Comparative analysis of single-cell RNA-sequencing methods

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Abstract

Single-cell mRNA sequencing (scRNA-seq) allows to profile heterogeneous cell populations, offering exciting possibilities to tackle a variety of biological and medical questions. A range of methods has been recently developed, making it necessary to systematically compare their sensitivity, accuracy, precision and cost-efficiency.

Here, we have generated and analyzed scRNA-seq data from 479 mouse ES cells and spike-in controls that were prepared with four different methods in two independent replicates each. We compare their sensitivity by the number of detected genes and by the efficiency with which they capture spiked-in mRNAs, their accuracy by correlating spiked-in mRNA concentrations with estimated expression levels, their precision by power simulations and variance decomposition and their efficiency by their costs to reach a given amount of power. While accuracy is similar for all methods, we find that Smart-seq on a microfluidic platform is the most sensitive method, CEL-seq is the most precise method and SCRB-seq and Drop-seq are the most efficient methods. Our analysis provides a solid basis to choose among four available scRNA-seq methods and to benchmark future method development.

Introduction

Genome-wide quantification of mRNA transcripts is a highly informative phenotype to characterize cellular states and understand regulatory circuits[1,2]. Ideally, this is collected with high temporal and spatial resolution and RNA-sequencing of single cells (scRNA-seq) is starting to reveal new biological and medical insights[3–5]. scRNA-seq requires to isolate intact single cells and turn their mRNA into cDNA libraries that can be quantified using high-throughput sequencing[4,6]. The sensitivity, accuracy, precision and throughput of this process determines how well single cell transcriptomes can be characterized. In order to choose among available scRNA-seq methods, it is important to estimate these parameters comparatively. It has previously been shown that scRNA-seq performed in the small volumes available via the automated microfluidic platform from Fluidigm (Smart-seq protocol on the C1-platform) performs better than Smart-seq or other commercially available kits in microliter volumes[7]. More recent scRNA-seq protocols have used unique molecular identifiers (UMIs) that tag mRNA molecules with a random barcode sequence during reverse transcription in order to identify sequence reads that originated during amplification[8–10]. This leads to a better quantification of mRNA molecules[11,12]. Another decisive recent development has been to add a barcode for each single cell during reverse

36 transcription allowing to process hundreds or thousands of scRNA-seq libraries in one
37 reaction, increasing the throughput of scRNA-seq library generation by one to three orders
38 of magnitude[9,13–15]. However, a systematic comparison of sensitivity, accuracy and precision
39 among such recently developed methods has not been performed yet. To this end, we have
40 generated and analysed 479 scRNA-seq libraries from mouse embryonic stem (mES) cells
41 cultured in two-inhibitor (2i/LIF) medium using four different methods run in two replicates
42 each (Fig. 1).
43

44 ## Results

45 ### Generation and processing of 479 scRNA-seq libraries

46 We have used the Smart-seq protocol on the C1 platform from Fluidigm (Smart-seq/C1)
47 that uses microfluidic chips to automatically separate up to 96 cells[7]. After lysis and the
48 addition of artificial mRNAs of known concentrations (ERCCs[16]), cDNA is generated by
49 oligo-dT priming, template switching and PCR amplification. These can then be turned into
50 scRNA-seq libraries using 96 Nextera reactions. Advantages of this system are that the
51 process of single cell isolation is automated, that captured cells can be imaged, that
52 reaction volumes are small and that the protocol generates full length cDNAs.
53 Disadvantages include that the protocol does not use UMIs and is expensive due to the use
54 of the Smart-seq kit, the microfluidic chip and the separate Nextera reactions.
55 For the second approach we isolated single cells in a 96-well plate by sorting them into
56 lysis buffer and used the SCRB-seq protocol to generate scRNA-seq libraries[13]. In this
57 protocol, cDNA from cells and ERCCs is generated by oligo-dT priming, template switching
58 and PCR amplification, similar to Smart-seq/C1. However, the oligo-dT primers contain
59 well-specific barcodes and UMIs and the resulting cDNA is turned into scRNA-seq using a
60 modified transposon-based fragmentation approach that enriches for 3' ends. The protocol
61 is optimized for small volumes and few handling steps, but it does not generate full length
62 cDNA profiles and its performance compared to the smaller reaction volumes in the C1 is
63 unclear.
64 The third method we tested is Drop-seq, a recently developed microdroplet-based
65 approach[15]. Similarly to SCRB-seq, each cDNA molecule is decorated with a cell-specific
66 multiplexing barcode and a UMI to count abundance. In the case of Drop-seq, these
67 barcoded oligo-dT primers are immobilized on beads and encapsulated with single-cells in
68 nanoliter-sized emulsion droplets. In Drop-seq, simultaneous inclusion of ERCC spike-ins
69 in the library preparation is not possible, as it would have to be included in the bead
70 suspension. However, most beads will not carry a cell transcriptome, as cell concentration

has to be relatively low to avoid doublets. To address the issue of lacking ERCCs in our data, we used a published dataset[15], where ERCC spike-ins were sequenced by the Drop-seq method without single-cell transcriptomes. Finally, we used data[12] generated using CEL-seq[9] for which also two different batches of scRNA-seq libraries were generated from the same cell type and culture conditions. Similarly to Drop-seq and SCRB-seq, cDNA is tagged with multiplexing barcodes and UMIs. As opposed to the three PCR-based methods described before, CEL-seq relies on linear amplification by in-vitro transcription (IVT) for the initial pre-amplification of single-cell material. An overview of the methods and their features is given in Supplementary Table 1.

For Smart-seq/C1, SCRB-seq and Drop-seq we generated 437, 253 and 432 million reads from 192, 192 and ~200 cells in the two independent replicates. Data from CEL-seq consisted of 51 million reads from a total of 74 cells (Fig. 1). After demultiplexing and assignment of cell barcodes, we could map >50% of all reads to the mouse genome and 30-40% of all reads to exons (Fig. 2a), comparable to previous results[7,14]. As expected, the 3' counting methods showed a strong 3' bias while Smart-seq/C1 showed a moderate 3' bias (Supplementary Fig. 1a). We used the Drop-seq pipeline[15] to quantify UMIs and found that between 2.6% (Drop-seq, replicate B) and 59.5% (SCRB-seq, replicate A) of all exon reads were unique, i.e. that the scRNA-seq libraries had been sequenced on average 1.7- to 38-fold over (Fig. 2a). We filtered the scRNA-seq libraries for possible cell doublets either by their microscope images (Smart-seq/C1) or by discarding transcriptomes with more than twice the mean total UMI content. Furthermore, we disregarded scRNA-seq libraries with less than 100,000 reads mapped to exons, resulting in 479 single cell transcriptomes across the four methods (Fig. 1). As expected, these transcriptomes cluster according to method in a principal component analysis, whereas the biggest difference is between the 3' counting methods and Smart-seq/C1 (Supplementary Fig. 1b). In summary, we obtained high-quality scRNA-seq data for 479 cells distributed equally across methods and replicates, allowing to compare the four different scRNA-seq methods with respect to their sensitivity, accuracy and precision.

SCRB-seq and Smart-seq/C1 show the highest sensitivity

As a relative measure of sensitivity we compared the number of detected genes per method and replicate (Fig. 2b). Clearly, Drop-seq had the lowest sensitivity with a median of 3158 genes per cell, CEL-seq had 6384 and SCRB-seq and Smart-seq/C1 were very similar with a median of ~8800 genes detected per cell (Fig. 2b). This relative ranking remains unchanged if one subsamples 100.000 reads for each cell, but CEL-seq performs then

106 almost as good as SCRB-seq and Smart-seq/C1 (Supplementary Fig. 2a). While the
107 number of detected genes per cell differs, the total number of detectable genes in all cell
108 converges probably around 20,000 of the 39,000 annotated mouse genes for all 3' counting
109 methods. In contrast, the Smart-seq/C1 method converges at more than 25,000 detected
110 genes (Fig. 2c). Furthermore, while the majority of genes is detected by all four methods,
111 Smart-seq/C1 detects 3974 genes not seen by the 3' counting methods (Supplementary
112 Fig. 2b,c). This pattern remains when subsampling 50 cells à 100,000 reads per method
113 (data not shown).
114 As an absolute measure of sensitivity, we compared the probabilities of detecting the 92
115 artificially spiked-in mRNAs (ERCCs) since their concentration per cell is known. We
116 recorded the number of cells with an expression value of zero for each ERCC per method
117 and applied a maximum likelihood method[17] to estimate the probability of successful
118 detection of an ERCC mRNA with a given number of molecules per cell as a binomial
119 process (Fig. 2d). This is more informative than non-zero detections of ERCC, as in that
120 case it is only known that some copies were successfully captured but not how many
121 exactly. Additionally, this method is also superior to other approaches as it does not try to
122 relate the expression level to the capture efficiency. For Smart-seq/C1, a gene is detected
123 in half of the cells when it has a concentration of ~8 molecules per cell. Drop-seq and
124 SCRB-seq show similar estimates of 11 molecules per cell. However, CEL-seq needs 92
125 molecules to reach the 50% detection probability (Supplementary Fig. 2d). Similar
126 estimates of 16, 17, 18 and 93 molecules per cell for Smart-seq/C1, SCRB-seq, Drop-Seq
127 and CEL-seq are made when subsampling 10,000 ERCC reads for 50 cells per method. The
128 drastically lower sensitivity of CEL-seq when using ERCCs contradicts the just slightly
129 lower sensitivity when considering the number of detected genes. This discrepancy was
130 also noted in the original CEL-seq publication and explained with a potential degradation of
131 the ERCCs[12]. Hence, the ERCC reads in this CEL-seq data set might underestimate the
132 sensitivity of this method considerably. The other discrepancy between the two sensitivity
133 estimates is seen for Drop-seq. While it detects less than half the number of genes than
134 Smart-seq/C1 and SCRB-seq, it shows a similar sensitivity for the ERCCs. However, it is
135 important to realize that ERCCs can not be spiked in when using the Drop-seq approach,
136 but have to be run separately from the cells[15]. It is unclear to what extend the lower
137 complexity in an "ERCC-only" run might lead to higher sensitivities and/or to what extent
138 this reflects a difference between data generated by Macosko et al and data generated by
139 us. For now, we conservatively assume that our data is representative and hence that Drop-
140 seq is less than half as sensitive as the other methods.

141 In summary, we find that Smart-seq/C1 is the most sensitive method as it detects at least
142 as many genes per cell as CEL-seq and SCRB-seq and detects in total ~ 4000 genes that
143 are not detected by the 3' counting methods.
144
145 **Accuracy is similar between Drop-seq, SCRB-seq and Smart-seq/C1**
146 In order to quantify the accuracy of transcript level quantifications, we compared observed
147 expression values with annotated molecule concentration of the 92 ERCC transcripts (Fig
148 3a). For each cell, we calculated the correlation coefficient (R^2) for a linear model fit (Fig 3b).
149 Clearly, CEL-seq performed worst in this measure, while Drop-Seq, SCRB-seq and Smart-
150 seq/C1 showed largely similar accuracy. This pattern remained with downsampled ERCC
151 data (data not shown). As discussed above, this low performance of CEL-seq could be
152 consistently explained with a potential degradation of the ERCCs. A previous study using
153 CEL-seq from 10 pg of total RNA input and ERCC spike-in reported a mean correlation
154 coefficient of R^2=0.87[9], similar to the ones reported for the other three methods. Assuming
155 that this is representative for CEL-seq, we find that the accuracy is similarly high across the
156 four methods.
157
158 **Precision is highest for CEL-seq and strongly increased by UMIs**
159 While a high accuracy is necessary to quantify absolute expression values, one most often
160 analyses relative differences among cells or samples in order to identify differentially
161 expressed genes or biological variation. Hence, the precision of the method, i.e. its
162 reproducibility or amount of technical variation matters more.
163 We use the probabilistic model proposed by Kim et al[18] that estimates the proportion of
164 technical variation from ERCCs. Since we use the same cells under the same culture
165 conditions we assume that the remaining fraction of biological variation is the same across
166 the data sets. We find that Smart-seq/C1 has the lowest proportion of technical variation,
167 which is surprising given that it is the only method that lacks UMIs that are expected to
168 increase the precision. Indeed, the total amount of variation is higher for Smart-seq/C1 than
169 for the other three methods (Supplementary Figure 3a) suggesting that the technical
170 variation is underestimated for Smart-seq/C1. Indeed, when we repeat the analysis without
171 considering the UMI information, Drop-seq and SCRB-seq have now more total variation
172 (Supplementary Fig. 3a) and less technical variation (Fig. 4a). Interestingly, the technical
173 variation of CEL-seq remains almost unaffected and is the lowest among the four methods
174 when considering the effect of UMIs (Fig. 4a). This pattern remains when filtering out lowly
175 expressed genes and ERCCs (data not shown). When using another variance

176 decomposition method[19], we do not observe the difference between using and not using
177 UMIs, probably due to filtering of genes whose variance composition can not be reliably
178 estimated. Although this filtering makes it difficult to compare the fraction of technical
179 variation, it is worth mentioning that CEL-seq has again the lowest amount of technical
180 variation (Supplementary Fig. 3b). Hence, when using variance decomposition methods
181 CEL-seq shows the highest precision. Maybe even more important, our analysis shows that
182 some variance decomposition method can severely underestimate the technical variation
183 from ERCCs for PCR-based amplification protocols.
184 In order to investigate precision in scRNA-seq methods independent of spike-in transcripts,
185 we used power simulation. Based on the method by Wu et al[20], we used the mean-
186 dispersion distributions of the data (Supplementary Fig. 4) to simulate the power and the
187 false discovery rate for differential gene expression by DESeq2[21]. While the three UMI-
188 based methods all had false discovery rates (FDRs) close to the expected 5%, Smart-
189 seq/C1 resulted in progressive FDRs considerably above 5% (Fig. 4b). Furthermore, CEL-
190 seq needed just 52 cells per group to detect small expression differences with 80% power,
191 followed by SCRB-seq (112) and Drop-seq (170), while Smart-seq/C1 reached 80% power
192 only for highly expressed genes (Fig. 4b and Supplementary Fig. 5a). Accordingly, when
193 simulating the power to detect larger expression differences ($\log_2$ fold-change of 0.5-3) with
194 a fixed sample size of 16 cells per group, CEL-seq performed best (Supplementary Fig. 6b),
195 followed by SCRB-seq and Drop-seq, while Smart-seq/C1 had the lowest power. Notably,
196 when analysing power without using UMIs, SCRB-seq and CEL-seq perform similar to
197 Smart-seq/C1, while Drop-seq performs very poorly (Supplementary Fig. 5b). Hence, UMIs
198 strongly increase the power to detect differentially expressed genes by scRNA-seq,
199 especially for methods that use PCR amplification.
200 In summary, when using power simulations to compare precision among methods, CEL-
201 seq performs best, followed by SCRB-Seq and Drop-Seq. Smart-seq/C1 performs worst,
202 probably because it does not include UMIs.
203
204 **Efficiency is highest for SCRB-seq and Drop-seq when considering costs and power**
205
206 Practically, the costs of a method also matter when judging the performance of different
207 scRNA-seq methodologies. Here, we estimate the cost-efficiency by calculating the costs
208 for generating scRNA-seq data at a given amount of power. Given the number of single
209 cells that are needed per group to reach 80% power (50% for Smart-seq/C1) as simulated
210 above (Figure 4b), we calculate the costs to generate these libraries and to sequence

211 enough to obtain 100,000 exon-mapped reads (Figure 2). For example, SCRB-seq requires
212 112 cells per group and generating 224 SCRB-seq libraries costs ~450€. To generate
213 100,000 reads mapping to exons for 224 SCRB-seq libraries requires ~64 million paired-
214 end reads generated with a 50 cycles single end kit that we assume cost 320€. When we
215 do analogous calculations for the three other methods (Table 1), we find that Drop-Seq and
216 SCRB-seq are equally cost-effective, closely followed by CEL-seq, while Smart-Seq/C1 is
217 almost ten-fold less efficient due its high library costs that arise from the microfluidic chips
218 and the costs for generating independent libraries. The estimate should be understood as a
219 lower boundary for the real costs since many factors are not considered such as costs to
220 set-up the methods, costs to isolate single cells, costs due to unequal pooling of libraries or
221 costs due to practical constraints in generating a fixed number of scRNA-seq libraries and
222 reads. Furthermore, power for differential gene expression is not the only factor that
223 matters and in addition to the metrics investigated above, methods differ with respect to full
224 length coverage of cDNAs (only possible for Smart-seq/C1), possibility of imaging cells
225 (CEL-seq and Smart-seq/C1) and the possibility to have ERCCs for each library (not
226 possible for Drop-seq).
227 Nevertheless, we think that our estimates allow a fair and transparent comparison of the
228 four methods and reveal that Drop-Seq and SCRB-seq are most efficient and Smart-
229 seq/C1 is almost ten-fold less cost-efficient.
230
231
232 **Discussion**
233 Single-cell RNA-sequencing (scRNA-seq) is a powerful technology to tackle biomedical
234 questions. To facilitate choosing among the many approaches that were recently
235 developed, we systematically compared four scRNA-seq methods and assessed their
236 sensitivity, accuracy, precision and cost-efficiency. We chose a leading commercial
237 platform (Smart-seq/C1), a method that uses in-vitro transcription for amplification from
238 manually isolated cells (CEL-seq), a PCR-based method with a very high throughput (Drop-
239 seq) and a PCR-based method that allows single cell isolation by FACS (SCRB-seq). All
240 these methods can be set up by a molecular biology lab, as has been done by us for
241 Smart-seq/C1, SCRB-seq and Drop-seq.
242 We find that SCRB-seq, Smart-seq and CEL-seq detect a similar number of genes per cell
243 while Drop-seq - at least in our hands - detects over 50% less than the other methods (Fig.
244 2b and Supplementary Figure 2a). Whereas we detect 3,158 genes per ES cell, 5,663 genes
245 per 3T3 cell are found in the original Drop-seq publication[15]. It is currently unclear whether

246 this discrepancy is due to biological or technical reasons. Importantly, Drop-seq does not
247 detect generally fewer genes since the total number of detected genes converges around
248 20,000, similar as for SCRB-seq and CEL-seq (Fig. 2c). While these three 3' counting
249 methods detect largely the same genes, Smart-seq/C1 detects almost 4000 additional
250 genes (Supplementary Fig. 2b). So while ERCCs are measured with similar high accuracy
251 by all four methods (Fig. 3), a considerable number of genes are missed by the 3' counting
252 methods. Hence, Smart-seq/C1 is the most sensitive method because it detects
253 considerably more genes in addition to its full length coverage of transcripts
254 (Supplementary Fig. 1a). However, it is difficult to gauge how much information this adds
255 for finding and defining cell types and characterizing their biological states and networks.
256 Probably more important than the sensitivity and accuracy for the performance of scRNA-
257 seq methods is their precision, i.e. their reproducibility of measuring gene expression levels.
258 We find that variance decomposition methods that use ERCCs to estimate precision can
259 severely underestimate the technical variance when PCR is used to amplify cDNA without
260 UMIs (Fig. 4a). Further work will be needed to investigate whether more ERCCs, other
261 ERCCs and/or different estimation methods could solve this. Assuming that variance
262 decomposition can accurately estimate precision when using UMIs, we find that CEL-seq
263 performs better than the PCR-based Drop-seq and SCRB-seq. When using power
264 simulations that use the entire data, we also find that CEL-seq is more precise since it
265 detects differentially expressed genes with fewer cells (Fig. 4b). These simulations also
266 show that UMIs increase the power considerably, especially for the PCR-based methods
267 (Supplementary Figure 5b). Hence, in-vitro transcription (IVT)-based amplification is more
268 precise than PCR-based amplification and - in contrast to bulk RNA-seq libraries[22] - UMIs
269 decisively increase the power for differential gene expression from single cell RNA-seq
270 libraries. However, the higher precision of CEL-seq comes with higher costs per library and
271 in its current form also requires manual isolation of single cells (Table S1). Indeed, when we
272 calculate the costs to reach a given power for differential gene expression, Drop-Seq and
273 SCRB-seq are both more efficient than CEL-seq (Table 1). Smart-seq/C1 is by far the least
274 efficient method due to its high costs and low precision. The efficiency could be improved
275 for all methods, e.g. when implementing UMI-based protocols on the C1 platform[11], or
276 using early barcoding to increase the number of cells per chip. The CEL-seq variant MARS-
277 seq combines the higher precision of IVT-based amplification with higher throughput[14] and
278 SCRB-seq is even more cost-efficient on a 384-well format[13].
279

280 In summary, we find that Drop-seq is probably the most efficient method when analysing
281 large numbers of cells and SCRB-seq might be preferable for analyzing smaller numbers,
282 especially when considering that ERCCs can be spiked in, cells can be preselected by
283 FACS and the same set-up can also be used for bulk RNA-seq.

284 **Methods**

285 **Published data**

286 CEL-seq data for J1 mESC cultured in 2i/LIF condition[12] were obtained under accession
287 GSE54695. Drop-seq ERCC[15] data were obtained under accession GSE66694. Raw fastq
288 files were extracted using the SRA toolkit (2.3.5).
289 We trimmed cDNA reads to the same length and processed raw reads in the same way as
290 data sequenced for this study (see below).
291

292 **Cell culture of mESC**

293 J1 mouse embryonic stem cells were maintained on gelatin-coated dishes in Dulbecco's
294 modified Eagle's medium supplemented with 16% fetal bovine serum (FBS, Sigma-Aldrich),
295 0.1 mM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine, 1x MEM non-essential amino
296 acids, 100 U/ml penicillin, 100 µg/ml streptomycin (PAA Laboratories GmbH), 1000 U/ml
297 recombinant mouse LIF (Millipore) and 2i (1µM PD032591 and 3µM CHIR99021 (Axon
298 Medchem, Netherlands). J1 embryonic stem cells were obtained from E. Li and T. Chen and
299 mycoplasma free determined by a PCR-based test. Cell line authentication was not recently
300 performed.
301

302 **Single cell RNA-seq library preparations**

303 *Drop-seq*

304 Drop-seq experiments were performed as published[15] and successful establishment of the
305 method in our lab was confirmed by a species-mixing experiment (data not shown). For this
306 work, J1 mES cells (100/µl) and barcode-beads (120/µl, Chemgenes) were co-flown in
307 Drop-seq PDMS devices (Nanoshift). Emulsions were broken by addition of perfluoroctanol
308 (Sigma-Aldrich) and mRNA on beads reverse transcribed. Unused primers were degraded
309 by addition of Exonuclease I (New England Biolabs). Washed beads were counted and
310 aliquoted for pre-amplification (2000 beads / reaction). Nextera XT libraries were
311 constructed from 1 ng of pre-amplified cDNA with a custom P5 primer (IDT).
312

313 *SCRB-seq*

314 RNA was stabilized by resuspending cells in RNAprotect Cell Reagent (Qiagen) and RNAse
315 inhibitors (Promega). Prior to FACS sorting, cells were diluted in PBS (Invitrogen). Single
316 cells were sorted into 5 µl lysis buffer consisting of a 1/500 dilution of Phusion HF buffer
317 (New England Biolabs), spun down and frozen at -80 °C. Plates were thawed and libraries
318 prepared as described previously[13]. Briefly, RNA was desiccated after protein digestion by

319 Proteinase K (Ambion). RNA was reverse transcribed using barcoded oligo-dT primers (IDT)
320 and products pooled and concentrated. Unincorporated barcode primers were digested
321 using Exonuclease I (New England Biolabs). Pre-amplification of cDNA pools were done
322 with the KAPA HiFi HotStart polymerase (KAPA Biosystems). Nextera XT libraries were
323 constructed from 1 ng of pre-amplified cDNA with a custom P5 primer (IDT).
324
325 *Smart-seq/C1*
326 Smart-seq/C1 libraries were prepared on the Fluidigm C1 system according to the
327 manufacturer's protocol. Cells were loaded on a 10-17 µm RNA-seq microfluidic IFC at a
328 concentration of 200,000/ml. Capture site occupancy was surveyed using the Operetta
329 (Perkin Elmer) automated imaging platform.
330
331 **DNA sequencing**
332 For each scRNA-seq method, final library pools were size-selected on 2% E-Gel Agarose
333 EX Gels (Invitrogen) by excising a range of 300-800 bp and extracting DNA using the
334 MinElute Kit (Qiagen) according to the manufacturer's protocol.
335 0.1 pmol of library pools were sequenced on a Illumina HiSeq1500 using High Output
336 mode. Smart-seq/C1 libraries were sequenced 45 cycles single-end, whereas Drop-seq
337 and SCRB-seq libraries were sequenced paired-end with 20 cycles to decode cell
338 barcodes and UMI from read 1 and 45 cycles into the cDNA fragment.
339
340 **Basic data processing and sequence alignment**
341 Smart-seq/C1 libraries (i5 and i7) and Drop-seq/SCRB-seq pools (i7) were demultiplexed
342 from the Nextera barcodes. All reads were mapped to the mouse genome (mm10) including
343 mitochondrial genome sequences and unassigned scaffolds concatenated with the ERCC
344 spike-in reference. Alignments were calculated using STAR 2.4.0[23] using all default
345 parameters.
346 For libraries containing UMIs, cell- and gene-wise count/UMI tables were generated using
347 the published Drop-seq pipeline[15]. We discarded the last 2 bases of the Drop-seq cell and
348 molecular barcodes to account for bead synthesis errors.
349 For Smart-seq/C1, features were assigned and counted using the Rsubread package[24].

350 **Power Analysis**

351 We utilized the R package PROPER [20] for statistical power evaluation. For each method, we
352 estimated mean baseline expression and dispersions using DESeq2[21] and all genes with
353 log2(dispersion) >-10 were considered for simulation. For each method, 100 RNA-seq
354 experiments were simulated with 5 percent differentially expressed genes between two
355 groups of sample size of 2^4, 2^5, 2^6, 2^7, 2^8 and 2^9 cells.

356

357 **ERCC capture efficiency**

358 To estimate the single molecule capture efficiency, we assume that the success or failure of
359 detecting an ERCC is a binomial process, as described before[17]. Detections are
360 independent from each other and are thus regarded as independent Bernoulli trials. We
361 recorded the number of cells with nonzero and zero expression values for each ERCC per
362 method and applied a maximum likelihood estimation to fit the probability of successful
363 detection. The fit line was shaded with the 95% Wilson score confidence interval.

364

365 **Variance decomposition**

366 Variance decomposition was done as implemented by Kim et al[18]. We estimate the variation
367 in capture efficiency (θ) and in sequencing efficiency (γ) separately for each replicate to
368 account for batch effects. We integrated the published Drop-seq ERCC data by scaling it
369 with size factors from our mESC data to make up 10% of counts in one cell.
370 We applied scLVM as described previously[19], both using the log-linear fit of mean and
371 variance relationship as well as the ERCCs for estimation of technical variation.

372

373 **Cost efficiency calculation**

374 We based our cost efficiency extrapolation on the power simulations performed in this
375 study (Fig. 4b). We determined the number of cells required per group for adequate power
376 (80% in UMI-based methods and 50% for Smart-seq/C1) by an asymptotic fit to the
377 median powers. Next, we determined the number of raw reads required to reach 100,000
378 exonic reads per cell. For this, we used the mean exon assignment rate from Fig. 2a (CEL-
379 seq: 36%, Drop-seq: 40%, SCRB-seq: 44%, Smart-seq/C1: 44%). To account for reads
380 that do not belong to desired cell-transcriptomes in early-barcoding methods, we assumed
381 a loss of reads of 20% (CEL-seq), 50% (Drop-seq) and 20% (SCRB-seq). For the
382 calculation of sequencing cost, we assumed 5€ per million raw reads, independent of
383 method. Although UMI-based methods need paired-end sequencing, we assumed a
384 50 cycle sequencing kit is sufficient for all methods.

385 **Data accession**

386 The raw and analysed data files can be obtained in GEO under accession number
387 GSE75790.

388

389 **Competing interests**

390 The authors declare that they have no competing interests.

391

392 **Author's contributions**

393 CZ and WE conceived the experiments. CZ prepared scRNA-seq libraries and analyzed the
394 data. SP helped in data processing and power simulations. BV estimated ERCC capture
395 efficiencies. MS performed cell culture of mESC. WE and HL supervised the experimental
396 work and IH provided guidance in data analysis. CZ and WE wrote the manuscript. All
397 authors read and approved the final manuscript.

398

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Tables

Method	Power	n_{cells} (per group)	Library cost (€)	Sequencing cost (€)	Experiment cost (€)
CEL-seq	0.8[a]	52	~820[b]	~180[c]	~1000
Drop-seq	0.8[a]	170	~30[b]	~840[c]	~870
SCRB-seq	0.8[a]	112	~450[b]	~320[c]	~870
Smart-seq/C1	0.5[a]	129	~6560[b]	~290[c]	~6850

Table 1 | Cost efficiency extrapolation for single-cell RNA-seq experiments.

[a] Based on simulations (Fig. 4b) for detection of $\log_2$(fold-change) = 0.25

[b] assuming per cell library preparation costs of 8€, 0.1€, 2€, 25€ respectively

[c] Taking into account the mapping and exon assignment rates found in this study (see Fig. 2a), as well as the fraction of reads that falls into undesired cell barcodes in early-barcoding methods.

Figures

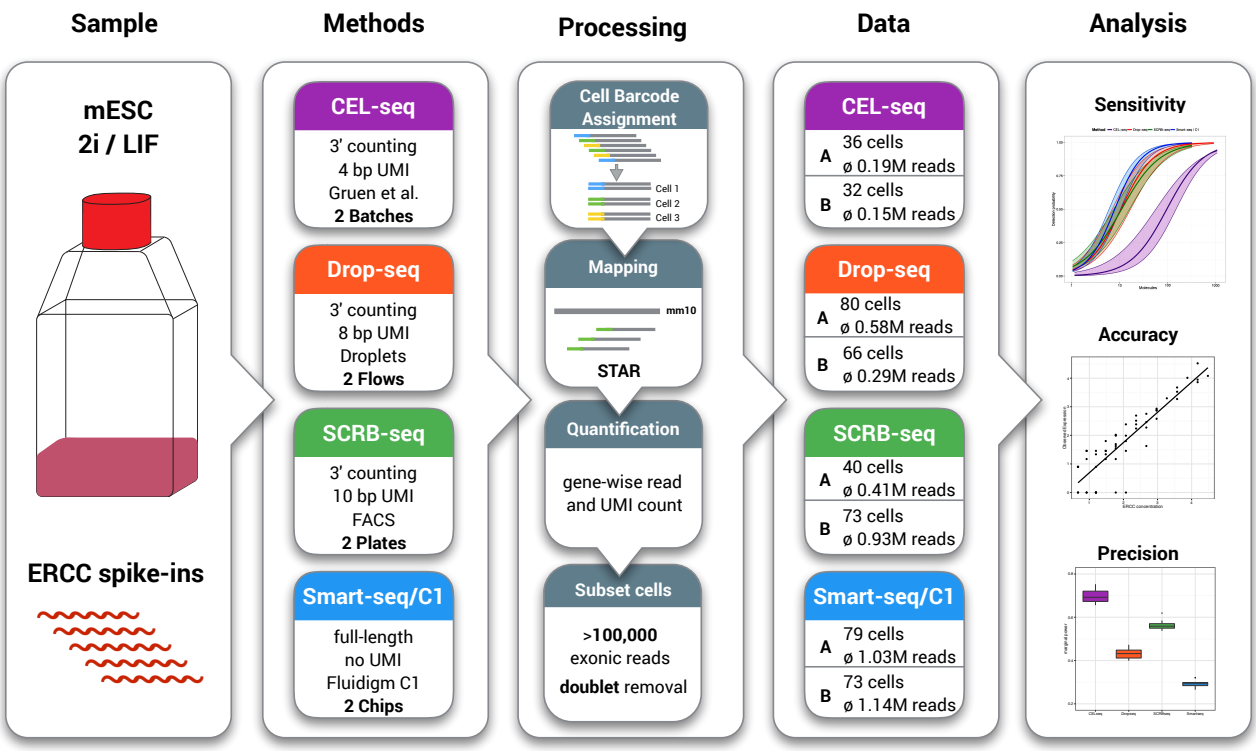

Figure 1 | Schematic of the experimental and computational pipeline. Mouse embryonic stem cells cultured in 2i/LIF and ERCC spike-in RNA were used to prepare single-cell RNA-seq libraries. The four methods differ by the presence and length of a unique molecular identifier sequence (UMI) allowing to identify reads generated during cDNA amplification. Data processing and subsetting of cells was done comparatively for all methods. Final cell numbers per method and replicate are shown with their mean sequencing depth. Colors represent the compared scRNA-seq methods: purple - CEL-seq, orange - Drop-seq, green SCRB-seq, blue - Smart-seq and are used throughout this study.

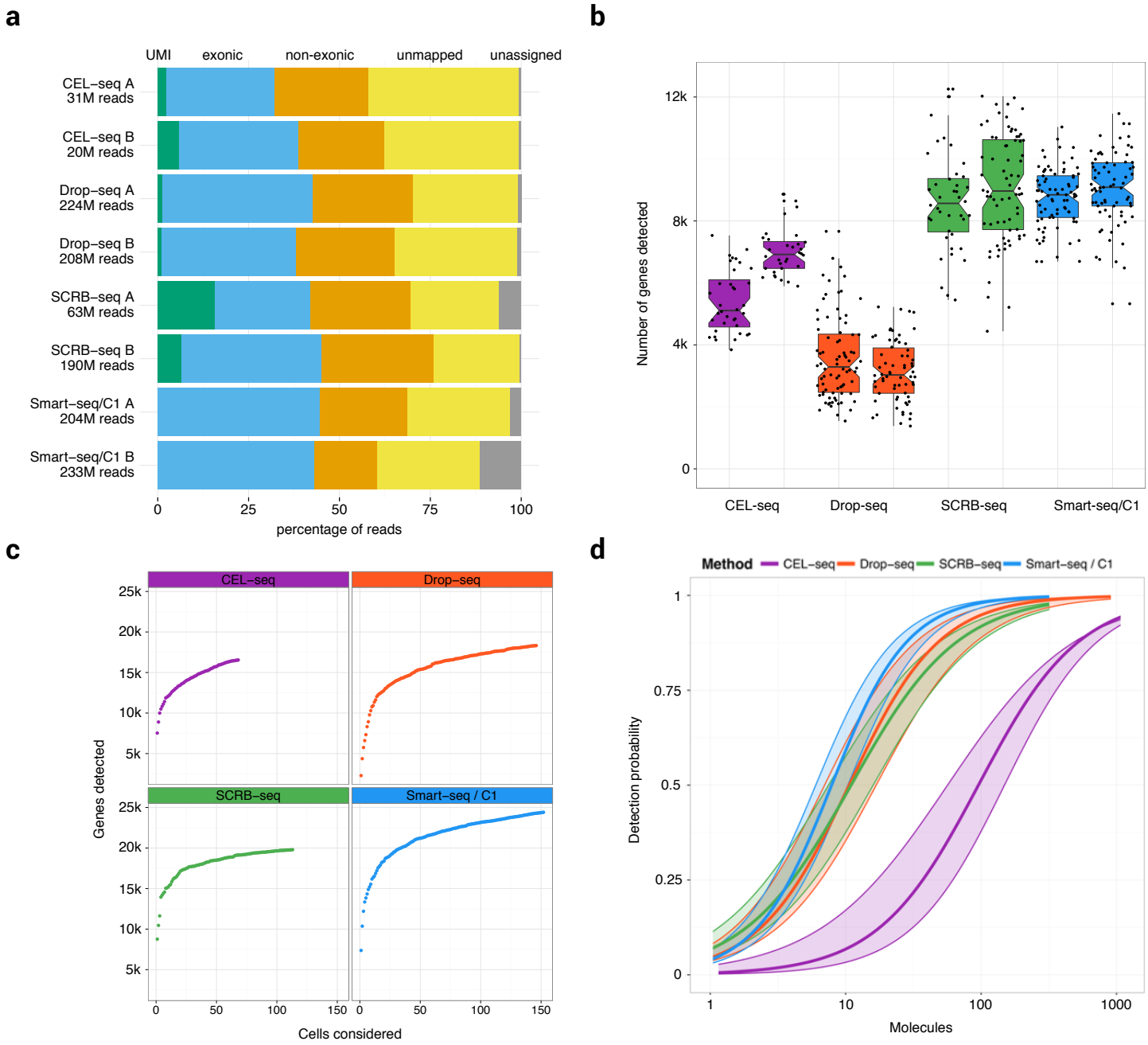

Figure 2 | Sensitivity of scRNA-seq methods.
(**a**) Percentage of the total reads that can not be assigned to a cellular barcode (grey), can not be mapped to the mouse genome (yellow), are mapped to regions outside exons (orange), inside exons (blue) and carry a unique UMI (green). (**b**) Number of genes with at least one read in all cells with more than 100,000 total reads in total. Each dot represents a cell and each boxplot represents the median, first and third quartile per replicate and method. (**c**) Cumulative number of genes detected as more cells are added. (**d**) Sensitivity per method estimated as the probability to detect ERCC transcripts dependent on their copy number per cell. The 95% confidence interval of an estimate is displayed as shaded area.

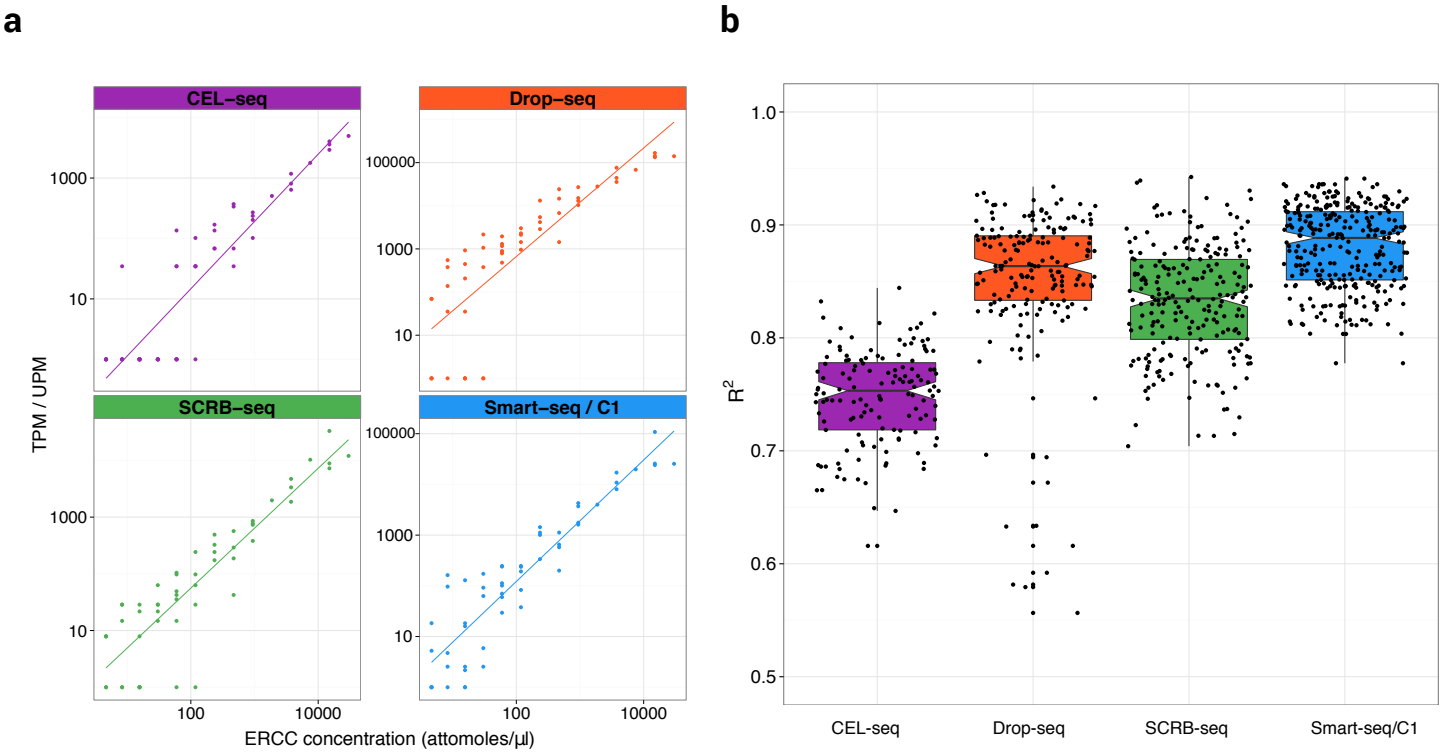

Figure 3 | Accuracy of scRNA-seq methods. (**a**) Representative correlations of single-cell / single-bead (Drop-seq) expression values for ERCC spike-ins and their annotated molarity. Expression is measured as transcripts per million reads (TPM, Smart-seq/C1) or UMIs per million reads (UPM). (**b**) Distribution of correlation coefficients (Pearson) across methods. Each dot represents a cell/bead and each boxplot represents the median, first and third quartile.

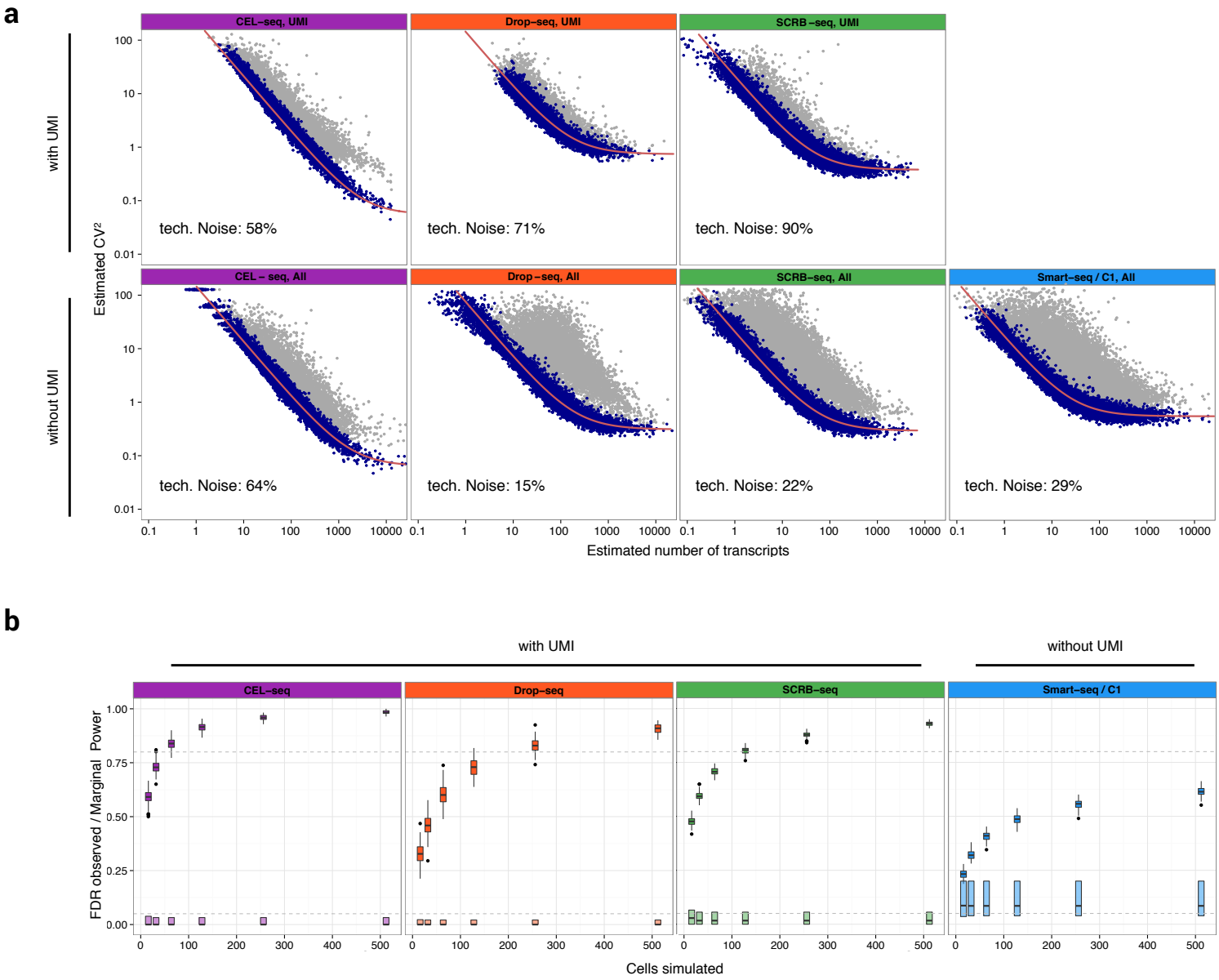

Figure 4 | Variance decomposition and power analysis. (**a**) Variance decomposition according to Kim et al. 2015. Technical and biological noise factors were estimated from complete read counts and UMI counts (where available) and further used to simulate mean / variance distributions per scRNA-seq method. Shown are simulated genes with technical and technical plus biological factor. Genes with technical factor only are shown in blue, with technical and biological factor in grey and the technical noise fit for ERCC spike-ins is shown in red. (**b**) Power simulations using empirical mean/ dispersion relationships. Boxplots represent the median, first and third quartile of 100 simulations. Power analysis of differentiation scenario with a small fixed effect size of 0.25 log2 fold-change and variable sample size n=16, n=32, n=64, n=128, n=256 and n=512. Shown are marginal power and observed FDR over 100 simulations with relevant cut-offs marked in dashed lines (marginal power = 0.8; FDR = 0.05).