Characterization of vulvar diseases: novel imaging tools, models and molecular targets

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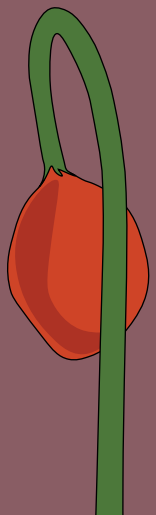
CHAPTER 1

General introduction and thesis outline



Vulvar cancer is a rare gynecological malignancy with a tremendous disease burden. Vulvar squamous cell carcinoma (VSCC) is the most common histologic type of vulvar cancer and constitutes 80–90% of all vulvar cancers.[1–4] Other histological subtypes include melanoma, basal cell carcinoma, Bartholin gland adenocarcinoma, sarcoma, and Paget disease.[5] The cornerstone of treatment for VSCC consists of surgery with or without radiochemotherapy. In addition, precursors of VSCC often require surgical or medical intervention as well.[6] Treatment of vulvar (pre)malignancies is a challenging balance act, as on the one hand clearance of all lesions is desired, while on the other hand normal vulvar anatomy and function must be preserved as best as possible. Utilizing the current treatment interventions, recurrence occurs in up to 40% of VSCC patients and no major improvements in 5-year survival rates were observed in the last decades.[1,7,8] Additionally, most treatments are accompanied by bothersome side-effects such as disfigurement, sexual dysfunction, and psychological problems in more than half of the patients.[9,10] This is partly caused by the difficulty to recognize vulvar (pre)malignant lesions for the medical specialist, either macroscopically or pathologically.[11,12] This highlights the high unmet medical need for preferably non-invasive and accurate diagnostics for vulvar (pre)malignancies. Further, effective therapies are needed with a favorable safety profile that encompass complete reduction of the affected tissue. Therefore, the aim of this thesis has been to search for disease-specific biomarkers to improve the clinical management of vulvar (pre)malignancies. This first chapter of this thesis summarizes the pathophysiology and current treatments of vulvar (pre)malignancies. In the subsequent chapters, the multimodal profiling approach to evaluate novel techniques for diagnosis and treatment evaluation of the affected vulvar area is introduced. Finally, the aims and outlines of this thesis are discussed.

PATHOPHYSIOLOGY AND CURRENT TREATMENTS OF VULVAR (PRE)MALIGNANCIES

The vulva is the outer female genital. The vulvar area is bordered by the mons pubis, the groins and the anus. It includes the clitoris, urethral meatus, labia majora and minora and the introitus of the vagina (Figure 1).[13] The vulva is important in many aspects of female life, as the entrance to the interval environment via the vagina, for urinating, sexual functioning and childbearing. Unfortunately, several diseases can occur in this area which may affect all these different domains of vulvar functioning.[14]

The studies included in this thesis are focusing on four diseases of the vulva

- vulvar squamous cell carcinoma (VSCC)[14];
- precursors vulvar intraepithelial neoplasia (VIN) consisting of
  a. vulvar high grade squamous intraepithelial lesion (vHSIL)
  b. differentiated vulvar intraepithelial lesion (dVIN)15;
- genital lichen sclerosus (LS)[16] (Table 1).

Table 1 Key characteristics of vulvar (pre)malignancies.

	VSCC[1,2]	vHSIL[15,17]	dVIN[15,17]	LS[16,18,19]
Incidence	2.6 : 100.000	3.3 : 100.000	0.08 : 100.000	10-20 : 100.000
Gradation	malignant	premalignant	premalignant	benign
Relative proportion of VIN-		95%	5%	-
Risk of malignant progression	-	9% (if treated)	33-86%	3-6%
Time to progression	-	41 months	9-23 months	unknown
Current treatment(s)	surgical excision, adjuvant (chemo) radiotherapy	surgical excision, ablative therapy and topical treatment	surgical excision	topical corticosteroids
Average age at diagnosis	68 years	30-50 years	60-80 years	69 years
Etiology	HPV infection/ unknown, TP-53 mutation early observed	HPV infection	unknown, TP-53 mutation early observed	unknown, an overactive immune system or an imbalance of hormones may play a role
Recurrence	up to 40%	common	13-32 months, less common	NA

VSCC=vulvar squamous cell carcinoma, vHSIL=vulvar high grade squamous intraepithelial lesion, dVIN=differentiated vulvar intraepithelial lesion, HPV=human papilloma virus, NA=not applicable

VULVAR SQUAMOUS CELL CARCINOMA Vulvar cancer is rare with an incidence of 2.6 per 100,000 women per year and only accounts for an estimated 0.4% of all cancers and 5% of gynecologic cancers.[1] As mentioned, of all vulvar cancer types, VSCC is the most common histologic type and focus of this thesis is on this subgroup. The average age at diagnosis is 68 years for VSCC patients. Remarkably, especially in younger women the incidence is rising.[7,21] There are two different pathophysiological pathways for VSCC: (I) an human papilloma virus (HPV)-independent type, accounting for 70% of all VSCC's, which is frequently associated with LS and/or dVIN and therefore mostly observed in older women and (II) a HPV-dependent type, accounting for 30% of all VSCC's, which is often associated with vHSIL and occurs mostly

in younger women. [16,22-25] Surgery with or without adjuvant (chemo)radiotherapy is the cornerstone of treatment for VSCC.[26,27] Positive surgical margins are associated with high local recurrence rates up to 40% and a corresponding poor 5-year survival of 25-50%.[28,29] In addition, difficult to identify precursor lesions as vHSIL and dVIN are often found adjacent to the tumor. Incomplete resection of these lesions may contribute to earlier recurrence of disease.[30]

VULVAR INTRA-EPITHELIAL NEOPLASIAS: VHSIL AND DVIN Vulvar intra-epithelial neoplasias are etiologically categorized into a HPV-dependent and HPV-independent variant (vHSIL and dVIN respectively).[25] 95% of all VIN cases concern vHSIL.[16] Both premalignant conditions have a distinct etiology and pathways towards VSCC.[23] vHSIL is caused by a persistent high-risk HPV infection, which in 90% of cases is identified as HPV 16. These HPV-dependent lesions are more frequently observed in premenopausal patients. vHSIL lesions are typically multifocal, can appear as a visible lesion and/or a palpable abnormality and diagnosis is confirmed by histological review of a lesional biopsy. Microscopic characteristics include loss of maturation of the squamous epithelium. This phenomenon is mainly observed in the middle and upper third layer of the epithelium to full thickness (formerly termed VIN 2 or 3). If minimized to basal atypia, the lesion is classified as vulvar low-grade squamous intraepithelial lesion (VLSIL, formerly termed VIN 1). vHSIL is subdivided in a basaloid and warty subtype, based on the morphologic and histologic features.[6] The main goals for treatment are to prevent progression towards VSCC and to relieve symptoms. The risk of malignant transformation in untreated vHSIL is estimated to be as high as 9%.[16,31] Symptoms include vulvar pruritis, pain and dyspareunia which lead to a substantial burden for the patient. Currently the choice of treatment for vHSIL depends on the level of invasiveness, prior treatments, and the location of the lesion(s).[11,32] Treatment options include surgical excision, ablative therapy and topical pharmacological treatment with e.g. imiquimod.[32,33] The pathogenesis of dVIN is less understood compared to vHSIL and mostly observed in postmenopausal women. dVIN is usually found as an unifocal and unicentric lesion developed in the background of lichen sclerosis or adjacent to VSCC. Lesions appear generally as grey-white discolorations with a rough surface, elevated nodules or white plaques. Identification is difficult due to lack of accurate and rePROducible diagnostic criteria.[34] If left untreated, dVIN has a high absolute risk of 33-86% malignant progression.[12] As a result, dVIN is rarely identified in advance of a diagnosis of invasive malignancy, despite being the precursor lesion of approximately 80% of VSCC's.[35] Symptoms of dVIN are similar as described for vHSIL. Primary treatment for dVIN patients consists of surgical excision.

LICHEN SCLEROSUS LS is a chronic, progressive, mutilating disease with a high impact on quality of life. It occurs mainly (85-98 % of all cases) in the anogenital region but can theoretically develop on any skin surface. Severe cases of vulvar LS can show scarring and narrowing of the vaginal introitus, along with disappearance of the labia minora and clitoris. This is all accompanied by severe itch and pain independent of disease severity. The cause of LS is unknown.[17] A genetic and autoimmune role has been suggested, as up to 12% of the patients report LS in their family history.[36] Histologically, LS is characterized by marked inflammation and epithelial thinning. LS treatment comprises of patient education and life-long intermittent ultrapotent topical corticosteroid application.[35] Disadvantages of this treatment consist of a burning or irritated sensation of the vulva. And if continuously applied, atrophy of the skin can occur, further worsening the effects of epithelial thinning. Daily use of topical emollients may help to relief symptoms or be used as vaginal lubricant for dyspareunia.[17] The risk of development of VSCC in patients with LS is up to 5 percent.[17,37] There is prospective evidence that proactive management of LS may lead to a reduction in risk of the development of squamous cell carcinoma.[38]

MULTIMODAL CHARACTERIZATION WITH BIOMARKERS FOR VULVAR HEALTHY AND DISEASED SKIN

At present, identification of VSCC, vHSIL, dVIN and LS is mainly based on visual and tactile skills of the medical specialist. No universal objective clinical scoring systems are present to guide these diagnostics. The current 'golden standard' used for confirmation of clinically suspected vulvar tissue is by histological examination of a biopsy. However, biopsies are invasive and uncomfortable. In addition, when dVIN is suspected, it is sometimes difficult to demonstrate the presence or absence of dysplasia. The difficulties faced during this convoluted diagnostic process can lead to delayed or incorrect vulvar tissue classification.[15,39] Accompanying consequences are re-excisions, local recurrences, regional metastases and associated

worse prognosis. Altogether a decreased quality of life often ensues patients with vulvar diseases. This underlines the high unmet medical need to preferably non-invasively and real-time discriminate vulvar abnormalities and improve therapeutic options. Disease specific biomarkers could aid for those needs. Biomarkers are quantifiable measurements of a biological process that can contribute to diagnosis, prognosis and therapy of diseases.[40] A valuable biomarker discriminates correctly normal and pathological conditions and/or the pharmaceutical response to a therapeutic intervention.[41,42]

A multimodal approach has been applied within this thesis to identify novel biomarkers and methods for vulvar disease characterization. The multimodal research approach included different domains: imaging, molecular and cellular complemented by patient reported outcomes and physician-based input. (Figure 2). The focus of this thesis was specifically on the molecular and imaging domains. The composition of this approach is based on the most important aspects of the blueprint for early phase clinical pharmacology studies in the field of clinical pharmacodermatology.[43,44] With this multimodal approach we aim to obtain a more complete patient profile regarding vulvar skin disease.

The following principles of this multimodal approach are described below in more detail:

- **Non-invasive clinical tools to discover vulvar biomarkers**, focusing on discrimination of aberrant vulvar tissue (Figure 1, all domains except drug development);
- **Development of healthy and diseased *in vitro* vulvar models,** to increase pathophysiologic knowledge and improve drug development (Figure 1, cellular domain);
- **Molecular target identification of vulvar carcinomas,** to guide staging and surgery with real time optical imaging (Figure 1, molecular and imaging domain).

SECTION I NON-INVASIVE CLINICAL TOOLS TO DISCOVER VULVAR SKIN BIOMARKERS

In the field of clinical (pharmaco)dermatology several non-invasive imaging tools are successfully applied to detect cutaneous biomarkers.[43,44] These biomarkers could potentially be translated to vulvar skin research. Therefore, discriminatory properties of several imaging tools are examined on the vulvar area in a clinical trial. Lesional tissue of vulvar HSIL and LS patients was compared to vulvar skin of healthy controls using the techniques described below.

A *dermatoscope* is used as an imaging tool to better visualize subsurface structures and identify patterns that help improve diagnostics for a wide range of dermatologic skin diseases.[46] The dermatoscope is a handheld device that functions as a magnifier and is routinely applied by dermatologists to enhance diagnosis of melanoma, basal cell carcinoma and other cutaneous disorders.[46] However, its application on the vulvar area is currently limited to vulvar pigmented lesions.[47]

Optical coherence tomography (OCT) is a non-invasive imaging technique that provides real-time cross-sectional images of biologic structures, based on differences in tissue optical properties. OCT can determine epidermal thickness, skin roughness and blood flow parameters based on algorithms These algorithms use visual information to determine numerical values. OCT has been incorporated in the daily practice of ophthalmology for the visualization and diagnosis of retinal diseases.[48] In addition, OCT is an established research tool in dermatology, mostly for recognition of non-melanoma skin cancer. In this setting, it has shown potential to reduce biopsy frequency in basal cell carcinomas.[49] OCT is not an established tool in the gynecologic clinic yet, but a handful of clinical and *ex vivo* studies in cervical, vulvar and ovarian tissue suggest potential for differentiation between healthy and (pre)malignant tissue of epithelial origin.[50,51]

A *reflectance confocal microscope* (RCM) is an *in vivo* confocal imaging tool that uses a low powered laser to provide non-invasive and real-time visualization of the epidermis and superficial collagen layers at a cellular level up to a depth of 250 µm.[52] This results in optical transversal sectioning of unstained epithelium and stroma. This technique has been applied for early and accurate diagnosis of skin diseases, including basal cell carcinoma, and reportedly may reduce unnecessary biopsies for the diagnosis of melanocytic lesions.[53,54] Despite these technological and clinical advancements in the improvement of diagnostic accuracy, RCM imaging has only sparingly been applied on the vulvar area.[55–57]

Mobile e-diary application for monitoring of patient-reported outcomes and treatment adherence has been successfully applied in interventional studies in several skin diseases.[45]

This method is convenient for both the patient and the investigator. Data can be digitally stored in a safe environment. This e-diary tool is therefore assessed in this clinical study to monitor symptoms and treatment adherence.

SECTION II DEVELOPMENT OF HEALTHY AND DISEASED IN VITRO VULVAR MODELS

Besides the importance of biomarkers for monitoring of disease status, focus should also be on novel drug development in the vulvar field. Prior to *in vivo* testing of novel drugs in human clinical trials, *ex vivo* testing in (animal) models is used to study e.g. toxicity, to define novel drug sensitivity, predict human safety and provide reliable toxicokinetic evidence. Unfortunately, no well-established experimental models are available that mimic human healthy skin or VSCC.[58]

Most vulvar cancer research and drug screening programs are performed on tumor biopsies or (transgenic) animal models.[59,60] However, animal models are time intensive, expensive, and obtained data are often difficult to extrapolate to the human situation. Human skin equivalents (HSE's) are *in vitro* 3D reconstructions which may form a solution for the lack of experimental models (Figure 4).[61-63] HSE's are tools that can be used to study biological processes in the skin including healthy and disease conditions.[64] In addition, these models can serve as a prediction model to determine the penetration profile of compounds/drugs across the skin. To mimic a VSCC *in vitro*, general knowledge of squamous cell carcinomas (SCC's) is essential and especially of the crucial tumor microenvironment.[65] In SCC's, the epidermal homeostasis gets disrupted by interactions between epidermal cancer cells and underlying stroma, allowing epidermal cells to invade into the stroma.[66] Stroma consists of basement membrane, immune cells, fibroblasts and extracellular matrix. It has been proven that papillary and reticular fibroblasts (PF'sand RF's, respectively) are important for both epidermal and dermal homeostasis in cutaneous models.[67,68]

In addition, cancer associated fibroblasts (CAF's) are key players in cutaneous squamous cell carcinoma (CSCC), as demonstrated in previous work with dermatologic skin models.[69] (Wu et al., submitted) CAF's are part of the tumor microenvironment (TME) and contribute functionally to the process of SCC progression (Figure 4).[58,70] While increasing knowledge on pathogenesis and drug development is obtained using cutaneous 3D models, these models are still lacking for healthy vulvar skin and VSCC.

SECTION III MOLECULAR TARGET IDENTIFICATION OF VULVAR CARCINOMAS

Along with development of the diagnostic process and drug intervention studies for vulvar (pre)malignancies, we have explored VSCC-specific targets that in the future might be used for real-time optical imaging. It has been shown in other oncological fields that real-time intraoperative guidance is of added value during surgery for complete and safe tumor resection.[71,72] Molecular imaging integrates advanced imaging modalities with probes targeting molecular biomarkers of interest.[73] An imaging probe consists partly of a contrast label, such as radionuclides for nuclear based imaging, paramagnetic or electron opaque substances for radiological techniques, or bioluminescent or fluorescent molecules for fluorescence guided imaging (Figure 5). This contrast label is conjugated to a molecular imaging agent with high affinity for a biomarker selectively expressed at the surface of tumor(-associated) cells (Figure 4A). Small molecules, peptides, aptamers, antibodies, protein fragments and nanoparticles have been used as molecular imaging agents.[74] After administration of an imaging probe to a cancer patient, real-time images of the tissue of interest can be obtained by a suitable camera system that generates optical contrast between tumor and surrounding healthy tissue. Optical imaging is a discipline within the molecular imaging field. Imaging agents applicable for optical imaging benefit of their high-spatial resolution and real-time localization. An example of optical imaging coupled with image-guided surgery is fluorescent guided surgery (FGS), which has been widely explored in the last decade (Figure 4B). Particularly, the use of near-infrared (NIR) fluorescence dyes can provide sufficient tissue penetration for vulvar carcinomas with up to 1cm ingrowth.[72] In this manner, FGS is a promising technique for real-time detection of occult tumor lesions and localization of cancer margins. Proper identification of tumor-specific targets for molecular imaging is the key to the success of FGS. The following characteristics define a potential protein marker for targeted imaging: extracellular biomarker localization, expression pattern, tumor-to-healthy tissue ratio, percentage and distribution of positive cells, and previous use of the biomarker for *in vivo* targeted imaging.[71,74,75] Although this technique is very promising, it is never generally explored for vulvar (pre)malignancies yet.

AIMS AND OUTLINE OF THIS THESIS

This thesis describes studies performed within a multimodal characterization approach of vulvar healthy and (pre)malignant skin. The focus is on the *physician, patient, cellular, molecular, and imaging* domains with the aim to find disease-specific biomarkers to improve the clinical management of vulvar (pre)malignancies. This thesis is divided into three sections:

Section I of this thesis discusses the evaluation of several non-invasive clinical tools on the vulvar healthy and diseased skin. **Chapter 2** covers the domains *physician- and patient reported outcomes and imaging*. A mobile e-diary application for monitoring of *patient-reported outcomes* and treatment adherence has been applied. Within the *imaging* domain, we investigated if outcome measures of the non-invasive imaging methods dermatoscopy and optical coherence tomography (OCT) could serve as biomarkers for vulvar diseased skin. Next, in **Chapter 3** reflectance confocal microscopy (RCM) has been assessed on the vulvar skin of healthy and diseased patients as part of the *imaging* domain. RCM shows great potential in other clinical fields, but has sparingly been applied on the vulvar area. The primary objective is to explore feasibility and tolerability of RCM imaging on premalignant vulvar skin. The *cellular* domain is discussed in **Section II**. **Chapter 4** describes how the experience with 3D human skin equivalents (HSE's) in dermatologic research has been used to set-up vulvar healthy and diseased 3D-HSE's. The cancer models were thereafter used to test the effect of standard-of care chemotherapeutics on the acquired tumors. In **Section III** the focus is on the *molecular* domain. In **Chapter 5** a systematic review is presented, elaborating on the search for potential molecular targets for fluorescence guided surgery (FGS) for vulvar cancer. Subsequently, in **Chapter 6** potential targets for FGS in VSCC are evaluated using immunohistochemistry on healthy and (pre)malignant vulvar tissue sections. The expression of each marker has been quantified using digital image analysis and expression scores are compared for all cohorts included. **Chapter 7** summarizes the results of all these chapters and highlights future perspectives.

Figure 1 Anatomy of the vulva.

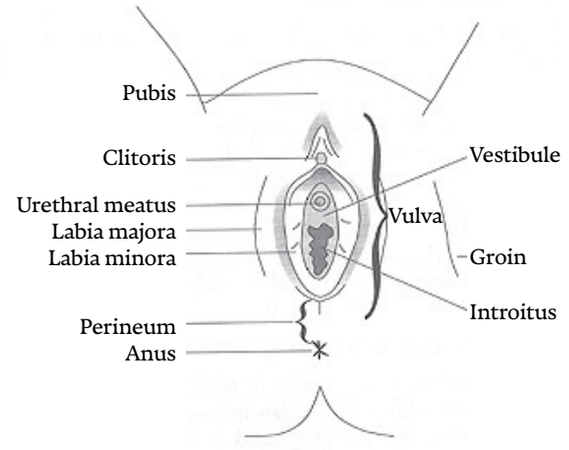


Figure 2 Multimodal profiling of vulvar healthy and diseased skin, to improve diagnostics and drug development.[41,42] Five domains are studied: physician, patient, molecular, cellular and imaging. Section numbering indicates the location in this thesis where a specific method is discussed.

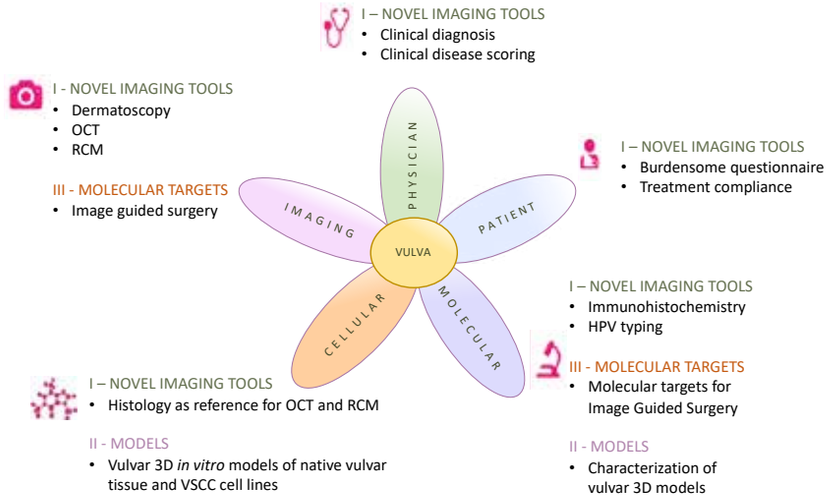
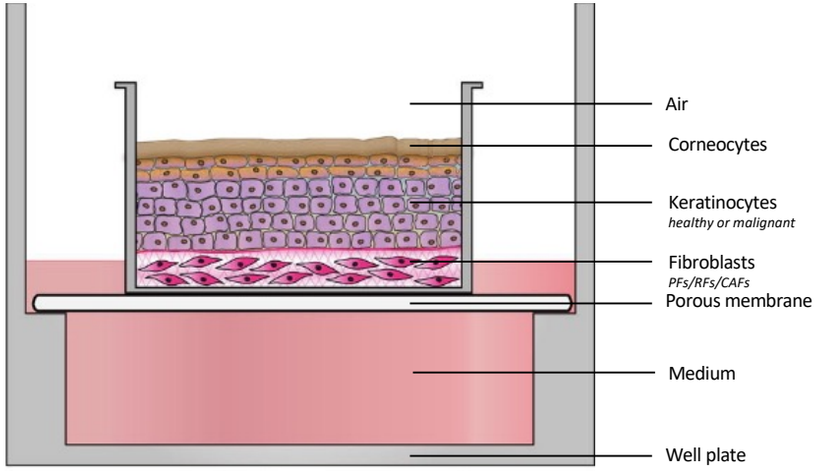
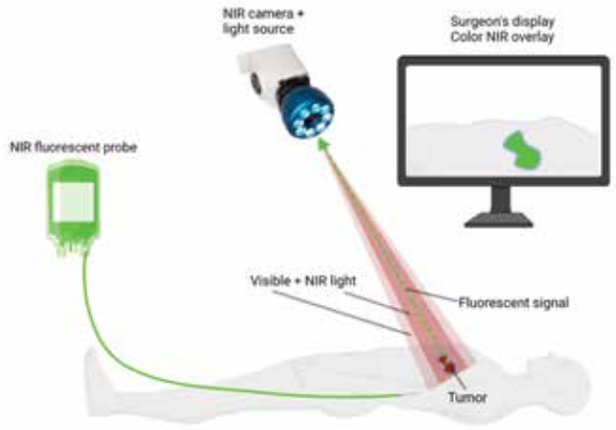


Figure 3 Cross-sectional view of a human skin equivalent (HSE) in an air-liquid well plate. Isolated healthy or squamous cell keratinocytes can grow on different type of fibroblasts. These HSE models shows an epidermis that includes all dermal layers observed in freshly obtained skin.

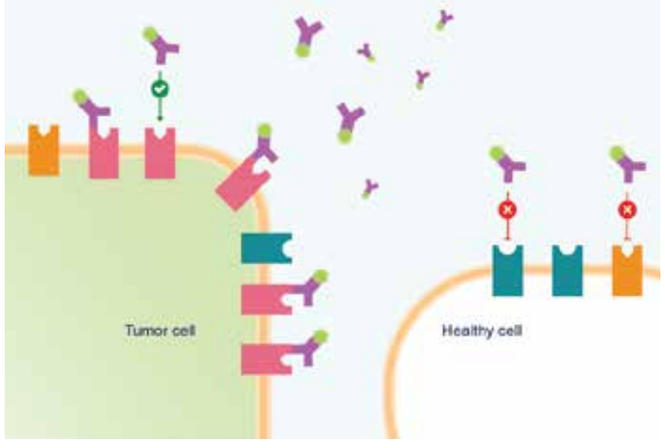


HSE=human skin equivalent, PF= papillary fibroblasts, RF=reticular fibroblasts, CAF'S=cancer associated fibroblasts

Figure 4 Image guided surgery using near-infrared (NIR) fluorescence.
A A NIR fluorescent contrast agent is administered e.g. intravenously or topically to a cancer patient. During surgery, the agent is visualized using a NIR fluorescence imaging system. This system must have adequate NIR excitation light, collection optics and filtration, and a camera sensitive to NIR fluorescence emission light. An optimal imaging system includes simultaneous visible (i.e., white) light illumination of the surgical field, which can be merged with NIR fluorescence images. The surgeon may see the camera records on a computer monitor, goggles, or a wall projector (monitor form factor shown).



B Graphical overview of binding of a probe, here a antibody (purple) conjugated to fluorescent label (fluorescent green), specifically to a target present at tumor cells and absent at healthy cells.



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