as in the AFR, but where excitation wavelengths could be varied: the Excitation-Emission Matrix Scanner (EEMS), as previously described by Graaff et al [25]. With this equipment, matrices of the amount of fluorescence as a function of excitation and emission wavelengths can be obtained from the skin in vivo to investigate the contributions of skin autofluorescence at excitation wavelengths within this range in various clinical conditions. This study will concentrate on EEMS measurements obtained from DM patients with and without DM-related chronic complications. Furthermore, the study will be limited to the excitation range of approximately 355 – 405 nm, as used in the AGE Reader, since this range already provides a very significant clinical parameter.

The aim of this study is first to determine whether specific excitation wavelengths within the currently used excitation range have to be preferred over the broad excitation peak for differentiation between diabetic and non-diabetic subjects or between diabetic subjects with and without DM-related chronic complications. And secondly, to evaluate whether differences in fluorescence emission occur that are associated with the presence of DM-related chronic complications.

2. Materials and methods

2.1. Subjects

EEMs were collected in groups of patients with type 1 and type 2 diabetes, with and without chronic complications, as well as in a control group, see Table 1. Diabetes patients were recruited from the University Medical Center Groningen. All participants gave informed consent to this study which was approved by the local medical ethics committee.

All participants had an age between 35 and 50 years. This limited range of age was chosen, since the amount of accumulated AGEs increases with age [13, 27]. Only Caucasian patients were included in this study, because dark skin may influence *AF* assessment [26]. We included 17–24 subjects in each group. Patients were classified as having chronic complications when retinopathy, neuropathy, microalbuminuria or macrovascular disease were present, all defined

according to definitions described in detail previously [27, 33]. Patients were classified as having no complications only if they were in good health, except for their DM status. Table 1 shows the characteristics of the five subject groups.

Table 1. Characteristics of the five groups. Where applicable, values are mean \pm 1 s.d.

Complications	DM type 1 no	DM type 1 yes	DM type 2 no	DM type 2 yes	Control -
N	19	17	21	16	24
Age (yr)	42.3 \pm 4.5	43.2 \pm 4.6	45.8 \pm 4.7	47.5 \pm 5.1	46.8 \pm 3.3
Gender (M:F)	8:11	6:11	15:6	10:6	7:17
Diabetes duration (yr)	20 \pm 11	28 \pm 7	5 \pm 1	9 \pm 6	-

2.2. Equipment and measurements

For generating excitation dependent emission spectra, an instrument had been developed where excitation wavelengths can be varied. This instrument was obtained by adaptation of a 0.2 m f/4 monochromator (PTI, New Jersey, USA), and was illuminated by a 75 W Xenon lamp. The monochromator has been connected to a measuring section, where a glass fiber (with a diameter of 200 μ m) is pointed at the measuring site (of approximately 4 cm²) under an angle of 45 degrees, as shown in Fig. 1. This glass fiber is connected to a spectrometer (USB2000, Avantes, Eerbeek, The Netherlands) with a spectral range of at least 300 to 750 nm and a resolution of approximately 0.3 nm. The spectral sensitivity equals that of the AFR and current AGE Reader. All spectra are immediately converted to a spectral resolution of 1 nm. The total setup, referred to as Excitation Emission Matrix Scanner (EEMS) has a similar setup as the previously described tool for measuring skin autofluorescence, the AFR [25]. Wavelength scanning of the monochromator was realized with a PC using a LabView program (National Instruments, Austin, USA). The spectra were obtained by this software as well. Measurements were performed on the volar side of the arm, approximately 10 cm below the elbow. A whole series of measurements, including measurements at 11 excitation wavelengths (355 – 405 nm, in steps of 5 nm) was performed in a few minutes. Each measurement provides the full spectrum, including the excitation and emission ranges. Dark spectra were also obtained and subtracted for each measurement to correct for dark current deviations in the applied CCD detector. The shape of the peak at each selected excitation wavelength has a standard deviation of 6.5 and the Full Width at Half Maximum (FWHM) is approximately 10 nm.

2.3. Analyses and statistics

Autofluorescence as obtained from the EEMS (AF_E) was defined for each measurement at a selected excitation wavelength separately and for all individual subjects as

$$AF_E(\lambda_{exc}, s) = 100 \frac{\langle I_{em} \rangle(\lambda_{exc}, s)}{\langle I_{exc} \rangle(\lambda_{exc}, s)}, \quad (1)$$

where $\langle I_{em} \rangle(\lambda_{exc}, s)$ is the mean measured light intensity in the range 420 – 600 nm and $\langle I_{exc} \rangle(\lambda_{exc}, s)$ is the mean intensity in the range 300 – 420 nm, both determined from the spectrum that was obtained with the selected excitation wavelength λ_{exc} and for subject s . By defining AF_E as a ratio of emission and reflected excitation light, AF_E is compensated for variations in lamp intensity and partly for changes in skin color. The same method is used in the AGE Reader to calculate AF from a spectrum [25, 26].

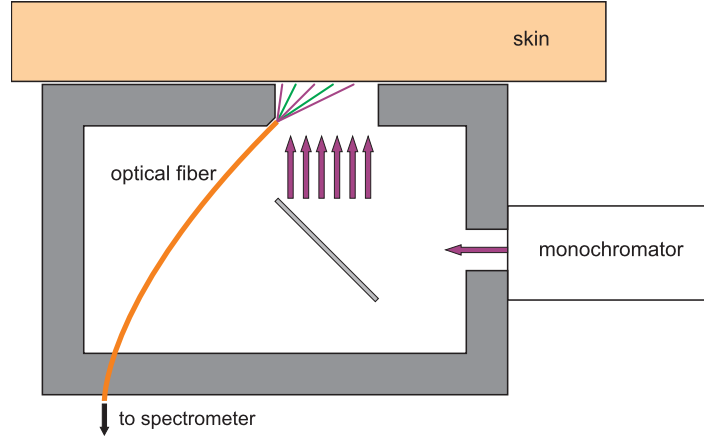


Fig. 1. Instrument schematic of the EEMS. UV excitation light from the monochromator is directed to the skin via a mirror. The emission and reflection light from the skin is guided to a spectrometer via an optical fiber, pointing at the measuring site at an angle of 45 degrees.

After calculating AF_E for all subjects and all excitation wavelengths, the mean, $\langle AF_E \rangle$, for subjects in the control group per excitation wavelength was used as a reference for all four DM subject groups.

For comparison between AF_E values of DM subject groups and the control group, we introduced relative autofluorescence for each excitation wavelength for the DM patients as

$$AF_{E,rel}(\lambda_{exc}, s) = \frac{AF_E(\lambda_{exc}, s)}{\langle AF_E \rangle(\lambda_{exc}, control)} \quad (2)$$

where $AF_E(\lambda_{exc}, s)$ is the AF_E of a patient in the DM subject group for excitation wavelength λ_{exc} and $\langle AF_E \rangle(\lambda_{exc}, control)$ is the mean AF_E of all subjects in the control group for that excitation wavelength. For each excitation wavelength and for all DM groups separately, a Mann-Whitney U-test was performed against the control group to evaluate the significance of the difference. These tests were performed using SPSS.

In the AGE Reader, a blacklight is used, emitting a broad band of UV light, as shown in Fig. 2. In order to derive an analysis for this light source as well, a weighted summation of measured responses on excitation at given wavelengths was used. The AF that would have resulted from the blacklight, AF_{BL} , per subject was calculated using

$$AF_{BL} = \frac{\sum w_i AF_i}{\sum w_i}, \quad (3)$$

where AF_i is AF_E as calculated from excitation peak i (where each i represents one of the excitation maxima between 355 and 405 nm) and the weight factor w_i was determined by multiple regression fitting of the separate peaks to the broad excitation peak. The broad excitation peak that can be reconstructed in this way is shown in Fig. 2 (dotted line) for comparison. The Mann-Whitney U-test calculations were also performed for AF_{BL} .

To analyze possible differences in the emission spectra between the groups, the spectra were all normalized. The mean value of intensity of the emission peak (in the range 450 – 600 nm) was taken as a reference. Subsequently, mean values were calculated (per nanometer) from the normalized spectra of all subjects in each group for each excitation wavelength. These mean spectra were used to observe whether emission peaks occurred that might be specific for a certain patient group.

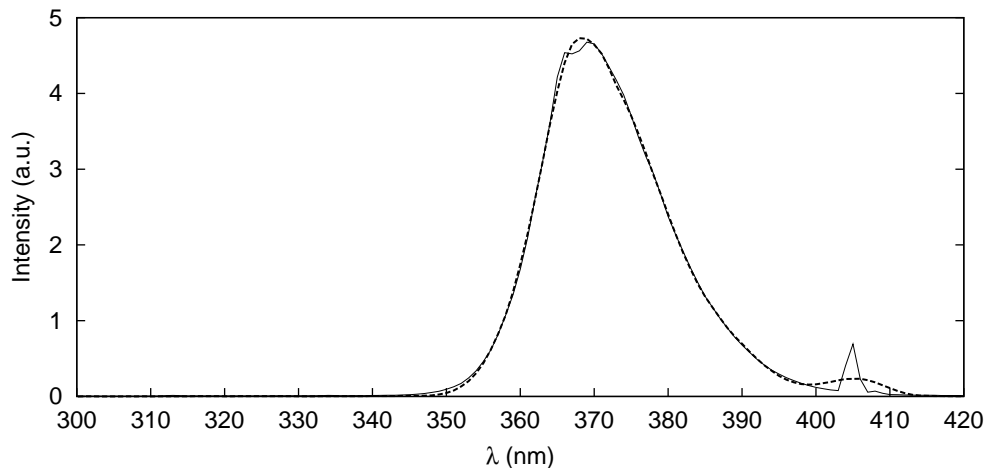


Fig. 2. Spectrum of the UV-A light source as used in the AFR and the AGE Reader (continuous line) and a fit using a weighted summation of EEMS responses of separate excitation peaks (dotted line). The small peaks at 366 nm and at 405 nm are caused by mercury emission within the lamp.

3. Results

3.1. Autofluorescence ratio

Autofluorescence values, $AF_E(\lambda_{exc}, s)$, were obtained using Eq. (1) for all subjects and for excitation wavelengths in the range 355 – 405 nm in steps of 5 nm. Figure 3(a) shows the mean relative $AF_E(\lambda_{exc})$ values for the four groups, as obtained by Eq. (2). The standard deviation of the AF values within each group ranged between 18% and 36% of the mean value. Instead of error bars, Fig. 3(b) shows the significance of the differences between groups. The figure shows a significantly increased mean AF_E for the two groups of DM subjects with chronic complications ($p < 0.01$) for all excitation wavelengths. Also the group of type 1 DM subjects without chronic complications tends towards an increased AF . It should be noted that the measurements with an excitation wavelength of 360 nm were discarded from this part of the study, because of technical problems during the measurements. Finally, Eq. (3) was used to calculate results for the broad excitation peak of the AGE Reader. Figure 3 shows similar results for the broad excitation peak as compared to the separate narrow excitation peaks.

No significant differences in mean autofluorescence values between subjects within each group were found for smoking, gender and age. The difference between the groups with DM type 1 and type 2 patients without complications that can be seen in Fig. 3 is not significant ($p > 0.05$) and may be caused by the longer diabetes duration of the DM type 1 patients.

3.2. Normalized spectra

The emission peaks of the normalized spectra for the control group are displayed in Fig. 4. The shape of the spectrum is slightly changing for different excitation wavelengths. However, the maximum intensity remains at approximately 500 nm, independent of excitation wavelength.

The normalized spectra of the four patient groups have a similar shape. The emission spectra of all five groups are shown in Fig. 5 for excitation wavelengths of 375 nm (a) and 400 nm (b). These figures clearly show the similarity of the emission spectra between groups. The examples are typical for all other excitation wavelengths as well.

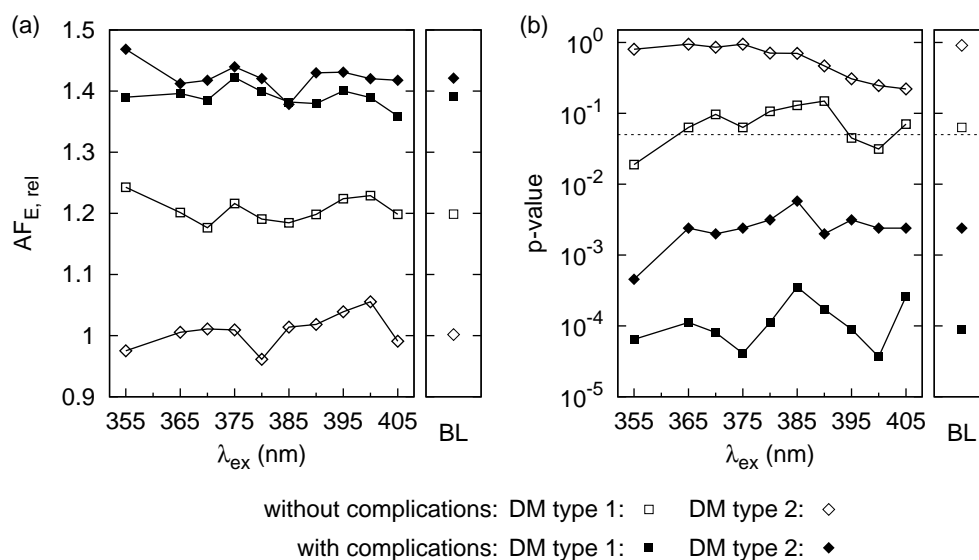


Fig. 3. (a) Mean relative autofluorescence values for the separate excitation peaks ($AF_{E,rel}(\lambda_{exc})$) and the broad excitation peak from the blacklight (BL) as used in the AGE Reader. (b) Statistical significance (Mann-Whitney U-test) of the differences between AF_E values of subjects in the respective subject groups and the control group. The dotted line in (b) represents a value of $p=0.05$.

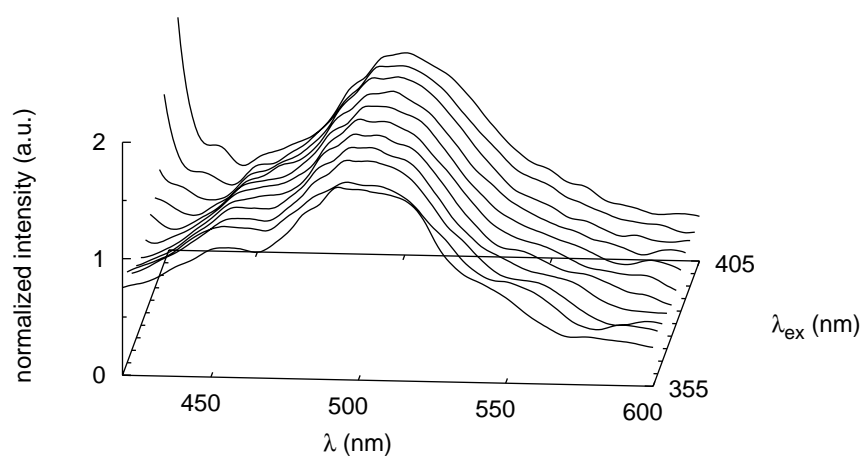


Fig. 4. Emission peaks from the average spectra of the control group for increasing excitation wavelength. The spectra are normalized for emission.

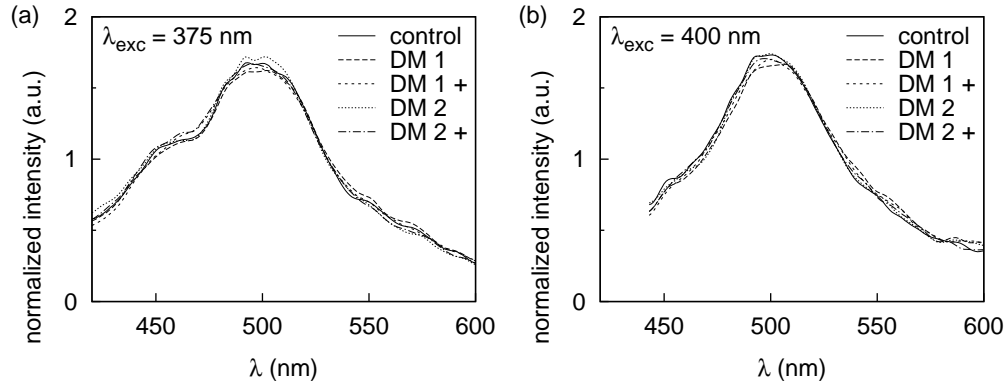


Fig. 5. Emission peaks from the average spectra of the five groups for $\lambda_{exc} = 375\text{nm}$ (a) and $\lambda_{exc} = 400\text{nm}$ (b). In (b), the spectrum starts at 443 nm as below that wavelength, the excitation peak is still present.

4. Discussion

No specific excitation or emission wavelengths were found that would yield an increased distinction between the groups of patients with both types of DM, with or without chronic complications, or the control group. In fact, our results show almost constant relative AF values and significances thereof for all excitation wavelengths. As the excitation spectrum of the AGE Reader is sufficiently modelled by a combination of the investigated excitation spectra (Fig 2), we may conclude that about the same relative AF values occur for the broad excitation band from the blacklight as used in the AGE Reader. From the observation that the emission spectra of the five groups are very similar for each separate excitation wavelength within the current range, it can be concluded that no fluorophores dictate the use of specific excitation or emission wavelengths in these patient groups. Therefore, the shapes of the spectra do not add more information to skin autofluorescence as determined by the AGE Reader for recognizing DM or chronic complications in DM. Moreover, from our results, the fluorophores that contribute to emission in this range seem to be equally increased in the groups of patients with complications.

Figure 3 shows some small variations in relative AF for both type 1 DM patient groups at 375 nm, which might be interpreted as an indication of influence of the excitation wavelength on $AF_{E,rel}$ for these groups. However, these variations in $AF_{E,rel}$ are much smaller than the influence of complications on $AF_{E,rel}$. Furthermore, it can be noted from the figure that the relative AF values are highest for the group of DM type 2 patients with complications, although these are not the most significant. This is mainly the result of somewhat more variation within this group.

The levels of glycated hemoglobin, HbA1c, have not been used in the present study. Earlier results have shown that AF is a much stronger marker than HbA1c in assessing chronic complications [24, 27, 33, 34], probably due to the high turnover of HbA1c in blood. For reference, the levels of HbA1c were $5.1 \pm 0.3\%$ in the control group (normal range 4.2 – 6.0 %) and $7.8 \pm 1.5\%$ in the various DM groups and had a variation between the DM groups of less than one standard deviation of the mean.

It is well-known that many fluorophores exist in the skin [22, 38]. From the field of AGEs, it has become clear that many AGE molecules may also contribute to autofluorescence [39]. Therefore, the observed broad emission spectra as seen in the normalized spectra could be expected, even within the limited range we investigated. It should be noted that, although various

fluorophores may have very different excitation maxima, variation of the excitation wavelength only yields a few changes in the shape of the emission spectrum. The emission spectra upon excitation with 375 nm (which are shown in Fig. 4(a) show a peak at approximately 460 nm, which is not as prominent in the emission spectrum upon excitation with 405 nm (Fig. 4(b)). However, this same shape is visible in the emission spectra from all 5 groups. This is probably due to the fact that the different skin fluorophores have at least overlapping excitation spectra. Our results suggest that the role of excitation wavelength may not be very important to detect clinical differences, a suggestion that also raised in the field of cancer detection [40].

It is a remarkable observation that the shapes of the emission spectra for a given excitation wavelength are similar for the different groups, even as compared to the controls. The high number of different fluorophores in the skin and their interwoven emission spectra as well as the turbid nature of tissue, make it very difficult to recognize specific fluorophores that might cause differences between the groups. Furthermore, the spectra may be influenced by the location of the fluorophores in the skin, the biochemical and biophysical environment, and the presence of other (non-fluorescing) chromophores [2, 22, 41]. We speculate that the same pool of fluorophores causes fluorescence in the current range in healthy subjects as in DM patients, since only the amount of fluorescence is different, not the composition.

Our initial expectation was that we might see specific AGE-related emission peaks in metabolic diseases like DM as compared to control subjects, since AGEs have an essential role in the development of chronic complications in DM [13,42,43]. We furthermore expected a possible difference between type 1 and type 2 DM, as these diseases with different metabolic conditions could generate different AGEs. Collagen-linked 370/440 nm fluorescence, as a marker of AGE accumulation, was a proven predictor of these complications in earlier studies on skin biopsies, and fluorescent AGEs such as pentosidine and argpyrimidine are also related to such conditions. These and some other fluorescent compounds (for example NADH) might result in clinically relevant specificities in excitation-emission pairs. However, attempts to derive specific emission peaks from our measurements between different groups have not yet been successful; the clinical differences manifest themselves proportionally for all excitation-emission pairs.

Even without an exact knowledge of the composition of the fluorophores, a correlation exists between the amount of total autofluorescence and the presence of DM-related chronic complications, as has been reported before [27, 34]. The study of Lutgers et al [27] reported an increased *AF* for type 2 DM patients without chronic complications, while our study showed no increased *AF* in this group. The type 2 DM population in that study was however a large unselected cohort. Our rather small group of type 2 DM patients without complications was specifically selected for having type 2 DM and being in perfect health otherwise. However, our results show a clear distinction of the groups of subjects with chronic complications in either DM type, with highly significant differences compared to controls and an almost constant increase of *AF* values for all excitation wavelengths. These significant differences remain present for a broad excitation band from a blacklight as used in the AGE Reader. This finding confirms the validity of using a broad excitation wavelength for distinguishing complications in conditions such as DM.

5. Conclusions

Our results show that skin autofluorescence at all excitation wavelengths in the range 355 – 405 nm equally distinguishes increased risk of DM-related chronic complications in Caucasian subjects. The fluorophores do not dictate the use of a specific wavelength or set of wavelengths in assessing this risk. The shape of the emission spectra did thereby not show any influence of DM or DM-related complications. These results therefore show the validity of a broad excitation wavelength range, such as applied in the AGE Reader.

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Competing interest

R. Graaff and A.J. Smit are also founders of DiagnOptics BV, manufacturer of the AGE Reader (www.diagnoptics.com).