

Clinical Internship Technical Medicine

# Objective quantification of fluorescence intensity during the creation of anastomoses and sentinel lymph node identification using Indocyanine green

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## ABSTRACT

**Background** Colon cancer is one of the most common cancer types in the Netherlands, with an incidence of 12,900 newly diagnosed patients in 2019. Fluorescence imaging using indocyanine green provides information of the vascular perfusion and identifies the Sentinel Lymph Node (SLN) in colorectal surgery. However, the evaluation of the fluorescence intensity is subjective and based on the surgeon's visual judgment. Therefore, this study aimed an objective quantification of the fluorescence intensity during the creation of the anastomosis or the SLN procedure.

**Material and Methods** The study population consists of patients who underwent an anastomotic or SLN procedure with the Da Vinci Xi at the Meander Medical Center in Amersfoort between March 2020 to January 2021. Segmentation based on thresholding calculates the maximum and mean intensity to quantify the vascular perfusion of the colon and detection of the SLN. Besides, the correlation between the intensity and patient characteristics is analyzed using multiple linear regression.

**Results** Twenty patients who underwent an anastomotic procedure and two patients who underwent an SLN procedure are included. The segmentation shows the vascularization of the anastomosis or SLN, without surrounding tissue, the reflection of light, and surgical equipment. The mean intensity of the anastomosis patients ranges from 30.9 to 130.8 arbitrary units (AU) and the maximum intensity ranges from 142.1 to 251.3 AU. There was no correlation found between patient characteristics and fluorescence intensity.

**Conclusion** This study aimed an objective quantification of the fluorescence intensity during the creation of the anastomosis or the SLN procedure. Due to the small sample size, there was no correlation found between patient characteristics and fluorescence intensity. For follow-up research, larger sample size is needed to determine a cut-off value for the maximum and mean intensity. These cut-off values can be used to determine whether the patient should receive a stoma.

**Keywords** Anastomosis, Da Vinci robot, Indocyanine Green, Objective Quantification, Sentinel Lymph Node

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## LIST OF ABBREVIATIONS

ASA	American Society of Anesthesiologist
AU	Arbitrary Units
BMI	Body Mass Index
CBR	Contrast-to-Background Ratio
CC	Colon carcinoma
GI	Gastrointestinal
HSV	Hue, Saturation, Value
ICG	Indocyanine green
NIR	Near-infrared
ROI	Region of interest
SLN	Sentinel-lymph-node
UInt8	Unsigned eight-bit integer

## I. INTRODUCTION

Colon cancer (CC) is one of the most common cancer types in the Netherlands, with an incidence of 12,900 newly diagnosed patients in 2019<sup>1</sup>. Moreover, CC caused more than 5,000 death in 2017<sup>3</sup>. Due to the nationwide bowel screening program, the incidence of CC in the Netherlands increased. This screening program contributes to an earlier diagnosis and more curative treatment options. Therefore, annual CC mortality is decreasing in the Netherlands.<sup>2</sup> The nationwide bowel screening program diagnosed asymptomatic CC patients. Symptomatic CC patients may present both bowel-specific symptoms, such as abdominal pain, rectal bleeding, and change in bowel habit, and systemic symptoms, as fatigue and loss of weight<sup>4,5</sup>. The gold standard for diagnosis is colonoscopy with biopsies, where the treatment depends on the pathological stage of the tumor<sup>6,7</sup>.

For staging a tumor, the TNM-staging system is used, where T describes the tumor (see Figure 1), N describes the lymph nodes that are involved, and M describes the distant metastases. Table 1 describes these stages with the corresponding TNM-classification.<sup>7</sup> The most important indication for adjuvant chemotherapy is regional lymph-node metastasis. For this reason, only stage III and IV patients receive adjuvant or neoadjuvant therapies. Adjuvant therapy aims to reduce the risk of recurrence and is an additional cancer treatment after the primary treatment. Neoadjuvant therapy aims to shrink the tumor and is treated before the main treatment. However, up to 30% of the patients with stage I or II will develop distant metastasis. The five-year survival rate for patients diagnosed with stage I is 90% and for those with stage II it is 75%. To improve the five-year survival rate for these patients, adequate lymph node detection is necessary. Since a tumor first

metastasizes into the sentinel-lymph-node (SLN), SLN detection can be used for understaging.<sup>9</sup>

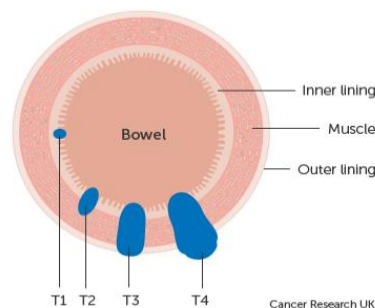


Figure 1 – T staging in CC.<sup>8</sup>

Table 1 – Staging system for colorectal cancer<sup>7</sup>.

Stage	TNM stage	Definition
<b>I</b>	T1;N0;M0	T1: tumor invades submucosa
<b>I</b>	T2;N0;M0	T2: tumor invades muscularis propria
<b>IIa</b>	T3;N0;M0	T3: tumor invades through muscularis propria into subserosa or non-peritonealised pericolic tissue
<b>IIb</b>	T4;N0;M0	T4: tumor directly invades other organs or structures and/or perforates visceral peritoneum
<b>IIIa</b>	T1 or T2;N1;M0	T1/T2; N1: metastases to 1-3 regional lymph nodes
<b>IIIb</b>	T3 or T4;N1;M0	T3/T4; N1: metastases to 1-3 regional lymph nodes
<b>IIIc</b>	Any T;N2;M0	N2: metastases to $\geq 4$ regional lymph nodes
<b>IV</b>	Any T;Any N; M1	M1: distant metastases

Fluorescence imaging can be used for SLN detection, by exposure to a near-infrared (NIR) (700-900 nm) light source and a fluorescent dye. The amount of fluorescent dye diffused in the tissue is proportional to the fluorescence intensity and is, therefore, a tissue perfusion marker<sup>10</sup>. Indocyanine green (ICG) is approved for human use by

the United States Food and Drug Administration and absorbs NIR light at 805 nm<sup>11,12</sup>. The fluorescence emission of ICG is at a slightly longer wavelength of 820 to 835 nm<sup>12</sup>. This emission can be visualized on the Firefly of the Da Vinci Xi (Intuitive Surgical Inc., Sunnyvale, USA).

Since ICG is a tissue perfusion marker, it can also be used during the creation of an anastomosis<sup>13</sup>. Anastomotic complications, such as anastomosis leakage are associated with significant morbidity and mortality<sup>14</sup>. For assessing anastomotic perfusion in colorectal surgery, ICG fluorescence imaging is useful. However, the evaluation of the fluorescence intensity is subjective and based on the surgeon's visual judgment. Therefore, an objective evaluation of fluorescence intensity is required.<sup>13</sup>

Previous research proposed an objective evaluation of the fluorescence images during colorectal surgery. Inge van den Berg<sup>15</sup> concluded that the fluorescence intensity varied within and between patients during surgical procedures. The correlation between the intensity and patient characteristics (gender, age, Body Mass Index (BMI), and American Society of Anesthesiologists (ASA) score) was analyzed for ten patients. No significant difference in the correlation between the intensity and patient characteristics was found since there was a small patient population. The variation in fluorescence intensity within patients can be explained by the variation of the camera angle and distance to the colon during surgery.<sup>15</sup> This is in accordance with Van den Bos *et al.*<sup>16</sup>, where a higher intensity is found when the camera is closer and perpendicular to the dye.

This study aimed an objective quantification of the fluorescence intensity during the creation of the anastomosis or the SLN procedure. Moreover, patient characteristics are analyzed to determine the correlation between these patient

characteristics and the fluorescence intensity. The remainder of this study is structured as follows: In Section 2, materials and methods, in Section 3, the results, in Section 4, the discussion, and in Section 5, the conclusion is given.

## II. MATERIALS AND METHODS

### 2.1 Study design and study population

This is a retrospective study of a prospective database of twenty patients who underwent an anastomotic procedure with the Da Vinci Xi at the Meander Medical Center in Amersfoort between March 2020 to January 2021. Of these twenty patients, fifteen patients have already been included and the other five patients are included during this internship for which the camera position during the creation of the anastomosis is standardized. Also, two patients who underwent an SLN procedure with the Da Vinci Xi surgical robot from Intuitive (Sunnyvale, California, USA) at the Meander Medical Center in Amersfoort between March 2020 to January 2021 are included. The study population consists of patients over 18 years in which a colon carcinoma is pathologically confirmed or have diverticulitis. Patients were enrolled in this study when ICG was used to visualize perfusion during anastomosis creation or when ICG was used to detect SLN. Patients were excluded if they are allergic to ICG or iodine. The SLN identification exclusion criteria were distant metastases, lymph node involvement, or preoperative suspicion of T3-T4 tumors. Written informed consent was obtained from all patients.

### 2.2 Surgical procedure

Two surgical procedures will be included, the creation of the anastomosis and the SLN procedure. During both procedures, the surgeon achieves intra-abdominal access with the Da Vinci Xi. The fluorescence signal is visualized using the Firefly. Robotic surgery involves the insufflation of carbon dioxide into the peritoneal cavity to

create a pneumoperitoneum. After creating the pneumoperitoneum and positioning the arms of the Da Vinci Xi, the surgery can be performed.

During the creation of the anastomosis, 25 mg ICG (ICG-Verdye, Diagnostic Green GmbH Aschheim-Dornach, Germany) diluted in 5 mL of water for injections (5 mg/ml), is injected intravenously to visualize the perfusion of the colon. The time between intravenous injection and ICG visualization is noted. The Firefly is switched on when the anastomosis is visible, so the camera does not have to be moved during the measurement. During the measurement, the camera is positioned 5 cm from the anastomosis, using a sterile ruler. After the camera is positioned 5 cm from the anastomosis, the ruler is removed, as autoscaling otherwise results in lower fluorescence intensity, and the Firefly can be switched on. The moment the fluorescence is visible on the Firefly, the time starts, and it is measured for 30 seconds. The surgery is recorded using RVC Clinical Assistant (RVC Medical IT B.V., Amersfoort, The Netherlands). Information about the surgery can be noted on the form, which is attached in Appendix A.

During the SLN procedure, a colonoscopy is performed by a gastroenterologist and at four sites (proximal, distal, lateral, and medial) the 25 mg ICG diluted in 5 mL of water for injection (5 mg/ml) is injected submucosally around the tumor. The time between submucosal injection and ICG visualization is noted. Immediately after the administration of ICG, the Firefly is activated to detect the SLN. After the SLN has been detected, the camera is positioned 5 cm from the SLN, using a sterile ruler. After the camera is positioned, the ruler is removed. The moment the SLN is visible on the screen, it is stably visualized for 30 seconds. The surgery is recorded and the information about the surgery can be noted on the form, which is attached in Appendix A.

### 2.3 Quantitative evaluation of ICG fluorescence imaging

To calculate the intensity of the fluorescence a segmentation is performed in Python version 3.8.5 (Python Software Foundation, Beaverton, USA). Anaconda Navigator version 1.10.0 (Anaconda Inc., Austin, USA) was used as a code editor. Since the intensity of the fluorescence is calculated, the image was manually trimmed before segmentation, using iMovie (Apple Inc., Cupertino, USA). The part where fluorescence is visible is saved with the same resolution. To decrease the number of frames, every 10<sup>th</sup> frame of the patient is included, resulting in three frames per second. First, a 3D scatter plot in Hue, Saturation, Value (HSV) color space was made to visualize the color distribution of the pixels in a frame (See Algorithm 1). In HSV color space the pixels are split into hue, saturation, and value. The hue represents the position of the color in the range  $0-2\pi$  radians. The saturation describes the intensity of the color in the range  $[0,1]$ . 0 corresponds to unsaturated and 1 corresponds to fully saturated. The value is a measurement of the brightness of the color in the range  $[0,1]$ , the brighter the color the closer to 1.<sup>17</sup> The hue, saturation, and value are shown in Figure 2.

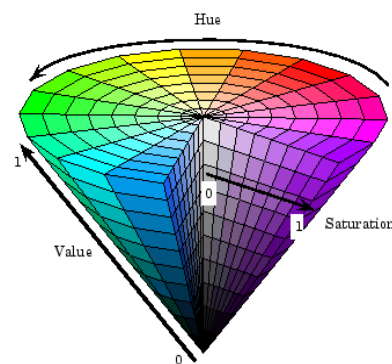


Figure 2 – HSV color space<sup>17</sup>

The 3D scatter plot is used to determine the threshold values for segmentation. Segmentation based on thresholding creates a binary mask image, which defines a region of interest (ROI) (fluorescent area) of the frame (See Algorithm 2). Pixels in the ROI have a value of 1 (white) and pixels outside the ROI have a value of 0 (black). Reflection of light is not included since this falls out of the threshold range. Then, the morphological opening operation is applied to the binary mask image, to remove small objects that do not belong to the ROI. The opening operation erodes ( $\ominus$ ) an image (A) first and then dilates ( $\oplus$ ) the eroded image. For the opening operation, an ellipse is used as a structuring element (B), see Formula 1.

$$A \circ B = (A \ominus B) \oplus B \quad (1)$$

This binary mask image is imposed on the original frame. Finally, the binary mask image is converted into a grayscale image with pixels in the range of 0 to 255. The maximum intensity is calculated by finding the maximum value of the grayscale image for all frames. The mean intensity is calculated by taking the average of the grayscale image of all frames. When calculating the mean intensity only non-zero elements are considered. In Appendix B the Python script is attached.

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*Algorithm 1: Converting RGB color space to HSV color space<sup>18</sup>*

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**Input:**  $\mathbf{F}(u, v, w) \rightarrow$  RGB image of the anastomosis or SLN, a matrix stored in Unsigned eight-bit integer (uint8), with  $u=540$ ,  $v=960$  and  $w=3$ .

**Output:**  $\mathbf{K}(u', v', w') \rightarrow$  HSV image of the anastomosis or SLN, a matrix stored in uint8, with  $u'=540$ ,  $v'=960$  and  $w'=3$ .

**Initialization:**

1. Rescale indices in  $\mathbf{F}$  from 1-256 to 0-1  
 $R' = R/255$   
 $G' = G/255$   
 $B' = B/255$
2.  $C_{\max} = \max(R', G', B')$
3.  $C_{\min} = \min(R', G', B')$
4.  $\Delta = C_{\max} - C_{\min}$
5. For  $\mathbf{K}(u', v', 1)$

$$H = \begin{cases} 0^\circ & \Delta = 0 \\ 60^\circ \times \left( \frac{G' - B'}{\Delta} \bmod 6 \right) & , C_{\max} = R' \\ 60^\circ \times \left( \frac{B' - R'}{\Delta} + 2 \right) & , C_{\max} = G' \\ 60^\circ \times \left( \frac{R' - G'}{\Delta} + 4 \right) & , C_{\max} = B' \end{cases}$$

6. For  $\mathbf{K}(u', v', 2)$   
 $S = \begin{cases} 0 & , C_{\max} = 0 \\ \frac{\Delta}{C_{\max}} & , C_{\max} \neq 0 \end{cases}$
7. For  $\mathbf{K}(u', v', 3)$   
 $V = C_{\max}$
8. Repeat at each pixel value

**End**

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*Algorithm 2: Creating a binary mask image using HSV color space*

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**Input:**  $K(u, v, w) \rightarrow$  HSV image of the anastomosis or SLN, a matrix stored in uint8, with  $u=540$ ,  $v=960$  and  $w=3$ .

**Output:**  $H(u, v) \rightarrow$  Binary mask showing the ROI's, a matrix stored in uint8, with  $u=540$ ,  $v=960$ .

**Initialization:**

1. Create HSV scatter plot to visualize color distribution in  $K$ .
2. Set threshold ( $T_{min}$ ) at lowest pixel value ( $t_1$ ).
3. Set threshold ( $T_{max}$ ) at highest pixel value ( $t_2$ ).
4. If pixel value  $\geq T_{min}$  and  $\leq T_{max}$ , it gets value 1. If not, it gets value 0.
5. Repeat at each pixel value.
6. Use morphological operation, area opening, to remove connected components with small number of pixels resulting in  $H$ .

**End**

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## 2.4 Statistical analysis

Multiple linear regression is performed to analyze the correlation of patient characteristics ( $X_i$ ) with the fluorescence intensity ( $Y$ ) during the creating of an anastomosis. The patient characteristics included in this study are age<sup>2,13,19-21</sup>, gender<sup>2,13,19,21</sup>, BMI<sup>22</sup>, ASA-score<sup>19-21</sup>, TNM-staging<sup>2,13,19,21</sup>, comorbidities<sup>11,19-21,23</sup>, smoker status<sup>11,23</sup>, consumption of alcohol<sup>11</sup>, type of anastomosis<sup>21</sup>, and blood pressure<sup>24</sup>. The measurement levels of these independent variables are categorical. Age is divided into  $\leq 59$ , 60-69, and  $\geq 70$  years. Gender is split into female or male. BMI is classified into three groups: underweight + normal weight ( $< 24.9$ ), pre-obesity (25.0-29.9), and obesity class I (30.0-34.9). The ASA-classification is classified into three groups: ASA-I, ASA-II, and ASA-III. The TNM-staging is subdivided into stage I+II, and stage III+IV. Comorbidity is subdivided into four groups: GI (Gastrointestinal) disease, Diabetes Mellitus, Hypercholesterolemia, and other diseases. Smoker status (yes or no) and alcohol use (yes or no) are also included. The type of anastomosis is split into end to end, side to side, and side to end. Finally, blood pressure measured during the pre-operative screening is classified according to the American Heart Association: normal/elevated ( $<129/<80$  mmHg) and

hypertensive ( $>130/>80$  mmHg). Multiple linear regression is calculated according to Formula (2). P values of  $< 0.05$  were considered statistically significant. Besides,  $R^2$  is calculated to determine the strength of the correlation between the dependent variable and the independent variables. Statistical analyses were conducted with SPSS (IBM SPSS Statistics, Chicago, USA).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_6 x_6 + \beta_7 x_7 + \beta_8 x_8 + \beta_9 x_9 + \beta_{10} x_{10} + \varepsilon \quad (2)$$

With:

$x_1$  = age,  $x_2$  = gender,  $x_3$  = BMI,  $x_4$  = ASA-score,  $x_5$  = TNM-staging,  $x_6$  = comorbidities,  $x_7$  = smoker status,  $x_8$  = consumption of alcohol,  $x_9$  = type of anastomosis,  $x_{10}$  = blood pressure.

Finally, a power analysis was performed to calculate the required sample size ( $N$ ). The required power is  $1 - \beta = 0.80$ , so  $\beta = 0.20$  and the required significance level is  $\alpha = 0.05$ . Since it is tested whether each independent variable has a significant relationship with the dependent variable, Green recommendation<sup>25</sup> (Formula 3) can be used.

$$N \geq 105 + m, \text{ where } m \text{ is the number of independent variables} \quad (3)$$

### III. RESULTS

#### 3.1 Patient's population

A total of twenty patients (seven male and thirteen female) who underwent an anastomotic procedure with the Da Vinci Xi at the Meander Medical Center in Amersfoort were analyzed in this study. The patients' characteristics are presented in Table 2. The median age was 65.4 years. The median BMI was 25.38 kg/m<sup>2</sup>. Most patients are in ASA-II (60.0%). Six patients (31.6%) had diverticulitis and in the remaining fourteen patients (68.4%) colon carcinoma was pathologically confirmed. Most of the patients were diagnosed with stage I + II (36.8%) colon cancer. The TNM staging of one patient was unknown, so the valid percentage was taken. Most patients (50.0%) were known with GI as comorbidity. 63.2% of the patients have never smoked and 42.1% of the patients have never drunk alcohol. Both smoker status and alcohol use had one missing, so the valid percentage was taken. A side to end anastomosis was most commonly created (50.0%). The type of anastomosis

had two missing values, so the valid percentage was taken here as well. Most patients (55.0%) had hypertensive blood pressure. Three patients (15.0%) received a double-barreled colostomy preoperatively. Two patients (10%) received a double loop ileostomy during surgery. Anastomotic complications did not occur for any patient. A total of two patients (one male and one female) who underwent an SLN procedure were analyzed in this study. The median age was 75.65 years. The median BMI was 26.45 kg/m<sup>2</sup>. One patient (50%) had ASA-II and one patient (50.0%) had ASA-score III. Both patients were diagnosed with stage I colon cancer (100%). All patients (100%) were known with other diseases as comorbidity. 100% of the patients had never smoked and 100% of the patients drank alcohol. Both patients (100%) had hypertensive blood pressure. A side-to-side anastomosis was created for both patients (100%). No patient (100%) received a stoma preoperatively or during surgery. Anastomotic complications did not occur for any patient.

Table 2 – Patient characteristics.

Patient characteristics	Anastomosis N (%)	SLN N (%)
Age (years)		
▪ ≤ 59	5 (25.0%)	1 (50.0%)
▪ 60-69	10 (50.0%)	0 (0.0%)
▪ ≥70	5 (25.0%)	1 (50.0%)
Gender		
▪ Male	7 (35.0%)	1 (50.0%)
▪ Female	13 (65.0%)	1 (50.0%)
BMI (kg/m <sup>2</sup> )		
▪ Normal weight	8 (40.0%)	1 (50.0%)
▪ Pre-obesity	8 (40.0%)	1 (50.0%)
▪ Obesity class I	4 (20.0%)	0 (0.0%)
ASA-score		
▪ ASA-I	4 (20.0%)	0 (0.0%)
▪ ASA-II	12 (60.0%)	1 (50.0%)
▪ ASA-III	4 (20.0%)	1 (50.0%)
TNM-staging		
▪ Stage I + II	7 (36.8%)	2 (100%)
▪ Stage III + IV	6 (31.6%)	0 (0.0%)
▪ None, diverticulitis	6 (31.6%)	0 (0.0%)

Comorbidity		
▪ GI disease	10 (50.0%)	1 (50.0%)
▪ Diabetes Mellitus	4 (20.0%)	1 (50.0%)
▪ Hypercholesterolemia	4 (20.0%)	0 (0.0%)
▪ Other diseases	7 (35.0%)	2 (100.0%)
Smoker status		
▪ Currently or ex	7 (36.8%)	0 (0.0%)
▪ Never	12 (63.2%)	2 (100%)
Alcohol use		
▪ Yes	5 (57.9%)	2 (100.0%)
▪ Never	8 (42.1%)	0 (0.0%)
Type of anastomosis		
▪ End to end	4 (22.2%)	0 (0.0%)
▪ Side to side	5 (27.8%)	2 (100%)
▪ Side to end	9 (50.0%)	0 (0.0%)
Blood pressure (mmHg)		
▪ Normal/elevated	9 (45.0%)	0 (0.0%)
▪ Hypertensive	11 (55.0%)	2 (100%)
Anastomotic complications		
▪ No	20 (100%)	2 (100%)
▪ Yes	0 (0%)	0 (0.0%)
Receive stoma preoperative		
▪ Yes	3 (15.0%)	0 (0.0%)
▪ No	17 (85.0%)	2 (100%)
Receive stoma		
▪ Yes	2 (10.0%)	0 (0.0%)
▪ No	18 (90.0%)	2 (100%)

### 3.2 Quantitative evaluation of ICG fluorescence imaging

In Figure 3 a 3D scatter plot of frame 940 of anastomosis patient 15 is shown. The green-colored pixels are positioned into a small range along the hue axis. In addition to the green-colored pixels, there are also blue-

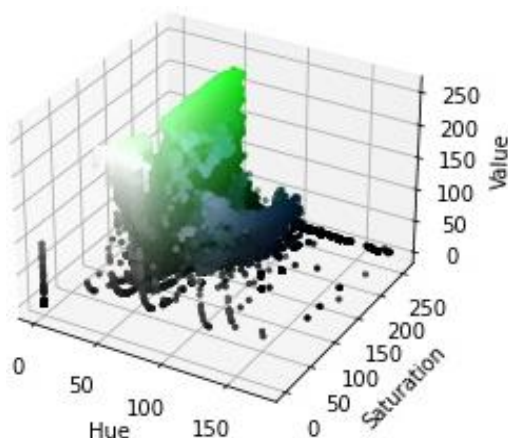


Figure 3 – 3D scatter plot in HSV color space.

colored pixels, which can be distinguished from the green-colored pixels on the hue axis. The segmentation steps of anastomosis patient 15 are shown in Figure 4. Figure 4 shows the vascularization of the anastomosis, without surrounding tissue, the reflection of light, and surgical equipment. In Figure 5 the original frame and the grayscale image are shown for patient 18. A rectosigmoid resection was performed because of diverticulitis. As can be seen in Figure 5, almost no fluorescence of ICG is visible. Figure 6 shows the original frame and the grayscale image for patient 19. Patient 19 underwent a low anterior resection because of a rectosigmoid carcinoma. As can be seen in Figure 6, fluorescence is visible in the surrounding tissue, but almost no fluorescence is visible in the anastomosis.

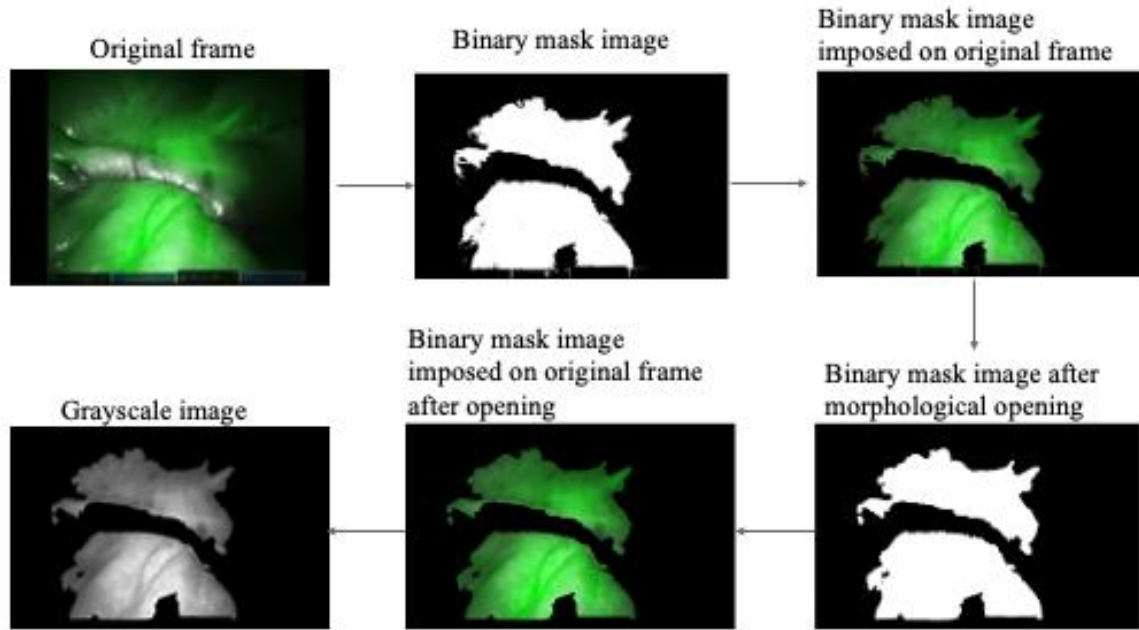


Figure 4 – Segmentation steps of anastomosis patient: form original frame to grayscale image.

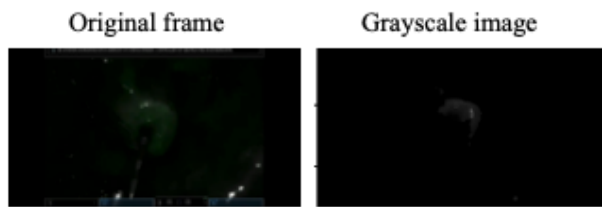


Figure 5 – Result of segmentation of patient 18.

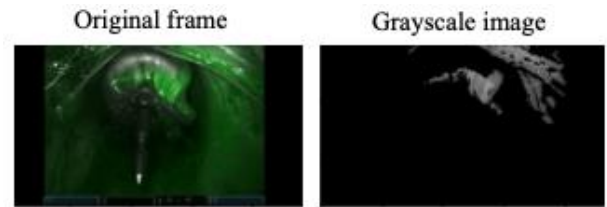


Figure 6 – Result of segmentation of patient 19.

In Figure 7 a 3D scatter plot of frame 500 of the SLN patient 2 is shown. The green-colored pixels are also positioned into a small range along the hue axis. The segmentation steps of 15 are shown in Figure 8. The last image in figure 8 shows

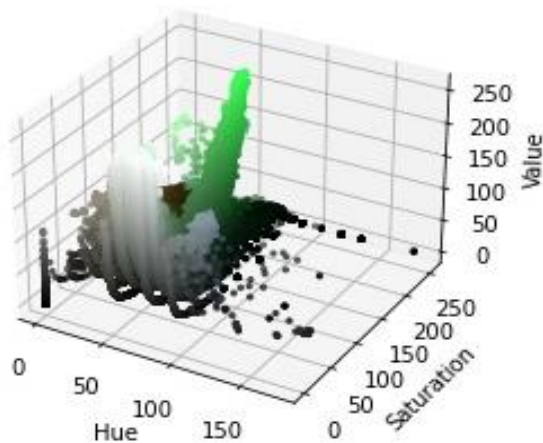


Figure 7 – 3D scatter plot in HSV color space.

the SLN, without surrounding tissue, the reflection of light, and surgical equipment. The fluorescence intensity is objectively analyzed by calculating the mean intensity, and the maximum intensity (Table 3). The mean intensity ranges from 30.9 to 130.8 arbitrary units (AU) and the maximum intensity ranges from 142.1 to 251.3 AU. Figure 9 shows the intensity curve of patient 15 during the creation of the anastomosis and Figure 10 shows the intensity curve of patient 18. The intensity curves of the other patients can be found in Appendix C. Figure 9 shows that the mean intensity varies. The maximum intensity remains steady around a certain intensity. Figure 10 shows that the mean intensity remains constant around a certain intensity. The maximum intensity is, in addition to a few outliers, also steady.

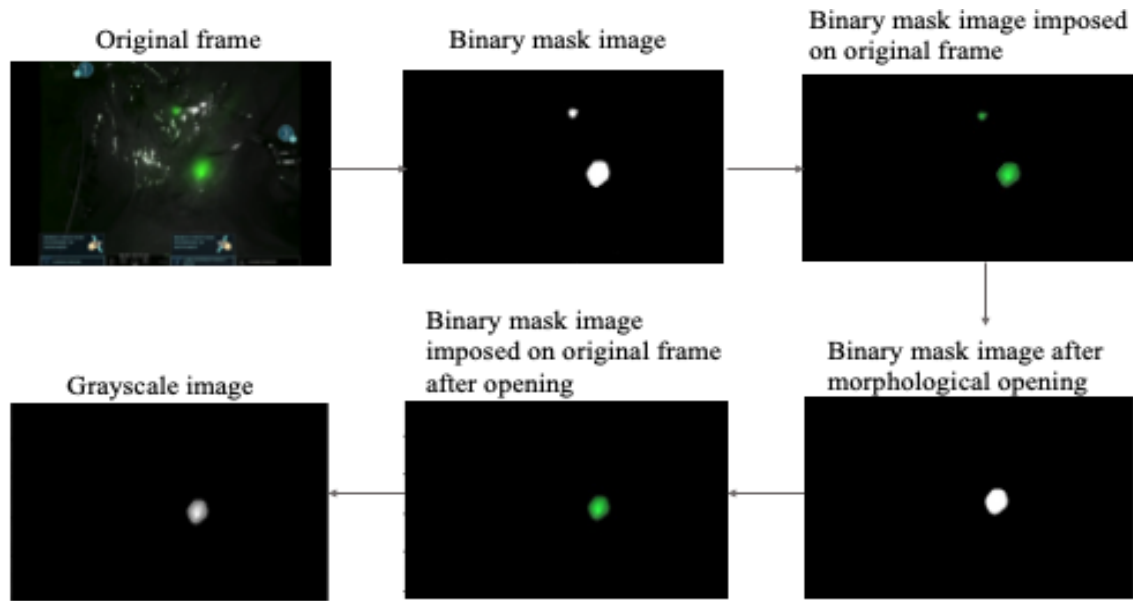


Figure 8 – Segmentation steps of SLN patient: form original frame to grayscale image

Table 3 – Mean intensity and maximum intensity of anastomosis and SLN.

Patient	Mean intensity (AU)	Maximum intensity (AU)
<b>Anastomosis</b>		
1	91.8	171.9
2	103.9	202.1
3	105.1	194.9
4	86.8	142.1
5	95.7	180.7
6	85.3	162.7
7	104.4	205.2
8	85.2	171.1
9	80.5	166.4
10	118.3	182.6
11	122.1	224.8
12	81.2	251.3
13	102.2	244.6
14	66.7	241.3
15	130.6	248.1
16	87.3	247.6
17	68.2	235.2
18	30.9	231.1
19	80.0	247.4
20	114.0	246.6
<b>SLN</b>		
1	103.0	161.2
2	115.0	179.0

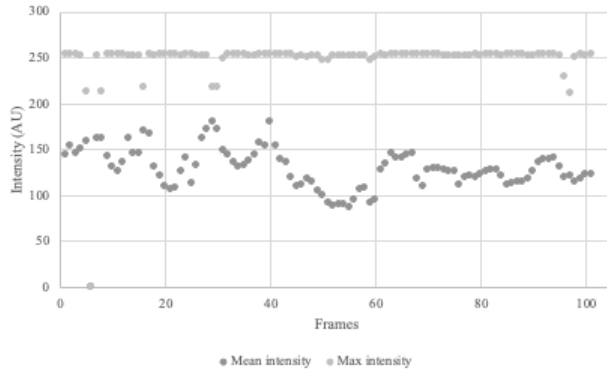


Figure 9 – Fluorescence intensity during the creation of an anastomosis of patient 15.

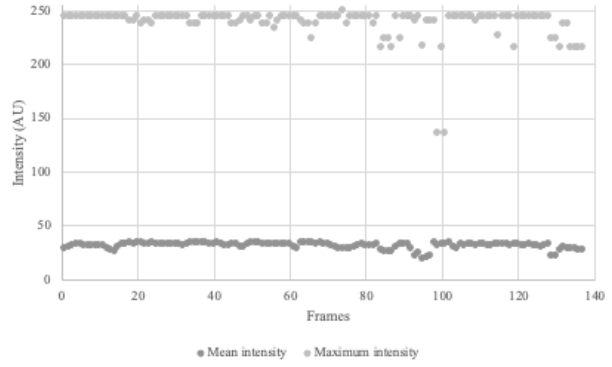


Figure 10 – Fluorescence intensity during the creation of an anastomosis of patient 18.

### 3.3 Statistical analysis

Table 4 shows the  $\beta$ -coefficients for the mean and the maximum intensity. For the mean intensity, age (60-69), tumor stage III + IV, diverticulitis, hypercholesterolemia, Diabetes Mellitus, other comorbidities, side to side anastomosis, and hypertensive blood pressure have negative  $\beta$ -coefficients. This indicates that these independent variables cause a lower intensity value. No independent variable is statistically significant, as all P values  $> 0.05$ . The  $R^2$  for the mean intensity is 0.962, which means that 96.2% of the dependent variable

can be explained by the independent variables. For the maximum intensity, age ( $\geq 70$ ), pre-obesity, obesity class I, ASA II, ASA III, tumor stage III + IV, diverticulitis, other comorbidities, never smoked, and no alcohol, cause a lower intensity, since the  $\beta$ -coefficients are negative. No independent variable is statistically significant, as all P values  $> 0.05$ . The  $R^2$  for the maximum intensity is 0.777, which means that 77.7% of the dependent variable can be explained by the independent variables.

Since 17 independent variables are included in the model, a sample size of 122 patients is required, to have a power of 80%.

Table 4 – Output Multiple linear regression for mean intensity and maximum intensity

Characteristic	$\beta$ -coefficients mean intensity	P-value	$\beta$ -coefficients maximum intensity	P-value
Intercept	-123.7	0.161	254.0	0.129
Female	21.6	0.483	16.7	0.746
Age 60-69	-8.2	0.830	43.4	0.543
Age $\geq 70$	239.7	0.067	-126.0	0.392
Pre obesity	54.8	0.165	-52.7	0.366
Obesity class I	362.7	0.152	-269.4	0.447
ASA II	188.1	0.208	-175.2	0.440
ASA III	259.7	0.206	-289.0	0.372
Stage III + IV	-89.2	0.100	-5.9	0.923
Diverticulitis	-109.1	0.109	-40.4	0.624
Hypercholesterolemia	-151.4	0.087	71.4	0.492
Diabetes Mellitus	-288.1	0.129	190.7	0.450
Other comorbidities	-96.9	0.129	-33.3	0.677
Never smoked	99.2	0.077	-13.3	0.822
No alcohol	171.9	0.192	-114.2	0.546
Side to side anastomosis	-178.1	0.332	323.0	0.326

Side to end anastomosis	21.2	0.742	88.4	0.471
Hypertensive blood pressure	-36.9	0.709	176.2	0.369

## IV. DISCUSSION

### 4.1 Interpretation of the results

This study aims an objective evaluation of the fluorescence intensity. The results of the segmentation show the vascularization of the anastomosis and SLN, without surrounding tissue, the reflection of light, and surgical equipment. Every 10<sup>th</sup> frame of the patient is included, for the segmentation. It was tested whether the inclusion of each frame gave a different result in terms of intensity. This showed that the intensity value did not differ, so it was decided to include every 10<sup>th</sup> frame, to decrease the segmentation time. This is important if it is to be applied in the clinic. The maximum and mean intensity is calculated to quantify the fluorescence intensity. Using the new surgical procedure, the intensity measurements are stable compared to the previous study<sup>15</sup>. However, when the surgical procedure is not fully followed and the camera position changes, the intensity measurements are no longer stable, as can be seen in Figure 9. During this measurement, the camera was not kept at a fixed distance. It is, therefore, essential to follow the surgical procedure. In Figure 5, almost no fluorescence of ICG is visible, so the lower and upper threshold for segmentation was adjusted manually. Perhaps the patient characteristics in follow-up research describe this low fluorescence intensity. In Figure 6, fluorescence is visible in the surrounding tissue, but almost no fluorescence is visible in the anastomosis. An explanation may be that the vascularization was closed due to the high pressure applied to the anastomosis. Should this situation occur again, it can be checked whether the fluorescence is more visible if slightly less pressure is exerted on the anastomosis.

Besides, it may also be possible that these patients received too little (patient 18) or too much (patient 19) ICG. However, the concentration of ICG administered is noted for each patient during surgery, and this was not the case. Bos *et al.*<sup>16</sup> describe that a weight-adjusted dose of ICG seems preferable over a fixed-dose, especially in overweight patients. This can be considered in follow-up research. Since the fluorescence intensity of patient 18 was low, it has been determined whether the contrast to background ratio (CBR) has an added value. For patient 18 the CBR was calculated and compared to the CBR of patient 15 (See Appendix D). The CBR can be calculated by:  $(\text{intensity ROI} - \text{intensity background}) / \text{intensity background}$ . The same segmentation steps as described above have been applied. Only the complement image of the binary image is used instead of the binary image. If patients 15 and 18 had a background intensity in the same range, the CBR would have been a solution for the low ROI intensity of patient 18. However, the background intensity of patient 18 was much lower compared to patient 15. As a result, the CBR of patient 18 was much higher compared to patient 15. Automatic thresholding can be a solution to avoid having to manually adjust the threshold for this patient. However, this makes comparing patients more difficult, as a different threshold is used for each patient. This influences the average pixel intensity. Finally, multiple linear regression is performed to analyze the correlation of patient characteristics with the fluorescence mean and maximum intensity during the creating of an anastomosis. No significant differences were found. The  $R^2$  of the mean and maximum intensity are respectively 96.2% and 77.7%, which indicated 96.2% and 77.7% of the dependent variable can be explained by the independent variables. However, this can be the result of



overfitting. The more independent variables added to the model, the higher  $R^2$ . Therefore, the adjusted  $R^2$  is calculated for the mean and maximum intensity. The adjusted  $R^2$  for mean intensity is 0.639 and for the maximum intensity -1.118, which indicated 63.9% and 0.0% of the dependent variable (mean intensity and maximum intensity) can be explained by the independent variables. This shows that the maximum intensity does not correlate with the independent variable. This can be explained by the fact that the maximum intensity only needs to have one pixel of a high intensity in each frame, which directly results in a high maximum intensity. The mean intensity takes the average intensity, which is expected to show a better correlation. This must be confirmed in follow-up research.

#### **4.2 Limitations and follow-up research**

This study has two important limitations. The first limitation is the small sample size. As a result, it was not possible to find statistically significant correlations between patient characteristics and fluorescent intensity. New patient characteristics have been included, based on the literature. Due to the small sample size, it cannot be determined whether these patient characteristics correlate with the fluorescence intensity. The small sample size can also explain the low adjusted R-squared. In this study, five patients are included for which the camera position during the creation of the anastomosis is standardized. For the other 15 patients this is not the case. In the statistical analysis, these two groups are seen as one group, while a different protocol concerning the camera position is used for both groups. Furthermore, due to the small sample size, it is not possible to compare both groups (non-standardized and standardized) with each other using the Chi-square test. This can be used to investigate whether standardizing the camera position is effective. Finally, too many independent variables are included in this linear

regression model. As a rule of thumb<sup>25</sup>, each independent variable should have at least 10 observations. With too many independent variables, the R-squared becomes 100%. Since 17 independent variables are included in this study, at least 170 patients must be included. The result of the power analysis showed that 122 patients are required for a power of 0.80. This means that in follow-up research, not all independent variables can be included. It will be necessary to investigate which patient characteristics predict the dependent variable. According to the rule of thumb, it may be a maximum of 12 independent variables, with a sample size of 122 patients. For follow-up research, larger sample size is needed to find statistically significant correlations between patient characteristics and fluorescent intensity. If 122 patients are included, a cut-off value can be determined from the maximum and mean intensities of fluorescence images indicating a high perfusion intensity. Besides, it is interesting for follow-up research to note whether the surgeon decided not to create a stoma through the use of ICG and would have done it without the use of ICG.

The second limitation is related to standardization. In the current surgical procedure, the measurement is started the moment the ICG is visible on the Firefly of the Da Vinci robot. The disadvantage of this is that starting the measurement is subjective. So, no measure is used for when ICG is visible. For follow-up research, it is recommended to investigate when the measurement can be started objectively. This can be done based on an intensity value, so if the intensity is above a certain value, the measurement is started.

#### **4.3 Future perspectives**

This research describes a new method of quantifying the fluorescence intensity objectively. There are several studies where an objective quantification is achieved. Wada *et al.*<sup>13</sup> evaluated the quantitative



assessment of ICG fluorescence imaging of the proximal colon during laparoscopic colorectal surgery, using NIR fluorescence. A retrospective analysis of intestinal perfusion was performed on the data using analysis software. In this study, the intensity was measured at only one point: the site of transection of the proximal colon at the serosal surface. Kudzusz *et al.*<sup>26</sup> used laser fluorescence angiography to calculate the mean pixel intensity at four points: post anastomotic transverse colon, unaffected small bowel as internal standard, external standard with constant fluorescence, and preanastomotic ileum. Hayami *et al.*<sup>21</sup> aimed to evaluate the visualization and quantification of bowel perfusion around the anastomosis using near-infrared fluorescence. They calculated the intensity at five points around the site of anastomosis on the bowel surface. Protyniak *et al.*<sup>27</sup> aimed to objectively assess colon perfusion before a bowel anastomosis using intraoperative ICG fluorescence angiography. The intensity was measured at four random points near the terminal segments of the bowel. The absolute intensity was measured in a gray-scale image. In the aforementioned studies, the intensity is measured at certain points. The disadvantage of this is that these points have to be placed manually in the image. Moreover, this technique only works for a single frame. By performing the segmentation described in this study, the intensity of the entire region of interest is calculated for all frames. This way of segmenting does not contain the disadvantages described above. It is, therefore, a new method of quantifying the fluorescence intensity objectively.

Currently, ICG is not yet linked to a tumor marker, so fluorescence is visible in several

lymph nodes. If it is possible to connect ICG with a tumor marker, only fluorescence is visible in the SLN where tumor cells are located. This would allow intraoperative identification of CC cells.

After enough patients have been included in this study, the correlation of patient characteristics with the fluorescence intensity can be analyzed. Using the linear regression model, a formula can be drawn up which calculates the intensity. A Graphical User Interface can be used to segment the frames and to calculate the fluorescence intensity. Using the linear regression model, the intensity can be calculated based on the patient characteristics. Using the cut-off values, it can be decided during surgery whether the patient should receive a stoma. This objective quantification may result in fewer patients receiving a stoma.

## V. CONCLUSION

This study aimed an objective quantification of the fluorescence intensity during the creation of the anastomosis or the SLN procedure. Segmentation based on thresholding calculates the maximum and mean intensity to quantify the vascular perfusion of the colon and detection of the SLN. Due to the small sample size, there was no correlation found between patient characteristics and fluorescence intensity, using multiple linear regression. For follow-up research, a larger sample size is needed to determine a cut-off value for the maximum and mean intensity. Using a graphical user interface, the use of ICG can be a meaningful and safe step for the surgeon to decide whether to create a stoma.

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## APPENDIX A – SURGICAL PROCEDURE FORMS

*Table A1 – Form creation of the anastomosis.*

<b>Patient ID:</b>		
<b>ICG concentration</b>	25 mg ICG diluted in 5 mL of water for injections (5 mg/ml)	Otherwise, namely:
<b>Time between IV- injection and visualization of ICG</b>		Seconds
<b>Distance from the surface</b>	5 Centimeter	Otherwise, namely:
<b>Measurement time</b>	30 seconds	Otherwise, namely:

*Table A2 – Form SLN procedure*

<b>Patient ID:</b>		
<b>ICG concentration</b>	25 mg ICG diluted in 5 mL of water for injections (5 mg/ml)	Otherwise, namely:
<b>Time between submucosal injection and visualization of ICG</b>		Seconds
<b>Distance from the SLN</b>	5 Centimeter	Otherwise, namely:
<b>Measurement time</b>	30 seconds	Otherwise, namely:

## APPENDIX B – PYTHON SCRIPT

```
#Import packages
import matplotlib
from matplotlib import pyplot as plt
from matplotlib import cm, colors
from mpl_toolkits.mplot3d import Axes3D

import numpy
import numpy as np

import cv2

import skimage

from skimage import morphology

import os

#Change current path
path = '/Users/Eva/Desktop/Python/Anastomose 18'
os.chdir(path)

#Include every frame of the video and save as .jpg
# https://stackoverflow.com/questions/33311153/python-extracting-and-saving-video-frames
vidcap = cv2.VideoCapture("pt18_anastomose.mp4") # Create a VideoCapture object and
read from input file
success,image = vidcap.read()
count = 0
while True:
    success,image = vidcap.read()
    if not success:
        break
    if count % 10 == 0: # for every frame
        cv2.imwrite("pt18_anastomose_frame_" + str(count) + ".jpg", image) #save frame as
JPG file
    if cv2.waitKey(10) == 27: #exit if Esc is hit
        break
    count += 1
vidcap.release()

#Imread input image in RGB
image = cv2.imread('pt18_anastomose_frame_1290.jpg') # import one frame in BGR
image_RGB = cv2.cvtColor(image, cv2.COLOR_BGR2RGB) # change frame to RGB
plt.imshow(image_RGB)
plt.show()

#Create 3D scatter plot in RGB color space
r, g, b = cv2.split(image_RGB)
fig = plt.figure()
```

```

axis = fig.add_subplot(1, 1, 1, projection="3d")

pixel_colors = image_RGB.reshape((np.shape(image_RGB)[0]*np.shape(image_RGB)[1],
3))
norm = colors.Normalize(vmin=-1.,vmax=1.)
norm.autoscale(pixel_colors)
pixel_colors = norm(pixel_colors).tolist()

axis.scatter(g.flatten(), r.flatten(), b.flatten(), facecolors=pixel_colors, marker=".")
axis.set_xlabel("Green")
axis.set_ylabel("Red")
axis.set_zlabel("Blue")
plt.title('3D scatter plot in RGB color space of Anastomosis patient')
plt.savefig('3D_scatter_pt20_rgb_frame_670.jpg')
plt.show()

#Changing Color-space (RGB to HSV)
hsv_img = cv2.cvtColor(image_RGB, cv2.COLOR_RGB2HSV) # change frame to HSV
plt.imshow(hsv_img)
plt.show()

#Create 3D scatter plot in HSV color space
pixel_colors = image_RGB.reshape((np.shape(image_RGB)[0]*np.shape(image_RGB)[1],
3))
norm = colors.Normalize(vmin=-1.,vmax=1.)
norm.autoscale(pixel_colors)
pixel_colors = norm(pixel_colors).tolist()

h, s, v = cv2.split(hsv_img)
fig = plt.figure()
axis = fig.add_subplot(1, 1, 1, projection="3d")
axis.scatter(h.flatten(), s.flatten(), v.flatten(), facecolors=pixel_colors, marker=".")
axis.set_xlabel("Hue")
axis.set_ylabel("Saturation")
axis.set_zlabel("Value")
plt.title('3D scatter plot in HSV color space of anastomosis patient')
plt.savefig('3D_scatter_pt20_hsv_frame_670.jpg')
plt.show()

#Include all frames of the patient (adjust manually)
all_frames = []
for i in range(0, 137):
    frame = cv2.cvtColor(cv2.imread("pt18_anastomose_frame_" + str(i*10) + ".jpg"),
cv2.COLOR_BGR2RGB)
    all_frames.append(frame) #Inserts at the end of the Python list

#Perform segmentation and morphological opening of image in HSV
def segmentation(image):
    hsv_img = cv2.cvtColor(image,cv2.COLOR_RGB2HSV) # change to HSV image
    low_threshold = (20,110,90) # lower threshold value in HSV

```

```

high_threshold = (80,255,255) # upper threshold value in HSV

#Creating binary mask image, using basic thresholding operation
mask = cv2.inRange(hsv_img, low_threshold, high_threshold)
#plt.title('Binary mask image')
#plt.imshow(mask, cmap='gray')
#plt.savefig('Mask_image.jpg')

#Imposed binary mask image on original frame
result = cv2.bitwise_and(image_RGB, image_RGB, mask = mask) #take only region of
logo from logo image
#plt.title('Binary mask image imposed on original frame')
#plt.imshow(result)
#plt.savefig('Mask_imposed_image.jpg')

#Creating structuring element
kernel = cv2.getStructuringElement(cv2.MORPH_ELLIPSE, (10,10)) # create ellipse with
size 8x8
#plt.title('Kernel')
#plt.imshow(kernel, cmap='gray')
#plt.savefig('Kernel.jpg')

#Applying morphological operator: opening
opened_mask = cv2.morphologyEx(mask, cv2.MORPH_OPEN, kernel)
#plt.title('Binary mask image after morphological oopening')
#plt.imshow(opened_mask, cmap='gray')
#plt.savefig('Mask_opening_image.jpg')

#Imposed binary mask image after opening on original frame
masked_img = cv2.bitwise_and(image_RGB, image_RGB, mask = opened_mask)
#plt.title('Binary mask image imposed on original frame after opening')
#plt.imshow(masked_img)
#plt.savefig('Mask_imposed_opening_image.jpg')

#Convert to gray scale image
gray_hsv = cv2.cvtColor(masked_img,cv2.COLOR_BGR2GRAY) # change to grayscale
image
#plt.title('Grayscale image')
#plt.imshow(gray_hsv, cmap='gray')
#plt.savefig('Grayscale_image.jpg')

return gray_hsv

#Apply segmentation for all frames
results = [segmentation(frame) for frame in all_frames]

#Show all original frames and segmentation of frames
for i in range(0, len(all_frames)):
    plt.subplot(1, 2, 1)
    plt.imshow(all_frames[i])

```

```

plt.subplot(1, 2, 2)
plt.imshow(results[i], cmap = "gray")
plt.show()

#Calculate mean pixel value of segmentation
for i in range(0, len(all_frames)):
    gray_hsv = results[i]
    nonzeros = gray_hsv[gray_hsv != 0] # calculate mean pixel value of segmentation (without
background)
    print('0' if len(nonzeros) == 0 else round(numpy.mean(nonzeros)))

#Calculate maximum pixel value of segmentation
for i in range(0, len(all_frames)):
    max_pixel = results[i].max() # calculate max pixel value of segmentation
    print(max_pixel)

#Segmentation of background
def segmentation_background(image):
    hsv_img = cv2.cvtColor(image,cv2.COLOR_RGB2HSV) # change to HSV image
    low_threshold = (20,110,75) # lower threshold value in HSV
    high_threshold = (80,255,255) # upper threshold value in HSV

    #Creating binary mask image of background, using basic thresholding operation
    mask = cv2.inRange(hsv_img, low_threshold, high_threshold)
    mask_b = cv2.bitwise_not(mask)

    #Imposed binary mask image (background) on original frame
    result_b = cv2.bitwise_and(image_RGB, image_RGB, mask = mask_b) #take only region
of logo from logo image

    #Creating structuring element
    kernel_b = cv2.getStructuringElement(cv2.MORPH_ELLIPSE, (10,10)) # create ellipse
with size 8x8

    #Applying morphological operator: opening (background)
    opened_mask_b = cv2.morphologyEx(mask_b, cv2.MORPH_OPEN, kernel_b)

    #Imposed binary mask image after opening on original frame (background)
    masked_img_b = cv2.bitwise_and(image_RGB, image_RGB, mask = opened_mask_b)

    #Convert to gray scale image (background)
    gray_hsv_b = cv2.cvtColor(masked_img_b,cv2.COLOR_BGR2GRAY)# change to
grayscale image

    return gray_hsv_b

#Apply background segmentation for all frames
results_b = [segmentation_background(frame) for frame in all_frames]

#Show all original frames and segmentation of frames

```



```

for i in range(0, len(all_frames)):
    plt.subplot(1, 2, 1)
    plt.imshow(all_frames[i])
    plt.subplot(1, 2, 2)
    plt.imshow(results[i], cmap = "gray")
    plt.show()

#Calculate mean pixel of background value of segmentation
for i in range(0, len(all_frames)):
    gray_hsv_b = results_b[i]
    nonzeros_b = gray_hsv_b[gray_hsv_b != 0]# calculate mean pixel value of segmentation
    (without background)
    print('0' if len(nonzeros_b) == 0 else round(numpy.mean(nonzeros_b)))

#Calculate maximum pixel of background value of segmentation
for i in range(0, len(all_frames)):
    max_pixel_b = results_b[i].max() # calculate max pixel value of segmentation
    print(max_pixel_b)

```

## APPENDIX C – FLUORESCENCE INTENSITY CURVES

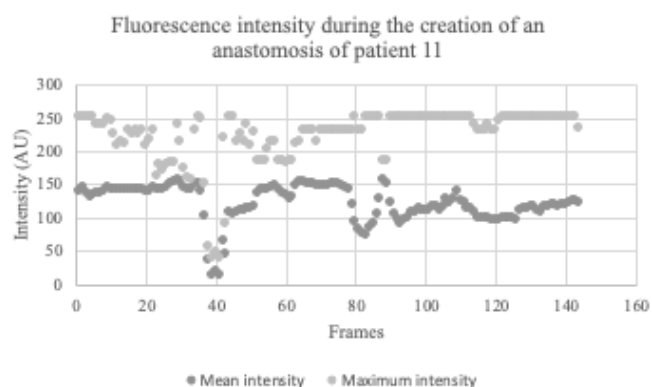


Figure C1 – Fluorescence intensity during the creation of an anastomosis of patient 11.

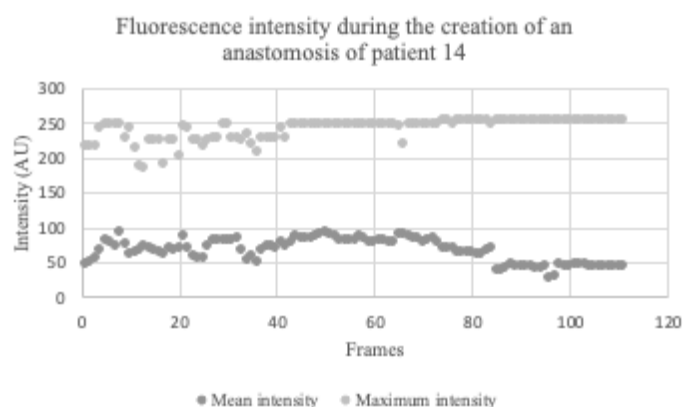


Figure C4 – Fluorescence intensity during the creation of an anastomosis of patient 14.

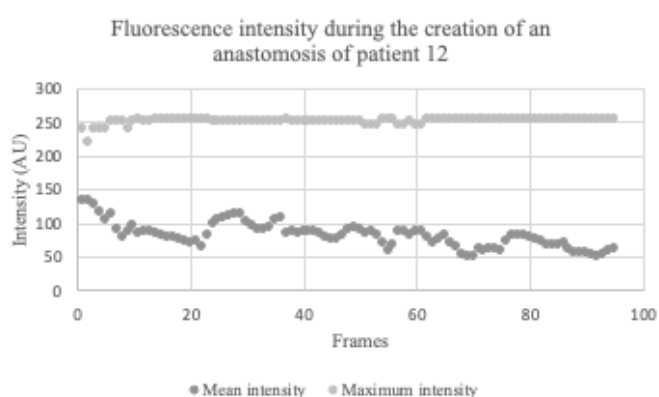


Figure C2 – Fluorescence intensity during the creation of an anastomosis of patient 12.

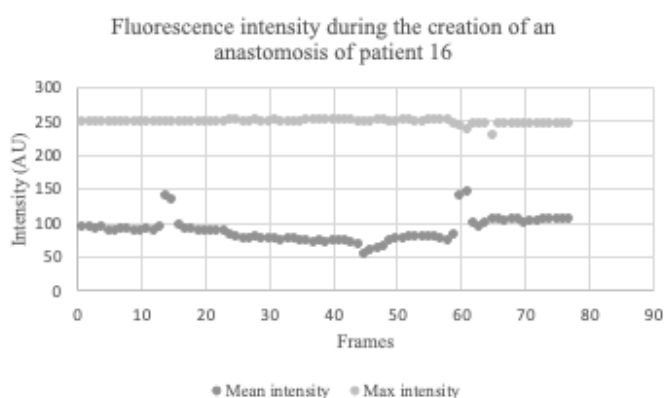


Figure C5 – Fluorescence intensity during the creation of an anastomosis of patient 16.

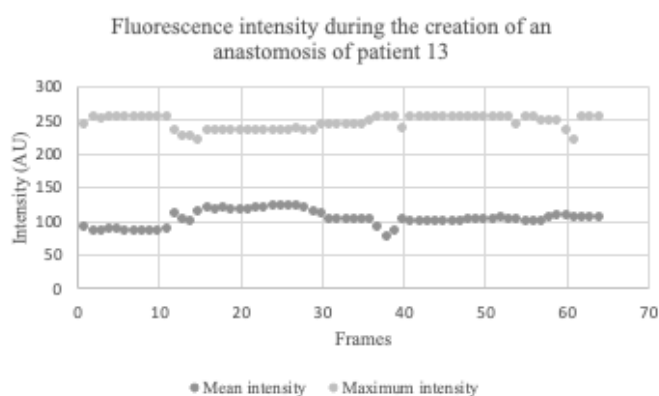


Figure C3 – Fluorescence intensity during the creation of an anastomosis of patient 13.

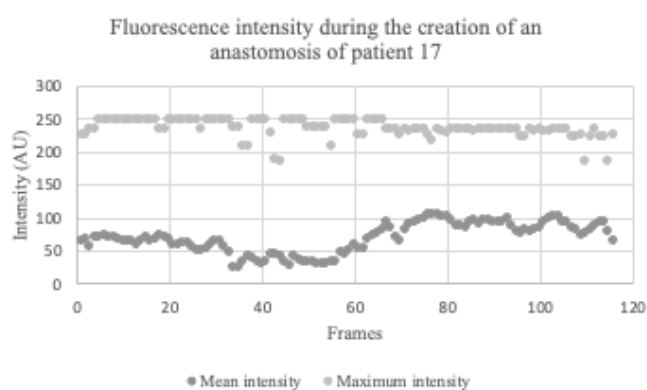


Figure C6 – Fluorescence intensity during the creation of an anastomosis of patient 17.

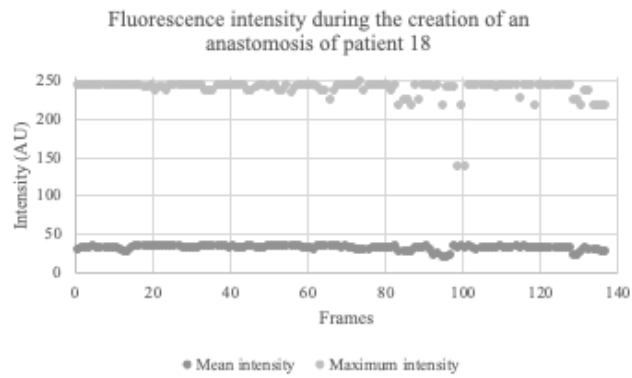


Figure C7 – Fluorescence intensity during the creation of an anastomosis of patient 18.

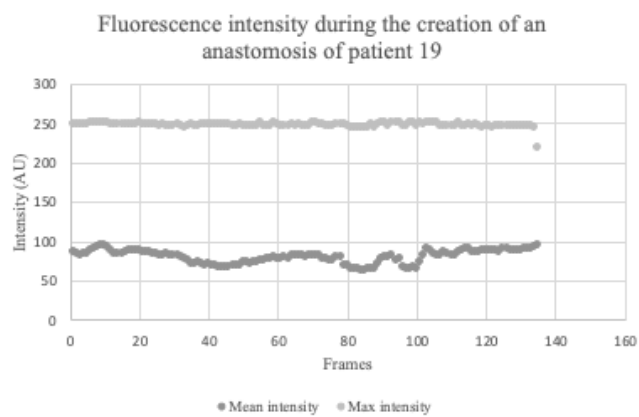


Figure C8 – Fluorescence intensity during the creation of an anastomosis of patient 19.

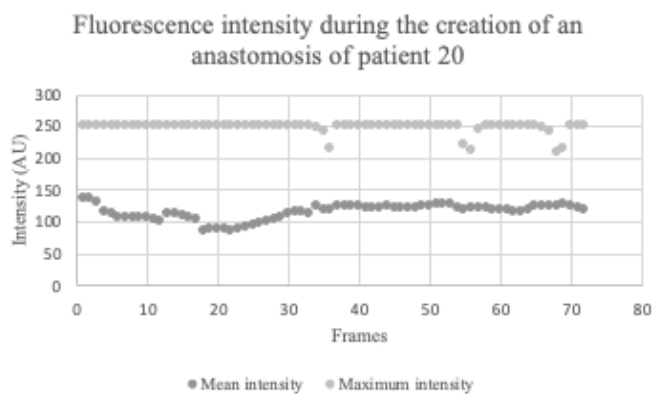


Figure C9 – Fluorescence intensity during the creation of an anastomosis of patient 20.

## APPENDIX D – CONTRAST TO BACKGROUND RATIO

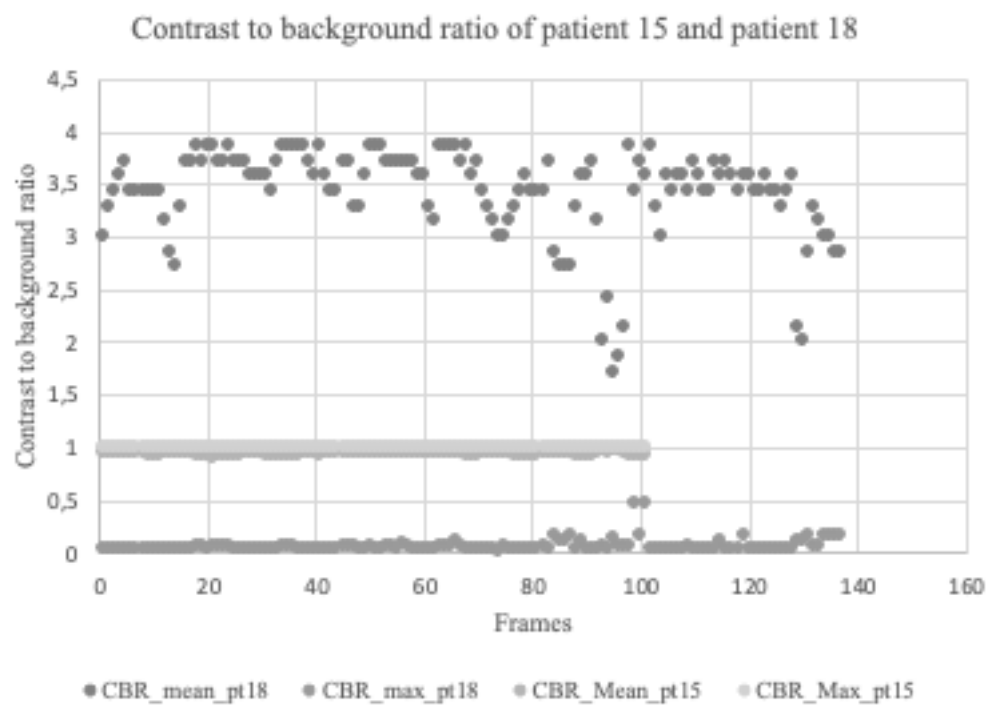


Figure D1 – Contrast to background ratio of patient 15 and patient 18.