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REVIEW

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Approaches for single-cell RNA sequencing across tissues and cell types

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ABSTRACT

Single-cell sequencing of RNA (scRNA-seq) has advanced our understanding of cellular heterogeneity and signaling in developmental biology and disease. A large number of complementary assays have been developed to profile transcriptomes of individual cells, also in combination with other readouts, such as chromatin accessibility or antibody-based analysis of protein surface markers. As scRNA-seq technologies are advancing fast, it is challenging to establish robust workflows and up-to-date protocols that are best suited to address the large range of research questions. Here, we review scRNA-seq techniques from mRNA end-counting to total RNA in relation to their specific features and outline the necessary sample preparation steps and quality control measures. Based on our experience in dealing with the continuously growing portfolio from the perspective of a central single-cell facility, we aim to provide guidance on how workflows can be best automatized and share our experience in coping with the continuous expansion of scRNA-seq techniques.

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Introduction

The first report on single-cell RNA sequencing (scRNA-seq) in 2009 literally focused on just one single cell [1] but nevertheless provided the key steps for single-cell cDNA amplification with a poly-(dT) primer and tailing of the 5'-end to add a PCR handle. After rapid advancements in terms of throughput and efficiency, single-cell sequencing (sc-seq) of RNA and DNA was named method of the year in 2013 [2]. Six years later, the selection of multi-omics sc-seq highlighted the extension of these techniques to additional readouts that are frequently used in combination with scRNA-seq [3]. Meanwhile, continuous progress has been made, especially in terms of sensitivity, throughput, and cost reduction [4]. The application of scRNA-seq techniques provides a wealth of information on RNA biology in general and transcriptional programs in development and their deregulation in disease (Figure 1). It ranges from resolving heterogenous tissues [5,6], dissecting cell fate decisions of hematopoietic stem cells [7], resolving intratumor heterogeneity, and the interactions of tumor cells with their microenvironments [8–10] to comprehensive cell atlases that provide reference transcriptomes for human cell types [11–13]. For instance, analysis of the developing human brain yielded a high diversity of progenitor cells with gradual transitions across the cell-type spectrum [14]. A similar model of a continuous landscape of progenitor cells has been suggested for the hematopoietic system [15]. Coupling single-cell RNA sequencing with T-cell receptor profiling revealed clonal T-cell replacement under checkpoint blockade in melanoma [16] and squamous cell carcinoma [17].

Today, scRNA-seq has become a "must-have" technique but various issues need to be considered in order to obtain high-quality data using this technique. Most importantly, samples have to be prepared in a way that the transcriptome is still intact and unchanged. In addition, a scRNA-seq technique that accounts for the specific requirements of a given project needs to be selected. The available methods can be broadly classified into plate-based techniques that separate cells into wells or the encapsulation of cells into droplets (Figure 2). Several benchmark papers concluded that plate-based scRNA-

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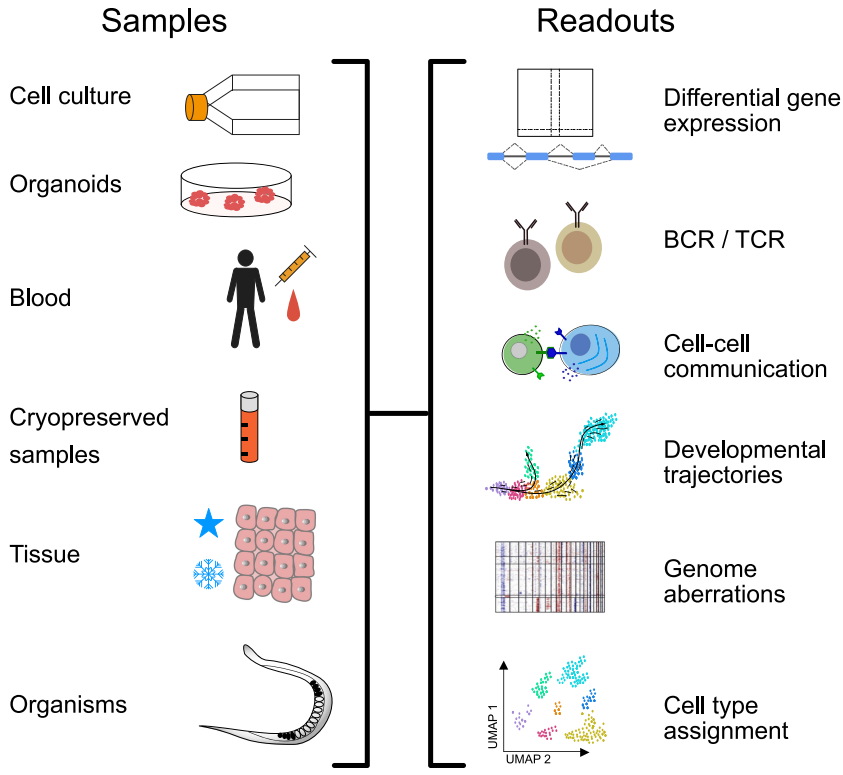

Figure 1. Overview on cell input material and analyses. Single-cell RNA sequencing can be performed using a variety of source materials including cell culture, blood, cryopreserved cells, model organisms, and frozen/fixed tissue. Exemplary analyses that can be conducted are depicted such as differential expression including splice variants, B/T-cell receptor repertoire, cell–cell interactions, differentiation trajectories, variant calling, and cell type clustering/annotation.

seq usually performs better in terms of number of genes detected per cell [18,19]. This increased sensitivity could arise from a combination of factors, for example, purification after cDNA amplification or mapping across the whole transcript. Even though the usually higher sequencing depth also plays a role, droplet-based methods still show lower gene yield when sequenced with the same read depth. On the other hand, they may yield a higher throughput. However, these general classifications are fraught with difficulties due to the rich technology landscape of scRNA-seq methods and also by combining plate- and droplet-based steps [20]. Here, we would like to give a broad technical overview on the experimental scRNA-seq techniques summarized in Table 1 including sample preparation procedures. It is noted that various multi-omics readouts exist that combine scRNA-seq with, for instance, genomic DNA, DNA methylation, protein via barcoded antibodies, nucleosome occupancy, histone modifications, and chromosome capture. These technologies are described in a number of excellent reviews [33–35] and are not further discussed. Our comparisons aim to provide guidance on selecting the optimal scRNA-seq methodology as well as considerations on their implementation via setting up a dedicated central laboratory.

Preparation of samples prior to single-cell sequencing

Efficient sample preparation is an essential prerequisite for any single-cell study. Irrespective of the type of cells or tissue, an optimal quality sample for scRNA-seq is a single-cell suspension with high viability (>90%) and as little alterations in their inherent gene expression profiles as possible. Sample preparation is relatively straightforward for cells from peripheral blood, e.g., mononuclear cells among others [36,37] as they are already present in a liquid suspension that can be easily obtained. For most of the complex tissues, however, specific dissociation methods need to be employed. A typical dissociation workflow

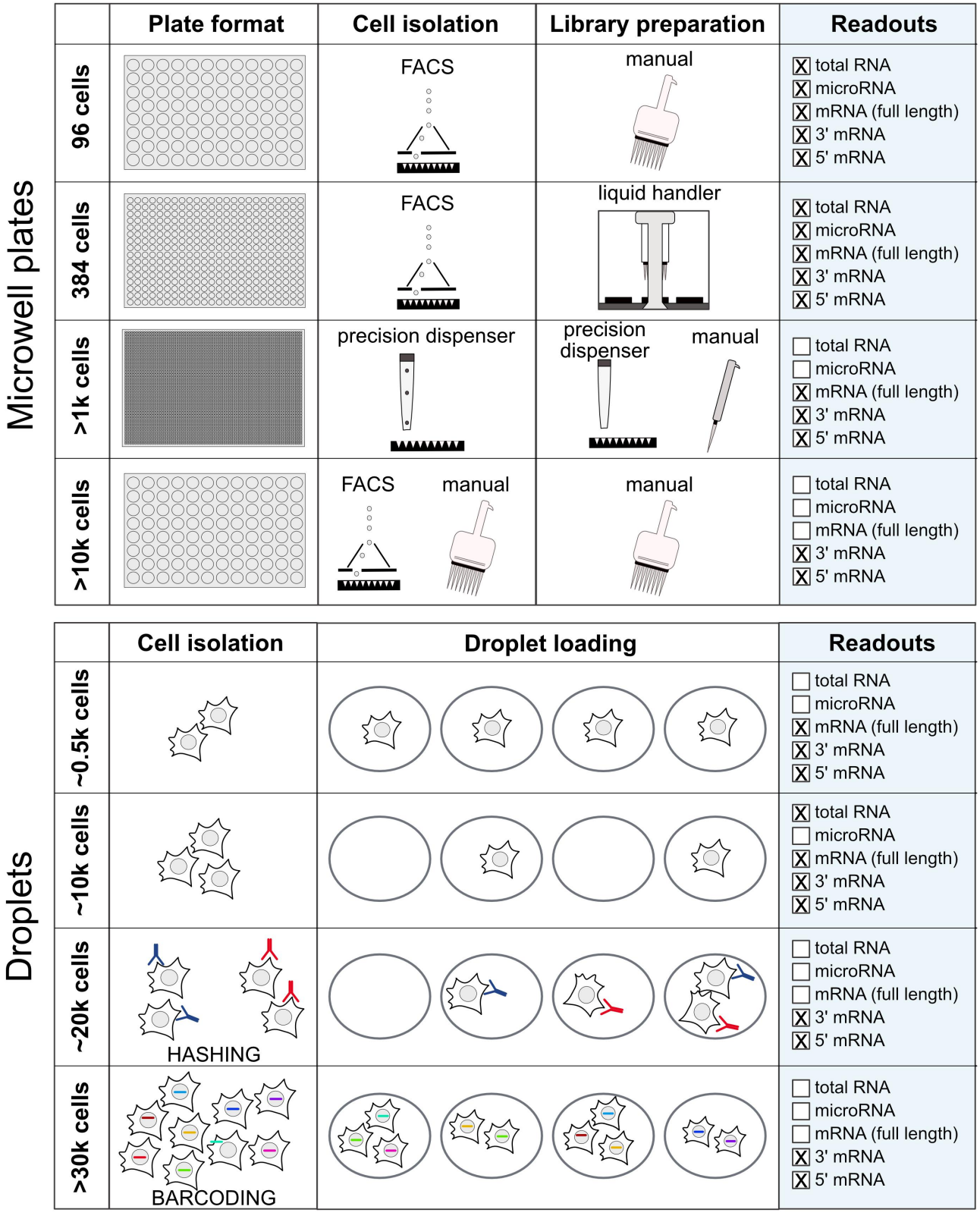

Figure 2. Overview on well- and droplet-based methods. For classical plate-based methods, protocols for all RNA species exist with cell isolation by FACS and library preparation by manual or automated workflows. Micro- or nanowells can be used to increase throughput with dedicated flowcells or dispensers. Molecular barcoding on plates allows manual handling of thousands of cells with limited read-out capabilities. Droplet-based methods mainly differ in how the cells are captured and pre-processed. Self-build systems allow for optimal loading with low-input samples as well as total RNA as a readout. Increasing throughput can be achieved by super loading using hashing or in-situ barcoding.

Table 1. Overview of scRNA-seq techniques and their characteristic features.

	Cell capture	Custom reagents / systems	Storage after capture	Single-cell sorting required	Commercial kits	Commercial instruments	Robotics advisable	Full length with short reads	High cell throughput	Reference
scRNA-seq GEM	Droplet				■	■			■	(21)
Scifi-RNA-seq		Primers				■			■	(20)
DisCo-seq		System								(22)
HyDrop		System							■	(23)
BD Rhapsody	Nanowell				■	■			■	(24)
HIVE			■		■					
SCOPE-seq					■	■			■	(25)
SMART-seq 2.5	Plate-based		■	■			■	■		(26)
SMART-seq3			■	■			■	■		(27)
SPLIT-seq		Primers			■				■	(28)
SMART-seq3xpress			■	■			■	■		(29)
FLASH-seq			■	■			■	■		(30)
sciRNA-seq		Transposase			■				■	(5)
VASA-seq			■	■			■	■		(31)
SMART-seq-total		Cas9	■	■			■			(32)

comprises mechanical dissociation/mincing of the tissue and enzymatic removal of extracellular matrix components, followed by washing and filtration to eliminate dead cells, debris, and cellular aggregates (Figure 3). Depending on the tissue type, its cell composition, extracellular matrix composition, and stiffness, the dissociation methods need to be optimized. This includes selecting the right enzymes for dissociation, optimizing enzymatic-digestion times, washing and centrifugation conditions, and selecting the right resuspension buffers. Resuspension buffers containing EDTA (>0.1 mM) and excess Mg^{2+} and Ca^{2+} ions interfere with reverse transcription (RT) reaction, thus reducing cDNA yield. The degradation of RNA during the sample processing steps can be minimized by using nuclease-free reagents and by addition of RNase inhibitors.

Several excellent reviews have discussed different tissue dissociation protocols and sample processing for single-cell analysis [38–40]. Most single-cell sequencing methods generally use fresh, viable samples. However, processing of fresh material can sometimes be challenging, especially in case of clinical samples which are frequently collected at places and times when immediate downstream processing is not possible. To address this issue, currently two types of preservation techniques compatible with scRNA-seq workflows are used [37,41–43]: one is cryopreservation by freezing cells in presence of a cryoprotectant such as DMSO. The other is fixation of cell suspensions with 80% methanol and storage at −80°C, if cryopreservation is not well tolerated for a given sample. In addition, fixation of cells/nuclei with 4% formaldehyde with subsequent storage (up to 3 months at −80°C) has recently been demonstrated to be compatible with the single-cell whole-transcriptome profiling by 10× Genomics in a manufacturer's protocol.

An alternative strategy is to purify nuclei from snap-frozen tissue and to perform single-nucleus RNA sequencing (snRNA-seq) [38,40,44] (Figure 3). Following the first reports of snRNA-seq in neural progenitor cell lines and

hippocampal tissue [45], there have been numerous studies applying this technique for different tissues. It is particularly useful for complex and/or fragile tissues such as brain, heart, or lung where isolation of intact cells from the tissue is difficult [44,46–48]. In some cases, it might be better to start from snap frozen samples than from cryopreserved cell suspensions due to a high fraction of dead cells. It is also an efficient alternative for cells larger than 40 µm, for instance, cardiomyocytes (up to 100 µm in length) that can, otherwise, not be captured on droplet-based microfluidic platforms [49–51]. In comparison with single-cell sample preparation, single-nuclei preparation is known to be robust with little dissociation-induced alterations to nuclear gene expression profiles [40,52,53]. Several comparative studies between single-nucleus and scRNA-seq reported that similar number of genes were detected between nuclei and whole cells, allowing identification of diverse cell types present in the tissue [46,54,55], even though nuclei contain lower amounts of mRNA and are enriched in lncRNAs and unspliced transcripts [56].

Some key technical issues are generally relevant for sample preparation: (i) dead/dying cells with nucleic acids leaking out of compromised cell membranes may cause RNA contamination. If captured together with a healthy cell, this can confound subsequent gene expression analysis. In our hands, dead cell exclusion with columns did not prove beneficial. Thus, gradient centrifugation or sorting with cell viability dyes to eliminate dead cells from the suspension is necessary. In addition, it has to be ensured that the dissociated single-cell suspension captures the entire cell population diversity of the tissue. (ii) Stressed cells frequently display an increased expression of pro-apoptotic or stress-related genes [53]. Cold dissociation could help to minimize these effects [57] (Figure 4). Dissociation procedures may introduce such biases that need to be assessed by FACS or, in case of nuclei, by sequencing, which has been done extensively across tissues [40]. (iii) Further, sample

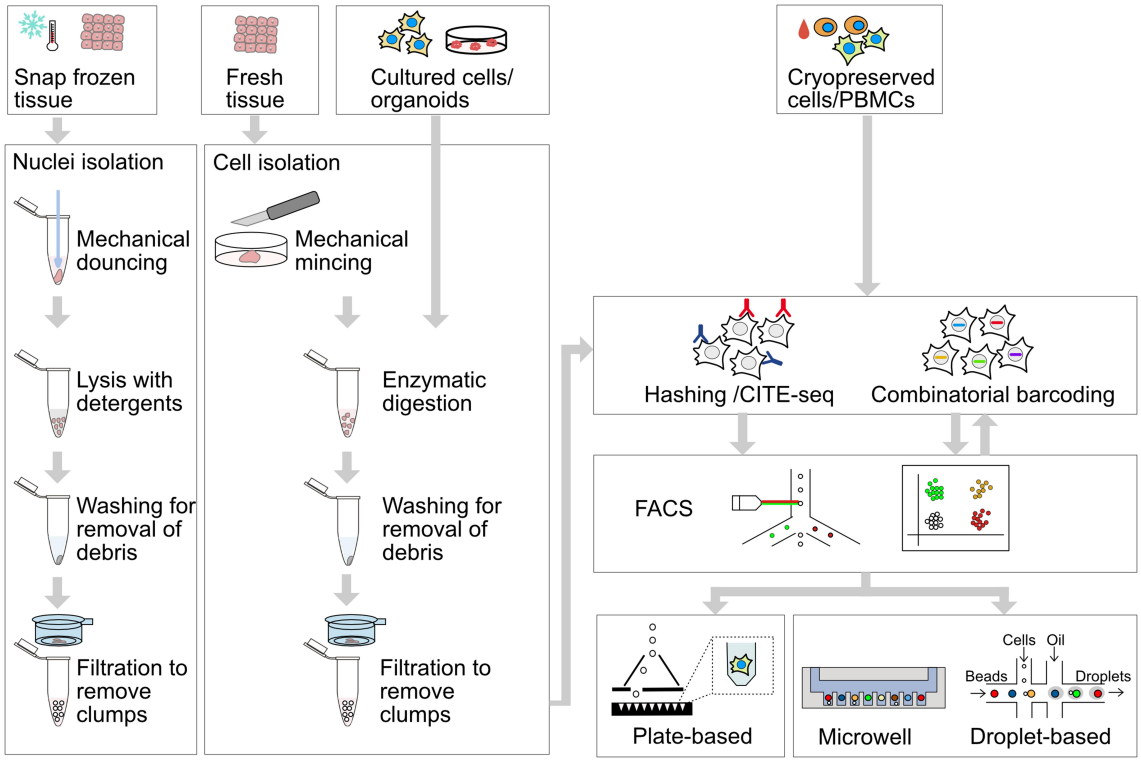

Figure 3. Wet lab procedures from sample to cell/nuclei capture. Depending on the sample types as source material, steps are depicted on how to obtain suspensions of single cells or single nuclei. The protocols include washing and filtering steps to remove debris and clumps. Optionally, hashing and barcoding for pooling can be performed followed by FACS to enrich/deplete certain cell types if needed. Subsequently, cells are captured in single-reaction compartments.

acquisition and storage can also introduce technical variations, which is especially relevant for clinical samples [58]. Standard operation procedures are required, and the history of sample processing should be carefully recorded. (iv) Due to the complexity of cell/nuclei isolation technical variation and batch effects may be introduced. Thus, a "balanced experimental design" is recommended, wherein different experimental conditions, and appropriate controls, are evenly distributed across the different stages of the experiment (from sample to library preparation). For instance, all experimental conditions are evenly included on each multi-well plate or droplet chip to ensure identification and mitigation of any batch effects [59,60]. For droplet-related techniques, pooling samples by using hashtags or utilizing SNPs for demultiplexing can be used to detect and also correct batch effects bioinformatically [61–63] (Figure 3).

Once cell suspensions with optimal quality have been obtained, the next steps are the capturing of cells and addition of cell-specific barcodes to the RNA or cDNA to individual cells so that they can be identified by sequencing after pooling and library preparation. In the following sections, we will review workflows that employ separating cells on plates, nanochambers, or droplets and using in-situ combinatorial barcoding.

Droplet-based single-cell workflows

Overview of droplet technologies

Droplet-based microfluidic technologies, comprising compartmentalization of cells into water-in-oil droplets for single-cell reactions in picoliter-scale volumes, provide many advantages in terms of throughput of cells as well as reaction efficiency. Current platforms provide the possibility to process thousands to up to tens of thousands of cells simultaneously in a single reaction, thus immensely reducing the processing time and cost [64–66]. The corresponding Drop-seq [65] and inDrop (indexing droplets [64]) systems as well as commercial implementations like the Chromium platform [21] share the same design and workflow principles. They typically start with encapsulation of single cells into water-in-oil droplets using microfluidic devices. Together with the cell, barcoded beads and reagents for cell lysis and RT are included in the droplet. Barcoded beads have also been used in combination with microwell plates where cells are captured by gravity [67]. Variations of this technique were commercialized as HIVE scRNA-seq, SCOPE-chip, and BD Rhapsody [24,25]. These systems have the practical advantage that large volumes can be loaded without the need to concentrate cells, as needed for droplet techniques, which may cause clumping.

The original nDrop platform encapsulates cells into droplets, with beads, also called barcoded hydrogel microspheres [64]. These beads are self-designed and their primers contain a photocleavable moiety at the 5'-end. Accordingly, the primers can be cleaved by UV light to capture the mRNA transcripts via the oligo(dT) sequence and to initiate cDNA synthesis. cDNA amplification, similar to the CEL-seq (cell expression by linear amplification and sequencing) protocol [68], is performed by in vitro transcription through a T7 promoter that is part of the primer sequence. For library preparation, the in vitro transcribed RNA needs to be again reverse transcribed into cDNA, which prolongs the total library processing time.

Drop-seq, another self-build microfluidic system, also encapsulates cells with barcoded beads ("microparticles") in water-in-oil droplets [65]. The barcodes on beads contain a common PCR handle. Following cell lysis in the droplets, mRNA gets captured onto the primers, while they are still attached to the microparticles, forming STAMPs (single-cell transcriptomes attached to microparticles). Here, cDNA is amplified exponentially by PCR via the PCR handle sequence and libraries are generated as per standard procedures, for example, with the Nextera XT kit. In 2017, Habib et al. adapted the Drop-seq protocol for single-nucleus RNA sequencing (DroNc-seq) of frozen mouse brain and archived human brain samples. They reduced the droplet size and volume to adapt the protocol for the smaller size and lower mRNA content of nuclei [69]. Improvements to the first version of Drop-seq, in terms of bead design and

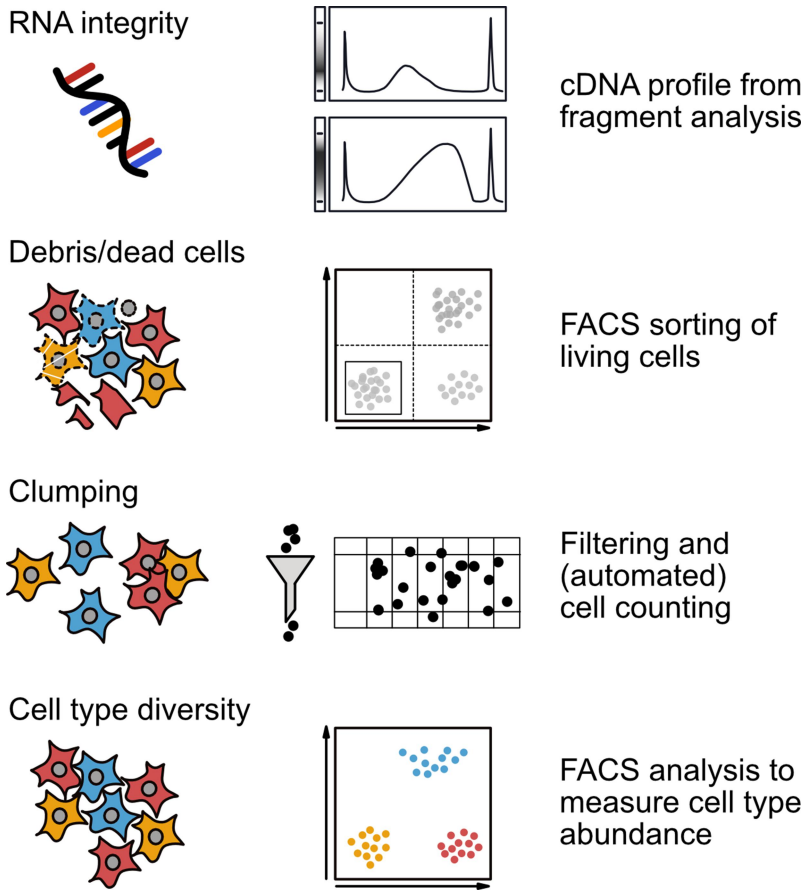

Figure 4. Quality control measures for sc/snRNA-seq. Quality of cell/nuclei suspensions can be assessed in different ways to further improve the sample preparation protocols. This includes measurement of RNA integrity and yield that can be impacted by mechanical or enzymatic digestions or high endogenous RNase levels. Checking for debris and dead cells is important as the presence of cell-free RNA will confound the analysis. FACS can be used to remove dead cells. Some cell types and especially nuclei tend to clump, and accurate cell counts are needed for e.g., droplet-based techniques. Depending on the sample preparation (e.g., mild or harsh digestions) some cell types might be enriched or depleted from the sample, which should be verified by FACS.

capture efficiencies were introduced when Dolomite Bio adopted the technique and commercialized its automated encapsulation platform [70].

The frequently used commercial Chromium platform for high-throughput droplet-based RNA sequencing from 10× Genomics is built on the GemCode technology and employs a gel bead in emulsion approach. It encapsulates barcoded gel beads and cells into droplets in an 8-channel microfluidic chip with each channel containing a sample with thousands of cells [21]. The gel beads are dissolvable. As in case of Drop-seq, the barcodes contain sequencing primers and adapter sequences. cDNA amplification is PCR-based, and libraries are generated using the Illumina short-read sequencing library preparation procedure. As an end-to-end solution, the Chromium platform includes a pipeline termed Cell Ranger for initial data processing and analysis. Over the years, this platform has been optimized in terms of protocols and chemistry to increase precision and reproducibility.

Barcodes, hashing, and library generation

Most barcode systems used with beads share common features. They usually have a cell-specific barcode that aids in tracing the cell from which the mRNA transcripts originate. In addition, each captured transcript is labeled with a unique molecular identifier (UMI) to allow transcript counting and to reduce PCR amplification-induced biases. The barcodes frequently also have an oligo(dT) sequence, which positions the cell barcodes and UMI at the 3'-ends of polyadenylated mRNA transcripts, thus making these techniques 3'-end

specific [21,64–66,71]. On encapsulation, the cells are lysed, either on intact beads (Drop-seq) or on broken or dissolved beads (inDrop and Chromium, respectively). The mRNA transcripts are captured by the oligo(dT) sequence and subjected to RT. The resulting cDNA is then amplified, and sequencing libraries are generated by standard procedures. Since the template switch technique is used to combine the RT and tailing in step [29,72,73], the template switching oligo can also be utilized to introduce cell and UMI barcodes. In this way, the cell barcode is placed at the 5'-ends of the mRNA transcripts. Using specific primers targeting the TCR/BCR constant regions, enrichment of the barcoded 5'end cDNA for V(D)J segments can be performed, followed by V(D)J sequencing, thus allowing transcriptomic profiling of the TCR and BCR receptor repertoires and clonotype analysis [74,75]. This has provided important insights into immune responses and skewed immune receptor repertoires during disease progression, such as in case of COVID-19 [76–78], systemic lupus erythematosus (SLE) [74], progression of cancers as well as responses to anti-cancer therapies [28,79–81]. Modifying the primer sequences to comprise additional capture sequences in addition to the oligo(dT) sequence facilitates capturing of certain non-poly(A) sequences. These include, for example, DNA barcoded antibodies for detecting cell surface proteins or cell multiplexing oligo (CMO) tags. This also allows one to combine scRNA-seq with multiple other readouts, including CITE-seq for cell surface protein phenotyping [82,83], CRISPR Screening to capture sgRNA via capture sequences binding to protospacers [84], and cell hashing [85,86]. CITE-seq and cell hashing are also compatible with other droplet-based platforms, including Drop-seq [87].

Cell hashing or CMO tags allow sample multiplexing prior to single-cell capture, thereby reducing the processing cost and time, and avoiding unwanted batch effects. In addition, sample multiplexing approaches also make use of natural genetic variations, specifically SNPs, or by artificially introducing barcodes within cells or nuclei using viral vectors and liposomal transfections [88]. All of these methods allow flagging and computational removal of cross-sample doublets from the analysis, thus enabling overloading of the droplet-based systems with cells [85]. However, they do not resolve individual transcriptomes of the doublets and thus the number of cells that can be analyzed per sample remains limited to ~20,000 cells, which is a challenge for large-scale studies like cell atlas projects that require millions of cells. To this end, Datlinger et al. introduced single-cell combinatorial fluidic indexing (scifi) RNA-seq, which included a single round of combinatorial indexing of permeabilized cells prior to using them as input for droplet-based systems. With this approach, individual transcriptomes from multiplets can be separated computationally, which makes overloading of droplets up to a 100-fold possible to largely increase the number of cells analyzed per run [20].

Advantages and disadvantages of different droplet-based methods

Selection of the right platform largely depends on the research goal and is always a trade-off between the sensitivity of the method, capturing efficiencies, and processing cost. The single-cell capturing efficiencies for Drop-seq are relatively low and between 5% and 12%, while they range around 75% and 50% for inDrop and Chromium, respectively. This can be partly attributed to the bead design. As Drop-seq uses non-deformable beads, encapsulation of beads and cells follows a double Poisson distribution, causing the majority of droplets to remain empty, thereby reducing the capture efficiencies [65,66,89]. InDrop and Chromium use deformable beads, which are packed closely together during encapsulation. Accordingly, the encapsulations follow a super-Poisson distribution with increased single cell/bead capture efficiencies [21,64,66,71,89]. In terms of sensitivity, Chromium was found to be the most sensitive system of the three approaches, detecting roughly twice as many UMIs as those detected with the other two systems [66]. In inDrop, the high capture efficiency was accompanied with a high cell barcode error rate, causing elimination of more than 50% of the sequencing data due to failure in matching the cell barcodes. In terms of operational costs, Drop-seq proved to be the most efficient platform. Cost of the entire setup and the per-cell processing costs were much

smaller than for inDrop and Chromium, with the latter being the most expensive [66].

Important limitations of these systems are high costs per cell for low cell input numbers and the low single-cell capture rate for samples with low cell numbers. Further, cell hashing, to pool different low-input samples together, is impeded by the huge losses of cells during the washing steps. Here, the recently developed DisCo-seq platform provides a solution [22]. DisCo-seq uses a self-build microfluidic system to closely regulate the flow of cells, beads, and oil. Simultaneously, using machine-vision, single-cell/bead encapsulated droplets are detected and isolated for further processing. By regulating the cell concentrations, DisCo-seq increased the capturing efficiencies up to 90%, thus making it a suitable platform for processing low input samples (<500 cells). High capture rates were also reported for HyDrop, which is an open-source platform using hydrogel beads [23].

Even though droplet-based methods are usually employed with short read 5'- or 3'-end sequencing on Illumina systems, full-length information can nevertheless be generated by switching to long-read sequencing platforms, such as PacBio or Nanopore sequencing [90]. This is possible since full-length cDNA is initially generated. Library preparation needs to be adjusted for the respective platform. While PacBio provides sufficient read quality to use most of the cellular barcodes, its throughput of several million reads per chip is somewhat low to capture the full transcriptome variation beyond cell types [91,92]. Error correction pipelines were established for nanopore sequencing that are required to call cells form cell barcodes and also count transcripts confidently from UMI sequences [93,94]. Both methods provide insights in splicing and also RNA editing. The number of reads per cell currently, however, does not appear to reach the high resolution on cell states/types by Illumina systems.

Plate-based techniques

Approaches for distributing cells into micro-/nanowells

Single-cell RNA-sequencing was first developed in regular PCR tubes and then progressed to multi-well plates and nanoliter chips. One of the first commercial systems, the Fluidigm C1, has used microfluidics that integrated the capturing of single cells into array-based chambers (nanowell, integrated fluidic circuits) with downstream lysis and cDNA amplification. It could process hundreds of cells simultaneously in miniaturized reaction volumes. In an integrated quality control step, quantification and viability analysis of the captured cells could be performed [95]. Xin et al. have used the C1 platform to capture and process 622 pancreatic cells from mice [96]. They identified distinct subtypes of islet cells based on good quality gene expression profiles but also highlighted technical limitations in the capture process of the platform.

Single-cell isolation by FACS/MACS (fluorescence/magnetic-activated cell sorting) allows direct sorting of cells into microwell plates that can be directly processed for downstream reactions for library preparation, such as SMART-seq2 or CEL-Seq2 among others. While the throughput is lower than that obtained with droplet methods, plate-based methods have the advantage that cells can be stored after isolation without quality loss by freezing cells in lysis buffer. As cells need to be placed in wells, characteristics of each cell can be recorded, such as their shape, size, ploidy, or expression markers by index sorting, which provides an additional layer of information. An important technical limitation is the requirement of relatively high input volumes (in the range of hundred microliters), which makes isolation from low input volumes challenging [97].

An improvement in terms of throughput for plate-based techniques is provided by Takara Bio's iCell8 Single-cell system that captures cells into a 5,184 nanowell chip. With the iCell8 chip, wells containing viable single cells can be visualized and selected for downstream reactions and library preparation using SMARTer chemistry [98]. By capturing and sequencing >1000 human and mouse cultured cells and 468 pancreatic islet cells from mice on the iCell8 chip, Goldstein et al. were able to identify distinct islet cell subtypes with a low multiplet and cross-contamination rate between wells. A major advantage of the iCell8 is its multi-sample nano-dispenser for dispensing cells as large as

cardiomyocytes (100 µm) or even complete 3D spheres into the nanowells [99,100]. The system is, however, limited by its low capture efficiency of 800 to 1,400 cells per run (15% to 27% of input cells) depending on the tissue type [98,101]. To overcome this limitation, researchers have combined the CellenONE system from Cellenion with iCell8 chips. Cells are "printed" with the CellenONE into the nanowells of the iCell8 chip with a higher recovery rate as compared to the original iCell8 system [101]. Due to efficient doublet and multiplet detection at the cell capture stage, the doublet rate is very low. Thus, it is no longer required to employ dedicated analysis pipelines for computational doublet detection. Further, the system is compatible with full length-based chemistry for sequencing, which makes it a full-length-based platform with high-throughput, in the range of ~3,000 cells. In general, if the starting material has very small cell numbers, cell dispensers such as the CellenONE are advantageous for plate-based techniques over FACS.

Protocols for plate-based scRNA-seq library generation

The protocols that are used to generate cDNA are very similar in plate-based techniques as for droplet-based approaches, using either PCR or linear amplification. Since cDNA from each cell is synthesized in individual wells, there is some freedom in the downstream processing. Full-length transcriptome and splice information can be obtained by usual short read sequencing. As discussed above, cDNA can be sequenced at full length also with Nanopore or Pacbio systems.

Rare cell types that require enrichment are usually placed in lysis buffer during sorting. The following steps are protocol-dependent and can include 3'-counting with cell barcodes as for mcSCRB-seq. In this case, cDNA from all cells can be pooled after cDNA synthesis and all downstream steps are performed in one tube very similar to droplet-based protocols [102]. While this approach is cost efficient (barcoded primers being the major investment), full-length transcript information cannot be recorded with short read sequencers.

SMART-seq has been quickly adopted by many labs and has seen several improvements since. SMART-seq2 introduced a stabilized template switching oligo and RT buffer conditions that were further improved using molecular crowding as well as optimized strategies for nuclei [26] and inclusion of UMIs [27]. In an attempt to simplify the workflow, SHERRY2 was developed that omits cDNA amplification and tagmentation is performed on the RNA/cDNA hybrid [103]. In the FLASH-seq protocol, cDNA synthesis and amplification are combined in one step [30], while SMART-seq3Xpress uses nanoliter reaction volumes overlaid with oil to save chemistry and also drops cDNA purification prior to library preparation [29].

The above methods typically are used with 384-well plates. Considerably higher throughput can be achieved by combinatorial barcoding that can be regarded as yet another plate-based methodology. However, here the cells themselves are the single reaction compartment during cDNA synthesis, while barcodes are added in the intact cells over multiple rounds. This way thousands or even millions of cells can be analyzed in one experiment. Several protocols exist that employ this multi-step barcoding protocol starting with well-specific RT oligos, similar to mcSCRB-seq. Additional barcodes are then introduced by ligation [104], PCR, or transposomes [5]. Both, SPLIT-seq and sci-RNA-seq have been commercialized recently by parse biosciences and scale biosciences, respectively. These split-and-pool approaches work without any special equipment and allow multiplexing of high cell numbers. Due to the serial enzymatic steps, these techniques showed a similar performance as compared to commercial droplet-based chemistry. As large numbers of cells or nuclei can be profiled, many projects utilizing these methods focused on developmental processes such as brain development [105,106].

Profiling of total RNA or small RNAs cannot be done with oligo(dT) priming methods as RNAs lacking a poly(A) tail would be missed. Thus, a ligation and masking-based approach is used to obtain small RNA information [107] with the possibility to enrich specifically for microRNAs, if needed [108]. It was shown that small RNAs, especially miRNAs, are well suited to deconvolve

highly complex cell-type compositions, and even unannotated small RNAs could be identified with this technique [107,109]. In 2019, a half-cell approach was used to analyze both microRNAs and mRNAs from the same cell [110]. This provided insights in the regulatory connection between miRNA and mRNA targets. Recently, VASA-seq [31] has been published that allows profiling of total RNA by including a fragmentation and A-tailing step prior to cDNA synthesis. Due to UMI-tagging along the whole transcript, the resulting libraries are strand-specific. rRNA depletion is performed by hybridization and RNaseH digestion. In addition to a 384-well-based protocol, the authors provided details to implement the required steps and barcoding in oil droplets, which requires a self-build system [31]. The authors demonstrated the potential to detect cell-type specific splicing events, high resolution of cell cycle states and cell trajectories by a higher intron coverage.

The inclusion of mRNA, non-poly(A) RNA, and small RNAs has been demonstrated in cell lines by SMART-seq-total [32]. Here, RNAs are polyadenylated in situ and reverse transcribed. The template switching oligo is digested and mRNA (long RNA) libraries are generated by tagmentation, whereas the small RNA fraction is directly indexed in a separate reaction. rRNA depletion is done by CRISPR. Assessment of the regulatory function of the non-coding transcriptome could be envisioned with this approach. Since the authors used SMART-seq2 as a basis for their protocol, it would be interesting to see how the changes to the SMART-seq workflow will influence the power of this technique.

Advantages of plate-based techniques

The main reasons why researchers choose plate-based techniques are (i) low number of (fragile) cells, for example rare cell types in complex cell suspensions, (ii) detection of low abundant transcripts, (iii) detection of mutations, and (iv) the need for a custom workflow for multi-omics, especially DNA methylation or inclusion of small RNAs. Mutational analysis is especially important for cancer samples. Several methods that dissect tumor evolution, both computationally and experimentally, have been published. Droplet data can be used to call copy number variations, for example, with inferCNV or copyKAT [111,112]. However, the analysis of point mutations can be limited by location (for example, away from the 5'- or 3'-end) or detection sensitivity [113]. TARGET-seq has been developed to call mutations both on the RNA and the DNA level, which allows detection of mutations in silent alleles [114,115]. It requires the addition of cDNA and gDNA-specific primers as it is a targeted approach. Similarly, MutaSeq uses target-specific primers during cDNA amplification to increase the probability of sequencing interesting regions at the required depth [116]. Long-read sequencers, such as PacBio, can be used for full-length transcript sequencing. In this way, gene fusions and mutations can be detected, as stated above for droplet-based methods.

Selecting and implementing scRNA-seq workflows

Identifying suitable scRNA-seq workflows

In Table 1 and Figure 5, central features of droplet- and multi-well-based methods are depicted in a simplified manner with the aim to provide some guidance for selecting a suitable scRNA-seq method. Together with the discussion in the preceding part, it provides a starting point to identify a method that meets the essential requirements, for example, in terms of number of cells/samples or additional RNA-based readouts needed together with the transcriptome.

Establishing a technology hub for scRNA-seq work

The numerous scRNA-seq techniques have their specific advantages and disadvantages, which raises the question on how they can be efficiently implemented and how one can keep up with the rapid technological advancements in the field. Based on our experience with setting up the Single-Cell Open Lab (https://www.dkfz.de/en/single-cell-sequencing/open-lab.html) at the German Cancer Research Center (DKFZ), we favor an "open lab" approach. A core infrastructure with

respect to the instrumentation and dedicated core personnel (staff scientists, technicians) is provided, but samples are not processed in a service-like manner. Rather, the scientists from different research groups conduct their experiments on the available instrumentation after booking. They are supported in project design and conducting the experiment by the core staff. In this manner, a technology hub is generated that serves as both the instrument and knowledge base for running scRNA-seq experiments for specific research questions. It is noted that it is crucial to ensure that the bioinformatic analysis workflows are also established and available, that follow the experimental data generation, which is not covered here. By creating a technology hub for scRNA-seq, different issues can be addressed in a synergistic manner:

(i) A large variety of scRNA-seq technologies (Figure 1, 2) with different cell input material (Figure 3) and appropriate quality controls (Figure 4) can be provided to allow optimal method selection, as depicted in Table 1 and Figure 5.

(ii) New technologies can be implemented, and novel workflows can be developed through joint pilot projects of the core staff and scientists from a research group to cope with typical budgetary constraints for wet/dry lab personnel. Joining forces in method development and implementation should include both experimentalists and bioinformaticians from the beginning. This is an important point to ensure correct processing of raw data. Thus, new methods are implemented in the centralized facility by collaborations with research groups. Furthermore, all new methodologies can be quickly rolled out via the open lab structure.

(iii) The complete workflow, from sample preparation and optimization, quality control up to library preparation can be considered. For example, it is important to obtain high-quality single-cell or single-nuclei suspensions with adjusted cell-type specific fixation procedures, wherever needed.

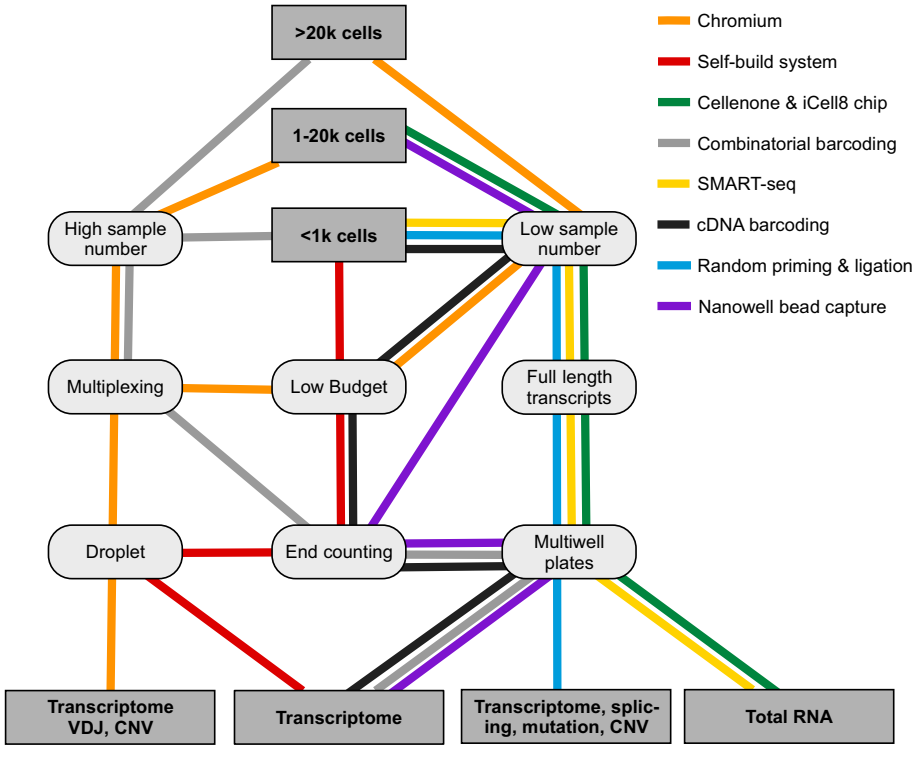

Figure 5. Simplified scheme for selection of scRNA-seq techniques. The scheme captures the possibilities and strengths for several scRNA-seq techniques including throughput, cell number, read-out and cell capture as central parameters. This representation does not illustrate all workflows but can serve as a guide to select or look into specific methods.

Quality of such a preparation can be monitored by using leftover chemistry to generate cDNA and by including other facilities to perform QC by flow cytometry or shallow sequencing.

(iv) A constantly updated repository of protocols that have worked well for others can be built up, which represents a valuable asset in the experimental design for new projects.

(v) Efficient training of users is conducted with their real samples and chemistry once the optimizations are complete. PhD students and postdocs acquire crucial expertise for their scientific work and at the same time become independent users, which increases the throughput of the facility. Importantly, this also enables out-of-the-box projects requiring specific adjustments that can be tested and carried out by the users.

Practical considerations to implement a single cell open lab/technology hub

Most of the technologies discussed in this review can be implemented with a relatively compact core technical infrastructure. A few practical considerations are warranted on how to implement workflows like the one depicted in Figure 6:

(i) Guidance in project design and the selection of the most suitable technology needs to be provided, coupled with standard operation procedures for comparable data sets, facilitating the FAIR principle (findability, accessibility, interoperability, and reusability) in connection with data management services.

(ii) Facility management software to book instruments, bench space, training, and project discussions is crucial [117]. Comparable tools can also provide the possibility to order centralized chemistry by users, track projects, and record protocol optimizations/failures.

(iii) The experimental work is facilitated by providing centralized chemistry. A reagent pool, especially for plate and combinatorial barcoding techniques, that is provided by the central lab is advantageous over individual reagent purchases.

(iv) In order to avoid cross-contamination, a strict separation of pre- and post-PCR areas/equipment is recommended.

(v) Standard workflows using robotics can minimize the human-based heterogeneity in library quality. Classical liquid handling systems, such as the Bravo system (Agilent), are used extensively in many labs due to its accuracy and flexibility [32,118]. The transfer of programs from different systems, however, can be challenging, and scripting new ones requires experience. Easy-to-use systems, capable of working with very low volumes that are suitable for 384-well plates are, for instance, the Mosquito dispensers (SPT Labtech) [32]. Due to high costs of tips, contact-free dispensing with the Mantis (Formulatrix) or i.dot (Dispendix) systems is worth considering [29,119].

(vi) A fast-track sample processing option for proof-of-concept and experiments for manuscript revisions with short turnaround times, supported by the lab staff, can provide additional benefits.

(vii) Implementing shared data analysis pipelines can save resources and time.

(viii) Further synergies can be created by intramural calls for pilot projects. They bring together groups with similar interest/problems to generate synergies, help identify current needs, streamline experimental and data analysis workflows, and provide financial support for exploring new methods (see above).

(ix) Promoting and engaging in scientific exchange on scRNA-seq technologies on different levels is highly valuable to constantly optimize and advance the workflows. This includes local more informal activities (e.g. the Single Cell Center Heidelberg, https://single-cell-center-hd.de), national platforms (Single Cell Omics Germany, https://www.singlecell.

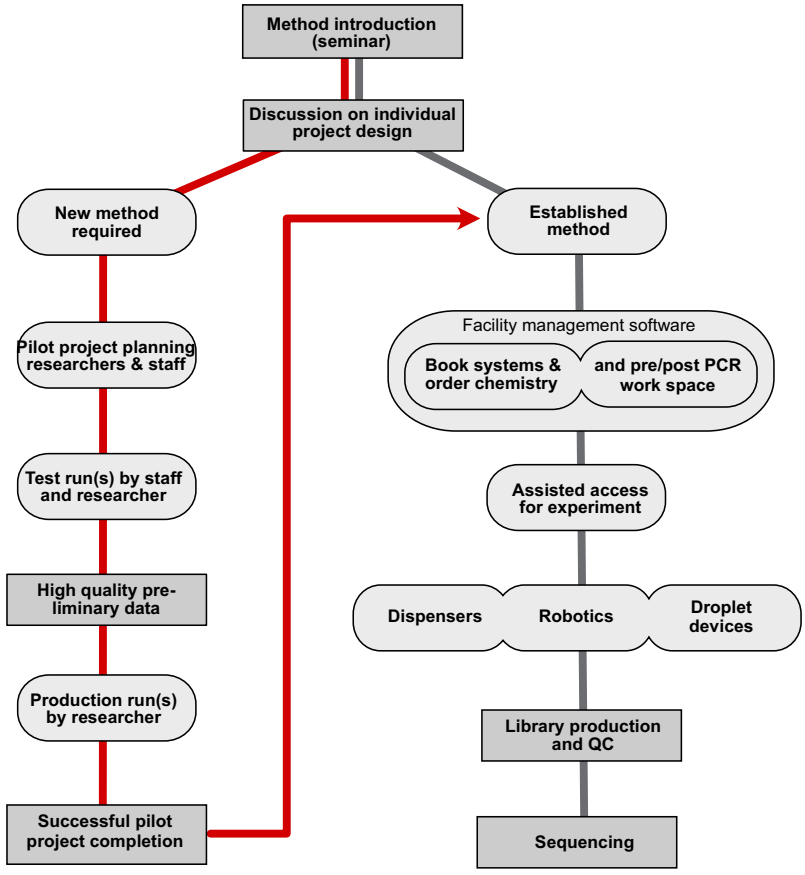

Figure 6. Overview workflow in central facility. After mandatory seminar and project discussions either (i) new protocols will be established by experienced staff members and rolled out to users quickly for further testing or (ii) standard methods are introduced by training users with their real samples in an organized environment. A tracking system allows booking of infrastructure, training requests and ordering of central chemistry.

de) as well as global networks (Human Cell Atlas, https://www.humancellatlas.org/).

Conclusion

The field of scRNA-seq technologies remains highly dynamic. In particular, the area of multi-omics that typically combines scRNA-seq with additional readouts continues to expand. While this review was written, further method developments like improvements for snRNA-seq, usage of FFPE material or analysis of allele-specific expression were published as pre-prints [120–122]. In addition, methods to conduct spatial transcriptomics experiments represent an emerging area to analyze cell transcriptomes within the tissue context [123,124]. Persisting limitation of scRNA-seq data analysis is data sparsity, especially for cell types of low RNA content, as well as sample dropouts due to technical issues [125]. Improvements in capturing the full transcriptome will facilitate experiments that follow cellular responses and functional transitions. In particular, research topics like RNA splicing or modifications require increased sequencing depth and highly sensitive protocols, respectively [126,127]. Thus, it will be important to continuously reevaluate the scRNA-seq workflows that are being used for a given application. Nevertheless, a number of methodological principles have emerged that range from pre-processing of source material, cDNA protocols up to the generation of sequencing libraries. By highlighting and comparing them in relation to

the desired readouts and sample features, we hope to provide some guidance to the experimental design and performance of scRNA-seq experiments.

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