



Regulatory guidelines and preclinical tools to study the biodistribution of RNA therapeutics



P. Vervaeke^a, S.E. Borgos^b, N.N. Sanders^{c,1}, F. Combes^{d,*,1}

^a Laboratory of Gene Therapy, Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium

^b SINTEF Industry, Dept. of Biotechnology and Nanomedicine, Research Group Mass Spectrometry, Sem Sælands v. 2A, N-7034 Trondheim, Norway

^c Laboratory of Gene Therapy, Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium

^d SINTEF Industry, Dept. of Biotechnology and Nanomedicine, Research Group Mass Spectrometry, Sem Sælands v. 2A, N-7034 Trondheim, Norway

ARTICLE INFO

Article history:

Received 20 December 2021

Revised 9 February 2022

Accepted 23 March 2022

Available online 26 March 2022

Keywords:

Guidelines
Biodistribution
Distribution
RNA therapeutic
mRNA vaccine
mRNA
siRNA
Vaccine
Lipid nanoparticle
LNP
Imaging
FLI
BLI
QWBA
RT-qPCR
FISH
bDNA
Mass spectrometry
MS
Regulatory
Onpatro
Patisiran
Givlaari
Givosiran
Leqvio
Inclisiran
Oxlumo
Lumasiran
Comirnatmy
Pfizer/BioNTech
Spikevax
Moderna

ABSTRACT

The success of the messenger RNA-based COVID-19 vaccines of Moderna and Pfizer/BioNTech marks the beginning of a new chapter in modern medicine. However, the rapid rise of mRNA therapeutics has resulted in a regulatory framework that is somewhat lagging. The current guidelines either do not apply, do not mention RNA therapeutics, or do not have widely accepted definitions. This review describes the guidelines for preclinical biodistribution studies of mRNA/siRNA therapeutics and highlights the relevant differences for mRNA vaccines. We also discuss the role of *in vivo* RNA imaging techniques and other assays to fulfill and/or complement the regulatory requirements. Specifically, quantitative whole-body autoradiography, microautoradiography, mass spectrometry-based assays, hybridization techniques (FISH, bDNA), PCR-based methods, *in vivo* fluorescence imaging, and *in vivo* bioluminescence imaging, are discussed. We conclude that this new and rapidly evolving class of medicines demands a multi-layered approach to fully understand its biodistribution and *in vivo* characteristics.

© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

* Corresponding author.

E-mail addresses: niek.sanders@ugent.be (N.N. Sanders), francis.combes@sintef.no (F. Combes).

¹ These authors contributed equally.

Contents

1. Introduction	2
2. Regulatory framework	2
3. Regulatory guidelines	3
3.1. Validation of analytical techniques	3
3.2. Choice of animal species and animal model	4
3.3. Number of animals	4
3.4. Duration of longitudinal animal studies	4
3.5. Minimal tissue panels	4
3.6. Compliance to good laboratory practice	5
4. Techniques used for authorized RNA therapeutics	5
4.1. Quantitative whole-body autoradiography and microautoradiography	6
4.2. Mass spectrometry-based assays	8
4.3. Hybridization techniques	9
5. Other techniques that could reach EMA/FDA requirements	9
5.1. Reverse transcription quantitative polymerase chain reaction	9
5.2. Fluorescence and bioluminescence	10
5.2.1. <i>In vivo</i> bioluminescence imaging	10
5.2.2. <i>In vivo</i> fluorescence imaging	11
6. Final remarks	12
Declaration of Competing Interest	13
Acknowledgements	13
References	13

1. Introduction

The success of the messenger RNA (mRNA)-based COVID-19 vaccines of Moderna and Pfizer/BioNTech marks the beginning of a new chapter in modern medicine. Within weeks, any therapeutic protein of choice can now be encoded on mRNA, encapsulated in lipid nanoparticles (LNPs) and be supported by preclinical data. Within months, the mRNA COVID-19 vaccines were tested in clinical trials and within a year, were brought to the market [1–3]. The COVID-19 pandemic is considered a public health emergency by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), because the immediate availability of vaccines outweighs the risk associated with less comprehensive pharmaceutical and clinical data at the moment of authorization [3–5]. Consequently, SARS-CoV-2 vaccines became eligible for conditional marketing authorization in Europe and emergency use authorization in the United States (US) [3–5]. The shortened timeline associated with these approvals contrasts strongly with the mean duration of 10 years to develop and authorize a new drug [6]. Moreover, the rapid rise of mRNA therapeutics has resulted in a regulatory framework that is somewhat lagging. The current guidelines either do not apply, do not mention RNA therapeutics, or do not have widely accepted definitions [7–11].

In this review, “RNA therapeutics” refers to small interfering RNA (siRNA) and mRNA. It is important to note that prophylactic and therapeutic vaccines against infectious diseases are currently not considered “gene therapy medicinal products” or “gene therapy products”, according to EMA and FDA, respectively [8,9,12,13]. However, the guidelines for vaccines and RNA therapeutics are similar for many of the discussed points. This review describes the guidelines for biodistribution studies of RNA therapeutics and highlights the relevant differences for mRNA vaccines. We also discuss the role of *in vivo* RNA imaging techniques to fulfill and/or complement the regulatory requirements issued by FDA [9,10], EMA [7,8], the International Council for Harmonisation (ICH) [11], and the International Pharmaceutical Regulators Programme (IPRP) [14]. For vaccines, EMA refers to the WHO guidelines [15], whereas FDA has issued its own guidance document in response to the COVID-19 pandemic [16].

Consequently, this FDA guidance document contains the most up-to-date guidelines on mRNA vaccines and will be further updated after the pandemic [16]. Additionally, new mRNA-specific guidelines are expected by the WHO [17] and possibly also national regulatory agencies, such as EMA and FDA [18]. All biodistribution guidelines (summarized in Table 3) are nonbinding and generally less strict than those for e.g., toxicology studies, but applicants should consider that additional, binding national and international legislation may apply.

2. Regulatory framework

The goal of preclinical biodistribution studies is to characterize the presence, persistence, and clearance of the drug at the molecular level both in target tissues and an array of non-target tissues [7,9,10,14]. They are an important component of preclinical pharmacokinetic studies and help interpret nonclinical pharmacology and toxicology findings [8,9,11]. The requirement for preclinical biodistribution studies to initiate first-in-human studies is decided by regulatory agencies on a per-product basis. However, the default approach differs for RNA therapeutics and mRNA vaccines (Table 1). For RNA therapeutics, EMA advises that biodistribution studies are always performed, unless the design and type of the RNA therapeutic justifies otherwise [7,8,12]. FDA also advises that biodistribution studies are performed, but only for new vector classes and when significant changes are made in vector backbones, formulations, routes of administration, dose levels, and dosing schedules [9]. In contrast, mRNA vaccines do not require pharmacokinetic studies (which encompass biodistribution studies) to initiate first-in-human studies (both in Europe and the US) unless the vaccine uses novel adjuvants, formulations, additives, or routes of administration [11,15]. Because of the per-product-approach, developers of RNA therapeutics or mRNA vaccines are strongly advised to request assistance from the relevant regulatory body before initiating preclinical studies [7–10,19]. Moreover, the specific requirements for how the biodistribution study should be designed will be tailored to each product. For example, RNA therapeutics are often delivered through some carrier or delivery system, such as lipid nanoparticles

Table 1
Different requirement for biodistribution studies for RNA therapeutics and mRNA vaccines.

RNA therapeutics
Europe European Directive 2001/83/EC [12], "Biodistribution studies shall include investigations on persistence, clearance and mobilisations. Biodistribution studies shall additionally address the risk of germline transmission." United States FDA (2013) [9], "Prior to administration in humans, biodistribution studies should be considered for: - Investigational gene therapy products that belong to a new vector class. - Established vectors with significant changes in the vector backbone. - Established vectors with a significant formulation change. - Established vectors with a significant change in route of administration. - Established vectors with a significant change in the dosing schedule and/or the vector dose levels."
mRNA vaccines
Europe and United States WHO (2005) [15], "Pharmacokinetic studies ... are normally not needed. Distribution studies should be considered in the case of new formulations, novel adjuvants or when alternative routes of administration are intended to be used..."

(LNP) or polymers. Consequently, preclinical biodistribution studies may be requested for the therapeutic RNA construct, the carrier/delivery system, individual components of the carrier/delivery system, and the combined final product [8,11,14]. For mRNA-based therapeutics, biodistribution of the produced protein product should also be investigated.

Biodistribution studies can in some cases be avoided. For example, in the preclinical studies of the Pfizer/BioNTech COVID-19 vaccine [20] and the Moderna COVID-19 vaccine [21], no biodistribution studies were included for the final mRNA-LNP formulation. Instead, these applications relied on biodistribution studies from LNP formulations encapsulating a different nucleoside-modified RNA (modRNA) sequence [20,21]. Similarly, the guidelines also imply that no additional biodistribution and pharmacokinetic studies are required for new mRNA vaccines which only alter the modRNA component, but use the same carrier and route of administration as either the Pfizer/BioNTech or the Moderna COVID-19 vaccine [15]. In theory, applicants from other companies can thus avoid biodistribution studies by referring to the data in the Pfizer/BioNTech and Moderna applications. In practice however, access to the experimental data may be subject to company secrecy. In addition, FDA guidelines even state that new vaccines using the COVID-19 vaccine (mRNA) platform technologies can rely on the existing toxicology data [16]. The same rationale applies to non-vaccine mRNA therapeutics, i.e., biodistribution studies can be avoided when only the protein coding sequence of mRNA is changed and adequate justification for not performing the study is provided [8,10,11].

Apart from the RNA component, novel carrier components such as the lipids in LNPs need to be supported by individual preclinical studies as well [7–10]. For example, the LNP formulation used in the Pfizer/BioNTech COVID-19 vaccine contains four lipids, of which only the ionizable lipid (ALC-0315) and the polyethylene glycol (PEG)-phospholipid conjugate (ALC-0159) were novel components. Indeed, cholesterol and distearoyl phosphatidylcholine (DSPC) comply with the European Pharmacopoeia and are used in several already approved products [20]. More specifically, the provided justification referred to the use of DSPC in the LNP of Onpattro®, which is an EU-approved siRNA drug administered intravenously and at a much higher dose than the intended intramuscular dose of the Pfizer/BioNTech COVID-19 vaccine [22]. In addition, the structurally related dioleoyl phosphatidylcholine (DOPC) is also used in intramuscular products and approved by the EU [20,23]. EMA therefore only requested new studies for the novel components (ALC-0315 and ALC-0159) and their combined use in fully formed mRNA-LNPs [20].

Both for RNA therapeutics and mRNA vaccines, the preclinical product should reflect the intended clinical product and its applications as much as possible. However, deviations can be justified. For example, adjusting dosages to different species or using a different RNA molecule in a certain carrier [7–11,14–16]. Because changing the coding sequence of the mRNA is unlikely to have a significant impact on the physicochemical properties and distribution of mRNA-LNPs, luciferase-encoding mRNA may be used to monitor the distribution of a novel formulation. This justification was provided for the COVID-19 vaccines of Pfizer/BioNTech and Moderna [20,21]. Finally, although the marketing authorization of these mRNA vaccines was conditional, no additional non-clinical *in vivo* data (including biodistribution) was requested in the European Public Assessment Reports, indicating that the provided data was sufficient for a future full marketing authorization [4,20,21].

3. Regulatory guidelines

3.1. Validation of analytical techniques

The analytical techniques that are used in preclinical studies of RNA therapeutics should be validated [8,10,11,14]. This also applies to mRNA vaccines [15,16]. Applicants must demonstrate that the used techniques, procedures, equipment, and materials are appropriate to detect the target (i.e., the RNA product, a carrier component, or the expressed protein) at the molecular level and in the relevant biological matrix [8,9,15]. This can be demonstrated either through own experimental data or by referring to published data. Guidelines on validation of analytical techniques are available from multiple agencies [24–27]. Limits of quantification should be specified, as well as techniques used to obtain test samples and the order in which samples are obtained. The latter is important to prevent, for example, cross-contamination between samples [8,10,11,14].

Regarding DNA detection, guidelines from EMA, ICH, IPRP, and FDA refer to quantitative Polymerase Chain Reaction (qPCR) [7,9–11,14]. Note that the European guidelines use the term 'Nucleic Acid Amplification Testing', which is a broader term and might include alternative methods such as loop-mediated isothermal amplification [8]. EMA, ICH and IPRP guidelines add Reverse Transcription qPCR (RT-qPCR) for RNA [8,11,14]. ICH also lists techniques that can be used to monitor the RNA product or the expressed protein product in nonclinical biodistribution studies: enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), western blot, *in situ* hybridization (ISH), digital PCR, flow cytometry, *in vitro* and *in vivo* imaging techniques and "other evolving technologies" (which still require validation) [11].

3.2. Choice of animal species and animal model

The chosen animal species used in preclinical studies on RNA therapeutics should be as biologically relevant as possible and show a pharmacological response that is similar to the expected response in humans. This also applies to mRNA vaccines [7–11,14–16]. For siRNA therapeutics, this means that the animal species should ideally express an mRNA target that has an identical sequence to that expressed in humans. For mRNA therapeutics, this suggests that the delivered mRNA should be translated in a similar manner (i.e., amount, duration, and distribution) to that expected in humans, and that the animals should show a similar biological response to the produced protein product. To achieve comparable translation kinetics of mRNA, the innate immune response is also an important factor to consider, because a strong response can significantly reduce mRNA expression [28–31]. This might imply that rodents are not suited for all RNA therapeutics. For example, mice have a toll-like receptor 8 (TLR8) that functions differently from the TLR8 in humans [32]. This receptor plays an important role in triggering an innate immune response, upon recognition of foreign single-stranded uridine-rich, unmodified RNA [28]. This response can potentially lead to differences in translation kinetics, and toxicological effects, resulting in unexpected drug responses in humans [29–31,33]. Additionally, recent reports demonstrate that mice, and even non-human primates, do not reliably predict human systemic inflammatory events after RNA administration. Compared to humans, it was shown that the expression levels of anti-inflammatory interleukin-1 receptor antagonist (IL-1ra) were much higher in mice, both at baseline and after multiple RNA doses [34]. Similarly, non-human primates had a higher IL-1ra response at all RNA dose levels (but not at baseline), while the pro-inflammatory IL-1 β was lower than in humans. These data support the use of adapted models, such as IL-1ra knock-out mice for the evaluation of RNA therapeutics and vaccines [34].

The used animals can be wild-type, immunocompromised, knock-out, knock-in, humanized or transgenic animals [8]. In fact, the use of disease/injury models is encouraged to obtain a better estimation of the risk–benefit ratio of testing the RNA therapeutic in humans and to improve the biological relevance of the preclinical data [8,9]. An example of a deviation from wild-type rodents are the preclinical studies for COVID-19 vaccines. SARS-CoV-2 uses human Angiotensin Converting Enzyme 2 (hACE2) to infect cells. Since rodents do not express hACE2, transgenic hACE2-positive mice and non-human primates were used [20]. Another approach is to use a mouse adapted SARS-CoV-2 virus [21], or to select alternative SARS-CoV-2 infection models, such as cats, ferrets, and hamsters [35,36].

When a single animal species cannot answer all preclinical questions, a panel of studies in various species should be used [8,11]. Furthermore, regulatory agencies also consider practical limitations. For example, the small size and short lifespan of rodents can be an issue. Similarly, studies in non-human primates can have sample size limitations and require qualified facilities, personnel, and expertise [8]. Importantly, preliminary biodistribution studies can help identify relevant species for subsequent pharmacokinetic, pharmacodynamic, and toxicology studies, by determining intracellular gene delivery efficiency or assay methodologies [11].

3.3. Number of animals

FDA and ICH advise to use at least 5 animals per sex per group per sacrifice timepoint for rodents and correspondingly 3–5 animals for non-rodents [10,11]. EMA does not specify numbers in their guidelines [7,8], nor does WHO in the vaccine guidelines [15]. Remarkably, this is in contrast with WHO guidelines for tox-

icology, which advise to use 10 rodents per sex and per experimental group [15]. To reduce the number of animals, endpoint analysis can sometimes be complemented or replaced by non-invasive imaging methods in the same animal. This is important for mRNA therapeutics, which typically require many assessments at short interval timepoints post-injection. The number of animals can also be reduced by performing multiple studies within one animal. To allow correlation of possible toxicity to the presence or absence of the investigated compound, it is recommended to use the same animal model in both toxicology and biodistribution studies [8,9,14]. Thus, combining aspects of both studies in one experiment avoids unnecessary use of animals and provides a better correlation between toxicology and biodistribution [8,11]. In general, both genders should equally be represented in the experiments, but deviations are allowed when adequately justified [8,10,11,14].

3.4. Duration of longitudinal animal studies

The duration of longitudinal animal experiments depends on the RNA therapeutic, the dose, the encoded protein, the carrier, and the route of administration [7–10]. For example, the bioluminescence of intravenously administered LNP-mRNA (encoding luciferase) was no longer detectable in the liver after 3 days but remained detectable at the injection site for up to 7 days [37]. When the same product was injected subcutaneously or intradermally, no signal was seen in the liver but bioluminescence at the injection sites endured for 6 and 10 days, respectively [37]. In contrast, intradermal administration of self-amplifying RNA results in observable bioluminescence for up to 28 days post-injection [29]. To address these marked differences between RNA platforms, EMA and IPRP specify that the study should continue until the signal becomes undetectable or until a long-term plateau phase is reached [8,14]. For modRNA or unmodified mRNA, an appropriate duration ranges from a few days to a (few) week(s), depending on the formulation and route of administration. For self-amplifying or *trans*-amplifying mRNA, this period is more likely to approach several weeks. Similarly, subcutaneously injected siRNA can have a long half-life, with persistence of up to 40 days being reported [38]. Newer RNA platforms, such as circular RNA have demonstrated *in vitro* and *in vivo* expression profiles of up to a week [39,40]. Not only the type of therapeutic RNA will influence the duration of the study, but also the encoded protein product. For example, proteins with short half-lives are expected to disappear shortly after the mRNA translation stops. On the other hand, stable proteins can persist for a longer period, which should be considered when designing a preclinical study. When mRNA is used to deliver CRISPR/Cas9 or other gene-editors, preclinical studies will have to be conducted much longer. Additionally, the risk of vertical germline transmission of the induced genome modification must be examined. More details on guidance for products with genome-altering effects or expected long-term persistence, can be found in FDA and EMA guidelines [8,10]. For vaccines, no appropriate duration is mentioned [15,16]. Finally, the elimination profiles of carrier components, such as ionizable lipids in LNPs should be considered. Especially with repeated dosing (e.g., long-term protein replacement, (booster) vaccination), accumulation can occur. It has been demonstrated that cationic lipids, which can cause inflammatory and hepatic toxicity, adsorb serum proteins, aggregate and accumulate significantly in the lung, liver and spleen [41,42]. This is less pronounced (but not completely abolished) with next-generation ionizable lipids [43]. Additionally, the use of biodegradable lipids can aid in reducing accumulation of these drug components [44,45]. Still, these effects should be considered when determining an appropriate duration of a longitudinal study.

Table 2

Minimal tissue panels to be examined in preclinical biodistribution studies, according to FDA, ICH, IPRP (identical to FDA's panel) and EMA.*

FDA/IPRP	ICH	EMA*
Blood	Blood	Blood smears
Brain	Brain	Brain (coronal sections at three levels to include cerebrum, cerebellum and brain stem)
Gonads	Gonads	Epididymides, Ovaries, Seminal vesicles (rodents), Testes
Heart	Heart	Heart
Injection site(s)	Injection site(s)	Application site (when relevant)
Kidneys	Kidney	Kidneys and ureters
Liver	Liver	Liver
Lung	Lung	Lungs with bronchi and bronchioles
Spleen	Spleen	Spleen
	Adrenal gland	Adrenal glands
	Spinal cord (cervical, thoracic, lumbar)	Spinal cord
		Aorta
		Eyes and optic nerves
		Gallbladder (when relevant)
		Gross lesions
		Joint with bone
		Large intestines (when relevant including Peyer's Patches)
		Larynx
		Lymph nodes (mesenteric and any peripheral)
		Mammary glands
		Oesophagus
		Pancreas
		Peripheral nerves
		Pituitary gland
		Prostate
		Salivary glands (mandibular, parotid, sublingual)
		Skeletal muscle
		Skin and subcutaneous tissue
		Small intestines
		Sternebrae, femur or vertebrae (including bone marrow)
		Stomach
		Thymus
		Thyroid / Parathyroid glands
		Tissue masses of tumours
		Tongue
		Trachea
		Urinary bladder
		Uterus with uterine cervix and oviducts
		Vagina

* The EMA refers to the tissue panel for repeated dose toxicological studies and is therefore considerably longer.

3.5. Minimal tissue panels

The minimal panel of tissues to be examined mainly depends on by the RNA product, the expressed protein, and the route of administration. However, prespecified tissue panels have been determined and can be used as a general starting point (Table 2) [10,11,14,46]. For example, when subcutaneous or intramuscular injection is used, FDA requires that the draining lymph node and contralateral site are examined as well [10]. Similarly, when inhalation is used, inclusion of tissues such as the nasal mucosa or larynx may be required. As mentioned, applicants should always consult with the regulatory agency to obtain a definitive list of tissues to be included in preclinical biodistribution studies. For pharmacokinetic studies of mRNA vaccines, no minimal tissue panel is available. Finally, it should be noted that the interpretation of these panels can be species-specific. For instance, when "Sternebrae,

Table 3

Guidelines for preclinical biodistribution studies of RNA therapeutics and mRNA vaccines.

1.	Applicants are strongly advised to request assistance from the relevant regulatory body before initiating preclinical studies.
2.	Biodistribution studies for RNA therapeutics should encompass both the RNA molecule(s), the individual components of the carrier, the combined RNA-carrier drug, and the produced protein.
3.	In all preclinical studies, the administered drug should reflect the intended clinical product as much as possible, including quality aspects, dose, dosing regimen, formulation, and route of administration. Deviations are allowed when adequate justification is provided.
4.	Applicants can avoid biodistribution studies by referring to previously performed studies for identical components, dosing, routes of administration, etc.
5.	Analytical techniques must be validated. This can be done by referring to previous validation or by providing experimental data. Dedicated guidelines are available.
6.	The experimental animal species and disease models must be as relevant as possible to the expected situation in humans. When necessary, multiple species/models can be used.
7.	FDA and ICH advise to use at least 5 animals per sex, per experimental group, and per sacrifice timepoint for rodents. For non-rodents, 3–5 animals are advised. Biodistribution studies must include both genders, but deviations are possible when adequately justified.
8.	The appropriate duration will depend on the RNA therapeutic, the dose, the encoded protein, the carrier, and the route of administration. When no reference can be made to published data, preliminary studies should be performed to assess an appropriate duration.
9.	Minimal tissue panels are available but will vary on a per-product basis.
10.	Preclinical biodistribution studies do <u>not</u> have to comply with GLP.
11.	Preclinical biodistribution studies are <u>not</u> needed for vaccine products, unless new administration routes, novel adjuvants or novel additives are used (Table 1).

femur or vertebrae (including bone marrow)" is required, this can be sternum or femur with cartilage for rodents, while the femur may be less suited for non-rodents (due to the less uniform presence of active marrow and increased collection difficulty) [47].

3.6. Compliance to good laboratory practice

Preclinical *in vitro* and *in vivo* pharmacology (including biodistribution of mRNA therapeutics and mRNA vaccines) do not have to comply with GLP regulations [9,11,15,16,48,49]. However, when toxicology data is obtained alongside other information (e.g., in preliminary dose finding studies), GLP-compliance should be respected in the procedures which yield toxicological data [9,11,49]. For example, when performing histopathology at the end of a non-GLP biodistribution study, every step from organ sampling to the histopathological procedures should be GLP-compliant. Drug developers can choose to perform preliminary non-GLP-compliant toxicology studies for screening purposes. However, GLP-compliant toxicology is a mandatory dataset for the final authorization application of a chosen drug candidate. WHO also mentions that dedicated safety pharmacology studies can be included in repeat dose toxicity studies, and these can replace single dose toxicity studies [15].

4. Techniques used for authorized RNA therapeutics

Preclinical biodistribution data of therapeutics that have received authorization, can be found in either FDA Application Review Files (<https://www.accessdata.fda.gov/scripts/cder/daf/>) or the European Public Assessment Reports (<https://www.ema.europa.eu/en/medicines>). siRNA therapeutics that have received authorization by EMA and/or FDA at the time of writing this review are Onpattro® (patisiran), Givlaari® (givosiran), OxLumo® (lumasiran), and Leqvio® (inclisiran). Onpattro® is formulated as an

Table 4
RNA-containing therapeutics that received authorization by EMA/FDA.

Name	Type	ROA	FDA-authorized	EMA-authorized	Techniques used
Spikevax® – INN COVID-19 vaccine Moderna	modRNA LNP-delivered	IM	EUA	Conditional	QWBA bDNA assay
Comirnaty® – INN COVID-19 vaccine Pfizer/BioNTech	modRNA LNP-delivered	IM	Yes	Conditional	QWBA LC-MS BLI
Leqvio® – INN inclisiran	naked siRNA	SC	Yes	Yes	QWBA LC-MS
Oxlumo® – INN lumasiran	naked siRNA	SC	Yes	Yes	QWBA LC-MS
Givlaari® – INN givosiran	naked siRNA	SC	Yes	Yes	QWBA LC-MS
Onpattro® – INN patisiran	siRNA LNP-delivered	IV	Yes	Yes	HPLC-probe assay QWBA LC-MS HPLC-probe assay

ROA = route of administration, IM = intramuscular, SC = subcutaneous, IV = intravenous infusion, modRNA = nucleoside modified messenger RNA, EUA = emergency use authorization, INN = international non-proprietary name, QWBA = quantitative whole-body autoradiography, bDNA = branched DNA, LC-MS = liquid chromatography – mass spectrometry, BLI = *in vivo* bioluminescence imaging, HPLC = high-performance liquid chromatography, “naked” = not formulated as LNP.

Table 5
Radioactive isotopes used to label RNA or carrier components.

Isotope	Decay type	Decay energy (KeV) maximum – average	half-life	Epidermal penetration	Range in tissue (mm)	Source
³ H	β [−]	16.8–5.7	12.3 years	0.00	0.006	[59]
¹⁴ C	β [−]	156–49	5730 years	0.11	0.27	[60]
³² P	β [−]	1710–700	14.3 days	0.95	8.00	[61]
³³ P	β [−]	249–76	25 days	0.35	0.60	[62]
³⁵ S	β [−]	167–49	87.6 days	0.12	0.30	[63]
¹²⁵ I*	γ	35	60.1 days	0.99	HVL = 20 mm	[64]

KeV = kiloelectronvolt, Epidermal penetration is defined as the fraction of emitted particles that are transmitted through the human epidermis, Range in tissue = distance an emitted particle travels in tissue, HVL = half-value layer (the amount of tissue needed to reduce the radiation intensity by 50%).

* ¹²⁵I is not used to label RNA, but is included here as a reference.

LNP, while Givlaari®, Oxlumo®, and Leqvio® are chemically modified and are conjugated to a nitrogen-containing moiety. This moiety is connected to N-Acetylgalactosamine to mediate the siRNA delivery into hepatocytes [50]. Like Onpattro®, both COVID-19 vaccines are delivered in LNPs and the mRNA COVID-19 vaccines from Moderna and Pfizer/BioNTech are currently the only mRNA therapeutics on the market. The techniques that were used to obtain biodistribution data for these siRNA and mRNA drugs are either based on radiolabeling (e.g., quantitative whole-body autoradiography (QWBA)), liquid chromatography with online mass spectrometry (LC-MS) or hybridization assays (Table 4 and Fig. 2).

4.1. Quantitative whole-body autoradiography and microautoradiography

Quantitative whole-body autoradiography (QWBA) determines the distribution of radiolabeled materials in tissues. It is considered the industry standard for preclinical biodistribution studies and can be used to assess the distribution and concentration of the RNA product itself as well as carrier components and degradation products [51,52]. For example, the preclinical studies of Onpattro®, Oxlumo®, Leqvio®, Givlaari® and the COVID-19 vaccine of both Pfizer/BioNTech and Moderna relied on QWBA in rats [20–22,38,53,54]. Because both localization and quantification are possible, researchers can obtain tissue-specific pharmacokinetic data [52]. First, a radioactive isotope is incorporated in the investigated compound. The isotopes that were used in authorized RNA therapeutics are ³H and ¹⁴C, but ³²P and ³³P are options as well. Other isotopes, like ³⁵S and ¹²⁵I are used to label peptides and proteins [52]. When using ³²P, it is recommended to use labeled [α-³²P]UTP or [α-³²P]CTP during *in vitro* transcription, since labeled [α-³²P]ATP and [α-³²P]GTP are generally less efficiently incorpo-

rated and the resulting RNA is more subject to decomposition in storage [55]. All isotopes emit β[−] particles (except for ¹²⁵I, which emits gamma radiation) but the isotopes have varying half-lives and decay energies (Table 5) [56]. Compared to gamma radiation, beta particles have lower energy and can consequently only be detected in superficial tissues (hence the need for tissue slices). However, beta-particles provide superior resolution [52]. Likewise, ³³P provides superior resolution compared to ³²P, but is harder to produce and therefore more expensive. Other factors should be considered as well. For example, ¹⁴C is strongly preferred over ³H because it cleaves off less readily than a ³H-carbon bond. Additionally, although ³H-labeling is cheaper, easier to incorporate, and provides a better resolution than ¹⁴C, it requires a 10-fold higher dose and the phosphor plates to image ³H-labeled drugs can only be used once [52,57,58]. Contrastingly, phosphor plates for ¹⁴C-imaging can be used multiple times.

Researchers should first determine the *in vivo* label stability when using ³H or ¹²⁵I (see the review of Solon (2012, 2015)) [52,58]. The importance of assessing the stability of the label is demonstrated by Christensen et al. (2013). These authors showed that ³H-labeled siRNA lost 9% of the label after 2 h and 26% after 48 h post-intravenous injection [65]. Nevertheless, ³H-labeling was used to assess the biodistribution of Givlaari® siRNA. The European Public Assessment Report on Givlaari® states that: “Other peaks (comprising up to 14% of total radioactivity) were shown to be dose formulation impurities” (The report does not mention whether *in vivo* probe dissociation was examined) [54]. This may be in part attributable to probe dissociation events but is definitely worse than the advised maximum of 3% formulation impurities (i.e., a “radiopurity” of 97%) [52,58]. This can lead to over- or underestimations of drug and metabolite quantification in different tissues [58].

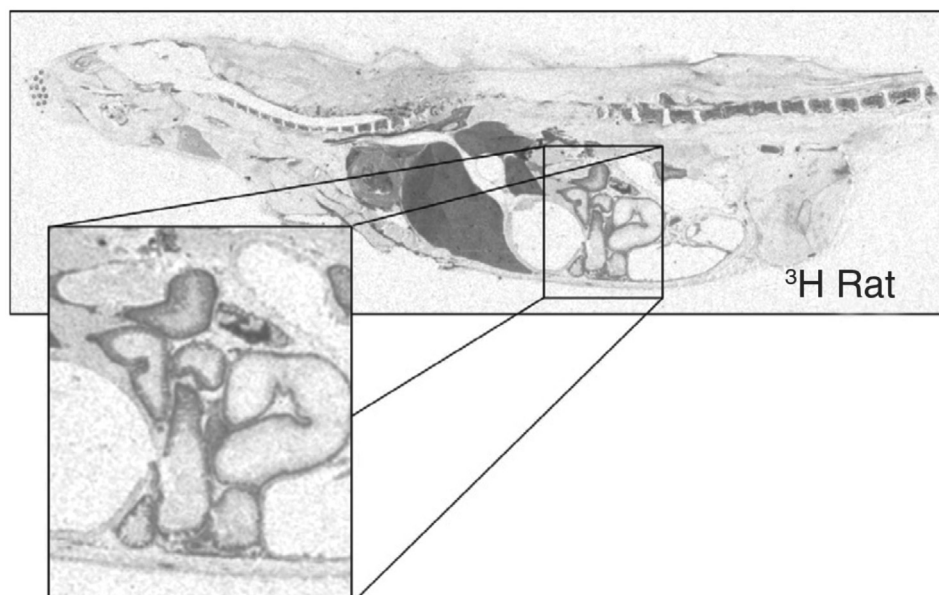


Fig. 1. Example of QWBA imaging, using ^3H -labeling in a rat. Reprinted with permission from Bioanalysis (2015) 7(5), 557–568 as agreed by Newlands Press Ltd.

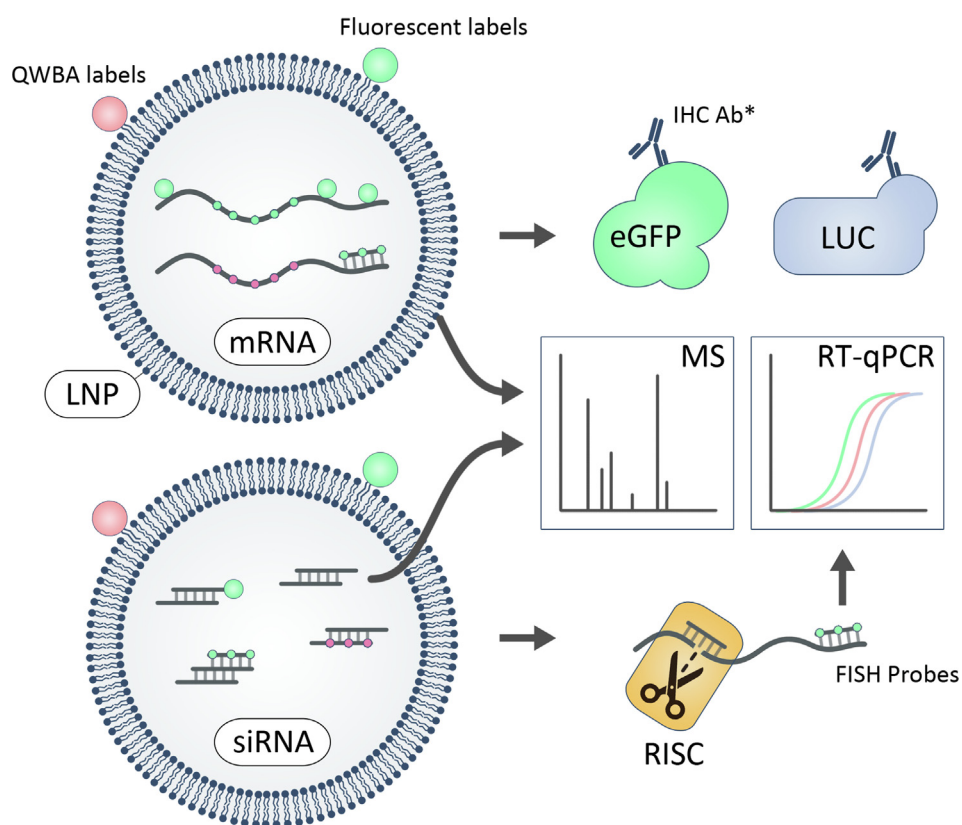


Fig. 2. Schematic overview of common labeling and detection methods for (m)RNA, proteins and LNPs. QWBA = quantitative whole body autoradiography, IHC Ab = immunohistochemistry antibody, eGFP = enhanced green fluorescent protein, LUC = luciferase, MS = mass spectrometry, RT-qPCR = reverse transcription quantitative polymerase chain reaction, RISC = RNA-induced silencing complex, FISH = fluorescence *in situ* hybridization. *IHC can also be used to demonstrate target protein knockdown after siRNA-LNP administration.

After administration and tissue distribution of the labeled compounds, animals are euthanized at specified timepoints and snap-frozen, for example in a hexane-dry ice bath [51,52]. The frozen carcasses are embedded in supporting medium and sectioned in thin tissue slices [51,52,58,66]. Typically, five to ten sagittal sec-

tions of about 20–50 μm thickness at predefined positions (depending on the study goals) will be analyzed [51,52]. The selected sections are freeze-dried and placed on a phosphor plate for imaging. Quantification can be performed by including standards with a known radioactive dose and standards with a known

thickness. Signal acquisition usually happens inside a lead box over 1–2 weeks. The phosphor plate can then be scanned and imaged [51,52].

In typical QWBA studies, both albino rats (e.g., Sprague-Dawley) and pigmented rats (e.g., Long-Evans) are used. The albino rats are included because they are also used in toxicology studies and thus allow for better correlation between distribution and toxicity. Pigmented rats are required to determine melanin binding, thereby predicting the radiation burden in humans [52,58,67]. This is because melanin can bind radiolabeled compounds, resulting in accumulation and a longer exposure than expected [68]. QWBA can also be applied to other species, including other rodents, rabbits, dogs, pigs, and non-human primates [52,58,69]. For example, QWBA was performed on liver samples of *Cynomolgus* monkeys to examine the elimination of Leqvio® siRNA [38].

QWBA can obtain tissue resolution (pixel size of about 25–100 μm^2), while preserving the tissue architecture (Fig. 1). QWBA is therefore preferred over techniques that require homogenization of an organ, such as LC-MS [51,52]. Moreover, the distribution data is obtained *in situ*, in samples that are minimally manipulated. This bypasses exsanguination effects that inevitably occur during organ excision [66]. For the same reason, cross-contamination is also much less of an issue compared to techniques that require excision of organs [66].

Distribution data at cellular resolution can be obtained with microautoradiography (MAR). Similar to QWBA, MAR relies on radiolabeling. Different procedures, such as the coating of microscope slides with photosensitive nuclear emulsion under darkroom conditions, making cryomicrotomic sections (4–10 μm thickness), and mounting the sections under darkroom conditions are needed for MAR. The nuclear emulsion is exposed to the radioactive sections during 3 days to 2 months. Images must be acquired in light-tight boxes at 4 °C or –20 °C (depending on which protocol is used) where the nuclear emulsion will blacken locally in response to the radiation [51,52,70]. Additionally, MAR requires higher radioactive doses compared to QWBA, due to the additional magnification. Finally, the freezing process needs to be accelerated to prevent the formation of ice crystals that disrupt cellular morphology [58,66]. Researchers can use different tissue samples from a single animal for both QWBA and MAR, by preparing them separately. Alternatively, different animals can be used for each procedure, for example when a higher radioactive dose is needed for MAR, when exsanguination is performed prior to MAR (which is not optimal for QWBA) [52,69], or when MAR samples are chosen based on the QWBA data [51]. Importantly, MAR is prone to user artifacts and typically does not permit reliable quantification [52]. Preclinical studies for Onpatro® most likely used MAR, as they contain data on intracellular radioactivity in hepatocytes, hepatic vascular cells (cell type not specified), and liver sinusoid lumen [22]. Obtaining cellular resolution has a more prominent role when cell-targeting moieties are used on LNPs, for example [71–76]. In this regard, MAR can sometimes (depending on reagent compatibility) be coupled to immunohistochemistry to verify targeted delivery [52].

The use of radioactive substances requires special handling and dedicated infrastructure. In addition, reliable quantification requires extensive training and/or experience, and the required equipment is very expensive [52,58]. Consequently, QWBA and MAR are typically outsourced. Using QWBA or MAR, it is impossible to distinguish between the drug (or carrier) itself, its metabolites, or its degradation products [52,58,66]. However, QWBA is non-destructive and can therefore be used in tandem with other techniques (e.g., LC-MS) to further characterize the obtained radioactive signals [51,52].

4.2. Mass spectrometry-based assays

Molecular mass spectrometry (MS) identifies analytes based on the measured ratio between the molecular mass and the charge of the ionized fragments. So-called tandem mass spectrometry (MS/MS) significantly improves the confidence of identification and analytical resolution by fragmenting the ions in a controlled fashion and quantifying the characteristic molecular fragments. Furthermore, coupling the mass spectrometer to an “online” separation step (commonly liquid chromatography (LC)) and online detectors (e.g., fluorescence or optical absorbance) dramatically increases the analytical resolution. Despite the challenges related to costs, skilled personnel, and its implementation in a validated/GLP pharmaceutical context, LC-MS/MS has become a *de facto* gold standard for many bioanalytical chemistries due to its unmatched combination of sensitivity, selectivity, and specificity. Most organic molecules can be analyzed by LC-MS/MS without requirements for external labeling or specific functional groups (e.g., chromophores or fluorophores). MS-based assays are currently used to determine the presence and concentration of small molecule drugs, metabolites, or carrier components in biological matrices (most commonly plasma). By performing serial sampling, pharmacokinetics can be determined. Regarding RNA therapeutics, the high molecular weights, the high anionicity, and the chemical instability of the phosphate linkages generally leads to higher detection limits than are seen for most small molecules.

siRNAs are generally assumed to have a molecular weight <15 kDa, largely making them amenable to the same analytical workflows as small molecule therapeutics (with siRNA quantification limits in the low ng/ml range [77]). For example, the European Public Assessment Reports of siRNA drugs Onpatro® and Givlaari® state that LC-MS/MS was used for quantification of siRNA in plasma and/or tissue samples [22,54]. The various MS modalities for detailed analysis of oligonucleotides beyond biodistribution have recently been extensively reviewed [78,79] and is beyond the scope of this review. In contrast to siRNA, mRNA molecules can exhibit a molecular weight into the MDa range. Consequently, MS-based quantification of an intact mRNA molecule is currently not practically feasible but the recent progress in native mass spectrometry could change this in the relatively near future [80]. Up to now, the longest intact RNA investigated in detail (top-down sequencing) by mass spectrometry seems to be the 76nt tRNA in the Breuker lab [81]. Interestingly, intact viral particles, including two protein-coding RNAs of 3.1 kb and 1.4 kb, respectively, have been detected by Orbitrap MS as single ion species in the MDa range [82].

Biodistribution analysis of RNA therapeutics adds significant challenges as compared to quantification and structure elucidation of neat RNA. Indeed, RNA therapeutics in a biological matrix contains high concentrations of endogenous RNA, as well as proteins and other biomolecules that can form strong interactions with RNA. MS-based RNA analysis (or their metabolites) then becomes a challenge of robust and efficient sample preparation and analyte extraction prior to analysis (e.g., through trizol extraction, protein digestion, solid-phase/bead-based extraction, and online trapping columns) [83].

MS-based detection of siRNA in biological samples usually does not require modifications or labeling methods. Indeed, most therapeutic siRNAs incorporate one or more non-endogenous chemical modifications to enhance stability or therapeutic effect [38,53,54]. This provides very good signal-to-noise ratios (SNR) and low detection limits in LC-MS/MS after forced depolymerization (unpublished data from our laboratories at SINTEF). The same principle would apply to mRNA therapeutics if one or more incorporated nucleotides are non-endogenous. An interesting approach would be to incorporate stable (non-radioactive) isotope-labeled nucleo-

tides in the mRNA. For example, an isotope-labeled 5' cap would generate non-endogenous heavy-labeled (typically ^2H , ^{13}C , ^{15}N) monomers upon nuclease digestion. Such stable isotope analogues, widely used in MS-based small molecule- and proteomics studies, are chemically indistinguishable from their non-labeled counterparts yet easily discernible in MS. The limiting factor of this approach would be the cost of the stable isotope nucleotides. Theoretically, MS-based detection of radioactive isotopes (as used in QWBA and MAR) is also possible but would require sufficient dedicated facilities. It was recently shown [84] that a non-endogenous phosphorothioate fragment of an antisense oligonucleotide could be used as a marker for biodistribution in matrix assisted laser desorption/ionization-Fourier transform-ion cyclotron resonance-MS (MALDI-FT-ICR-MS) imaging. The use of MALDI ionization for direct on-tissue, spatially resolved quantification for therapeutics has brought exciting results for small molecule drugs [85] and even antibodies [86], and it remains to be seen if this applicability can be extended fully to RNA therapeutics. This would, however, provide a significant improvement, as sample manipulation is drastically reduced, thereby avoiding cross-contamination and exsanguination effects [52,58]. Moreover, because organ homogenization is not needed, MALDI also provides spatial information [58].

Most RNA therapeutics are currently delivered in LNPs containing one or more non-endogenous lipids, often with intramolecular groups that ionize very well. This provides very good detection limits in LC-MS/MS. For example, the European Public Assessment Reports of Onpattro® and the Pfizer/BioNTech COVID-19 vaccine state that LC-MS/MS was used to assess the pharmacokinetics and biodistribution of the synthetic lipid components [20,22]. The same assay methodology was used to optimize the pharmacokinetics of several LNP formulations of Moderna [87]. The biodistribution of LNPs can also be used to approximate the biodistribution of the RNA payloads [88]. In addition, MS-based targeted proteomics approaches can help detect elevated therapeutic protein concentrations after mRNA translation.

4.3. Hybridization techniques

Fluorescence *in situ* hybridization (FISH) is the gold standard for single-molecule RNA visualization on fixed samples and is described for both siRNA [89–92] and mRNA [93–104]. Detection of single RNA molecules is typically achieved by hybridizing multiple fluorescently labeled probes on the target RNA [93,105,106]. As a possible advantage over QWBA, probes targeting multiple regions on the mRNA can be labeled with different fluorochromes thereby providing information on the integrity of the target mRNA. Moreover, different target mRNAs (e.g., in multivalent mRNA vaccines) can be visualized simultaneously [99,100,107–109]. The additive nature of FISH makes it an interesting technique that could be used in conjunction with other imaging modalities such as *in vivo* bioluminescence imaging (BLI) or *in vivo* fluorescence imaging (FLI). For example, a protocol was published which enables the *ex vivo* imaging and colocalization of endogenous mRNA (through FISH) and transgenic eGFP on the same hippocampal tissue section [110]. Moreover, selective probes can be designed for codon optimized synthetic mRNAs (even when encoding endogenous proteins). Similarly, high-fidelity FISH allows for detection of single-nucleotide variation in target mRNA [111]. The broad variety of available fluorescent compounds also allow co-staining with structural or functional markers for cellular identification and even subcellular localization. Interestingly, FISH can also be combined with immunohistochemistry to visualize both the mRNA and its translated protein [93,112] or (sub)cellular colocalization markers. Importantly, quantification of FISH data requires software that must be validated. Finally, it should be noted that that autofluorescence or aberrant probe-binding can

cause an unwanted background signal (Section 5.2) [96,109,113], that FISH is time-consuming and requires some experience, and that the necessary fixation prevents using the sample in other downstream assays [96,114].

The preclinical studies for Givlaari® and Onpattro® utilized hybridization techniques in tandem with high-performance liquid chromatography (HPLC) [22,54]. A fluorescently labeled dye was attached to a synthetic oligonucleotide, complementary to the target siRNA. After hybridization, HPLC was performed to isolate hybridized probes from e.g., unbound probes. Finally, fluorescence was used to quantify the siRNA.

Branched DNA (bDNA) amplification relies on a series of inter-connecting DNA probes which amplify a fluorescent signal after binding the target RNA [93]. The signal amplification is linear and therefore allows reliable quantification [115] and even allows for multiplexing [116]. A multiplexed bDNA assay was used in the preclinical studies of Moderna's COVID-19 vaccine to simultaneously examine the biodistribution of 6 mRNAs [21]. Although initially developed for detecting nucleic acids in solution, bDNA amplification has been used on frozen tissue samples mounted on slides [117].

5. Other techniques that could reach EMA/FDA requirements

QWBA is the gold-standard for preclinical biodistribution studies. However, this technique has some critical limitations for RNA therapeutics and especially for mRNA therapeutics. Among these limitations are the fact that the expressed protein is not tracked and the inability to discriminate metabolites and degradation products. The techniques described in this chapter (Fig. 2) avoid the use of radioactive materials. However, they have the common downside that they are currently not thoroughly validated and that they often require additional processing steps.

5.1. Reverse transcription quantitative polymerase chain reaction

Although the EMA and the FDA recommend RT-qPCR for biodistribution studies, the exact criteria for performing these studies are not yet defined. In addition, no acceptance criteria for assay validation have been determined. Experts in the field recommend testing 3 primer pairs and using probe-based qPCR analysis over DNA-binding fluorescent dyes due to their superior specificity and possibility to multiplex (the added cost of probes is compensated by fewer labor hours on method development) [118]. Evidently, PCR-based methods require careful sample extraction from tissue homogenates and bodily fluids to avoid sample cross-contamination. Sample collection is typically performed in "RNAlater" reagent to minimize RNA degradation, which can be evaluated by e.g., quantifying host housekeeping genes or by capillary gel electrophoresis. Each 96-well plate should include appropriate quality controls and a 10-fold dilution series of target RNA to allow absolute quantification over a wide dynamic range. These controls also indicate the efficiency and accuracy of the reverse transcription reaction and should be evaluated in the presence of total RNA from negative control samples. Although one-step RT-qPCR minimizes the risk of cross-contamination and technical errors, the need for reverse transcription always introduces variability and should therefore be carefully interpreted [118].

An RNA biodistribution assay is expected to have a lower limit of quantification of less than 50 copies per μg of input RNA [118]. However, small quantities of target RNA can sometimes be masked by large quantities of endogenous RNA. To address this problem, conventional qPCR can be replaced by digital droplet PCR (ddPCR) which partitions a particular sample into many small droplets where individual PCR reactions occur. The number of positive over

Table 6

A comparative summary of FLI and BLI.

<i>In vivo</i> fluorescence imaging (FLI)	<i>In vivo</i> bioluminescence imaging (BLI)
Light source Light at a particular wavelength excites fluorophores which in turn emit light of a longer wavelength	A chemical substrate is enzymatically converted. This reaction also produces light.
Pros	
Wide variety of excitation/emission combinations, facilitating multicolor imaging	High SNR (minimal photon production in the absence of substrate)
High spatial and temporal resolution	No phototoxicity or physiological responses due to excitation light, no photobleaching
Any light source can excite fluorophores, thereby facilitating tandem dyes, FRET, BRET, etc.	Growing range of luciferase enzymes with different substrate combinations (e.g., requiring various cofactors thereby serving as biosensors) Glow and flash luciferases available to match experimental needs
Cons	
Sometimes high background due to tissue-autofluorescence (reduces SNR)	Long-term imaging requires continuous substrate supplementation and substrate kinetics need to be considered
Absorption of light by water, hemoglobin, melanin etc. (reducing SNR). Using nude, shaved or albino mice can help mitigate this effect.	Relatively dim compared to FLI (about 1:100), requiring longer exposure times thereby limiting temporal resolution. When binning is required, spatial resolution is also limited
Phototoxicity can hamper sequential imaging	Broad emission spectra thereby limiting multicolor imaging. In addition, spectral unmixing is only possible on the emission spectrum
Excitation light can cause photon-induced, unwanted physiological responses	No optical sectioning, causing blurry images when thick samples are used
Photobleaching	
Required equipment	
Excitation light source with series of filters. Camera with emission filters.	Dedicated luminescence imager and software. A fluorescence microscope is often not sensitive enough due to inefficient transmission of light
Many compatible machines and setups such as flow cytometers, (confocal) fluorescence microscopes, FLI systems, multi-photon excitation	Appropriate substrate and cofactors
Most fit for	
Non-living samples, multiplexing, very high spatial and/or temporal resolution	Long-term or repetitive imaging of live samples (e.g., low-abundance proteins or fast dynamics), photo-sensitive samples

SNR = signal-to-noise ratio, FRET = Förster or fluorescence resonance energy transfer, BRET = bioluminescence resonance energy transfer.

negative droplets is then analyzed via Poisson distribution to determine the target RNA concentration. Benefits of ddPCR include improved sensitivity, precision, and reproducibility. However, preparation and analysis of ddPCR samples takes approximately 7 times longer than conventional qPCR and often involves more method optimization [118].

RT-qPCR could be an important tool in determining tissue biodistribution of RNA therapeutics. It is however currently unclear to what extent this technique discriminates between intact and degraded RNA [119]. Moreover, there are no publications available which utilize this technique in an RNA biodistribution context at the time of writing. In addition, not all amplified mRNA necessarily derives from the intracellular compartment as most

mRNA remains encapsulated in LNPs or never escapes the endosomes [120]. This could result in an over-estimation of synthetic RNA in tissues. Especially for protein replacement therapies, PCR-based detection might even be unable to discriminate between exogenous and endogenous mRNA, although careful codon selection could help address this issue.

5.2. Fluorescence and bioluminescence

In vivo fluorescence imaging (FLI) and *in vivo* bioluminescence imaging (BLI) are two very common imaging modalities (Table 6). In the case of FLI, light at a particular wavelength excites fluorogenic dyes or proteins which in turn emit light of a longer wavelength. In contrast, BLI is based on an enzymatic reaction that generates light while converting a chemical substrate [121].

BLI and FLI require photons to pass through an animal or tissue sample before reaching a detector. These tissues absorb and scatter light primarily at wavelengths below 600 nm and above 900 nm (Optical window, Fig. 3a). This has two important consequences. First, both excitation light and emission light get absorbed and scattered, which results in dimmer images as the tissue thickness increases [122]. The absorption of high-intensity excitation light can also result in phototoxicity, especially with repeated exposure [121]. Secondly, naturally occurring fluorogenic compounds also produce light that coincides with the emission of the fluorochrome under investigation. BLI does not require high intensity excitation light and its dim emission light causes minimal tissue fluorescence [121]. Contrastingly, the strong light required to excite fluorophores typically causes autofluorescence in the blue-green spectrum [123].

To minimize absorption, phototoxicity, and tissue fluorescence, emission in the near-infrared (NIR, wavelength 700–900 nm) can be used (Fig. 3b) [124]. Note that a second NIR-window exists (NIR-II, wavelength 1000–1700 nm), that can also be used for *in vivo* imaging [125]. Additionally, spectral unmixing can help filter out the tissue fluorescence. Background fluorescence from dietary components (e.g., chlorophyll) in the gut can be minimized by feeding the rodents with low-fluorescent diets [126,127]. In mice, the effect of a diet change is visible within 1–2 days, but the intake of feces can prolong this period. In practice, it is therefore advised to combine the dietary change with a cage change or to extend the waiting period [126]. As mentioned, melanin can contribute significantly to the absorption of light (Fig. 3a). Using albino mice, nude mice, or shaved mice should therefore be considered as well [128].

Finally, FLI and BLI are not mutually exclusive and can be used in parallel [129], as fusion proteins [130], and as BRET partners (Section 5.2.2).

5.2.1. *In vivo* bioluminescence imaging

BLI is characterized by a very high SNR and does not require excitation by an external light source. Prolonged imaging is easily achievable, but substrate kinetics should be taken into consideration. Regarding preclinical biodistribution studies, BLI can be used to characterize the translation kinetics of mRNA therapeutics. By exchanging the coding sequence of the therapeutic protein with the sequence of a luciferase, a luminescence signal can be acquired that is proportional to the amount of protein produced. This cannot be achieved with QWBA and is particularly useful to evaluate the distribution and performance of a new mRNA carrier [131–139]. The argument that the sequence of the mRNA is unlikely to have a meaningful effect on the mRNA-LNP biodistribution was used for the Pfizer/BioNTech COVID-19 vaccine [20]. After intramuscular administration to mice, *in vivo* bioluminescence was determined using an *In Vivo* Imaging System (IVIS) at six time points (i.e., 6 h, 24 h, 48 h, 72 h, 6d, 9d post-injection) [20]. These longitudinal measurements were performed in only 6 anesthetized mice. Addi-

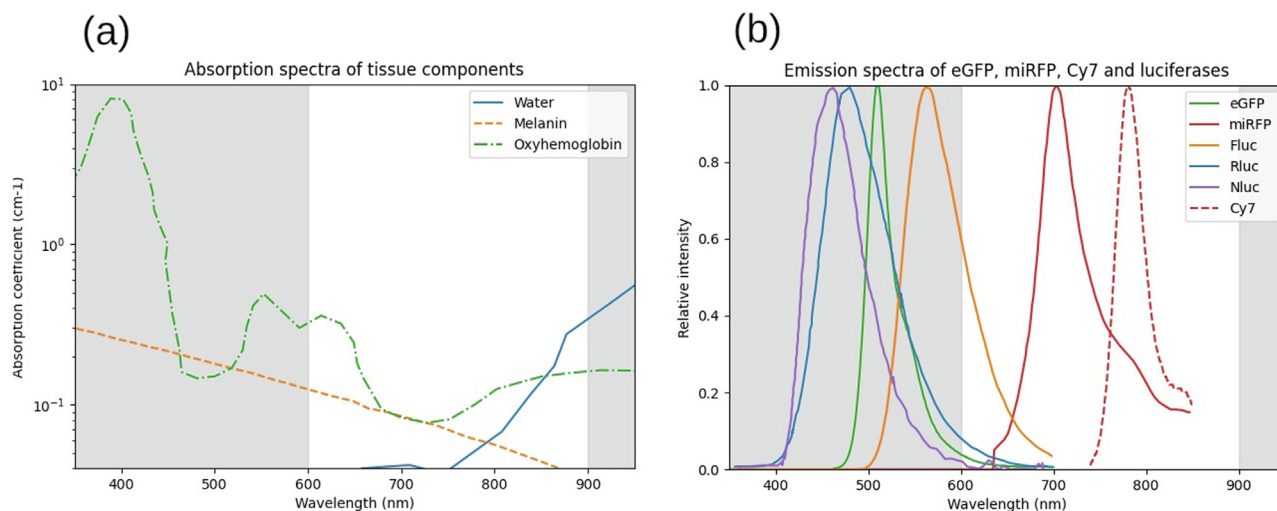


Fig. 3. (a) Absorption spectra of water, melanin, and oxyhemoglobin. Absorption is minimal in the optical window (white section in both (a) and (b)). (b) Emission spectra of eGFP, mRFP, Cy7, Firefly luciferase (Fluc), Renilla luciferase (hRluc), and NanoLuc (Nluc).

tionally, hepatic delivery efficiency of siRNA with LNPs can be examined by evaluating knock-down of luciferase mRNA expressed in the liver of mice [140].

A broad range of luciferases are available and are discussed in detail elsewhere [121,124,141–145]. Different luciferases require different cofactors (e.g., ATP, O₂, or Mg²⁺) and substrates with, for example, increased brightness or red-shifted emission spectrum. The kinetics of the substrate-dependent chemical reaction is another important factor. Some luciferases (e.g., Firefly luciferase (Fluc)) are characterized by light emission that steadily increases before reaching a plateau phase (where quantification should be performed). This kinetic profile is referred to as “glow” kinetics. Other luciferases (such as Renilla luciferase (Rluc) and Gaussia luciferase (Gluc)) will convert the substrate much quicker, reaching a higher peak luminescence within seconds and rapidly decaying afterward. Quantification of these “flash” kinetics should be performed immediately after the substrate is provided and are much more prone to technical variability. In practice, glow type kinetics are preferential *in vivo*, while the brighter flash kinetics of Rluc can be beneficial *in vitro*. Apart from the luciferase itself, the substrate can also have an influence on the reaction kinetics. The recently developed NanoLuc is based on bright flash kinetics (reported to be 150-fold higher than that of Fluc) that can be stabilized to mimic glow kinetics [146]. This enzyme and the accompanying substrate have been commercialized by Promega (Fitchburg, WI, US). Unfortunately, the wavelength of the emitted light is relatively short and therefore not yet optimal for *in vivo* use (Fig. 3). Finally, it should be noted that no apparent toxicity has been reported for the substrates of, Firefly or Renilla luciferase, apart from skin and mucosal irritation [147,148].

Despite the excellent SNR, BLI is known to be relatively dim [121]. This is also mentioned in the preclinical studies of the Pfizer/BioNTech COVID-19 vaccine. When the same luciferase mRNA containing LNP was radiolabeled and re-examined in rats, a broader biodistribution was determined. Although this was attributed to the higher sensitivity of the QWBA assay, it is important to note that both techniques were not compared directly. There were many differences between both studies, including animal species and administered dose. Moreover, QWBA is determined on sagittal tissue sections, while BLI images are acquired in whole animals thereby significantly diminishing and scattering the signal (but providing longitudinal data in the same animal). In addition, QWBA exposure times (days to weeks) are much longer than BLI

exposure times (seconds to minutes) [149]. Moreover, the observed broader distribution in the QWBA assay can also be attributed to radiolabeled degradation products or metabolites, especially since unstable ³H-labeling was used [52,57,58,66].

The limited spatial resolution of BLI is an important drawback in biodistribution studies. Moreover, the relative dimness of BLI often necessitates “binning”. This methodology combines the signal of multiple adjacent pixels into one exponentially brighter signal. Unfortunately, binning results in a further reduction of spatial resolution. Possible workarounds for the lower spatial resolution include decreasing the distance between the luminescent light source and the detector (which are exponentially related). Another workaround is to image individual organs *ex vivo*. However, this requires additional tissue handling, which can then influence quantification (e.g., due to variable exsanguination) and introduce cross-contamination. Alternatively, BLI can be used to sensitively detect signal in a crudely demarcated region, followed by a second technique (e.g., FLI) to determine the exact location of the signal origin. BLI assays can also be followed-up with, for example, flow cytometry and immunohistochemistry based on anti-luciferase antibodies. Finally, more accurate 3D spatial information can be obtained by using BLI tomography [150,151].

5.2.2. *In vivo* fluorescence imaging

LNPs can be fluorescently labeled with lipophilic tracers (e.g., DiD, DiR) [137,152–154], but this can potentially influence their physicochemical properties and biodistribution. Additionally, lipophilic tracers can leach out the LNPs. Therefore, this approach is not optimal for biodistribution studies as regulatory agencies might reject the data [8,9,11,14]. Fortunately, the coding sequence of mRNA therapeutics or vaccines can be replaced without significantly influencing mRNA-LNP distribution (Section 5.2.1) [20]. By introducing the coding sequence of a fluorescent protein (e.g., eGFP, mRFP) [133,155] or by fusing the therapeutic/antigenic protein to a dye-binding tag (e.g., HaloTag) [156], the distribution of an mRNA-LNP and its translation kinetics can be examined. Additionally, fluorescence/Förster resonance energy transfer (FRET) can also be used, for example, by including a FRET fusion protein in the coding sequence of mRNA. In a second example, labeled siRNA and nanoparticles formed a FRET-pair. Once the siRNA escape the confines of the LNP, fluorescence could no longer occur [157]. FRET increases the total brightness and/or shifts the emission spectrum to NIR by using the emission from a primary fluores-

cent protein to excite a second fluorescent protein [155]. Similarly, bioluminescence resonance energy transfer (BRET) uses a luciferase to excite an adjacent fluorescent protein [121,124,141,142,155]. Consequently, BRET does not require external excitation light, thereby significantly reducing tissue autofluorescence and potential phototoxicity.

To limit the number of animals needed, developers can co-encapsulate fluorescent protein-encoding mRNA and luciferase-encoding mRNA in a single LNP, which mimics multivalent mRNA vaccines [158,159]. Alternatively, it is also possible to encode an eGFP-Fluc fusion protein on a single mRNA instead [130].

mRNA and siRNA can also be labeled directly. This approach is however accompanied by some challenges. For example, cyanine dyes such as Cy5 and Cy7 (Fig. 3b) can be chemically linked to RNA molecules [160–163], but these bulky and hydrophobic groups can significantly interfere with translation dynamics [164]. Other dyes, such as the more hydrophilic Alexa Fluor® 647 and 750, have also been used to label siRNA [120,165]. The label can then be imaged *in vivo* using fluorescence tomography, coupled to micro-CT [165]. Alternatively, the organs can also be imaged *ex vivo* [166]. Other labeling approaches include the introduction of fluorescent nucleosides, 5'-cap, 3'-polyA tail, or even introducing dye-binding aptamers [164]. Unfortunately, these approaches either introduce insufficient labels for *in vivo* imaging, or they still perturb transcription and/or translation [164]. An interesting direct RNA labeling solution was recently developed by Baladi et al. (2021) [164]. These authors used a fluorescent tricyclic cytosine analogue, which can comprise up to 100% of the cytosines in a 1.2 kb-long GFP-encoding mRNA [164]. Unlike other labeling methods, direct incorporation of the fluorescent cytosine analogue had minimal influence on transcription and translation kinetics [164]. Evidently, this labeling method still requires further examination and validation before use in preclinical biodistribution studies.

An important aspect is the *in vivo* stability of a fluorescent label. This is the main reason that hybridization probes, such as FISH probes cannot be used *in vivo* [164]. Still, Kirschman et al. (2017) have used a multi-labeled probe and a handheld NIR fluorescence camera to successfully track intramuscularly injected mRNA *in vivo* [106]. The *in vivo* probe-mRNA interaction was verified 2 h after injection through a co-localization FISH assay [106]. This time-frame is however not yet long enough for longitudinal mRNA distribution studies. A similar attempt to design an RNA binding probe was recently published by Wu et al. (2020), where a tripartite DNA probe was injected intravenously and intratumoral to image a micro-RNA target [167]. Unfortunately, the current probe design resulted in very limited spatial resolution *in vivo*.

Not all fluorescently labeled RNA is directly accessible for translation (e.g., in LNPs, phagosomes, or endosomes [120]). To monitor translatable mRNA, Ai14 reporter mice can be used. This mice strain carries a fluorescent protein transgene (e.g., tdTomato) of which the transcription is inhibited due to an upstream LoxP-flanked stop cassette. When Cre recombinase mRNA is translated in these mice, the stop cassette is removed and the cells become permanently fluorescent through expression of the tdTomato protein [168–170]. This reporter model has been used to investigate tissue distribution and cytosolic mRNA delivery of altered LNPs [168,169]. Of note, this version of the system is less suitable for evaluation of expression dynamics, as the fluorescence observed is not expected to correlate well to the amount of Cre recombinase translated.

Lastly, additional molecular imaging tools have been designed for cell cultures and can therefore also be used to image target mRNA on tissue sections obtained during biodistribution studies. These techniques include aptamers (e.g., Spinach, Broccoli, and Mango) in the 3' untranslated region (3'UTR) of an mRNA, which

should have minimal impact on the biodistribution or on the translation dynamics of the mRNA but can potentially influence mRNA stability [171]. These aptamers selectively bind fluorescent dyes but currently lack the ability to visualize single mRNA molecules [93,171]. Background fluorescence by unbound dyes can be reduced, by using aptamer-binding fluorophore-quencher pairs which only become fluorescent when bound to the aptamer [93,171]. Alternatively, unique protein binding motifs (e.g., MS2, PP7, λ N) can be introduced in the 3'UTR of mRNA. These motifs interact with RNA-binding proteins that are fused to fluorescent proteins and allow visualization of single mRNA molecules [93,171]. Another interesting system to visualize single RNA molecules is based on a catalytically inactivated Cas13 protein (fused to a fluorescent protein). When supplied with a custom-made guide RNA, the Cas13 will sequence-specifically bind a target mRNA of choice [171]. Finally, molecular beacons are oligonucleotides that contain a fluorophore on one end of a stem loop, and a quencher on the other end. The stem loop opens upon binding its target RNA, elevating the influence of the quencher [93,171].

6. Final remarks

Biodistribution studies intend to gain insight into the whereabouts of injected drugs. This knowledge is then used to help interpret the drug's pharmacological or toxicological interactions. Interpreting all these interactions is a daunting task for mRNA therapeutics since these novel medicines contain many components and are processed on multiple levels. For example, PEGylated LNPs can potentially elicit immune responses [28], LNPs can temporarily saturate the scavenging systems in the liver [172], impurities such as dsRNA can trigger the production of pro-inflammatory cytokines and cause antiviral states in cells [30,31], therapeutic mRNA can act as miRNA sponges [173], and the expressed therapeutic protein can have local or even distant effects in the body. In addition, current regulations for preclinical biodistribution data of (m)RNA therapeutics are vague and ill-defined without concrete specification on e.g., thresholds for sensitivity. Evidently, expanding the clinical applications and public acceptance of this very promising platform technology would greatly benefit from a more robust regulatory framework. Pivoting from a per-product approach to more general guidelines may become a necessity, as the number of (m)RNA therapeutics applying for clinical approval increases rapidly.

mRNA vaccines against infectious diseases are currently not considered as gene therapeutics by regulatory agencies such as EMA and FDA. Instead, they are regarded as vaccines despite their identical composition and production process as mRNA therapeutics for protein replacement. Indeed, many mRNA-based therapeutics (including mRNA vaccines) rely on recombinant DNA technology for *in vitro* transcription template production. Based on this property, almost all mRNA-based therapeutics should be classified as “gene therapy medicinal products”, as defined by the EMA [174] (of note, this does not apply to siRNA-based therapeutics as these molecules are typically chemically manufactured). Additionally, mRNA vaccines against non-infectious diseases such as cancer are not regarded as mRNA vaccines, but as gene therapy products. Strikingly, this means that an mRNA vaccine against human papilloma virus (HPV)-induced malignancies is classified as a gene therapy, whereas using the same mRNA for HPV vaccination classifies it as a vaccine [174]. The rationale (and implications) for this distinction is unclear but may rely on the added effect of adjuvants and the antigenic nature of the translated exogenous proteins of mRNA vaccines. Although these exogenous proteins are unlikely to have a physiological function in the body, these vaccines are meant to elicit robust long-lasting immune responses and

are therefore not biologically inert. Moreover, tissue biodistribution and off-target interactions of all non-mRNA components of mRNA vaccines is identical to those of other (m)RNA therapeutics. As a final note, viral-based RNA platforms such as self-amplifying or *trans*-amplifying mRNA encode both a replicase complex comprising viral non-structural proteins and a therapeutic protein of choice [28,29]. The former is exogenous and elicits a vaccine-like immune response [29,175] while the latter can be both exogenous or endogenous in future applications.

Biodistribution studies are considered of lesser importance for mRNA vaccines because they are generally administered locally in the skin or in the muscle. However, intramuscularly injected luciferase mRNA also generates luminescence in the liver [20,37]. Hence, either the mRNA-LNPs or their protein product is distributed systemically and should therefore require pharmacokinetic studies, including biodistribution. Likewise, biodistribution studies are not strictly mandatory for (non-vaccine) mRNA therapeutics. With the advent of mRNA-based CRISPR/Cas9 therapies [76], this view should be reconsidered. Moreover, conducting pre-clinical biodistribution studies can save time, money, and animals by optimizing other, mandatory experiments (e.g., toxicology).

Quantitative whole-body autoradiography is currently the method of choice for most RNA biodistribution studies. This technique provides a valuable whole-body overview of the drug's distribution while still preserving tissue-level resolution. However, the used radioactive substances require specialized facilities and trained personnel. In addition, the inability to discriminate between parent compounds and their metabolites/degradation products is a considerable limitation, especially for unstable molecules such as RNA. Lastly, most of the labeled RNA will never gain cytosolic access and is therefore not necessarily relevant in interpreting toxicological findings. This is perhaps the most important difference of the novel RNA therapeutics compared to small molecules. Therefore, multi-modal imaging and quantification techniques are advised to adequately evaluate biodistribution of RNA therapeutics. For example, the distribution and translation of cytosolic mRNA can be examined by using the mRNA coding sequence of a luciferase or a fluorescent protein. Tissue slices from the same animals can be used to determine subcellular RNA location with hybridization techniques and (single-cell) RT-qPCR can be used to detect minute quantities of mRNA. These data can also be coupled to immunohistochemistry findings. Alternatively, RNA molecules can be visualized by directly labeling them with e.g., fluorescent cytosine and cytosolic mRNA delivery can be assessed with transgenic Ai14 reporter mice. Mass spectrometry is a powerful technique for quantifying intact and degraded siRNA and *in situ* ionization techniques (e.g., MALDI-FT-ICR-MS) couple this information to distribution data. We conclude that this new and rapidly evolving class of medicines demands a multi-layered approach to fully understand its biodistribution and *in vivo* characteristics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was made possible thanks the funding of the Research Foundation – Flanders (FWO) grant 1172121N, the Research Council of Norway grant 325243, and the European Union's Horizon 2020 Research and Innovation Programme grant 825828.

References

- [1] BioNTech, Project Lightspeed, 2021. <https://www.biontech.de>.
- [2] Moderna, Inc., Moderna's Work on our COVID-19 Vaccine, (n.d.). <https://www.modernatx.com/modernas-work-potential-vaccine-against-covid-19>.
- [3] A. Fortner, D. Schumacher, First COVID-19 Vaccines Receiving the US FDA and EMA Emergency Use Authorization, *Discoveries (Craiova)*. 9 (1) (2021) e122. <https://doi.org/10.15190/d.2021.1>.
- [4] European Medicines Agency, Conditional marketing authorisation, 2018. <https://www.ema.europa.eu/en/human-regulatory/marketing-authorisation/conditional-marketing-authorisation>.
- [5] Food and Drug Administration, Emergency Use Authorization, 2021. <https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization>.
- [6] J. Bossart, DEVELOPMENT TIMELINES - Drug Development Times, What it Takes - Part 2, Drug Development and Delivery, 2020. <https://drug-dev.com/development-timelines-drug-development-times-what-it-takes-part-2/>.
- [7] European Medicines Agency, Guideline on the Non-Clinical Studies Required before First Clinical Use of Gene Therapy Medicinal Products (EMA/CHMP/GTWP/125459/2006), 2008.
- [8] European Medicines Agency, Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014), 2018.
- [9] Food and Drug Administration, Preclinical Assessment of Investigational Cellular and Gene Therapy Products, 2013. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/preclinical-assessment-investigational-cellular-and-gene-therapy-products>.
- [10] Food and Drug Administration, Long Term Follow-up After Administration of Human Gene Therapy Products, 2020. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/long-term-follow-up-after-administration-human-gene-therapy-products>.
- [11] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, DRAFT: ICH guideline S12 on nonclinical biodistribution considerations for gene therapy products (EMA/CHMP/ICH/318372/2021), 2021.
- [12] European Commission (EC), Commission Directive 2009/120/EC of 14 September 2009 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products, 2009.
- [13] European Medicines Agency, Reflection paper on classification of advanced therapy medicinal products (EMA/CAT/600280/2010 rev.1), 2015. https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-classification-advanced-therapy-medicinal-products_en-0.pdf.
- [14] International Pharmaceutical Regulators Programme, Expectations for Biodistribution (BD) Assessments for Gene Therapy (GT) Products, 2018. https://admin.iprp.global/sites/default/files/2018-09/IPRP_GTWG_Reflection_Paper_BD_Final_2018_0713.pdf.
- [15] World Health Organization, WHO Technical Report Series, No. 927, 2005 - Annex 1 WHO guidelines on nonclinical evaluation of vaccines, 2005. https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf?ua=1.
- [16] Food and Drug Administration, Development and Licensure of Vaccines to Prevent COVID-19; Guidance for Industry, 2020.
- [17] World Health Organization, DRAFT Evaluation of the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases: regulatory considerations (WHO/BS/2021.2402), 2021. https://cdn.who.int/media/docs/default-source/biologicals/call-for-comments/bs.2021.bs2402_who-regulatory-considerations-for-mrna-vaccines_final.pdf?sfvrsn=c8623b32_5.
- [18] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines – a new era in vaccinology, *Nat. Rev. Drug Discov.* 17 (4) (2018) 261–279. <https://doi.org/10.1038/nrd.2017.243>.
- [19] European Medicines Agency, Scientific advice and protocol assistance, 2018. <https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-advice-protocol-assistance>.
- [20] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR Comirnaty, INN-COVID-19 mRNA Vaccine (nucleoside-modified) (EMA/707383/2020 Corr.1), 2021. https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf.
- [21] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR COVID-19 Vaccine Moderna, INN-COVID-19 mRNA Vaccine (nucleoside modified) (EMA/15689/2021 Corr.1), 2021. https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf.
- [22] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR Onpattro, INN - patisiran (EMA/554262/2018), 2018. https://www.ema.europa.eu/en/documents/assessment-report/onpattro-epar-public-assessment-report_en.pdf.
- [23] European Medicines Agency, EPAR Shingrix, INN - herpes zoster vaccine (recombinant, adjuvanted) (EMA/88588/2018), 2018. https://www.ema.europa.eu/en/documents/assessment-report/shingrix-epar-public-assessment-report_en.pdf.

- [24] European Medicines Agency, Guideline on bioanalytical method validation (EMA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2), 2011. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.
- [25] Food and Drug Administration, Bioanalytical Method Validation Guidance for Industry, 2018.
- [26] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, DRAFT - M10 Bioanalytical Method Validation, 2018.
- [27] World Health Organization, WHO Technical Report Series, No. 1019, 2019 - Annex 3 Good manufacturing practices: guidelines on validation, 2019. https://www.who.int/medicines/areas/quality_safety/quality_assurance/WHO_TRS_1019_Annex3.pdf.
- [28] Z. Zhong, S. Mc Cafferty, F. Combes, H. Huysmans, J. De Temmerman, A. Gitsels, D. Vanrompay, J. Portela Catani, N.N. Sanders, mRNA therapeutics deliver a hopeful message, *Nano Today* 23 (2018) 16–39, <https://doi.org/10.1016/j.nantod.2018.10.005>.
- [29] H. Huysmans, Z. Zhong, J. De Temmerman, B.L. Mui, Y.K. Tam, S. Mc Cafferty, A. Gitsels, D. Vanrompay, N.N. Sanders, Expression Kinetics and Innate Immune Response after Electroporation and LNP-Mediated Delivery of a Self-Amplifying mRNA in the Skin, *Mol. Ther. Nucleic Acids* 17 (2019) 867–878, <https://doi.org/10.1016/j.omtn.2019.08.001>.
- [30] Z. Zhong, S. McCafferty, L. Opsomer, H. Wang, H. Huysmans, J. De Temmerman, S. Lienenklaus, J.P. Portela Catani, F. Combes, N.N. Sanders, Corticosteroids and cellulose purification improve, respectively, the in vivo translation and vaccination efficacy of sa-mRNAs, *Mol. Ther.* 29 (4) (2021) 1370–1381, <https://doi.org/10.1016/j.ymthe.2021.01.023>.
- [31] Z. Zhong, F. Combes, N.N. Sanders, Corticosteroids and mRNA Vaccines: A Word of Caution, *Mol. Ther.* 29 (3) (2021) 893–894, <https://doi.org/10.1016/j.ymthe.2021.01.029>.
- [32] O. Demaria, P.P. Pagni, S. Traub, A. de Gassart, N. Branzk, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, R.A. Flavell, L. Alexopoulou, TLR8 deficiency leads to autoimmunity in mice, *J. Clin. Invest.* 120 (2010) 3651–3662, <https://doi.org/10.1172/JCI42081>.
- [33] S. Linares-Fernández, C. Lacroix, J.-Y. Exposito, B. Verrier, Tailoring mRNA Vaccine to Balance Innate/Adaptive Immune Response, *Trends Mol. Med.* 26 (3) (2020) 311–323, <https://doi.org/10.1016/j.molmed.2019.10.002>.
- [34] I. Mellman, S. Tahtinen, A.-J. Tong, P. Himmels, J. Oh, A. Paler-Martinez, L. Kim, S. Wichner, Y. Oei, M. McCarron, E. Freund, Z.A. Amir, C. de la Cruz, B. Haley, C. Blanchette, J. Scharfner, W. Ye, M. Yadav, U. Sahin, L. Delamarre, IL-1 β and IL-1 α are key regulators of the inflammatory response to RNA vaccines, 2021. <https://doi.org/10.21203/rs.3.rs-378991/v1>.
- [35] S.J. Cleary, S.C. Pitchford, R.T. Amison, R. Carrington, C.L. Robaina Cabrera, M. Magnen, M.R. Looney, E. Gray, C.P. Page, Animal models of mechanisms of SARS-CoV-2 infection and COVID-19 pathology, *Br. J. Pharmacol.* 177 (2020) 4851–4865, <https://doi.org/10.1111/bph.15143>.
- [36] C. Muñoz-Fontela, W.E. Dowling, S.G.P. Funnell, P.-S. Gsell, A.X. Riveros-Balta, R.A. Albrecht, H. Andersen, R.S. Baric, M.W. Carroll, M. Cavaleri, C. Qin, I. Crozier, K. Dallmeier, L. de Waal, E. de Wit, L. Delang, E. Dohm, W.P. Duprex, D. Falzarano, C.L. Finch, M.B. Frieman, B.S. Graham, L.E. Gralinski, K. Guilfoyle, B.L. Haagmans, G.A. Hamilton, A.L. Hartman, S. Herfst, S.J.F. Kaptein, W.B. Klimstra, I. Knezevic, P.R. Krause, J.H. Kuhn, R. Le Grand, M.G. Lewis, W.-C. Liu, P. Maisonnasse, A.K. McElroy, V. Munster, N. Oreshkova, A.L. Rasmussen, J. Rocha-Pereira, B. Rockx, E. Rodríguez, T.F. Rogers, F.J. Salguero, M. Schotsaert, K.J. Stittelaar, H.J. Thibaut, C.-T. Tseng, J. Vergara-Alert, M. Beer, T. Brasel, J.F. W. Chan, A. García-Sastre, J. Neyts, S. Perlman, D.S. Reed, J.A. Richt, C.J. Roy, J. Segalés, S.S. Vasan, A.M. Henao-Restrepo, D.H. Barouch, Animal models for COVID-19, *Nature* 586 (7830) (2020) 509–515, <https://doi.org/10.1038/s41586-020-2787-6>.
- [37] N. Pardi, S. Tuyishime, H. Muramatsu, K. Kariko, B.L. Mui, Y.K. Tam, T.D. Madden, M.J. Hope, D. Weissman, Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes, *J. Control. Release* 217 (2015) 345–351, <https://doi.org/10.1016/j.jconrel.2015.08.007>.
- [38] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR Leqvio, INN - inclisiran (EMA/696912/2020), 2020. https://www.ema.europa.eu/en/documents/assessment-report/leqvio-epar-public-assessment-report_en.pdf.
- [39] R.A. Wesselhoeft, P.S. Kowalski, D.G. Anderson, Engineering circular RNA for potent and stable translation in eukaryotic cells, *Nat Commun.* 9 (2018) 2629, <https://doi.org/10.1038/s41467-018-05096-6>.
- [40] R.A. Wesselhoeft, P.S. Kowalski, F.C. Parker-Hale, Y. Huang, N. Bisaria, D.G. Anderson, RNA Circularization Diminishes Immunogenicity and Can Extend Translation Duration In Vivo 508.e4–520.e4 *Mol. Cell* 74 (3) (2019), <https://doi.org/10.1016/j.molcel.2019.02.015>.
- [41] D.C. Litzinger, Limitations of Cationic Liposomes for Antisense Oligonucleotide Delivery in Vivo, *J. Liposome Res.* 7 (1) (1997) 51–61, <https://doi.org/10.3109/08982109709035481>.
- [42] Y.-N. Zhang, W. Poon, A.J. Tavares, I.D. McGilvray, W.C.W. Chan, Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination, *J. Control. Release* 240 (2016) 332–348, <https://doi.org/10.1016/j.jconrel.2016.01.020>.
- [43] S. Rietwyk, D. Peer, Next-Generation Lipids in RNA Interference Therapeutics, *ACS Nano* 11 (8) (2017) 7572–7586, <https://doi.org/10.1021/acsnano.7b04734>.
- [44] M.A. Maier, M. Jayaraman, S. Matsuda, J.u. Liu, S. Barros, W. Querbes, Y.K. Tam, S.M. Ansell, V. Kumar, J. Qin, X. Zhang, Q. Wang, S. Panesar, R. Hutabarat, M. Carioto, J. Hettinger, P. Kandasamy, D. Butler, K.G. Rajeev, B.o. Pang, K. Charisse, K. Fitzgerald, B.L. Mui, X. Du, P. Cullis, T.D. Madden, M.J. Hope, M. Manoharan, A. Akinc, Biodegradable Lipids Enabling Rapidly Eliminated Lipid Nanoparticles for Systemic Delivery of RNAi Therapeutics, *Mol. Ther.* 21 (8) (2013) 1570–1578, <https://doi.org/10.1038/mt.2013.124>.
- [45] S. Sabnis, E.S. Kumarasinghe, T. Salerno, C. Mihai, T. Ketova, J.J. Senn, A. Lynn, A. Bulychiev, I. McFadyen, J. Chan, Ö. Almarsson, M.G. Stanton, K.E. Benenato, A Novel Amino Lipid Series for mRNA Delivery: Improved Endosomal Escape and Sustained Pharmacology and Safety in Non-human Primates, *Mol. Ther.* 26 (6) (2018) 1509–1519, <https://doi.org/10.1016/j.ymthe.2018.03.010>.
- [46] European Medicines Agency - Committee for Medicinal Products for Human Use, Note for guidance on repeated dose toxicity (CPMP/SWP/142/99 corr.), 2000.
- [47] W.J. Reagan, A. Irizarry-Rovira, F. Poutout-Belissent, A.P. Bolliger, S.K. Ramaiah, G. Travlos, D. Walker, B. Bounos, G. Walter, Best Practices for Evaluation of Bone Marrow in Nonclinical Toxicity Studies, *Toxicol. Pathol.* 39 (2) (2011) 435–448, <https://doi.org/10.1177/0192623310396907>.
- [48] H. Wan, What ADME tests should be conducted for preclinical studies?, *ADMET DMPK* 1 (2013) 19–28, <https://doi.org/10.5599/admet.1.3.9>.
- [49] European Medicines Agency, Good Laboratory Practice (GLP) principles in relation to ATMPs, 2017. https://www.ema.europa.eu/en/documents/other/good-laboratory-practice-glp-principles-relation-advanced-therapy-medicinal-products-atmps_en.pdf.
- [50] J.K. Nair, J.L.S. Willoughby, A. Chan, K. Charisse, M.R. Alam, Q. Wang, M. Hoekstra, P. Kandasamy, A.V. Kel'in, S. Milstein, N. Taneja, J. O'Shea, S. Shaikh, L. Zhang, R.J. van der Sluis, M.E. Jung, A. Akinc, R. Hutabarat, S. Kuchimanchi, K. Fitzgerald, T. Zimmermann, T.J.C. van Berkel, M.A. Maier, K.G. Rajeev, M. Manoharan, Multivalent N-Acetylgalactosamine-Conjugated siRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing, *J. Am. Chem. Soc.* 136 (49) (2014) 16958–16961, <https://doi.org/10.1021/ja505986a>.
- [51] A. McEwen, C. Henson, Quantitative whole-body autoradiography: past, present and future, *Bioanalysis*. 7 (5) (2015) 557–568, <https://doi.org/10.4155/bio.15.9>.
- [52] E.G. Solon, Autoradiography techniques and quantification of drug distribution, *Cell Tissue Res.* 360 (1) (2015) 87–107, <https://doi.org/10.1007/s00441-014-2093-4>.
- [53] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR Oxluo, INN lumasiran (EMA/568312/2020), 2020. https://www.ema.europa.eu/en/documents/assessment-report/oxluo-epar-public-assessment-report_en.pdf.
- [54] European Medicines Agency - Committee for Medicinal Products for Human Use, EPAR Givlaari, INN - givosiran (EMA/CHMP/70703/2020), 2020. https://www.ema.europa.eu/en/documents/assessment-report/givlaari-epar-public-assessment-report_en.pdf.
- [55] New England Biolabs (NEB), Instruction Manual – HiScribe™ T7 High Yield RNA Synthesis Kit (Version 4.0.4/20), n.d. <https://www.neb.com/-/media/nebus/files/manuals/manuale2040.pdf?rev=8d4d39754e624bc9b425c3c52c21a159&hash=185F47CDABACA9F60250BA0342CEE74E>.
- [56] G. Audi, F.G. Kondev, M. Wang, W.J. Huang, S. Naimi, The NUBASE2016 evaluation of nuclear properties, *Chinese Phys. C* 41 (3) (2017) 030001, <https://doi.org/10.1088/1674-1137/41/3/030001>.
- [57] L. Kingston, C. Gu, J. Guo, S. Swallow, C.S. Elmore, The impact of radiochemistry in drug projects: The use of C-14 label in the AZD8529, AZD7325, and AZD6280 projects, *J. Label Compd. Radiopharm.* 64 (2) (2021) 65–72, <https://doi.org/10.1002/jlcr.3902>.
- [58] E.G. Solon, Use of Radioactive Compounds and Autoradiography to Determine Drug Tissue Distribution, *Chem. Res. Toxicol.* 25 (3) (2012) 543–555, <https://doi.org/10.1021/tx200509f>.
- [59] Hydrogen -3 (Tritium) | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/hydrogen-3-tritium>.
- [60] Carbon-14 | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/carbon-14>.
- [61] Phosphorus-32 | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/phosphorus-32>.
- [62] Phosphorus-33 | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/phosphorus-33>.
- [63] Sulfur-35 | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/sulfur-35>.
- [64] Iodine-125 | Office of Environmental Health and Safety, n.d. <https://ehs.princeton.edu/laboratory-research/radiation-safety/radioactive-materials/radioisotope-fact-sheets/iodine-125>.
- [65] J. Christensen, K. Litherland, T. Faller, E. van de Kerkhof, F. Natt, J. Hunziker, J. Krauser, P. Swart, Metabolism Studies of Unformulated Internally [3H]-Labeled Short Interfering RNAs in Mice, *Drug Metab. Dispos.* 41 (6) (2013) 1211–1219, <https://doi.org/10.1124/dmd.112.050666>.
- [66] E.G. Solon, A. Schweitzer, M. Stoekli, B. Prideaux, Autoradiography, MALDI-MS, and SIMS-MS Imaging in Pharmaceutical Discovery and Development, *AAPS J.* 12 (1) (2010) 11–26, <https://doi.org/10.1208/s12248-009-9158-4>.

- [67] I. Satoshi, Why You Need QWBA for Human Radiolabeled ADME Studies, 2019. <https://www.xenotech.com/blog/why-you-need-qwba-for-human-radiolabeled-adme-studies/>.
- [68] K. Shimada, R. Baweja, T. Sokoloski, P.N. Patil, Binding Characteristics of Drugs to Synthetic Levodopa Melanin, *J. Pharm. Sci.* 65 (7) (1976) 1057–1060, <https://doi.org/10.1002/jps.2600650725>.
- [69] E.G. Solon, L. Kraus, Quantitative whole-body autoradiography in the pharmaceutical industry: Survey results on study design, methods, and regulatory compliance, *J. Pharmacol. Toxicol. Methods* 46 (2) (2001) 73–81, [https://doi.org/10.1016/S1056-8719\(02\)00161-2](https://doi.org/10.1016/S1056-8719(02)00161-2).
- [70] A. Hirose, N.I. Kobayashi, K. Tanoi, T.M. Nakanishi, A Microautoradiographic Method for Fresh-Frozen Sections to Reveal the Distribution of Radionuclides at the Cellular Level in Plants, *Plant Cell Physiol.* 55 (2014) 1194–1202, <https://doi.org/10.1093/pcp/pcu056>.
- [71] M.M. Zak, L. Zangi, Lipid Nanoparticles for Organ-Specific mRNA Therapeutic Delivery, *Pharmaceutics* 13 (2021) 1675, <https://doi.org/10.3390/pharmaceutics13101675>.
- [72] E. Samaridou, J. Heyes, P. Lutwyche, Lipid nanoparticles for nucleic acid delivery: Current perspectives, *Adv. Drug Deliv. Rev.* 154–155 (2020) 37–63, <https://doi.org/10.1016/j.addr.2020.06.002>.
- [73] M. Yang, J. Li, P. Gu, X. Fan, The application of nanoparticles in cancer immunotherapy: Targeting tumor microenvironment, *Bioact Mater.* 6 (7) (2021) 1973–1987, <https://doi.org/10.1016/j.bioactmat.2020.12.010>.
- [74] M. Alavi, M. Hamidi, Passive and active targeting in cancer therapy by liposomes and lipid nanoparticles, *Drug Metab. Pers. Ther.* 34 (2019), <https://doi.org/10.1515/dmpt-2018-0032>.
- [75] R. Shankar, M. Joshi, K. Pathak, Lipid Nanoparticles: A Novel Approach for Brain Targeting, *Pharm. Nanotechnol.* 6 (2) (2018) 81–93, <https://doi.org/10.2174/2211738506666180611100416>.
- [76] Q. Tang, J.I. Liu, Y. Jiang, M. Zhang, L. Mao, M. Wang, Cell-Selective Messenger RNA Delivery and CRISPR/Cas9 Genome Editing by Modulating the Interface of Phenylboronic Acid-Derived Lipid Nanoparticles and Cellular Surface Sialic Acid, *ACS Appl. Mater. Interfaces* 11 (50) (2019) 46585–46590, <https://doi.org/10.1021/acsami.9b17749>.
- [77] L. Ramanathan, H. Shen, LC-TOF-MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues, *Bioanalysis* 11 (21) (2019) 1983–1992, <https://doi.org/10.4155/bio-2019-0134>.
- [78] I.C. Santos, J.S. Brodbelt, Recent developments in the characterization of nucleic acids by liquid chromatography, capillary electrophoresis, ion mobility, and mass spectrometry (2010–2020), *J. Sep. Sci.* 44 (1) (2021) 340–372, <https://doi.org/10.1002/jssc.202000833>.
- [79] S. Pourshahian, Therapeutic Oligonucleotides, Impurities, Degradants, and Their Characterization by Mass Spectrometry, *Mass Spectrom. Rev.* 40 (2) (2021) 75–109, <https://doi.org/10.1002/mas.21615>.
- [80] S. Tamara, M.A. den Boer, A.J.R. Heck, High-Resolution Native Mass Spectrometry, *Chem. Rev.* (2021), <https://doi.org/10.1021/acs.chemrev.1c00212>.
- [81] M. Taucher, K. Breuker, Characterization of Modified RNA by Top-Down Mass Spectrometry, *Angew. Chem.* 124 (45) (2012) 11451–11454, <https://doi.org/10.1002/ange.201206232>.
- [82] M. van de Waterbeemd, K.L. Fort, D. Boll, M. Reinhardt-Szyba, A. Routh, A. Makarov, A.J.R. Heck, High-fidelity mass analysis unveils heterogeneity in intact ribosomal particles, *Nat. Methods* 14 (3) (2017) 283–286, <https://doi.org/10.1038/nmeth.4147>.
- [83] J.M. Sutton, J. Kim, N.M. El Zahar, M.G. Bartlett, Bioanalysis and Biotransformation of Oligonucleotide Therapeutics by Liquid Chromatography-Mass Spectrometry, *Mass Spectrom. Rev.* 40 (4) (2021) 334–358, <https://doi.org/10.1002/mas.21641>.
- [84] F. Romero-Palomo, M. Festag, B. Lenz, S. Schadt, A. Brink, A. Kipar, B. Steinhuber, C. Huser, E. Koller, S. Sewing, Y. Tessier, P. Dzygiel, G. Fischer, M. Winter, U. Hetzel, M.J. Mihatsch, A. Braendli-Baiocco, Safety, Tissue Distribution, and Metabolism of LNA-Containing Antisense Oligonucleotides in Rats, *Toxicol. Pathol.* 49 (6) (2021) 1174–1192, <https://doi.org/10.1177/01926232211011615>.
- [85] S. Schulz, M. Becker, M.R. Groseclose, S. Schadt, C. Hopf, Advanced MALDI mass spectrometry imaging in pharmaceutical research and drug development, *Curr. Opin. Biotechnol.* 55 (2019) 51–59, <https://doi.org/10.1016/j.copbio.2018.08.003>.
- [86] G. Yagnik, Z. Liu, K.J. Rothschild, M.J. Lim, Highly Multiplexed Immunohistochemical MALDI-MS Imaging of Biomarkers in Tissues, *J. Am. Soc. Mass Spectrom.* 32 (4) (2021) 977–988, <https://doi.org/10.1021/jasms.0c00473>.
- [87] K.J. Hassett, K.E. Benenato, E. Jacquinet, A. Lee, A. Woods, O. Yuzhakov, S. Himansu, J. Deterling, B.M. Geilich, T. Ketova, C. Mihai, A. Lynn, I. McFadyen, M.J. Moore, J.J. Senn, M.G. Stanton, Ö. Almarsson, G. Ciaramella, L.A. Brito, Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines, *Mol. Ther. Nucleic Acids* 15 (2019) 1–11, <https://doi.org/10.1016/j.omtn.2019.01.013>.
- [88] G. Hawthorne, N. Henderson, M. Hölttä, S. Khan, J. Lindqvist, A. Wilson, Overcoming analytical challenges to generate data critical to understanding lipid nanoparticle-delivered modified mRNA biodistribution, *Bioanalysis* 11 (21) (2019) 1993–2001, <https://doi.org/10.4155/bio-2019-0138>.
- [89] M.O. Urbanek, A.U. Nawrocka, W.J. Krzyzosiak, Small RNA Detection by in Situ Hybridization Methods, *Int. J. Mol. Sci.* 16 (2015) 13259–13286, <https://doi.org/10.3390/ijms160613259>.
- [90] M. Takanashi, K. Oikawa, K. Sudo, M. Tanaka, K. Fujita, A. Ishikawa, S. Nakae, R.L. Kaspar, M. Matsuzaki, M. Kudo, M. Kuroda, Therapeutic silencing of an endogenous gene by siRNA cream in an arthritis model mouse, *Gene Ther.* 16 (8) (2009) 982–989, <https://doi.org/10.1038/gt.2009.66>.
- [91] B.A. Molitoris, P.C. Dagher, R.M. Sandoval, S.B. Campos, H. Ashush, E. Fridman, A. Brafman, A. Faerman, S.J. Atkinson, J.D. Thompson, H. Kalinski, R. Skalter, S. Erlich, E. Feinstein, siRNA Targeted to p53 Attenuates Ischemic and Cisplatin-Induced Acute Kidney Injury, *J. Am. Soc. Nephrol.* 20 (8) (2009) 1754–1764, <https://doi.org/10.1681/ASN.2008111204>.
- [92] O. Pontes, C.F. Li, P.C. Nunes, J. Haag, T. Ream, A. Vitins, S.E. Jacobsen, C.S. Pikaard, The Arabidopsis Chromatin-Modifying Nuclear siRNA Pathway Involves a Nucleolar RNA Processing Center, *Cell* 126 (1) (2006) 79–92, <https://doi.org/10.1016/j.cell.2006.05.031>.
- [93] E. Tutucci, N.M. Livingston, R.H. Singer, B. Wu, Imaging mRNA In Vivo, from Birth to Death, *Annu. Rev. Biophys.* 47 (1) (2018) 85–106, <https://doi.org/10.1146/annurev-biophys-070317-033037>.
- [94] A.M. Femino, F.S. Fay, K. Fogarty, R.H. Singer, Visualization of single RNA transcripts in situ, *Science* 280 (5363) (1998) 585–590.
- [95] A.M. Femino, K. Fogarty, L.M. Lifshitz, W. Carrington, R.H. Singer, Visualization of single molecules of mRNA in situ, *Methods Enzymol.* 361 (2003) 245–304, [https://doi.org/10.1016/S0076-6879\(03\)61015-3](https://doi.org/10.1016/S0076-6879(03)61015-3).
- [96] A.P. Young, D.J. Jackson, R.C. Wyeth, A technical review and guide to RNA fluorescence in situ hybridization, *PeerJ* 8 (2020) e8806, <https://doi.org/10.7717/peerj.8806>.
- [97] R. Arrigucci, Y. Bushkin, F. Radford, K. Lakehal, P. Vir, R. Pine, D. Martin, J. Sugarman, Y. Zhao, G.S. Yap, A.A. Lardizabal, S. Tyagi, M.L. Gennaro, FISH-Flow, a protocol for the concurrent detection of mRNA and protein in single cells using fluorescence in situ hybridization and flow cytometry, *Nat. Protoc.* 12 (6) (2017) 1245–1260, <https://doi.org/10.1038/nprot.2017.039>.
- [98] A.R. Buxbaum, B. Wu, R.H. Singer, Single β -Actin mRNA Detection in Neurons Reveals a Mechanism for Regulating Its Translatability, *Science* 343 (6169) (2014) 419–422.
- [99] H.M.T. Choi, C.R. Calvert, N. Husain, D. Huss, J.C. Barsi, B.E. Deverman, R.C. Hunter, M. Kato, S.M. Lee, A.C.T. Abelin, A.Z. Rosenthal, O.S. Akbari, Y. Li, B.A. Hay, P.W. Sternberg, P.H. Patterson, E.H. Davidson, S.K. Mazmanian, D.A. Prober, M. van de Rijn, J.R. Leadbetter, D.K. Newman, C. Readhead, M.E. Bronner, B. Wold, R. Lansford, T. Sauka-Spengler, S.E. Fraser, N.A. Pierce, Mapping a multiplexed zoo of mRNA expression, *Development* 143 (2016) 3632–3637, <https://doi.org/10.1242/dev.140137>.
- [100] H.M.T. Choi, J.Y. Chang, L.A. Trinh, C. Padilla, S.E. Fraser, N.A. Pierce, Programmable in situ amplification for multiplexed imaging of mRNA expression, *Nat. Biotechnol.* 28 (11) (2010) 1208–1212, <https://doi.org/10.1038/nbt.1692>.
- [101] C. Eliscovich, S.M. Shenoy, R.H. Singer, Imaging mRNA and protein interactions within neurons, *PNAS* 114 (2017) E1875–E1884, <https://doi.org/10.1073/pnas.1621440114>.
- [102] A. Lyubimova, S. Itzkovitz, J.P. Junker, Z.P. Fan, X. Wu, A. van Oudenaarden, Single-molecule mRNA detection and counting in mammalian tissue, *Nat. Protoc.* 8 (9) (2013) 1743–1758, <https://doi.org/10.1038/nprot.2013.109>.
- [103] A.F.M. Moorman, A.C. Houweling, P.A.J. de Boer, V.M. Christoffels, Sensitive Nonradioactive Detection of mRNA in Tissue Sections: Novel Application of the Whole-mount In Situ Hybridization Protocol, *J. Histochem. Cytochem.* 49 (1) (2001) 1–8, <https://doi.org/10.1177/002215540104900101>.
- [104] S.J. Neufeld, X. Zhou, P.D. Vize, J. Cobb, mRNA fluorescence in situ hybridization to determine overlapping gene expression in whole-mount mouse embryos, *Dev. Dyn.* 242 (9) (2013) 1094–1100, <https://doi.org/10.1002/dvdy.23993>.
- [105] A. Raj, P. van den Bogaard, S.A. Rifkin, A. van Oudenaarden, S. Tyagi, Imaging individual mRNA molecules using multiple singly labeled probes, *Nat. Methods* 5 (10) (2008) 877–879, <https://doi.org/10.1038/nmeth.1253>.
- [106] J.L. Kirschman, S. Bhosle, D. Vanover, E.L. Blanchard, K.H. Loomis, C. Zurla, K. Murray, B.C. Lam, P.J. Santangelo, Characterizing exogenous mRNA delivery, trafficking, cytoplasmic release and RNA-protein correlations at the level of single cells, *Nucl. Acids Res.* 45 (2017) e113, <https://doi.org/10.1093/nar/gkx290>.
- [107] K.H. Chen, A.N. Boettiger, J.R. Moffitt, S. Wang, X. Zhuang, Spatially resolved, highly multiplexed RNA profiling in single cells, *Science* 348 (2015) aaa6090, <https://doi.org/10.1126/science.aaa6090>.
- [108] C.-H. Eng, M. Lawson, Q. Zhu, R. Dries, N. Koulana, Y. Takei, J. Yun, C. Cronin, C. Karp, G.-C. Yuan, L. Cai, Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+, *Nature* 568 (7751) (2019) 235–239, <https://doi.org/10.1038/s41586-019-1049-y>.
- [109] A. Zirkel, A. Papantonis, Detecting Circular RNAs by RNA Fluorescence In Situ Hybridization, in: C. Dieterich, A. Papantonis (Eds.), *Circular RNAs: Methods and Protocols*, Springer, New York, NY, 2018, pp. 69–75. https://doi.org/10.1007/978-1-4939-7562-4_6.
- [110] A.A. Oliva, J.W. Swann, Fluorescence in situ hybridization method for co-localization of mRNA and GEP, *Biotechniques* 31 (74–76) (2001) 78–81, <https://doi.org/10.2144/01311st03>.
- [111] S.A.E. Marras, Y. Bushkin, S. Tyagi, High-fidelity amplified FISH for the detection and allelic discrimination of single mRNA molecules, *PNAS* 116 (28) (2019) 13921–13926, <https://doi.org/10.1073/pnas.1814463116>.
- [112] A. Kula-Pacurar, J. Wadas, A. Suder, A. Szczepanski, A. Milewska, M. Ochman, T. Stachel, K. Pyrc, Visualization of SARS-CoV-2 using Immuno RNA-Fluorescence In Situ Hybridization, *J. Vis. Exp.* (2020), <https://doi.org/10.3791/62067>.

- [113] L. Xiao, J. Labaer, J. Guo, Highly Sensitive and Multiplexed In Situ RNA Profiling with Cleavable Fluorescent Tyramide, *Cells* 10 (2021) 1277, <https://doi.org/10.3390/cells10061277>.
- [114] S. Eglöf, N. Melnychuk, E. Cruz Da Silva, A. Reisch, S. Martin, A.S. Klymchenko, Amplified Fluorescence in Situ Hybridization by Small and Bright Dye-Loaded Polymeric Nanoparticles, *ACS Nano* 16 (1) (2022) 1381–1394, <https://doi.org/10.1021/acsnano.1c09409>.
- [115] S. Zheng, M. Sieder, M. Mitterer, M. Reth, M. Cavallari, J. Yang, A.J. Garcia-Saez, A new branched proximity hybridization assay for the quantification of nanoscale protein–protein proximity, *PLoS Biol.* 17 (12) (2019) e3000569, <https://doi.org/10.1371/journal.pbio.3000569>.
- [116] C. Xia, H.P. Babcock, J.R. Moffitt, X. Zhuang, Multiplexed detection of RNA using MERFISH and branched DNA amplification, *Sci. Rep.* 9 (2019) 7721, <https://doi.org/10.1038/s41598-019-43943-8>.
- [117] V.P. Yin, In Situ Detection of MicroRNA Expression with RNAscope Probes, in: I. Gaspar (Ed.), *RNA Detection: Methods and Protocols*, Springer, New York, NY, 2018, pp. 197–208. https://doi.org/10.1007/978-1-4939-7213-5_13.
- [118] H. Ma, K.N. Bell, R.N. Loker, qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies, *Mol. Ther. Methods Clin. Dev.* 20 (2021) 152–168, <https://doi.org/10.1016/j.omtm.2020.11.007>.
- [119] B.K. Padhi, M. Singh, M. Rosales, G. Pelletier, S. Cakmak, A PCR-based quantitative assay for the evaluation of mRNA integrity in rat samples, *Biomol. Detect. Quantif.* 15 (2018) 18–23, <https://doi.org/10.1016/j.bdq.2018.05.029>.
- [120] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Kotliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape, *Nat. Biotechnol.* 31 (7) (2013) 638–646, <https://doi.org/10.1038/nbt.2612>.
- [121] K. Saito, T. Nagai, Recent progress in luminescent proteins development, *Curr. Opin. Chem. Biol.* 27 (2015) 46–51, <https://doi.org/10.1016/j.cbpa.2015.05.029>.
- [122] H. Zhao, T.C. Doyle, O. Coquoz, F. Kalish, B.W. Rice, C.H. Contag, Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection in vivo, *JBO* 10 (4) (2005) 041210, <https://doi.org/10.1117/1.2032388>.
- [123] J.R. Mansfield, K.W. Gossage, C.C. Hoyt, R.M. Levenson, Autofluorescence removal, multiplexing, and automated analysis methods for in-vivo fluorescence imaging, *J. Biomed. Opt.* 10 (4) (2005) 041207, <https://doi.org/10.1117/1.2032458>.
- [124] J.K. Tung, K. Berglund, C.-A. Gutekunst, U. Hochgeschwender, R.E. Gross, Bioluminescence imaging in live cells and animals, *Neurophotonics* 3 (02) (2016) 1.
- [125] Y. Miao, C. Gu, Y. Zhu, B. Yu, Y. Shen, H. Cong, Recent Progress in Fluorescence Imaging of the Near-Infrared II Window, *ChemBioChem* 19 (24) (2018) 2522–2541, <https://doi.org/10.1002/cbic.201800466>.
- [126] Y. Inoue, K. Izawa, S. Kiryu, A. Tojo, K. Ohtomo, Diet and Abdominal Autofluorescence Detected by in Vivo Fluorescence Imaging of Living Mice, *Molecular Imaging* 7 (2008) 7290.2008.0003. <https://doi.org/10.2310/7290.2008.0003>.
- [127] S.K. Cool, K. Breyne, E. Meyer, S.C. De Smedt, N.N. Sanders, Comparison of In Vivo Optical Systems for Bioluminescence and Fluorescence Imaging, *J. Fluoresc.* 23 (5) (2013) 909–920, <https://doi.org/10.1007/s10895-013-1215-9>.
- [128] S. Kwon, E.M. Sevik-Muraca, Effects of Depilation-Induced Skin Pigmentation and Diet-Induced Fluorescence on In Vivo Fluorescence Imaging, *Contrast Media Mol. Imaging* 2017 (2017) 1–7, <https://doi.org/10.1155/2017/7659242>.
- [129] F. Combes, A.M. Sofias, S. Mc Cafferty, H. Huysmans, J. De Temmerman, S. Hak, E. Meyer, N.N. Sanders, Mononuclear but Not Polymorphonuclear Phagocyte Depletion Increases Circulation Times and Improves Mammary Tumor-Homing Efficiency of Donor Bone Marrow-Derived Monocytes, *Cancers (Basel)* 11 (2019) E1752, <https://doi.org/10.3390/cancers11111752>.
- [130] D. Olmeda, D. Cerezo-Wallis, E. Riveiro-Falkenbach, P.C. Pennacchi, M. Contreras-Alcalde, N. Ibarz, M. Cifdaloz, X. Catena, T.G. Calvo, E. Cañón, D. Alonso-Curbelo, J. Suarez, L. Osterloh, O. Graña, F. Mulero, D. Megías, M. Cañamero, J.L. Martínez-Torrecuadrada, C. Mondal, J. Di Martino, D. Lora, I. Martínez-Corral, J.J. Bravo-Cordero, J. Muñoz, S. Puig, P. Ortiz-Romero, J.L. Rodríguez-Peralto, S. Ortega, M.S. Soengas, Whole-body imaging of lymphovascular niches identifies pre-metastatic roles of midkine, *Nature* 546 (7660) (2017) 676–680, <https://doi.org/10.1038/nature22977>.
- [131] M.M. Billingsley, N. Singh, P. Ravikumar, R. Zhang, C.H. June, M.J. Mitchell, Ionizable Lipid Nanoparticle-Mediated mRNA Delivery for Human CAR T Cell Engineering, *Nano Lett.* 20 (3) (2020) 1578–1589, <https://doi.org/10.1021/acs.nanolett.9b04246>.
- [132] R.L. Ball, K.A. Hajj, J. Vizelman, P. Bajaj, K.A. Whitehead, Lipid Nanoparticle Formulations for Enhanced Co-delivery of siRNA and mRNA, *Nano Lett.* 18 (6) (2018) 3814–3822, <https://doi.org/10.1021/acs.nanolett.8b01101>.
- [133] R.C. Ryals, S. Patel, C. Acosta, M. McKinney, M.E. Pennesi, G. Sahay, A.S. Lewin, The effects of PEGylation on LNP based mRNA delivery to the eye, *PLoS ONE* 15 (10) (2020) e241006, <https://doi.org/10.1371/journal.pone.0241006>.
- [134] P.P.G. Guimaraes, R. Zhang, R. Spektor, M. Tan, A. Chung, M.M. Billingsley, R. El-Mayta, R.S. Riley, L. Wang, J.M. Wilson, M.J. Mitchell, Ionizable lipid nanoparticles encapsulating barcoded mRNA for accelerated in vivo delivery screening, *J. Control Release* 316 (2019) 404–417, <https://doi.org/10.1016/j.jconrel.2019.10.028>.
- [135] F. Rizvi, E. Everton, A.R. Smith, H. Liu, E. Osota, M. Beattie, Y. Tam, N. Pardi, D. Weissman, V. Gouon-Evans, Murine liver repair via transient activation of regenerative pathways in hepatocytes using lipid nanoparticle-complexed nucleoside-modified mRNA, *Nat. Commun.* 12 (2021) 613, <https://doi.org/10.1038/s41467-021-20903-3>.
- [136] H. Zhang, J. Leal, M.R. Soto, H.D.C. Smyth, D. Ghosh, Aerosolizable Lipid Nanoparticles for Pulmonary Delivery of mRNA through Design of Experiments, *Pharmaceutics* 12 (2020) E1042, <https://doi.org/10.3390/pharmaceutics12111042>.
- [137] J. Di, Z. Du, K. Wu, S. Jin, X. Wang, T. Li, Y. Xu, Biodistribution and Non-linear Gene Expression of mRNA LNPs Affected by Delivery Route and Particle Size, *Pharm. Res.* 39 (1) (2022) 105–114, <https://doi.org/10.1007/s11095-022-01366-5>.
- [138] R. Zhang, R. El-Mayta, T.J. Murdoch, C.C. Warzecha, M.M. Billingsley, S.J. Shepherd, N. Gong, L. Wang, J.M. Wilson, D. Lee, M.J. Mitchell, Helper lipid structure influences protein adsorption and delivery of lipid nanoparticles to spleen and liver, *Biomater. Sci.* 9 (4) (2021) 1449–1463.
- [139] M.J.W. Evers, W. Du, Q. Yang, S.A.A. Kooijmans, A. Vink, M. van Steenberg, P. Vader, S.C.A. de Jager, S.A. Fuchs, E. Mastrobattista, J.P.G. Sluiter, Z. Lei, R. Schiffelers, Delivery of modified mRNA to damaged myocardium by systemic administration of lipid nanoparticles, *J. Control Release* (2022), <https://doi.org/10.1016/j.jconrel.2022.01.027>.
- [140] W. Tao, J.P. Davide, M. Cai, G.-J. Zhang, V.J. South, A. Matter, B. Ng, Y.e. Zhang, L. Sepp-Lorenzino, Noninvasive imaging of lipid nanoparticle-mediated systemic delivery of small-interfering RNA to the liver, *Mol. Ther.* 18 (9) (2010) 1657–1666, <https://doi.org/10.1038/mt.2010.147>.
- [141] A. Takai, M. Nakano, K. Saito, R. Haruno, T.M. Watanabe, T. Ohyanagi, T. Jin, Y. Okada, T. Nagai, Expanded palette of Nano-lanterns for real-time multicolor luminescence imaging, *Proc. Natl. Acad. Sci.* 112 (14) (2015) 4352–4356, <https://doi.org/10.1073/pnas.1418468112>.
- [142] H.-W. Yeh, O. Karmach, A. Ji, D. Carter, M.M. Martins-Green, H.-W. Ai, Red-shifted luciferase-luciferin pairs for enhanced bioluminescence imaging, *Nat. Methods* 14 (10) (2017) 971–974, <https://doi.org/10.1038/nmeth.4400>.
- [143] L. Mezzanotte, M. van't Root, H. Karatas, E.A. Goun, C.W.G.M. Löwik, In Vivo Molecular Bioluminescence Imaging: New Tools and Applications, *Trends Biotechnol.* 35 (7) (2017) 640–652, <https://doi.org/10.1016/j.tibtech.2017.03.012>.
- [144] M.A. Paley, J.A. Prescher, Bioluminescence: a versatile technique for imaging cellular and molecular features, *Med. Chem. Commun.* 5 (3) (2014) 255–267, <https://doi.org/10.1039/C3MD00288H>.
- [145] J.E. Kim, S. Kalimuthu, B.-C. Ahn, In Vivo Cell Tracking with Bioluminescence Imaging, *Nucl Med Mol Imaging* 49 (1) (2015) 3–10, <https://doi.org/10.1007/s13139-014-0309-x>.
- [146] C.G. England, E.B. Ehlerding, W. Cai, NanoLuc: A Small Luciferase is Brightening up the Field of Bioluminescence, *Bioconjug Chem.* 27 (5) (2016) 1175–1187, <https://doi.org/10.1021/acs.bioconjchem.6b00112>.
- [147] ThermoFisher Scientific, Safety Data Sheet D(-)-Luciferin, 2010. <https://www.fishersci.com/store/msds?partNumber=AC337050100&productDescription=D%28-%29-LUCIFERIN+10MG&vendorId=VN00032119&countryCode=US&language=en>.
- [148] Cayman Chemical, Safety Data Sheet Coelenterazine H, 2020. <https://cdn.caymanchem.com/cdn/msds/168944.pdf>.
- [149] E. Roncali, M. Savinaud, O. Levey, K.L. Rogers, S. Maitrejean, B. Tavitian, New device for real-time bioluminescence imaging in moving rodents, *JBO* 13 (5) (2008) 054035, <https://doi.org/10.1117/1.2976426>.
- [150] G. Wang, W. Cong, H. Shen, X. Qian, M. Henry, Y. Wang, Overview of bioluminescence tomography—a new molecular imaging modality, *Front. Biosci.* 13 (2008) 1281–1293, <https://doi.org/10.2741/2761>.
- [151] C. Darne, Y. Lu, E.M. Sevik-Muraca, Small animal fluorescence and bioluminescence tomography: a review of approaches, algorithms and technology update, *Phys. Med. Biol.* 59 (1) (2014) R1–R64, <https://doi.org/10.1088/0031-9155/59/1/R1>.
- [152] Y. Liu, L. Wang, Q. Song, M. Ali, W.N. Crowe, G.L. Kucera, G.A. Hawkins, S. Soker, K.W. Thomas, L.D. Miller, Y. Lu, C.R. Bellinger, W. Zhang, A.A. Habib, W. J. Petty, D. Zhao, Intrapleural nano-immunotherapy promotes innate and adaptive immune responses to enhance anti-PD-L1 therapy for malignant pleural effusion, *Nat. Nanotechnol.* 17 (2) (2022) 206–216, <https://doi.org/10.1038/s41565-021-01032-w>.
- [153] M. Tiffany, F.C. Szoka, Co-localization of fluorescent labeled lipid nanoparticles with specifically tagged subcellular compartments by single particle tracking at low nanoparticle to cell ratios, *J. Drug Target.* 24 (9) (2016) 857–864, <https://doi.org/10.1080/1061186X.2016.1233976>.
- [154] H. Liu, D. Wu, In vivo Near-infrared Fluorescence Tumor Imaging Using DiR-loaded Nanocarriers, *Curr. Drug Deliv.* 13 (1) (2016) 40–48, <https://doi.org/10.12174/1567201812666150703114908>.
- [155] T. Ozawa, H. Yoshimura, S.B. Kim, Advances in Fluorescence and Bioluminescence Imaging, *Anal. Chem.* 85 (2) (2013) 590–609, <https://doi.org/10.1021/ac3031724>.
- [156] G.V. Los, L.P. Encell, M.G. McDougall, D.D. Hartzell, N. Karassina, C. Zimprich, M.G. Wood, R. Learish, R.F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J.J. Zhu, A. Darzins, D.H. Klaubert, R.F. Bulleit, K.V. Wood, HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis, *ACS Chem. Biol.* 3 (6) (2008) 373–382, <https://doi.org/10.1021/cb800025k>.

- [157] C.A. Alabi, G. Sahay, R. Langer, D.G. Anderson, Development of siRNA-probes for studying intracellular trafficking of siRNA nanoparticles, *Integr. Biol.* 5 (2013) 224–230, <https://doi.org/10.1039/c2ib20155k>.
- [158] H. Zhang, J. Bussmann, F.H. Huhnke, J. Devoldere, A.-K. Minnaert, W. Jiskoot, F. Serwane, J. Spatz, M. Röding, S.C. De Smedt, K. Braeckmans, K. Remaut, Together is Better: mRNA Co-Encapsulation in Lipoplexes is Required to Obtain Ratiometric Co-Delivery and Protein Expression on the Single Cell Level, *Adv. Sci.* 9 (4) (2022) 2102072, <https://doi.org/10.1002/adv.202102072>.
- [159] S. Chivukula, T. Plitnik, T. Tibbitts, S. Karve, A. Dias, D. Zhang, R. Goldman, H. Gopani, A. Khanmohammed, A. Sarode, D. Cooper, H. Yoon, Y. Kim, Y. Yan, S.T. Mundle, R. Groppo, A. Beauvais, J. Zhang, N.G. Anosova, C. Lai, L. Li, G. Ulinski, P. Piepenhagen, J. DiNapoli, K.V. Kalnin, V. Landolfi, R. Swearingen, T.-M. Fu, F. DeRosa, D. Casimiro, Development of multivalent mRNA vaccine candidates for seasonal or pandemic influenza, *NPJ Vaccines* 6 (2021) 1–15, <https://doi.org/10.1038/s41541-021-00420-6>.
- [160] B. Shi, M. Abrams, Technologies for Investigating the Physiological Barriers to Efficient Lipid Nanoparticle–siRNA Delivery, *J. Histochem. Cytochem.* 61 (6) (2013) 407–420, <https://doi.org/10.1369/0022155413484152>.
- [161] P. Khare, K.M. Dave, Y.S. Kamte, M.A. Manoharan, L.A. O'Donnell, D.S. Manickam, Development of Lipidoid Nanoparticles for siRNA Delivery to Neural Cells, *AAPS J.* 24 (2021) 8, <https://doi.org/10.1208/s12248-021-00653-2>.
- [162] A. Reiser, D. Woschée, N. Mehrotra, R. Krzysztoń, H.H. Strey, J.O. Rädler, Correlation of mRNA delivery timing and protein expression in lipid-based transfection, *Integr. Biol.* 11 (2019) 362–371, <https://doi.org/10.1093/intbio/zyz030>.
- [163] B. Shi, E.d. Keough, A. Matter, K. Leander, S. Young, E.d. Carlini, A.B. Sachs, W. Tao, M. Abrams, B. Howell, L. Sepp-Lorenzino, Biodistribution of small interfering RNA at the organ and cellular levels after lipid nanoparticle-mediated delivery, *J. Histochem. Cytochem.* 59 (8) (2011) 727–740, <https://doi.org/10.1369/0022155411410885>.
- [164] T. Baladi, J.R. Nilsson, A. Gallud, E. Celauro, C. Gasse, F. Levi-Acobas, I. Sarac, M.R. Hollenstein, A. Dahlén, E.K. Esbjörner, L.M. Wilhelmsson, Stealth Fluorescence Labeling for Live Microscopy Imaging of mRNA Delivery, *J. Am. Chem. Soc.* 143 (14) (2021) 5413–5424, <https://doi.org/10.1021/jacs.1c00014>.
- [165] K. Thanki, D. van Eetvelde, A. Geyer, J. Fraire, R. Hendrix, H. Van Eygen, E. Putteman, H. Sami, C. de Souza Carvalho-Wodarz, H. Franzyk, H.M. Nielsen, K. Braeckmans, C.-M. Lehr, M. Ogris, C. Foged, Mechanistic profiling of the release kinetics of siRNA from lipidoid-polymer hybrid nanoparticles in vitro and in vivo after pulmonary administration, *J. Control. Release* 310 (2019) 82–93, <https://doi.org/10.1016/j.jconrel.2019.08.004>.
- [166] T. Asai, S. Matsushita, E. Kenjo, T. Tsuzuku, N. Yonenaga, H. Koide, K. Hatanaka, T. Dewa, M. Nango, N. Maeda, H. Kikuchi, N. Oku, Dicityl Phosphate-Tetraethylenepentamine-Based Liposomes for Systemic siRNA Delivery, *Bioconjugate Chem.* 22 (3) (2011) 429–435, <https://doi.org/10.1021/bc1004697>.
- [167] H. Wu, T.-T. Chen, X.-N. Wang, Y. Ke, J.-H. Jiang, RNA imaging in living mice enabled by an in vivo hybridization chain reaction circuit with a tripartite DNA probe, *Chem. Sci.* 11 (1) (2020) 62–69.
- [168] C.D. Sago, M.P. Lokugamage, K. Paunovska, D.A. Vanover, C.M. Monaco, N.N. Shah, M.G. Castro, S.E. Anderson, T.G. Rudoltz, G.N. Lando, P.M. Tiwari, J.L. Kirschman, N. Willett, Y.C. Jang, P.J. Santangelo, A.V. Bryksin, J.E. Dahlman, High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing, *PNAS* 115 (2018) E9944–E9952, <https://doi.org/10.1073/pnas.1811276115>.
- [169] Q. Cheng, T. Wei, L. Farbiak, L.T. Johnson, S.A. Dilliard, D.J. Siegwart, Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing, *Nat. Nanotechnol.* 15 (4) (2020) 313–320, <https://doi.org/10.1038/s41565-020-0669-6>.
- [170] M. Kim, M. Jeong, S. Hur, Y. Cho, J. Park, H. Jung, Y. Seo, H.A. Woo, K.T. Nam, K. Lee, H. Lee, Engineered ionizable lipid nanoparticles for targeted delivery of RNA therapeutics into different types of cells in the liver, *Sci. Adv.* 7 (9) (2021), <https://doi.org/10.1126/sciadv.abf4398>.
- [171] E. Braselmann, C. Rathbun, E.M. Richards, A.E. Palmer, Illuminating RNA Biology: Tools for Imaging RNA in Live Mammalian Cells, *Cell Chem. Biol.* 27 (8) (2020) 891–903, <https://doi.org/10.1016/j.chembiol.2020.06.010>.
- [172] X. Hou, T. Zaks, R. Langer, Y. Dong, Lipid nanoparticles for mRNA delivery, *Nat. Rev. Mater.* 6 (12) (2021) 1078–1094, <https://doi.org/10.1038/s41578-021-00358-0>.
- [173] R.O. Bak, J.G. Mikkelsen, miRNA sponges: soaking up miRNAs for regulation of gene expression, *WIREs RNA* 5 (2014) 317–333, <https://doi.org/10.1002/wrna.1213>.
- [174] T. Hinz, K. Kallen, C.M. Britten, B. Flamion, U. Granzer, A. Hoos, C. Huber, S. Khleif, S. Kreiter, H.-G. Rammensee, U. Sahin, H. Singh-Jasuja, Ö. Türeci, U. Kalinke, The European Regulatory Environment of RNA-Based Vaccines, *Methods Mol. Biol.* 1499 (2017) 203–222, https://doi.org/10.1007/978-1-4939-6481-9_13.
- [175] G. Maruggi, J.B. Ulmer, R. Rappuoli, D. Yu, Self-amplifying mRNA-Based Vaccine Technology and Its Mode of Action, in: Springer, Berlin, Heidelberg, n.d., pp. 1–40. https://doi.org/10.1007/82_2021_233.