1 Introduction

The discovery and development of new drugs is a key process in the fight against diseases. However, it is a time- and cost-intensive cycle. From the identification of a lead structure to the clinical trials, 14 years and about 800 million US dollars are estimated to be consumed.[1] Hence, every step of the process is subject to optimization and fluorescence-based imaging techniques are useful instruments in their optimization by visualization. Typically, the different steps are defined as target identification, target validation, lead identification, lead optimization, preclinical stage, and finally clinical trials.[2] The ideal drug target is a macromolecule, closely linked to the tackled disease that binds a small molecule.[3] Proteins and especially enzymes are therefore often chosen as valuable targets but also nucleic acids with a defined secondary structure have been successfully employed as drug targets.[4-7] Furthermore, G-protein coupled receptors (GPCRs) are popular target structures.[8-10] The aim of the interaction with the target is often the alteration of pathological protein functions, or in case of drugs targeting pathogens, the elimination of a unique target that leads to cell death. Especially in cancer, the identification of a valid target can be challenging since the physiological counterparts of dysfunctional proteins present in degenerated cells, often fulfill crucial functions in healthy tissue. Therefore, careful target validation is of tremendous significance to decrease the risk of interference with physiological functions or other pathways, the occurrence of side effects, and down-stream cost incurrence.[11,12] Besides biological and genetic approaches, the verification of a target in an *in vivo* disease model is a central step, often employing highly sophisticated fluorescence-based imaging techniques.[13] After successful identification and validation of a valuable drug target, small molecules interacting with the target are identified as potential leads. In the discovery of lead structures, two general strategies are applied: the high throughput screening (HTS) of large compound libraries or a structure-based drug design (SBDD). A broad variety of HTS has been developed over the last years, enabling the screening of up to 100,000 compounds per day in micro-titer scale assays based on fluorescence, radiometric, or colorimetric methods in a fully automated manner.[14-17] The recent development of DNA-encoded libraries (DELs) enables the investigation of thousands of different molecules with a target in a one-pot approach providing even more cost-efficiency in screenings. A whole library of molecules, each of them labeled with a specific DNA sequence, can be incubated within a protein binding assay for example, with a target protein and upon binding of one of the molecules, the lead can be identified using the DNA tag (**Figure 1.1**).[18-20]

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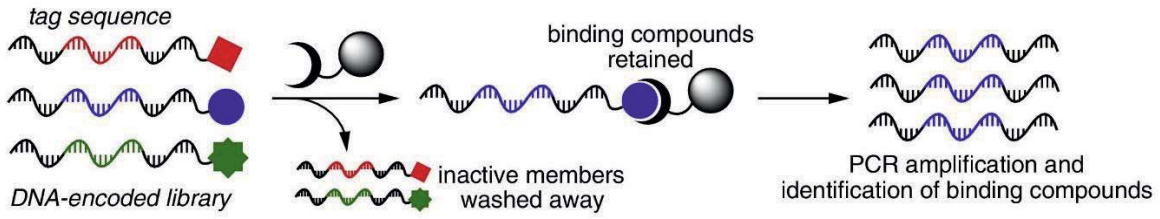

Figure 1.1: Simple example of a DNA encoded library assay with a target on solid phase.[18] [i]

The quality of large compound libraries has been significantly improved with defining the chemical space in which a potential lead is most likely situated. Lipinski proposed in 1996, after scanning and analyzing various libraries and positive lead hits, that poor permeation and absorption, which are critical characteristics for potential leads, are more likely if a compound exhibits a) more than 5 H-bond donors (expressed as the sum of OHs and NHs), b) a molecular weight greater than 500 g/mol, c) a log P (as a measure for hydrophilicity/lipophilicity) over 5, d) more than 10 H-bond acceptors (expressed as the sum of Ns and Os). Compound classes that are substrates for biological transporters have been defined as an exception to that rule.[21,22] By limiting compound libraries according to criteria like Lipinski's rule of 5 or the supposed ADME (absorption, distribution, metabolism and excretion) criteria, an increased efficiency in drug screenings has been achieved. A great advantage of HTS is that the binding site of the target does not have to be known *a priori*. However, if the binding site is known, often by resolving the protein crystal structures, a more rational approach to lead identification can be taken by the structure based drug design. Computational methods such as DOCK, aiming at finding the correct conformation of a ligand and its receptor, often in combination with molecular dynamic simulations, can result in exact lead predictions.[23,24] Iterative cycles and evaluations of these processes, together with the identification of structure-function relationships, are providing lead optimization. In the following preclinical phase, a synthetic strategy is usually developed that allows to synthesize the optimized lead efficiently in large scales and high purity. Additionally, a formulation is developed as a way to administer the drug (pill, spray, ointment etc.). Toxicity studies in animals are the basis of the permission process with regulatory authorities before entering the clinical phase and administering the potential drug to humans.[25] The final clinical development consists of four different phases: in phase 0, minimal concentrations of the investigated drug, significantly smaller than the therapeutic range, are administered to a small group of healthy individuals to gather preliminary ADME data in humans. In phase I, safety, tolerability, pharmacokinetic properties, and pharmacological effects are tested in a group of 20-100, usually healthy, volunteers. In phase II, the effectiveness of the potential drug is tested in a group of a few hundred patients while monitoring side effects and in case of success, the test group is increased to several thousand patients in phase III.[26]

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1.1 Targeted drug delivery systems

Whenever a drug is administered to a body, if systemically, locally or orally, there are various obstacles a drug has to face before it reaches its target. In general, the degradation of drugs by different enzymes is a major problem, as well as occurring side effects, or the provocation of an undesired immune response. When systemically applied, renal or hepatic clearance, vascular heterogeneity, and the access to the targeted tissue or cells is an issue. Local administration is limited by anatomical barriers, additional risks are material-induced tissue damage or an uncontrolled (burst) release combined with then occurring toxicity. The success of oral drug uptake is highly depending on variations in pH values and enzymes in the gastrointestinal tract, as well as on mucosal or epithelial barriers. Additionally, the residence time of the drug in the body is usually shorter than 30 h, the time the passage from intake to excretion takes in average (**Figure 1.2**).[27]

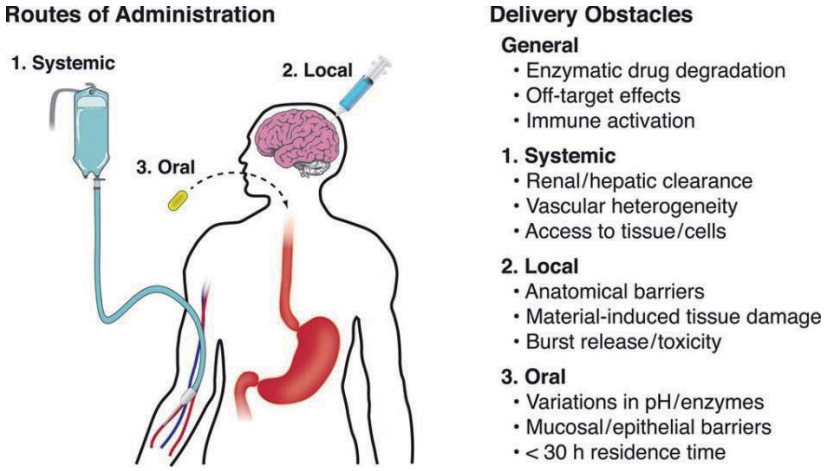

Figure 1.2: Different routes of drug administration and the various hurdles of drug delivery.[ii 27]

With the development of various drug delivery systems (DDSs), scientists have tried to overcome these obstacles in recent years. The aim of those systems is either the transport of a small molecule to a specific tissue with a targeted release to reduce undesired systemic effects, as well as to increase local drug concentration, and therefore drug efficacy. Or the aim is a controlled release in a specific tissue over time to avoid peaks and valleys in drug concentration, such as in implants or drug-covered stents.[28,29] For the former, either classic carrier molecules are utilized with a transport depending on the molecular structure, or by applying modern nanocarrier techniques, with effects mostly relying on the particle size.[30] Among the conventional carrier molecules are naturally occurring biomolecules, including lipid-based carriers and cell penetrating peptides (CPPs).[31-34] Both show the ability of transporting a cargo into a

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target cell by interacting with cell membranes. While lipids can easily pass membranes due to their hydrophobicity, the mechanism is not completely clear for CPPs. However, it is proposed that they strongly interact with negative charges on cell surfaces due to their polycationic character.[35] Unfortunately, the half-life of CPPs in biological systems is limited by their high sensitivity towards various enzymes and peptidases. The development of peptidomimetics, such as cell penetrating peptoids (CPPos) that display a shift of peptide side chains from the peptide bond α-carbon to the nitrogen atom, significantly increases their stability.[35-37] In nanocarrier systems, biodegradable and biocompatible polymeric materials, various nanoparticles, dendrimers, self-assembling micelles, or polymer-biomolecule hybrids are popular approaches towards drug delivery. Depending on the particle size, many of these systems are applied in cancer therapy. Solid tumors are highly vascularized and interestingly, the blood vessels in a tumor environment show an enhanced permeability for large molecules up to 300 nm in diameter, due to a fenestration with gaps between endothelial cells and a discontinuous or absent basement membrane.[38] If a drug is connected to a nanocarrier transporter in the desired size, it will show a prolonged circulation time in the blood stream after systemic injection since the large molecular systems are not able to pass the barrier of a healthy endothelial layer. When arriving at the tumor site, however, the molecules are able to leave the blood vessels and therefore, a targeted tumor transport is achieved. This effect is widely known as the enhanced permeation and retention (EPR) effect (**Figure 1.3**).[39,40]

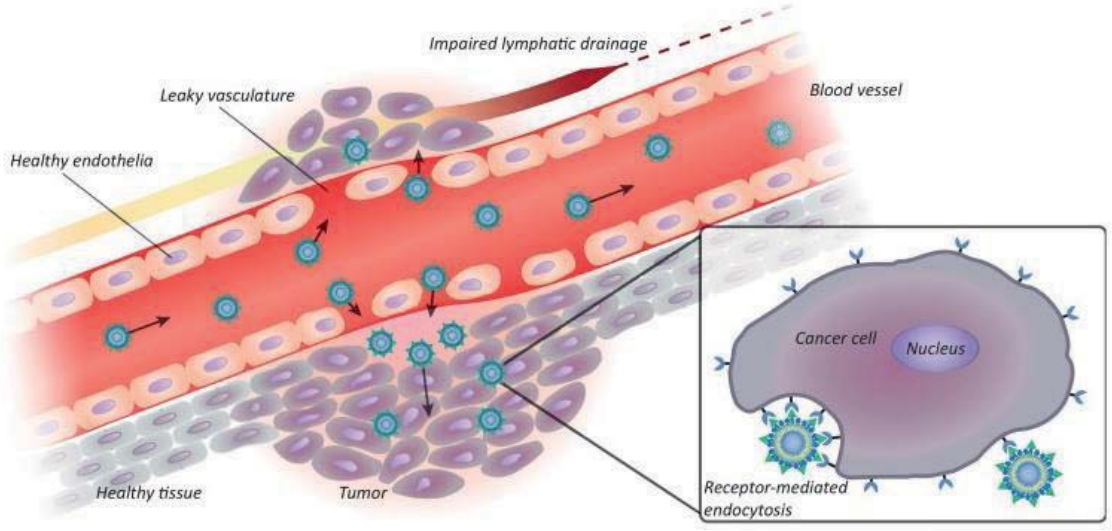

Figure 1.3: Passive tumor targeted drug delivery utilizing the EPR (enhanced permeation and retention) effect. Uptake of nanocarrier molecules through the damaged endothelial layer of blood vessels in tumor environments.[iii] [41]

A triggered release of the cargo from the nanocarrier can be achieved via different external stimuli such as the reduced pH value of tumor tissue.[29] With the application of

[iii] Reprinted from Trends in Biotechnology, Vol 32, Issue 1, Gavin T. Noble, Jared F. Stefanick, Jonathan D. Ashley, Tanyel Kiziltepe, Basar Bilgicer, igand-targeted liposome design: challenges and fundamental considerations, 32-45, Copyright (2014), with permission from Elsevier

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fluorescence-labeling and imaging techniques, a convenient tracking of successful delivery to the target site is possible.

1.2 Fluorescence-based imaging techniques

Along with the development of fluorescent labeling techniques, also the techniques of fluorescence imaging evolved since the first fluorescence-based microscopes were built in the early 1900s. In the basic set-up, the wide field fluorescence microscopy that is still a common tool in modern biology, a sample containing intrinsic or (most often) extrinsic fluorophores is excited as a whole specimen by a parallel beam of light combined with a filter set, allowing for specific fluorophore excitation. The simplicity of the instrumental set-up, together with the possibility of fast image acquisition when observing the whole sample at once, are of great advantage. However, due to diffraction and the projection of out-of-focus light, spatial resolution and contrast are often limited and the technique is only suited for thin specimen.[42] A possibility to improve the resolution of fluorescence microscopy came with the development of confocal laser scanning microscopy (CLSM). By using laser scanning excitation, the light is not directed to the whole specimen but to single points in the sample, and images are created by scanning the specimen point by point and subsequent computational reconstruction.[43] As in the wide-field method, the confocal set-up includes a dichroic mirror that directs the exciting light of a specific wavelength to the sample and the emitted light from the sample to a photomultiplier detector. However, by installing a pinhole between the mirror and the detector, the emitted light is bundled and out-of-focus light is widely excluded.[44] It is possible to detect specific layers within a thicker specimen in high resolution in a live-cell imaging approach. By shifting the focus level in z-direction and taking images of different layers, the single images can be combined afterwards to 3-dimensional structures. In extension of the single pinhole in CLSM, the spinning-disk confocal microscopy, often also referred to as Nipkow systems, was developed. Here, multiple pinholes are installed on a fast spinning disk, scanning multiple points within the specimen simultaneously and instead of a photomultiplier, a charge-coupled device (CCD) camera serves as a detector.[45] This method is especially valuable in the observation of dynamic processes such as fluorescence resonance energy transfer (FRET) between two different fluorophores, fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime imaging (FLIM). While FRAP is used to measure molecular dynamics within cells and cell surfaces, FLIM provides information from an ensemble of fluorophores about their local environment.[46] Despite all that progress, fluorescence-based imaging techniques were for a long time limited to the resolution borders Ernst Abbe stated already in 1873. According to his law, lateral resolution is limited to roughly half the wavelength of the illumination light.[47] Just recently, novel techniques in super-resolution microscopy have succeeded to overcome these limitations. The long-known resolution limit of about 200 nm could be decreased to about 10 nm, thus resolving structures on a subcellular level.[48] Different methods belong to this sophisticated class of imaging that can be

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broadly divided into those patterning the illumination light and those detecting and localizing single molecules. Structured illumination microscopy (SIM) and stimulated emission depletion microscopy (STED) belong to the former category, stochastic optical reconstruction microscopy (STORM) and photoactivation localization microscopy (PALM) belongs to the latter.[49] The structured illumination microscopy is taking advantage of an effect known as the moiré effect. If two patterns are superposed, a specific beat pattern, the moiré fringes, will appear in their product. In case of SIM, one pattern is the unknown structure of the fluorescent signal that is supposed to be resolved, and the other pattern is the structured illumination. By the known illumination pattern and detecting the resulting moiré fringes, conclusions about the unknown pattern are possible (**Figure 1.4**).[50] Advantages of the technique are the requirement of relatively low illumination power and the easy accessibility of multicolor imaging.[51]

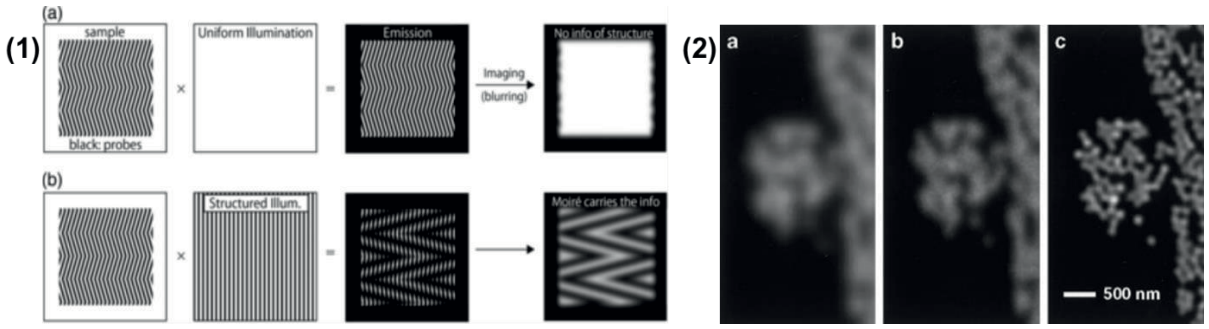

Figure 1.4: (1) Comparison of the image formation in a) conventional wide-field and b) structured illumination microscopy, producing moiré fringes.[iv] **(2)** Fluorescent microspheres, imaged a) as conventional wide-field, b) confocal and c) structured illumination microscopy.[v]

In 1994, Stefan Hell first proposed the principle of STED microscopy before he received the Nobel prize for his discovery in 2014.[52] As in confocal microscopy, the sample is illuminated by a point laser. However, besides the confocal laser that excites the fluorophores in the illuminated spot, a second, antagonistic STED laser is employed for specific de-excitation of every fluorescent signal except in the very center of the laser beam by stimulated emission at the long wavelength end of the spontaneous emission spectrum.[53] Since the STED system is very similar to confocal microscopy in the basic set-up, it exhibits several of the advantages present in confocal microscopy, such as the possibility of optical sectioning of thicker samples and the broad applicability for various organic dyes and fluorescent proteins. Furthermore, high imaging rates are achievable and, by tuning the STED laser, the tuning of resolution is possible.[54] In contrast to STED, the single molecule localization microscopy (SMLM) methods STORM and PALM are not relying on confocal microscopy as the technical basis of illumination but are similar to total internal reflection fluorescence (TIRF) microscopy in their set-up.[55] Here, the illumination is based on an evanescent field

[iv] Reprinted from: Yamanaka, Masahito; Smith, Nicholas I., Introduction to super-resolution microscopy, Microscopy, 2014, pp. 177-192, by permission of Oxford University press

[v] Reprinted with permission from M.G.L. Gustaffson, Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy, Journal of Microscopy, 2000, Vol. 198, Pt 2, pp. 82-87.

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produced by total internal reflection of light on a cover glass/substrate interface. Deeper tissue penetration and therefore out-of-plane light is avoided.[56] Both techniques are based on switching fluorescent molecules on and off, detecting the single distant signals, taking thousands of pictures and afterwards recombining them to a whole image. While PALM is applying fluorophores with direct control of their emitting stage such as fluorescent proteins, STORM is based on stochastically switching organic dyes by adapting buffer and illumination conditions.[57] In comparison to STED, SMLM techniques achieve a higher spatial resolution but require longer acquisition times.[58]

All the described methods, conventional as well as super resolution techniques, rely on the principle of exciting and detecting one fluorophore with one photon at a time. They are ideally suited to provide sophisticated images from a comparatively thin specimen. However, in the field of *in vivo* imaging, another advancement is of great significance: the development of 2-photon or multiphoton microscopy. In contrast to the before mentioned techniques, this method relies on the simultaneous absorption of two photons of a long wavelength in one fluorophore caused by the high photon density in a pulsed laser beam.[59] Typically, an excitation wavelength in the near infrared (NIR) range of about 700-1100 nm is applied, providing deep tissue penetration while causing minimum tissue damage.[60] Remarkably, the wavelength of the emitted photon is shorter. The simultaneous absorption of two (or more) red/NIR photons under addition of their energy can cause the emission of a green wavelength photon thus providing a great opportunity in imaging living animals with broad applications in biological and medical research as well as in the various stages of drug development.[61-64]

1.3 Fluorescence and related energy transfer mechanisms

The phenomenon of fluorescence was first described by Sir Frederik William Herschel in 1845, when he stated an “extremely vivid and beautiful celestial blue color” in a quinine solution in sunlight, an effect that could not be explained with the knowledge of that time.[65] The general effect of light emission after absorption of a photon, called luminescence, can be divided into the two categories fluorescence and phosphorescence and is explained in the Jablonski diagram (**Figure 1.5**).[66] When a (typically) aromatic molecule absorbs a photon, an electron is excited from the S_0 singlet ground state to the first or second excited singlet state (S_1 or S_2). For every singlet state there are several vibrational states (0, 1, 2, 3 etc.). Electrons are usually excited to a higher vibrational state and relax very fast (typically 10^{-12} s or less) via vibrational relaxation (VR) and internal conversion (IC) to the lowest vibrational state of the S_1 level. The electron in the excited state orbital is paired to an electron in the ground-state orbital by opposite spin and therefore return to the ground state by emission of a photon (called fluorescence) is a spin-allowed process and occurs rapidly. The average fluorescence lifetime is in the range of 10 ns. Since the return to the ground state usually occurs to a higher vibrational level with subsequent relaxation

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to the lowest level, emission spectra show a vibrational structure similar to absorption spectra. Alternatively, electrons in the first excited state can migrate to an excited triplet state (T_1) under spin conversion via intersystem crossing (ISC). In the T_1 state, electron spins have the same orientation as in the ground state and transition from there to the ground state via photon emission (called phosphorescence) is a spin-forbidden process. The emission rates are slow and the average phosphorescence lifetime is with milliseconds to seconds several magnitudes higher than the one of fluorescence. Due to many potential deactivation processes like non-radiative decay and quenching, phosphorescence occurs less frequently than fluorescence.[67,68] Quenching processes such as collisional quenching, do not only influence the frequency of phosphorescence occurrence but also the occurrence and intensity of fluorescence. Molecules with electrons in excited states can interact with other molecules in solution in various electron exchange interactions and form non-fluorescent complexes with quenchers.[69,70] Besides these non-fluorescent interactions, two fluorescent molecules can interact in a resonance energy transfer (RET), if the emission spectrum of a donor molecule overlaps with the absorption spectrum of an acceptor molecule. This spectral overlap is described as the Förster distance. Since the efficiency of these interactions is depending on the Förster distance and the distance between the donor and acceptor molecule, the measurement of energy transfer efficiency between a known donor-acceptor pair is widely used to measure distances of molecules, for example in biological macromolecules.[71,72]

The emission of a photon via fluorescence (or phosphorescence) inhibits lower energy than the absorption and it is usually related to longer wavelengths, a phenomenon discovered by Stokes in 1852. Hence, the difference in wavelengths between absorption and emission is called the Stokes Shift, one of the most important characteristics of fluorescent molecules.[73] Other important factors in the description of fluorescent molecules are the quantum yield, described as the rate between absorbed and emitted photons and a measure of fluorescence brightness, and the fluorescence lifetime, described as the time frame in which the electrons in excited states relax to the ground state.

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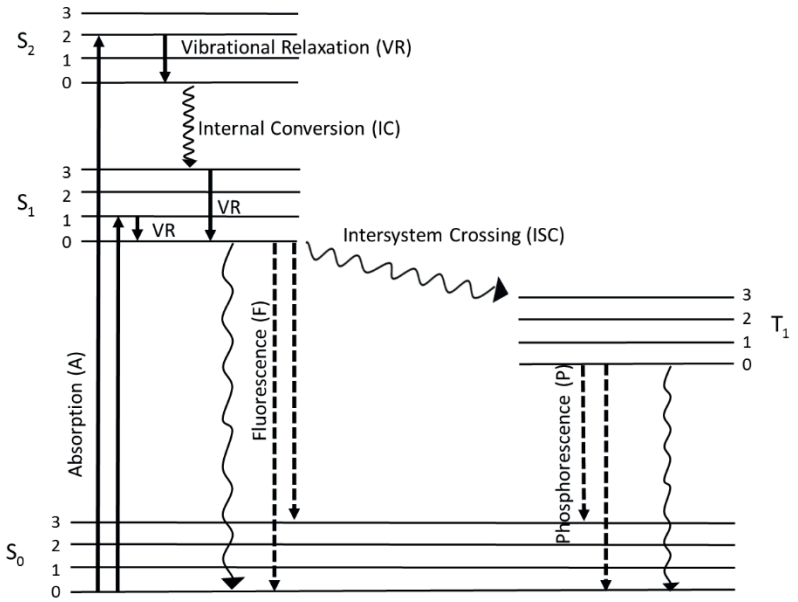

Figure 1.5: One form of a Jablonski diagram, depicting the processes of electron excitation by absorption (A) of a photon and relaxation into ground state by various processes, including vibrational relaxation (VR), internal conversion (IC), intersystem crossing (ISC), fluorescence (F) and phosphorescence (P).

1.4 Fluorophore classes

In general, fluorescent molecules can be divided into the categories of intrinsic or extrinsic fluorophores. While intrinsic fluorescence occurs naturally, extrinsic fluorescence is caused by the addition of a fluorescent molecule to a sample.[67] Important representatives of intrinsic fluorophores are the aromatic amino acids tryptophan[74], tyrosine[75], and phenylalanine[76], the fluorescent enzyme cofactors nicotinamide adenine dinucleotide (NADH)[77], pyridoxyl phosphate[78], riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD)[79-81] or chlorophyll.[82,83] Since these naturally occurring fluorescent molecules are absorbing and emitting mostly in the UV to blue light range and therefore are limited in their potential application in imaging, the synthesis of small molecule dyes is crucial to cover the area of higher absorption/emission wavelengths. Representatives of the various existing fluorophore classes are depicted in **Figure 1.6** with the archetypic quinine fluorophore indicated as number **1**. Starting in the range of UV-excited fluorophores, polycyclic aromatic compounds such as naphthalene, pyrene or anthracene derivatives are popular fluorophores and widely used scaffolds of commercially available dyes (i.e., “Lucifer yellow”, numbers **8** and **9**).[84-87] Their spectral properties are depending on the size of the aromatic system, as well as on electron density influencing substitutions. Besides this class, in many natural products and pharmaceuticals, the coumarin motif is a central building block and, upon substitution, exhibits fluorescence with a large

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Stokes shift in the UV-range (i.e., 4-methylumbelliferone (4-MU), , number **10**). A facile synthesis route, usually a Pechmann condensation from a phenol and a β-carbonyl ester, as well as flexible chemistry belong to the benefits of this still developing fluorophore class.[88] Indole and imidazole compounds, such as DAPI (number **12**) or Hoechst 33342 (number **13**) are widely used as DNA- intercalating dyes.[89,90] In the green to yellow wavelength range, fluorescein (number **17**) is one of the oldest fluorophore scaffolds (first synthesized by Baeyer in 1871) and until today, a widely used and extremely versatile dye.[91] It can be prepared from phthalic anhydride and resorcinol in the presence of zinc chloride and exists in two different forms: a closed, non-fluorescent lactone and an open, fluorescent quinoid form.[92]

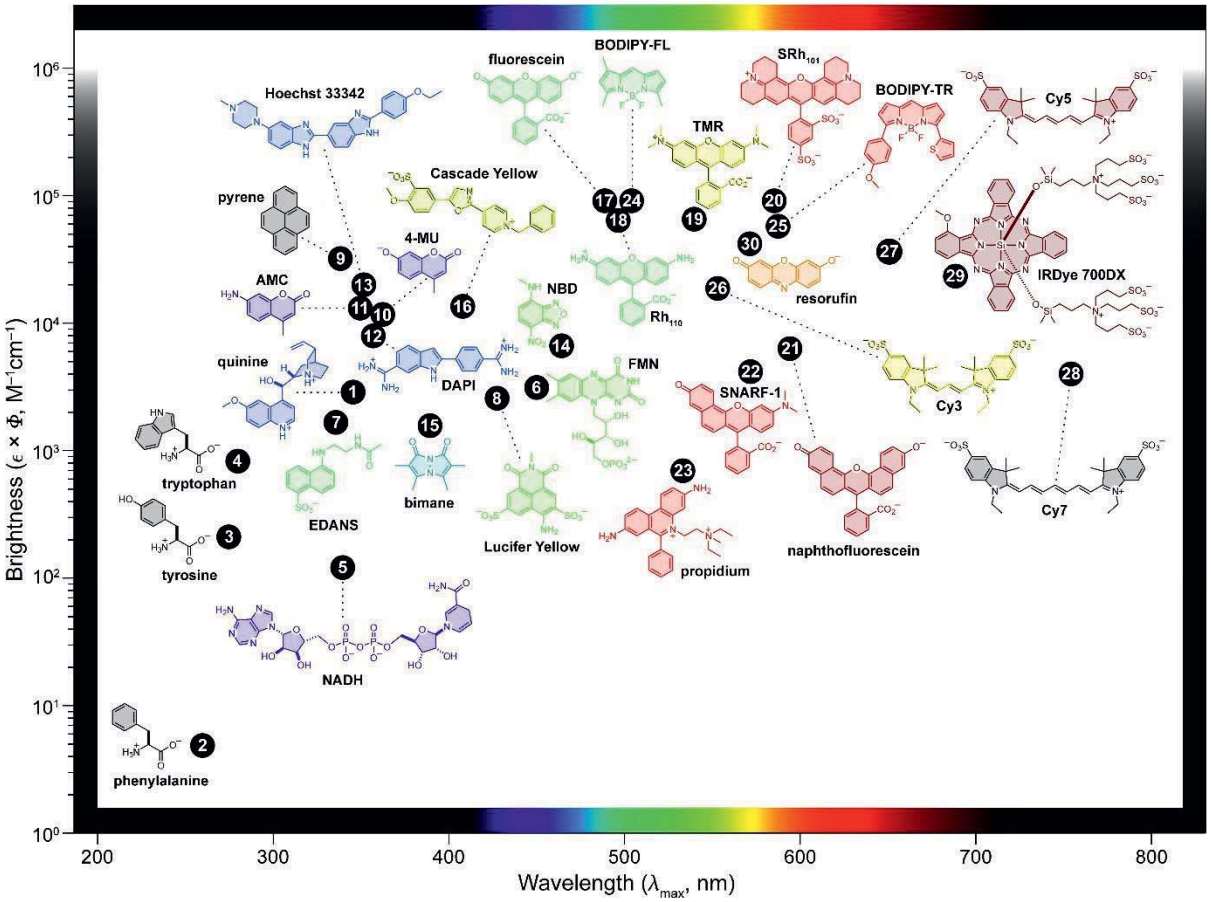

Figure 1.6: Common fluorophore classes plotted as brightness against maximum absorption wavelength. Reprinted with permission from Lavis et al.[93] Copyright © 2008, American Chemical Society.

Since the closed form can be locked by substituting the phenolic oxygens, which efficiently masks the fluorescein fluorescence, this equilibrium has been used to create photo- or enzyme-sensitive switchable dyes.[94,95] Additionally, fluorescein shows a fluorescence sensitivity towards surrounding pH values and therefore has been employed in the development of intracellular pH sensors.[96,97] An alteration and red-shift of the emission wavelength range of fluoresceins can be achieved by condensations of further phenyl units in Friedel-Crafts type reactions and results in naphthofluorescein or seminaphthofluorescein derivatives (number **22** and **21**).

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