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### Aspects of photodetection in cervical and ovarian neoplasia

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## **Chapter 8**

# **Localization and grading of cervical intraepithelial neoplasia using Double Ratio fluorescence imaging**

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## Abstract

A phase 0 evaluation was performed of a new fluorescence imaging technique for diagnosing cervical intraepithelial neoplasia. The fluorescence imaging prototype performed quantitative imaging of PpIX induced by topically applied ALA using Double Ratio (DR) fluorescence imaging technique developed in our group. In total 38 patients entered the protocol, 16 were colposcopically selected for biopsy. Of these 16 patients fluorescence images were taken and biopsies from 19 sites were taken and the disease was staged histopathologically. DR fluorescence imaging of the cervix using our general purpose prototype appeared to be cumbersome but feasible. In 4 cases strongly localized fluorescent hotspots were observed at the location where the disease was colposcopically visible. In the other cases the fluorescence showed a more diffuse multifocal image. The value of the DR determined at the site of biopsy correlated in a statistically significant way with the histopathologically determined stage of the disease (Spearman rank correlation,  $r = 0.881$ ,  $p < 0.001$  (confidence interval 0.7044 - 0.9552)). This suggests that non-invasive staging of CIN using this technique is feasible. We believe that the results of this study justify the development of a dedicated device that combines regular white light colposcopy with DR fluorescence imaging.

## Introduction

Cervical cancer is the leading cause of mortality in female cancer and the second most common cancer in females worldwide<sup>1</sup>. The introduction of new screening and treatment modalities led to a decrease in mortality of cervical cancer over the last 50 years<sup>2,3</sup>.

However, in the last two decades nation wide screening programs revealed an increase in the incidence of cervical cancer. Hornung *et al.*<sup>4</sup> estimated that the mortality of cervical cancer will rise by 20% in the next decade unless improvements are made in the current screening and detection techniques. Colposcopy has proved to be a useful diagnostic tool in identifying the most atypical site for biopsy on the cervix<sup>5,6</sup>. When women with an abnormal Pap-smear are referred for colposcopy, diagnosis and treatment of cervical intra-epithelial neoplasia (CIN) requires several visits to the doctor. With conventional colposcopy, using acetic-acid staining to select the most atypical site for taking a biopsy, only 53.6% of the removed biopsies contain histologic evidence of a dysplastic process<sup>6</sup>. In an overview of fourteen papers, by Hopman *et al.*, the positive predictive value of colposcopically directed biopsy was poor for 'no CIN' and increased with increasing stage of the disease<sup>8</sup>. Also a considerable inter-observer variability regarding diagnosing CIN is reported in the literature, for colposcopists as well as for histopathologists<sup>8,9,10,11</sup>. Fluorescence imaging and spectroscopy are relatively new experimental techniques for the detection of superficial epithelial changes. Fluorescence diagnostics is based on detecting *in vivo* differences in fluorescence between normal and cancerous tissues. Fluorescence is induced by excitation of fluorophores in the tissue, usually with blue or UV light. The area of interest is then imaged with a sensitive camera or a point measurement is performed with a spectrograph. The shallow penetration of the excitation light makes this tool particularly suitable for superficial lesions. Differences in fluorescence between normal and cancerous tissue can be naturally present due to different fluorescent molecules in normal and cancerous tissue or may lay in different absorption or scattering in tissue<sup>12,13,14,15</sup>.

The use of natural tissue fluorescence spectroscopy for CIN screening was extensively evaluated and reviewed by Mitchell *et al.* They concluded that fluorescence spectroscopy performs better than colposcopy<sup>16</sup>. Differences in fluorescence between normal and cancerous tissue can also be enhanced by administration of a tumor localizing fluorescent drug, preferentially accumulating in cancerous cells. Several fluorescent tumor localizers are currently under study. 5-Aminolevulinic acid (ALA) is currently a popular drug for photodynamic therapy. When administered topically it diffuses into the cells and converts to the fluorescent Protoporphyrin IX (PpIX). Selective accumulation in certain

cell types may be caused either by differences in cellular enzyme levels or by differences in the accessibility to ALA<sup>17,18,19,20</sup>. Hillemanns *et al.* used this approach for CIN screening based on fluorescence imaging. They showed that fluorescence imaging for CIN screening has similar results compared to colposcopy. However by using a more quantitative method, fluorescence spectroscopy with a fiber-optic probe at a fixed distance, they found a significant improvement over colposcopy<sup>21</sup>. Sinaasappel and Sterenberg described a more quantitative method for fluorescence measurements, the Double Ratio measurement technique<sup>22</sup>. Two excitation wavelengths ( $\lambda_i$  and  $\lambda_j$ ) and two detection fluorescence wavelength ranges ( $\lambda_m$  and  $\lambda_n$ ) are used to generate four different fluorescence images from which a DR image is calculated. The DR algorithm is designed to compensate for tissue color and tissue light scattering and produces an image that is dependent on the amount of the fluorescent tumor localizer only<sup>23</sup>

$$DR = \frac{F_{i,m} F_{j,n}}{F_{i,n} F_{j,m}} = \frac{1 + \alpha \frac{C}{C_0}}{1 + \frac{C}{C_0}} \quad (1)$$

where  $F_{x,y}$  stands for the fluorescence obtained with excitation wavelength  $\lambda_x$  detected at wavelength  $\lambda_y$  and  $C$  stands for the concentration of the photosensitizer. The parameters  $\alpha$  and  $C_0$  are unknown constants that are related to the fluorescence quantum yields of the autofluorophores and of PpIX. It can be shown<sup>24</sup> that  $\alpha$  for PpIX in tissue has a value of approximately 8. DR is a smooth function of  $C/C_0$ . At low values of  $C/C_0$  it is proportional to  $C/C_0$  and at higher values it saturates at a value of  $\alpha$ . The DR images therefore have a direct relation to the PpIX distribution of the imaged area, unbiased by the usual optical artifacts. The aim of this clinical pilot study is threefold. First, we will test our general purpose fluorescence imaging prototype in a clinical setting. Second, we will evaluate the feasibility of using the ALA- Double Ratio approach as fluorescence guide for taking biopsies. Third, we will test whether this quantitative fluorescence imaging approach is capable of non-invasive staging of CIN.

## Materials & Methods

### Patient selection.

Women with an abnormal Pap smear, routinely referred to the clinic for colposcopic examination, were asked to participate in this study. Pregnant or lactating women were excluded. A total of 30 volunteers enrolled in this study. Ages varied between 19 and 45 years with a mean age of 30 years. We excluded 6 volunteers with a history of previous

surgery, causing unknown changes to the epithelium. Another 8 volunteers were colposcopically unsuspect for CIN and consequently no biopsies could be taken. Finally, 19 biopsies from 16 volunteers were available for our analysis.

### **Application of ALA.**

The ALA was purchased from Medac GmbH (Hamburg Germany) in crystallized form. On the day of use it was dissolved in a concentration of 1% (weight/weight) in sterile saline solution with the pH adjusted to 5.5 using sodium hydrogen carbonate<sup>21</sup>. Sterile gauzes drenched with 10ml of this solution were applied to the cervix. The application time aimed for was 60 minutes, but in practice ranged from 60-120 minutes.

### **DR fluorescence imaging prototype.**

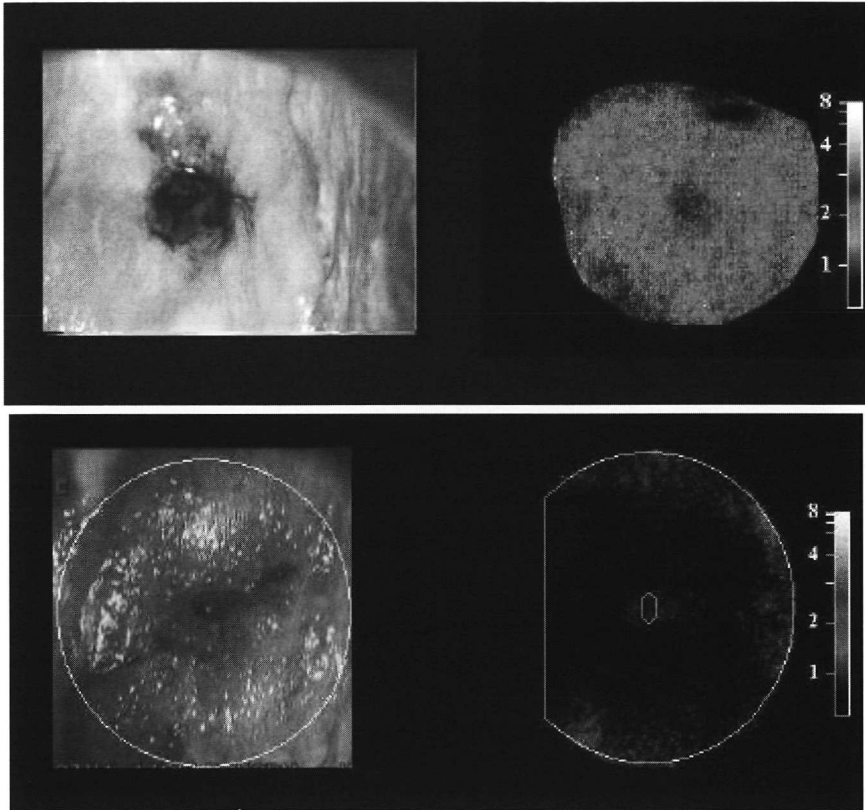
The DR fluorescence camera is a general purpose prototype developed by our group. In practical use it resembles an operating microscope. Preliminary clinical experiments have been performed for neurosurgery and dermatology. The device uses two violet wavelengths for excitation of the cervix, 405 nm and 435 nm at intensities of about 100 and 200 mW/cm<sup>2</sup>, respectively. These are not the optimum wavelengths for DR imaging of PpIX, but a compromise between contrast and signal to noise ratio. The excitation light is generated with a 200 Watt Hg-Xe lamp, two line filters and a chopper alternating the 2 excitation wavelengths. Fluorescence of the cervix is detected through with an image intensified camera (Philips IP 800) through a red and green filter (550nm, bandwidth 42 nm and 675nm, bandwidth 110 nm). In this manner 4 different fluorescence images are acquired. These are processed into DR images by ratioing the two red and green images and subsequently ratioing the two ratio images. A detailed description of this imaging device and the theory behind it can be found elsewhere<sup>23,24,25</sup>. For focusing and positioning a simple white light (monochrome) imaging mode is available.

### **Procedure.**

At the time of colposcopy the moist gauze containing the ALA is removed. Then a regular colposcopic procedure is performed, including acetic acid staining and photographic documentation of the cervix. In case the decision is made to take a biopsy, the position where the biopsy will be taken is marked on the photograph (Polaroid) that was taken to document the colposcopy procedure. Subsequently, the colposcope is removed and the fluorescence imaging device is positioned and focused. Positioning the device took 1-5 minutes, acquiring the fluorescence images approximately 60 seconds. The resulting DR image was not available to the clinician during the procedure. Next, the fluorescence

imaging device is removed and the colposcope is used to take the biopsy. The biopsy is then subjected to standard histopathological evaluation.

### Data analysis.



*Figure 1. White light image and DR fluorescence images for two cases where clinically no CIN was detected and consequently no biopsy was taken. A) normal cervix, fluorescence intensity is low, although some speckles are present. B) inflamed cervix the fluorescence intensity is extremely low.*

There were 2 blind steps in the procedure to avoid bias. First, as mentioned above, the clinician has not seen any fluorescence image before the biopsy is taken. Second, the results of the histopathologic evaluation of the biopsies were not available to us at the time of dataprocessing. Defining the location of the biopsy site in the DR image was not simple as it involved matching two different images taken with different devices (i.e. the colposcope and the fluorescence imaging device). The outcome of this procedure might be biased.

## Results

### General

Although the prototype functioned properly in the technical sense, its use in this particular clinical field proved to be very cumbersome. Especially positioning and focusing the device was difficult and time consuming. A typical example of a set of images is given

*Table 1 : The various CIN grades in our patient population*

Grade	Number
No CIN	3
CIN I	7
CIN II	3
CIN III	6

in figure 1b. The speculum, visible on the right side of the white light image, often caused severe artifacts in the fluorescence image that were not easy to avoid. These artifacts were related to reflections of excitation and fluorescence light on the stainless steel surface that were not completely blocked by the detection filters. In addition, the wall of the vagina (as far as it was not covered by the speculum) was out-of-focus and usually fluoresced intensely. In the fluorescence image this area is painted black, so only the fluorescence on the cervix remains

visible. DR fluorescence images were taken in 16 patients, on whom a total number of 19 biopsies were obtained resulting in various different grades of CIN (Table 1).

### DR fluorescence images of no CIN

Figure 1a shows the white light image and the corresponding DR image of the cervix of a patient that was colposcopically diagnosed as no CIN. Although some speckles are visible, DR values close to 1 are found throughout the cervix, suggesting a very minimal PpIX production. A similar situation is depicted in figure 1b, the main difference being that this cervix is severely inflamed. Nevertheless, the DR image shows a uniform low value, suggesting low PpIX levels throughout the cervix.

### Localization of CIN with DR fluorescence imaging

In 4 cases a region with clear boundaries could be recognized in the DR image. In all 4 cases this region matched the location where the biopsy was taken. The 4 lesions that showed localized fluorescence were all classified as CIN III. An example of this is shown in figure 2a. In the other 15 cases larger areas with unclear boundaries showed elevated DR values. An example of a more diffuse fluorescence image is given in figure 2b. In these cases the location with the highest DR never coincided with the location where a biopsy was taken.

### Staging of CIN with DR fluorescence imaging

Figure 3 shows the DR value averaged over a 16 by 16 pixel square located at the position

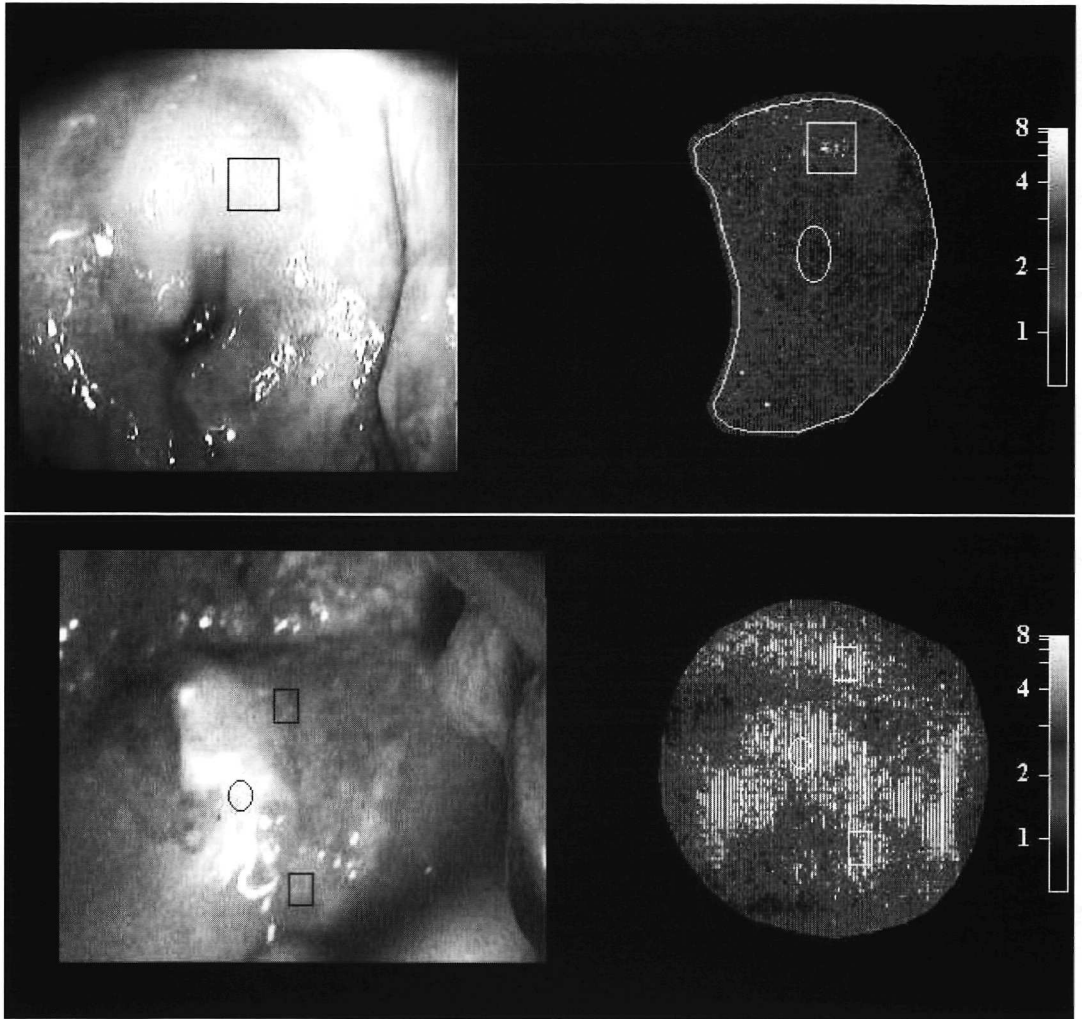


Figure 2. White light images and corresponding fluorescence images of two cases histopathologically confirmed as CIN III. The locations where the biopsies were taken are indicated with a square, the ellipse indicates the position of the portio in the fluorescence image. In the first case (a) a clearly localised spot is visible in the fluorescence image and corresponds to the location where the biopsy was taken. In the second case (b) a more diffuse increase in the DR is observed.

where the biopsy was taken versus the histopathological grade. Although some outliers are clearly present, there appears to be a significant correlation between DR value and histopathology (Spearman rank correlation,  $r = 0.881$ ,  $p < 0.001$  (confidence interval 0.7044 - 0.9552))

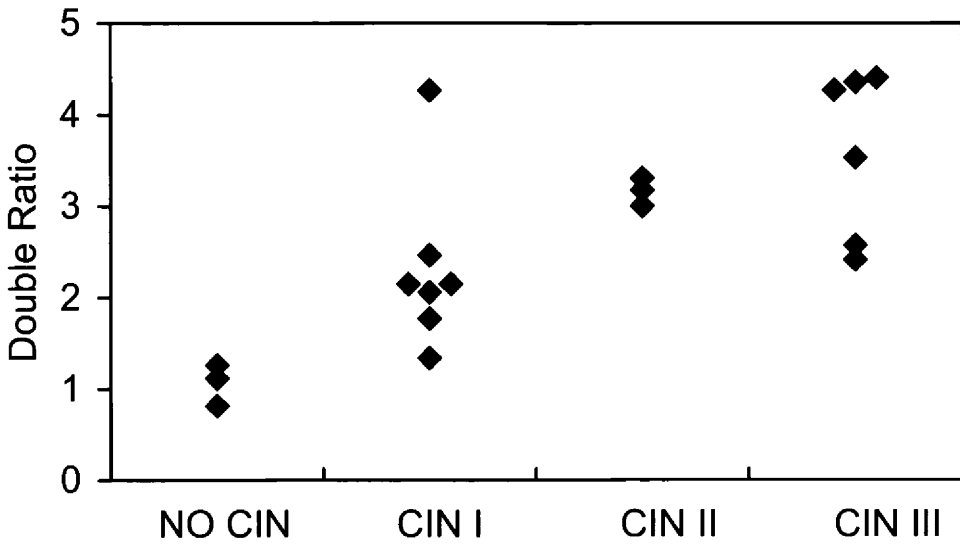


Figure 3. DR value calculated on the location where the biopsy was taken versus the histopathological staging performed on the biopsy.

## Discussion

### Localization

Fluorescence images were obtained of a total number of 16 cervixes. In 13 of these we have histopathological confirmation of intraepithelial neoplasia. In 4 (of these 13) a clearly localized lesion was visible in the fluorescence image on a location coinciding with the (previously determined) biopsy location. The biopsies from these sites were all classified as CIN III. In the other 9 cervixes the fluorescence images showed multiple hotspots of variable intensity or a more diffuse elevation of the fluorescence DR. This suggests a more multifocal nature of the disease in these cases.

### Grading

The strong correlation between the DR value and the histopathological classification as shown in figure 3 is quite striking. This might suggest that there is a relation between the accumulation of PpIX and the grade of the disease. However, we believe that a different mechanism is behind this phenomenon. In the derivation of eq. 1 a semi-infinite homogenous medium with a homogenous PpIX distribution is assumed. The clinical situation is obviously a lot more complex; In the case of topically applied ALA on a mucosal surface a PpIX containing layer may have a thickness of up to a few hundred micrometer; of the order of, or less than the penetration depth of the violet excitation light.

Furthermore, an epithelial tumor originates from the boundary between the stromal and epithelial layer and then progresses towards the top surface, into the monitored tissue volume. This will increase the average porphyrin concentration in this volume. As a consequence, we must seriously doubt the validity of the assumption of a semi-infinite homogenous distribution of PpIX. To gain more insight in the dependency of the DR on the layered clinical situation, we performed Monte Carlo simulations. The technique and the results are described in chapter 9

### **Methodology**

Tracing the location where the biopsy was taken on the fluorescence image was done by hand. Two different imaging devices were used and a topographical match between the two images was made based on visual cues and landmarks, like the portio and the edge of the cervix. Although a 'double blind' approach was used in this procedure, it remains the Achillesheel of the study. This problem could very simply be avoided by using an integrated device; performing both the photographic documentation and the DR imaging through the same optics. Technically this should not be a major problem, and the preliminary results obtained in this study certainly warrant such a development.

### **Conclusions**

DR fluorescence imaging of ALA induced PpIX of the cervix using our general purpose prototype appeared to be feasible. In 4 cases strongly localized fluorescent hotspots were observed at the location where clinically the disease was colposcopically visible. In the other cases the fluorescence showed a more diffuse multifocal image. The value of the DR determined at the site of biopsy correlated in a statistically significant way with the histopathologically determined grade of the disease. This suggests that non-invasive staging of CIN using this technique is feasible. We believe that the results of this study justify the development of a dedicated device that combines regular white light colposcopy with DR fluorescence imaging.

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