A novel and generic workflow of indocyanine green perfusion assessment integrating standardization and quantification towards clinical implementation

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Supplementary Content 1

METHODS

*Workflow of ICG-angiography integrating Standardization and Quantification (WISQ)*

WISQ is depicted in Figure 1 and consists of four steps. Step one: selecting suitable hardware, i.e., an open or laparoscopic fluorescence camera system appropriate for ICG imaging. The tissue and surgical procedure of interest determines the type of camera. Basic camera settings such as exposure time, frame rate, white balance and gain must be controllable by the user. These parameters are set and must remain unchanged for all study procedures. Step two: the standardization of the imaging setup in the operating theater. The positioning of the camera to the tissue of interest must be constant to quantify the data. The distance between the camera detector and the tissue of interest can be monitored using a ruler, or a laser distance indicator present on some commercial systems approved for clinical use. The camera should be placed perpendicular to the tissue to optimize the effective area of the sensor facing the region of interest (ROI), thereby maximizing the fluorescence signal reaching the observer (i.e., quantum efficiency). Furthermore, the camera’s position should closely recreate previous measurements’ composition since inhomogeneous field illumination affects the measured signal intensity. For open surgical systems (without using a laparoscope), the presence of an articulating arm is beneficial as it helps with the reproducibility and stability of the imaging. Step three: image acquisition. The ICG dose is predetermined for every patient, either a fixed dose or a corrected dose for the patient’s body weight or blood volume. The timing relative to imaging and rate of dye injection are vital for the reproducibility of the results as the inflow must be imaged entirely. It is crucial to limit ambient lighting to a consistent minimum during acquisition by turning off the overhead lights and headlights. Furthermore, movement artefacts should be minimized to obtain high-quality data. Step four: post-processing and data interpretation. It is pivotal to obtain raw data from the camera system. Data can be imported into analysis software according to available resources. ROIs are drawn on the corresponding tissue of
interest. Perfusion graphs are produced by plotting time on the x-axis and the mean fluorescence intensity (MFI) on the y-axis.

The intra-operative signal intensity of ICG is influenced by several factors, including the color processing mode, ambient light in the operating room, the dose of injected ICG, the distance/angle from the camera to the surgical field, and variable scattering properties of light. Given these multiple factors, it is understandable that perfusion cannot be determined by visual interpretation of the individual surgeon based on the absolute fluorescence intensity. As in other fields of diagnostic angiography, it should be determined by the dynamics of an inflow and outflow perfusion curve, characterized by the shape of the curve generated in a digital platform. Therefore, WISQ is based on the concept that the optimal perfusion curve can be split into two phases, i.e. the inflow and outflow phase as found in traditional CT and MRI angiography (Supplementary Figure 1). Hypothetically, the inflow phase displays a sharp increase of mean fluorescence intensity (MFI) and the outflow phase starts after the peak intensity has been reached, indicating a decrease in MFI (Supplementary Figure 1, panel A). We postulated that four different types of curves can be identified with the corresponding indication of inflow and outflow complications (Supplementary Figure 1, panel B). Curve A and B both suggest a compromised outflow. Curve A is characterized by rapid MFI increase during the inflow phase without any subsequent decrease throughout the entire outflow phase. Curve B shows an adequate inflow with only a partial reduction of MFI during outflow. Curve C and D both suggest a compromised inflow. Curve C is characterized by a slow increase in MFI over a longer period, thereby suggesting an arterial inflow problem. Curve D shows a limited increase of MFI during the inflow suggesting a problematic arterial inflow (Supplementary Figure 1, panel B). Perfusion patterns are analyzed based on descriptive perfusion graph characteristics and compared to the theoretical optimal perfusion curve (Supplementary Figure 1, panel A). The baseline fluorescence intensity was subtracted from MFI measurements over time to depict absolute inflow and outflow for analysis. We defined ingress slope and T_{in} as an inflow
quantification parameter and egress slope as an outflow quantification parameter. Fluorescence quantification parameters were MFI and AUC.

Supplementary Figure 1. Detailed description of step 4 of the WISQ model. (a). The optimal perfusion curve depicted by the dynamics of an inflow and outflow phase. The inflow phase is displayed as a sharp increase of fluorescence intensity. As the peak intensity is reached, the outflow phase starts. This phase comprises two parts, a steep fall of fluorescence intensity after which the second parts starts with a more gradual decline of fluorescence intensity. (b). Perfusion curves related to outflow and inflow problems. Curve A and B both suggest a compromised outflow. Curve A is characterized by rapid MFI increase during the inflow phase without any subsequent decrease throughout the entire outflow phase. Curve B shows an adequate inflow with only a partial reduction of MFI during outflow. Curve C and D both suggest a compromised inflow. Curve C is characterized by a slow increase in MFI over a longer period, thereby suggesting an arterial inflow problem. Curve D shows a limited increase of MFI during the inflow also suggesting problematic arterial inflow.

Surgical procedure

Three experienced endocrine surgeons (JP, LR and SK) performed a total thyroidectomy according to standard protocol. Neck dissection, where lymph nodes are sampled in the central neck region around the thyroid, was only performed if indicated (e.g., preoperative suspicion of lymph node metastasis). After excision of the thyroid gland, the specimen was examined for parathyroid glands. Parathyroid tissue which had been incidentally removed was confirmed by intraoperative pathology consultation and if present, auto-transplanted according to standard protocols. Furthermore, the surgeon reviewed the in situ parathyroid glands and auto-transplanted those glands that were subjectively deemed ischemic as
per judgement by the surgeons’ eye and experience. Those glands that looked congested but still vascularized on a pedicle were kept in-situ when possible. Parathyroid auto-transplantation involved transplanting parathyroid tissue from its usual location into small pockets within the sternocleidomastoid muscle in the neck. We recorded the number of parathyroid glands identified in each patient and the auto-transplantation rate. Of note, it takes, on average, four to eight weeks after auto-transplantation to have a functioning autograft, so postoperative calcium requirements would not be affected by a parathyroid transplant graft until after the initial one-month follow-up. ICG-angiography was blinded from the surgeon and did not influence intraoperative decision making.

Patients underwent standard follow-up consisting of measurement of PTH levels after a biochemical steady state (30-60 minutes after surgery or the following day). Serum calcium levels were measured if patients were symptomatic for hypocalcemia or if PTH levels were lower than the reference range. Calcium and calcitriol supplementation were monitored in the ambulatory outpatient setting.

Post-processing
Images were recorded in AVI format at 7.5 frames per second. Post-processing was performed according to the proposed method and the raw data was imported into Image J (Fiji, version 1.52v).

Visual interpretation versus postoperative outcome
The surgeons were asked to score all parathyroid glands into three categories, according to the criteria of Vidal Fortuny et al. (i.e. devascularized; moderately well vascularized; and well vascularized). Hypothetical auto-transplantation rate was calculated according to the principle of Vidal Fortuny et al., that states that auto-transplantation should be performed if the LIG is scored as moderately well or devascularized on ICG-angiography.
Statistical analysis

MFI was defined as the average pixel intensity per ROI in parathyroid tissue. The AUC was calculated using Matlab 2020a (Mathworks, Natick, United States). Categorical variables are displayed as count (n) and percentage (%) and continuous variables as median with range. Statistical analyses with non-parametric tests for skewed data were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). For graph design, we used GraphPad Prism (version 8.0, GraphPad Software Inc, San Diego, California, USA).
Supplementary Content 2

RESULTS

Supplementary Table 1. Patient Characteristics.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Indication for surgery</th>
<th>Extend of surgery</th>
<th>Number of identified PTG intraoperatively</th>
<th>Number of identified PTG (surgery + pathology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>21</td>
<td>MNG</td>
<td>TTx</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>49</td>
<td>MSO</td>
<td>TTx</td>
<td>4*</td>
<td>4</td>
</tr>
<tr>
<td>3‡</td>
<td>F</td>
<td>38</td>
<td>Graves’</td>
<td>TTx</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4‡</td>
<td>F</td>
<td>36</td>
<td>Graves’ and PTC</td>
<td>TTx + cLND (unilateral)</td>
<td>4†</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>19</td>
<td>Cowden syndrome</td>
<td>TTx</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>60</td>
<td>PTC</td>
<td>TTx</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>7‡</td>
<td>F</td>
<td>53</td>
<td>MSO</td>
<td>TTx</td>
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<td>4</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
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<td>TTx</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>63</td>
<td>Follicular nodules</td>
<td>TTx</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>42</td>
<td>MNG</td>
<td>TTx</td>
<td>4*†</td>
<td>4</td>
</tr>
</tbody>
</table>

PTG= parathyroid gland; F= female; TTx= total thyroidectomy; MNG= multinodular goiter; MSO= malignant struma ovaria; PTC= papillary thyroid carcinoma; cLND= central lymph node dissection; NA= not available; *= two caudal parathyroid glands located in the thymus; †= parathyroid gland autotransplanted during surgery (no ICG-angiography was performed for this gland); ‡= diagnosed with postoperative hypoparathyroidism (PTH < 14.1 pg/mL or < 1.5 pmol/L, low serum calcium levels and started on calcium and calcitriol supplementation).
Supplementary Figure 2. Visual interpretation of ICG-angiography of four experienced endocrine surgeons of all identified parathyroid glands. 

$S1 =$ surgeon 1; $S2 =$ surgeon 2; $S3 =$ surgeon 3; $S4 =$ surgeon 4; $Pt =$ patient; ‡ = diagnosed with postoperative hypoparathyroidism; $\text{red} =$ devascularized; $\text{orange} =$ moderately well vascularized; $\text{green} =$ well vascularized; $R =$ right; $L =$ left.
ICG quantification - Perfusion curves

In 6/7 patients without postoperative hypoparathyroidism, the LIG showed a perfusion curve similar or near similar to the proposed optimal perfusion curve (Supplementary Figure 3, panel A, B, E, F, I and J). The last patient without hypoparathyroidism showed a sharp inflow peak, however, during the outflow phase the intensity did not decline, suggesting an outflow problem (Supplementary Figure 3, panel H). In this patient, only three parathyroid glands were identified intraoperatively, and no parathyroid gland was found during microscopy by the pathologist. This patient likely had a fully functioning parathyroid gland within the thymus and not found by the surgeon.

In contrast, the LIG of the three patients with postoperative hypoparathyroidism (Supplementary Figure 3, panel C, D and G) showed multiple different types of perfusion problems, as indicated in Supplementary Figure 1, panel B. The LIG of the first patient with hypoparathyroidism (blue curve in Supplementary Figure 3, panel C) showed a minimal increase of fluorescence intensity over time. After peak intensity was reached, the decrease of fluorescence intensity during the outflow phase was slow and gradual. The LIG in the two other patients with hypoparathyroidism showed a curve with a low ingress slope and an outflow phase consisting of only a slightly decreasing fluorescence intensity (Supplementary Figure 3, panel D and G).
ICG quantification - Quantification parameters

The median of the ingress slope and $T_{in}$ of the LIG in patients without postoperative hypoparathyroidism was 5.9 a.f.u./sec (range 3.2 – 15.5 a.f.u./sec) and 10.5 seconds (range 6.9 – 20.5 seconds), whereas in patients with hypoparathyroidism this was 4.0 a.f.u./sec (range 2.3 – 4.1 a.f.u./sec) and 14.4 seconds (range 14.1 – 18.0 seconds), respectively ($p=0.053$ and $p=0.305$, respectively) (Supplementary Figure 4, panel A and B).

In patients without postoperative hypoparathyroidism, the median egress slope of the LIG was -2.4 a.f.u./sec (range -0.1 – -7.2 a.f.u./sec) compared to -1.0 a.f.u./sec (range -0.7 – -1.7 a.f.u./sec) in patients with hypoparathyroidism ($p=0.210$) (Supplementary Figure 4, panel C).

In patients without postoperative hypoparathyroidism, the median peak MFI for each patient was 97.8 a.f.u. (range 50.6 – 145.1 a.f.u.) compared to 56.1 a.f.u. (range 55.2 – 79.7 a.f.u.) in patients with hypoparathyroidism ($p=0.267$) (Supplementary Figure 4, panel D). In patients without hypoparathyroidism, the median peak and trough AUC were 4093.34 (range 1929.07 – 6481.13 a.f.u.) and 1625.92 (range 1187.22 – 2598.71) a.f.u. versus 3240.02 (range 2229.37 – 3943.3 a.f.u.) and 1191.19 (range 646.92 – 2693.77) a.f.u. in patients with hypoparathyroidism ($p=0.210$ and $p=0.569$, respectively) (Supplementary Figure 4, panel E).
Supplementary Figure 4. Parathyroid perfusion quantification parameters per patient. (a). Ingress slope of the perfusion graph per patient in a.f.u./sec. (b). Time from start peak to maximum fluorescence signal ($T_{in}$) of the perfusion graph per patient in seconds. (c). Egress slope of the perfusion graph per patient in a.f.u./sec. (d). Mean fluorescence intensity of the perfusion graph per patient in a.f.u. (e). Area under the curve of the perfusion graph per patient in a.f.u.

And $\dagger$ = diagnosed with postoperative hypoparathyroidism; $T_{in}$ = time from start peak to maximum.