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Trending: Radioactive and fluorescent bimodal/hybrid tracers as multiplexing solutions for surgical guidance

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ABSTRACT

By contributing to non-invasive molecular imaging and radioguided surgery, nuclear medicine has been instrumental in the realization of precision medicine. During the last decade it has also became apparent that nuclear medicine (e.g., in the form of bimodal/hybrid tracers) can help empower fluorescence guided surgery. More specifically, when using hybrid tracers, lesions can be non-invasively identified and localized with a high sensitively and precision (guided by the radioisotope) and ultimately resected under real-time optical guidance (fluorescent dye). This topical review discusses early clinical successes, preclinical directions and key aspects that could impact the future of this field.

INTRODUCTION

With regard to the future implementation of image-guided surgery, there is a clear desire to support precision surgery via (receptor) targeted molecular imaging. This means that there is a growing need for input from nuclear medicine; next to hybrid modalities such as PET/MRI, combined use of radioactive and fluorescence signals can help strengthen image-guided surgery. This type of image-guidance can come in two forms: First, implementation of a radioactive and a fluorescent tracers for pre- and intraoperative imaging. To ensure surgical accuracy, in such an application one has to make sure both tracers independently allow delineation of the same lesions. Although such an approach supports the use of existing radiotracers, it is chemically extremely challenging to create fluorescent tracers that behave in an identical manner (on a molecular scale fluorescent dyes are inherently different than radiolabels). This is exemplified with the sentinel node tracers ^{99m}Tc-nanocolloid and Indocyanine green (ICG) (*1*). Second, and the focus of this review, a radioactive and fluorescent signature can be integrated in a single bimodal/hybrid tracer. Integration ensures co-localization of the two signatures and promotes an advanced form of symbiosis (“best of both worlds”) that empowers surgeons to improve intraoperative target delineation (Fig.1).

Hybrid tracers come in many flavors; not only can the platform on which they are based vary from small-molecules (*2,3*) to nanoparticles (including proteins and nanocolloids; (*4*), they may also use differently emitting radioisotopes or different luminescent signatures (e.g., light with different wavelengths; Table 1). While each individual hybrid tracer and administration route has been designed to serve a specific purpose, conceptually all use revolves around the notion that both signatures can be used to intraoperatively depict complementary features of the same target. Despite differences in signal intensities, there is a high level of overlap in the way multiplexing of the different imaging signatures occurs. In the context of surgical guidance, the radioactive signal allows identification and localization of a lesion by means of its radioactive signature (even in deeper tissue layers), while the optical feedback allows direct lesion visualization and delineation in exposed tissue in the surgical field and/or provides high-resolution pathological identification of the tracer accumulation (*5*).

In this review we discuss where surgical guidance with hybrid tracers stands in relation to fluorescence- and radio-guidance, describe the early clinical implementation of hybrid tracers and comment on the preclinical spin-off these efforts have created. Subsequently, we address key requirements in a product-target-profile that could support future hybrid tracer development.

The competitive and symbiotic aspects of radio- and fluorescence guided surgery

In the field of image-guided surgery, the hybrid concept coincides with the use of monomodal radiotracers (radioguided surgery) and fluorescent dyes/tracers (fluorescence-guided surgery; Fig.1). For some indications clinical guidelines state the use of radio- or fluorescence-guidance approaches, but monomodal radio- and fluorescent-tracers are also increasingly being implemented in experimental clinical trials, including “cocktail” formulations (*1*).

Based on the current scientific knowledge and technical possibilities it seems worthwhile to critically assess the pros and cons of the use of hybrid or monomodal radio-/fluorescent tracers (Fig.2). Due to the comparably low tissue attenuation of gamma radiation and its high detection-sensitivity, nuclear medicine has been able to use radiotracers for accurate non-invasive

diagnostics. Especially fused biochemical/anatomical information obtained through PET/CT, PET/MR and SPECT/CT help place suspected pathologies in their anatomical context (a "road-map") (*6*). Intraoperatively, real-time gamma probe-based tracing is currently the method of choice in radioguided surgery; however, in some cases this method is also complemented by intraoperatively acquired static images obtained using, e.g., a portable (gamma) camera or freehandSPECT scan (*7*). Intraoperative radioguidance has been successfully applied in a number of "targeted" applications (Fig.3), the most important ones being sentinel node biopsy (*4*), neuroendocrine tumors (*8*) and recurrent prostate cancer (*9*). In these applications, the high detection sensitivity has made use of microdosing (<100μg/patient). Decay characteristics, relatively low tracer-cost and broad availability, have made ^{99m}Tc the radionuclide of choice for radioguided surgery (*9,10*). It must be noted that although the spatial resolution of radioguidance modalities lies in the high mm to low cm range (*7*), the limited tissue-induced signal attenuation means lesion delineation can suffer from background signals coming from underlying tissues or organs.

Compared to radioguidance, optical-guidance has a superior spatial resolution and is even detectable down to the microscopic level (*5*); Cerenkov light can provide a resolution of <2 mm (*11,12*). Unfortunately, light suffers heavily from tissue-induced signal attenuation, which limits the in-depth signal penetration of even near-infrared dyes to < 1 cm (*13*). While fluorescence imaging is sensitive in the lab (*in vitro*, *ex vivo*, in mouse models), the aforementioned *in vivo* signal attenuation severely limits the detection sensitivity in humans subjects (*14*). This statement is couched by the fact that fluorescence imaging in rodents hardly is predictive for the clinical value. Despite these limitations, however, the major asset of fluorescence imaging is its capability to accommodate the surgeons desire to optically identify the lesions in real-time. In this context, Cerenkov imaging, with its low luminescence brightness, represents an exception, since it requires the surgeon to still rely on static images (*11*). Routine clinical implementation of fluorescence-guided surgery has primarily been focused on physiological indication, where "flow" information provides a valuable imaging read out (Fig.3). At doses well above the micro-dosing level, fluorescent tracers such as fluorescein and ICG have allowed for superficial

fluorescence-guidance in dynamic processes such as vascular perfusion (angiography and anastomosis), lymphangiography (lymphatic drainage), liver clearance (cholecystectomies and identification hepatobiliary metastases) and renal clearance (ureter visualization) (*5*). Metabolism of 5-ALA in lesions, however, provides an indirect "targeted" application for fluorescence imaging (*5*).

Early clinical implementation of hybrid tracers

Pioneering clinical trials with hybrid tracers (*15,16*) have helped to overcome some reservations among researchers and surgeons with regard to this innovative surgical guidance concept. Table 1 illustrates that the field has moved forward over the years and underscores that different combinations of isotopes and dyes can be used in the clinical setting. Analogues such as ICG-^{99m}Tc-nanocolloid (*4*), ^{131}I-fluorescein (*3*), $^{123,\ 125}$I -Methylene blue (*2,17*) as well as Cerenkov imaging (*12,18*), add to procedures previously performed with parental radio- or fluorescent tracers. Of all the hybrid tracers applied in patients to date, ICG-^{99m}Tc-nanocolloid is the most frequently and widely applied (*4,19-21*). A recent study with this hybrid tracer, also indicated that fluorescence-guidance without prior knowledge of the location of the lesion potentially results in incomplete resection (*1*).

Hybrid tracer development

The literature on the preclinical use and assessment of hybrid tracers can be considered substantial. Following the pioneering review by Culver et al (*22*), numerous others have addressed the topic (*5,16,23-26*). Next to oncological (imaging) targets, e.g., HER2, PSMA, CEA, $\alpha 5\beta 3$, sstr2 and CXCR4 and/or sentinel node (SN) imaging applications in, for example, infectious diseases have been reported. In preclinical studies, a plurality of imaging labels have been incorporated into hybrid tracer-designs: radiolabels (e.g., ^{99m}Tc, ^{111}In, ^{18}F, ^{68}Ga, and ^{89}Zr) and fluorescent labels (e.g., Cerenkov, FITC, Cy5, NiRDye800CW, ZW800, and ICG). In an attempt to cover the essence of these initiatives, we refer to key review articles according to their chemical scaffold: peptides (*24,25*), nanobodies (*26*), antibodies (*16,24*) and (bio)nanoparticles (*5*). From

this combined literature we concluded that there are essentially three ways of creating a hybrid tracer: i) functionalization of large proteins and nanoparticles generally occurs with separate radio- and fluorescent labels, ii) functionalization of peptides takes place with hybrid imaging labels that contain both a fluorescent- and a radiolabel or by functionalizing the C- and N-terminal ends with a different imaging label, and iii) for small molecules only hybrid imaging labels are an option.

Key features hybrid tracer design

Although addition of a radiolabel and fluorescent dye to a targeting vector (e.g., small molecule, peptide, nanobody, antibody or nanoparticle) may seem trivial, generation of hybrid tracers suitable for clinical translation certainly represents a challenge. Here some key design features that can be taken into consideration and may support decision making (*27*).

Needless to say, successful chemical- and technological- imaging innovations should address a specific clinical need. In doing so, incorporation of a complementary imaging signature should ideally help to realize a near-seamless extension of a existing nuclear medicine diagnostics. This implies selection of known targets with proven clinical value, e.g., high receptor overexpression as seen in neuroendocrine tumors or PSMA-positive lesions (*8,9*). Most preclinical efforts in fluorescent- or hybrid tracer design, however, do not bench-mark their work to the current medical standard or a reference radiotracer. In the clinic, structurally optimized and clinically established radiotracers should be used to select patients that will then benefit from surgery. Only when surgery is scheduled, a second scan (with the hybrid tracer) may be performed to ensure that the lesions targeted during surgery correspond with those defined on diagnostic imaging. Since the chemical composition of a hybrid tracer intrinsically differs from that of the (parental) radiotracers, such a two-step procedure also helps establish the reproducibility in tracer performance (*9,28*). Here the Cerenkov imaging concept holds a potential advantage as its optical signal is based on the parental PET tracer (*12*). It should be noted that the use of mono-labelled fluorescent tracers for surgical guidance does not support

such a critical reproducibility check and could mean the surgical resection deviates from its planning, which was based on the "roadmap" defined by the diagnostic scan *(1).*

One of the key questions regarding the application of hybrid tracers evolves around the "gap" in sensitivity between radioactive and fluorescence imaging *in vivo*; radiotracers with a high specific-activity and can be used for imaging at a pM dose whereas fluorescent agents are often used in the μM range. This sensitivity "gap" suggests relatively high quantities of hybrid tracer are required and specific activity should be reduced in these formulations. Clinical use of the hybrid tracer ICG-^{99m}Tc-nanocolloid, however, indicates that the hybrid concept supports the use of much lower quantities of fluorescent dye (*29*). A study with ICG-^{99m}Tc-nanocolloid (*14*), further implied that micro-dosing concept may well be compatible with fluorescence-guided surgery, an observation that has been underlined by others (*12,30*). The same study did, however, indicate that fluorescence-guidance became less accurate in areas with relatively low accumulation rates (*14*). This trend, combined with relatively low tumor receptor density expressions, could mean that fluorescence imaging will underestimate the number of lesions when not supported by nuclear medicine.

After saturation of all target sites *in vivo* an increase in dose may negatively influence the specificity. Hence, the optical "brightness" of fluorescence needs to be improved to make them fully compatible with micro-dosing. Fluorescent signals can be amplified by increasing the excitation light intensity (*11*) or by using dyes with a higher brightness (*13*). Given their low toxicity, low molecular weight (< 1000 Mw) and widespread clinical use, cyanine (Cy) dyes are the dyes of choice for fluorescence-guided surgery. It is, however, unfortunate that the near-infrared Cy7 analogues display a low brightness (*31*). Introduction of additional aromatic units on the indoles reduces the brightness, while increasing the rigidity of the polymethine bridge enhances the brightness (*32*). Far-red Cy5 analogues provide a substantially brighter alternative (*13*), which can even be improved further via dye sulphonation (*33*). This increase in brightness was shown to translate into a deeper signal penetration (*13*). Inorganic dyes such as quantum dots have been proposed as bright dyes for fluorescence-guided surgery. Unfortunately, this brightness comes at the cost of a large stokes-shift (possibly limiting tissue penetration of the

excitation light), a relatively high molecular weight which influences dosing, and the presence of potentially toxic elements. In Cerenkov imaging the brightness is directly related to the radioisotope used and is thus inherent to the radiotracer used (*11*).

As a rule of thumb, a signal to background ratio > 2 is essential for efficient surgical guidance using tracers, and thus specificity of tracer accumulation and well-balanced accumulation and clearance kinetics are key. It is well known that the fluorescent dye component of (hybrid) tracers, even when large proteins are used as targeting vectors, influences their pharmacokinetics (e.g., via the lipophilicity of fluorescent dyes, which can influence serum binding and non-specific interactions with non-target tissue) and receptor affinity. In a direct comparison, Baranski et al demonstrated that use of structurally very different fluorescent dye moieties in PSMA-tracers impacted the biodistribution (*34*), a finding that is underlined by studies that investigated how variation in cyanine dye can be used to tailor tracer performance (*13,35*). For example, reducing the dye lipophilicity can positively effect tumour/background ratio's, while increasing the number of charged moieties enhances renal uptake (*13*). Interstingly, these effects can vary between targeting moieties (*33*). Nevertheless, many chemists still adhere to a "one size-fits-all" approach that is often based on the potentially radio- and chemical- unstable dye NIRDye800CW (*24,32*). When (inorganic) nanoparticles are used as fluorescent label, their sheer size, relative to the targeting vector(s), means the dye properties dictate the in vivo kinetics (*36*).

Future prospects

While the field of fluorescence-guided surgery is growing, it is becoming more and more apparent that some inherent limitations of the photophysics can be overcome by integrating fluorescence imaging with nuclear medicine. Although ongoing, clinical implementation of such concepts still remains restricted. One obvious discrepancy between clinical implementation and the more widespread preclinical technology development is that rodent models are not very representative for the clinical situation regarding e.g. the dose, physical size, rate of excretion and metabolism, biodistribution or tumor growth and composition. Although often ignored, these differences also impact the compatibility of tracers with clinically available imaging

modalities. Hence, experiments in large animals (e.g., pig, veterinary patients or disease models; Fig. 4) are required to efficiently engineer matching modalities such as hybrid modalities or GPS-like surgical navigation strategies that facilitate multiplexing of different signatures (*7*). By extending the hybrid concept into engineering (Fig.4), the field is maturing and the chance of creating clinical impact is increasing.

CONCLUSION

The introduction of hybrid tracers has provided a valuable extension to the fields of molecular imaging, nuclear medicine and, in particular, radio- and fluorescence-guided surgery. Early clinical translations have already impacted clinical care. This helps shape a landscape that: 1) facilitates further development and translational efforts in the areas of chemistry and engineering, 2) makes the hybrid tracer concept more accessible in the surgical and nuclear medicine environment and 3) supports the spread of the hybrid concept to clinicians and researchers in different surgical disciplines.

CONFLICT OF INTEREST STATEMENT

No potential conflicts of interests relevant to this article exist.

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Table 1. Hybrid tracers applied for surgical guidance in the clinical setting

Name tracer	Application	Parental tracer	Year first clinical implementation	Number of patients	Clinical routine Y/N
ICG-^{99m}Tc-nanocoloid (*4,19-21*)	Sentinel node imaging, radioguided occult lesion localization, tumor margin deliniation	^{99m}Tc-nanocolloid (head-to-head reproducibility study performed (*28*))	2009	>1500	Y
^{131}I-Fluorescein (*3*)	brain tumour identification	Fluorescein	1950	104	N
Cerenkov (*18*)	Rectal cancer identification, Head and neck cancer, melanoma	^{18}F-FDG	2014	> 60$^{\#}$	N
^{111}In-DOTA-Girentuximab-IRDye800CW (*37*)	Clear cell renal cell carcinoma identification	PET tracer Girentuximab	2018	15	N
^{68}Ga-IRDye800CW-BBN (*38*)	Glioblastoma multiforme identification	Radiolabelled BBN	2018	14	N
^{125}I-methylene blue (*17*) **^{123}I-Methylene blue (*2*)**	Sentinel lymph node biopsy, Parathyroid adenoma identification	Methylene Blue	2007 1972	12 5	N N
FITC-^{125}I- CEA mAb (*39*)	Colorectal carcinoma	Radiolabelled CEA-mAb	1992	6	N
124I-cRGDY-PEG-C (*40*)	Metastatic melanoma identification	**cRGDY-PEG-C ***	2014	5	N

N.D. = not defined, * = not clinically used, $^{\#}$ number was provided in a personal communication

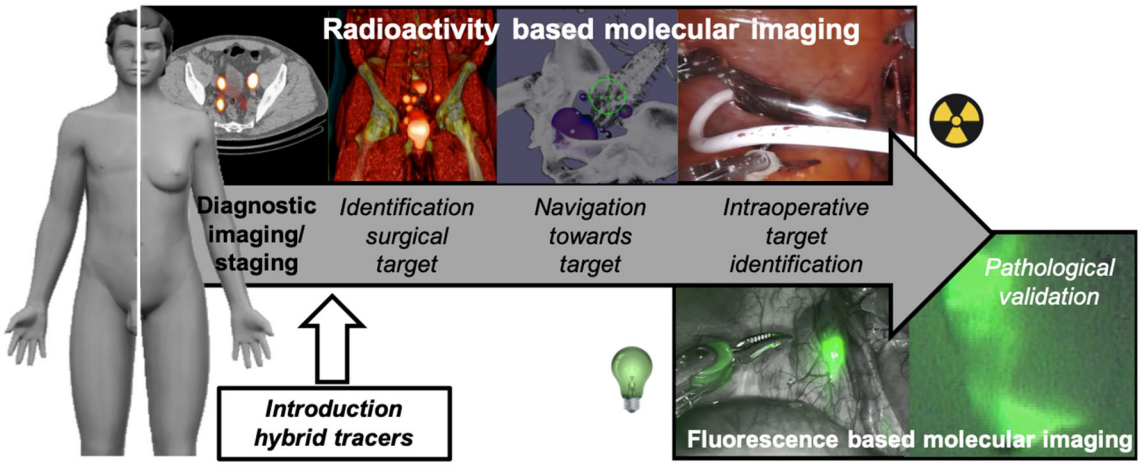

Fig.1. Schematic overview of the concept of molecular imaging-guided precision surgery (example robot assisted sentinel node procedure with the hybrid tracer indocyanine green-^{99m}Tc-nanocolloid).

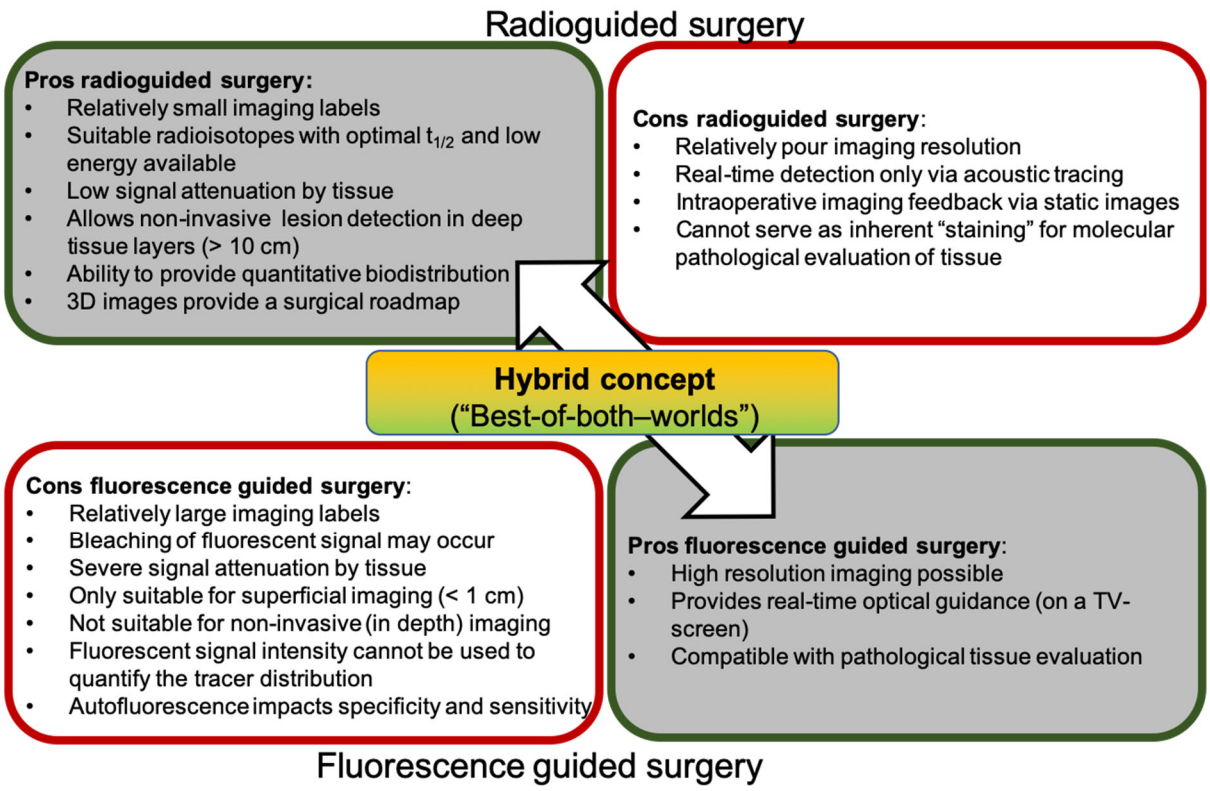

Fig.2. Pro's and Con's for radio- and fluorescence-guided surgery

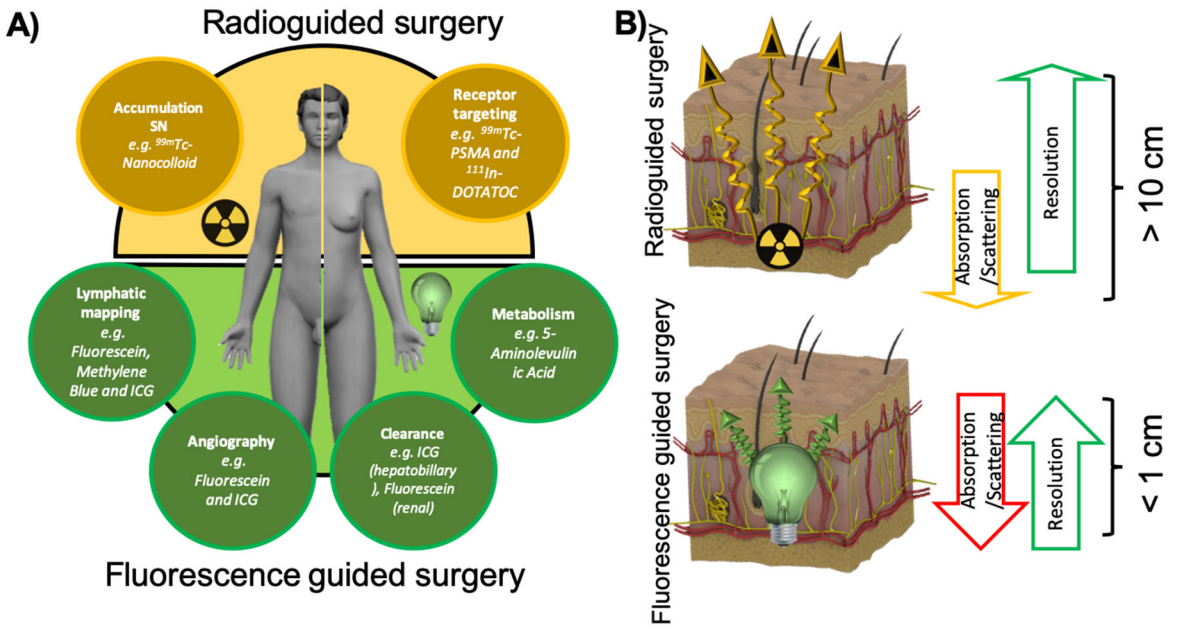

Fig.3. A) Overview of routine indications for radio- and fluorescence-guided surgery and B) Signal penetration vs. resolution.

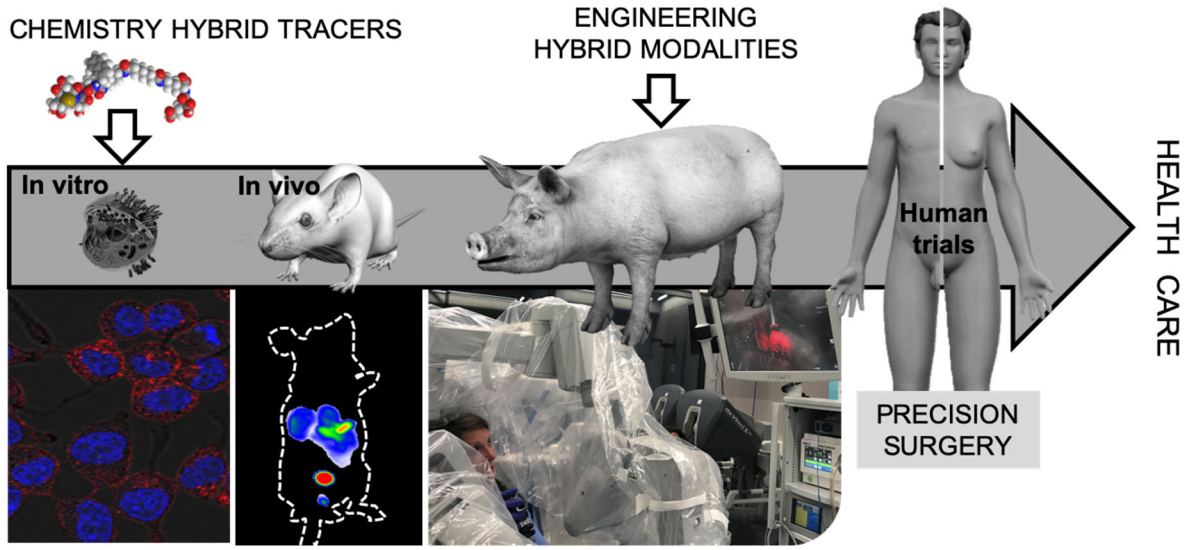

Fig.4. Translational pipeline towards routine health care for innovations in chemistry and engineering