

1 **Dissecting tumor cell programs through group biology estimation in clinical single-cell**
2 **transcriptomics**

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22

23 **Abstract**

24 Given the growing number of clinically integrated cancer single-cell transcriptomic studies, robust
25 differential enrichment methods for gene signatures to dissect tumor cellular states for discovery
26 and translation are critical. Current analysis strategies neither adequately represent the
27 hierarchical structure of clinical single-cell transcriptomic datasets nor account for the variability
28 in the number of recovered cells per sample, leading to results potentially confounded by sample-
29 driven biology with high false positives instead of accurately representing true differential
30 enrichment of group-level biology (e.g., treatment responders vs. non-responders). This problem
31 is especially prominent for single-cell analyses of the tumor compartment, because high intra-
32 patient similarity (as opposed to inter-patient similarity) results in stricter hierarchical structured
33 data that confounds enrichment analysis. Furthermore, to identify signatures which are truly
34 representative of the entire group, there is a need to quantify the robustness of otherwise
35 statistically significant signatures to sample exclusion. Here, we present a new nonparametric
36 statistical method, BEANIE, to account for these issues, and demonstrate its utility in two cancer
37 cohorts stratified by clinical groups to reduce biological hypotheses and guide translational
38 investigations. Using BEANIE, we show how the consideration of sample-specific versus group
39 biology greatly decreases the false positive rate and guides identification of robust signatures that
40 can also be corroborated across different cell type compartments.

41

42 **Introduction**

43 Single-cell transcriptomic profiling of patient tumors has enabled high-resolution dissections of
44 disease progression and treatment response. Building on seminal cellular atlases for specific
45 cancer types, many studies are increasingly focused on deriving hypotheses by evaluating groups
46 of patients (e.g., treated vs. untreated, responders vs. non-responders, and early- vs. late-stage)

47 for differences in gene signatures (which may be an experimentally and/or computationally
48 derived aggregation of related genes or pathways) between the two groups. For this purpose,
49 methods such as the Mann-Whitney U (MWU) tests and Generalised Linear Models (GLMs) have
50 been conventionally used in bulk RNA-sequencing (bulk RNA-seq) studies as well as single-cell
51 transcriptomic analyses;¹⁻⁴ however, they may have a number of limitations for the latter
52 application. First, these methods assume mutual independence of samples, and although this is
53 not problematic for bulk RNA-seq analyses, cells derived from the same patient in single-cell
54 analyses do not satisfy this criteria. Second, these methods fall short of representing the
55 hierarchical structure of tumor single-cell transcriptomic data, as tumor cells tend to exhibit more
56 intra-patient similarity as compared to inter-patient similarity due to the expression of patient-
57 specific transcriptional programs driven by DNA-level alterations and epigenetics.⁵⁻⁸ This
58 challenge, in turn, may lead to differential enrichment results being skewed by patient-specific
59 biology, instead of representing genuine group biology. Finally, the number of cells (and hence,
60 data points) sequenced in these single-cell transcriptomic datasets are typically large compared
61 to bulk RNA-seq datasets, thereby potentially increasing the power of statistical tests to detect
62 differences (by rejecting the null hypothesis) between the groups under consideration, which may
63 not reflect biologically or clinically relevant observations. These challenges exist for other cell
64 types as well, including the immune and stromal cells, albeit to a lesser extent. As a result of these
65 significant methodological challenges, single-cell transcriptomic case/control analyses of cancer
66 samples have thus far often not involved detailed assessments of the tumor compartments, which
67 has restricted the capability to learn from tumor cellular programs in increasingly complex clinical
68 contexts.

69

70 To maximize the utility of single-cell transcriptomic analyses between clinically relevant patient
71 populations and determine how tumor cell programs differ between groups of patients, we

72 developed a nonparametric statistical group biology estimation method (group **B**iology **E**stim**A**tion
73 **i**n **s**ingle **c**ell, “BEANIE”) inspired from *He et al.*,⁹ addressing the above-mentioned issues (Fig.
74 1, see Methods). This method first estimates the statistical significance (empirical p-value) of the
75 test signatures through a Monte Carlo approximation of the test signatures’ p-value distribution
76 (test distribution) and that of the random signatures’ p-value distribution (background distribution),
77 followed by contextualisation of the former with respect to the latter. It then uses the leave-one-
78 out cross-validation approach (sample exclusion) to infer robustness of the gene signatures (see
79 Methods). We used publicly available datasets to demonstrate the utility of this method, and
80 present suggested guidelines for the design of clinically embedded single-cell transcriptomic
81 studies in oncology.

82

83 **Results**

84 We evaluated single-cell transcriptomic data in two cancer types (melanoma and lung cancer)
85 that have the following clinical groups for comparison: (i) response to treatment; and (ii) disease
86 progression.^{1,2,10–12} We contextualised their tumor compartments with signatures from the
87 Molecular Signatures Database (MSigDB),^{13,14} including Hallmark (n = 50) and Oncogenic (n =
88 189) gene sets. We compared results obtained from MWU tests followed by Benjamini-Hochberg
89 (BH) corrections and GLMs with results obtained from BEANIE, and characterised our approach
90 relative to these methods (Table 1). Details regarding the implementation and comparisons are
91 available in the Methods section.

92

93 **Group biology analysis of Immune Checkpoint Blockade-naive vs. -exposed melanoma**

94 We first evaluated a melanoma dataset,¹ which included data for both immune checkpoint
95 blockade (ICB)-naive and ICB-exposed patients, to assess methodologies for comparing clinical
96 treatment states in tumor cells. All of the ICB-exposed samples were resistant to treatment and
97 were biopsied from the metastatic sites. We excluded samples having less than 50 tumor cells,
98 and, in total, there were 1891 tumor cells across 14 patients, with 7 patients per group (Fig. S1,
99 see Methods). We first assessed the data with Hallmark and Oncogenic gene set signatures from
100 MSigDb, to characterise treatment-driven biology within the tumor compartment (Fig. 2, Table 1).

101

102 We observed that the MWU test followed by a BH correction and GLMs predicted a large number
103 of differentially enriched signatures ($p\text{-value} \leq 0.05$), whereas BEANIE was more conservative,
104 detecting fewer signatures as differentially enriched (Fig. 2b, Table S1). Notably, a majority of the
105 signatures identified as significant by MWU test and GLMs were labelled as non-significant and
106 non-robust to sample exclusion by BEANIE.

107

108 Among signatures that were identified as statistically significant and robust to sample exclusion
109 by BEANIE (see Methods), signatures upregulated in the ICB-naive group include those for genes
110 upregulated by *STAT5* in response to *IL2* stimulation (HALLMARK_IL2_STAT5_SIGNALING),
111 genes regulated by NF- κ B in response to TNF (HALLMARK_TNFA_SIGNALING_VIA_NFKB),
112 and genes defining inflammatory response (HALLMARK_INFLAMMATORY_RESPONSE). We
113 also verified a previously identified T cell exclusion signature¹ upregulated in the ICB-exposed
114 group as statistically significant and robust to sample exclusion with the BEANIE method. To verify
115 gene-level differential expression, we used a MWU test and observed differential *IL2* gene
116 expression for the ICB-naive group in the T cell compartment ($p\text{-value} = 0.0078$), corroborating

117 our finding in the tumor compartment (differential HALLMARK_IL2_STAT5_SIGNALING). We
118 also identified the top constituent genes (ranked according to log2 fold change and robustness to
119 sample exclusion, see Methods) for these three signatures, and found that these genes were
120 differential in the tumor compartment uniformly across samples of a given group (Fig. 2e, Table
121 S2). Together, these results describe the tumor microenvironment of the ICB-exposed group
122 (consisting of treatment-resistant patients) as one depleted of T cells, with reduced IL2-STAT5
123 signaling, TNFA-NFKB signaling, and inflammatory response relative to the ICB-naive group.

124

125 Additionally, we found that the signature for genes upregulated by *IL6* via STAT3
126 (HALLMARK_IL6_JAK_STAT3_SIGNALING) was upregulated in the ICB-naive group and
127 statistically significant and robust to sample exclusion. Using a MWU test, we found differential
128 *STAT3* expression in the tumor compartment for the ICB-naive group (p-value = 3.28e-36).
129 Furthermore, we also found a positive correlation between the *STAT3* expression and
130 HALLMARK_IL6_JAK_STAT3_SIGNALING signature score in the tumor cells on an individual
131 cell basis (Fig. 2f). This observation supports the finding that *IL6* could potentially induce
132 downstream signaling via *STAT3* in the tumor cells of the ICB-naive group.^{15,16}

133

134 We further examined the cause for non-robustness of the signatures that were identified as
135 statistically significant but not robust to sample exclusion by BEANIE (Fig. 2c). We found that the
136 exclusion of one or more samples led to statistically non-significant results, in contrast to when
137 the sample was included, by shifting the empirical p-value to greater than 0.05 as a result of an
138 overlap between the test distribution and the background distribution as shown in Fig. 2d (see
139 Methods). For example, the signature ONCOGENIC_RAF_UP.V1_UP was not robust to the
140 exclusion of sample Mel106, and this particular sample was also the cause of non-robustness for

141 21 other signatures. This variability due to sample exclusion was also not explained by any of the
142 other available clinical variables (e.g., age, sex). Therefore, these signatures were driven by
143 sample-specific biology, and were consequently not representative of the group-level biology, but
144 would have otherwise been considered differentially enriched with statistical significance using
145 either of the conventional MWU test or GLM approaches.

146

147 We next investigated the methodological stability with respect to subsample size (Fig. 2g), and
148 accordingly repeated BEANIE's workflow using smaller subsample sizes. We found that a smaller
149 subsampling of cells led to fewer signatures that were identified as statistically significant and
150 non-robust to sample exclusion, and even fewer that were identified as both statistically significant
151 and robust to sample exclusion. However, the number of statistically significant and robust
152 signatures identified by BEANIE reached saturation around the subsample size of 30 cells per
153 sample, indicating that the subsample size of 60, which had been used for all of the
154 aforementioned results, could successfully capture all statistically significant signatures from the
155 test signature sets that were also robust to sample exclusion.

156

157 To assess the ability to detect noise from a true signal, we additionally used a curated set of
158 immune cell surface marker signatures¹⁷ (including signatures for T cells, NKT cells, NK cells, B
159 cells, mast cells, and a joint dendritic cell/macrophage signature), that should not be relevant to
160 tumor cells, to test the performance of the three methods (MWU test with a BH correction, GLMs,
161 and BEANIE). We observed that a MWU test with a BH correction led to a p-value ≤ 0.05 for all
162 signatures except the B cell signature and GLMs led to a p-value ≤ 0.05 for NKT cell, B cell, NK
163 cell, and the joint dendritic cell/macrophage signatures. By contrast, BEANIE correctly predicted

164 all of the immune cell surface marker signatures as both statistically non-significant and non-
165 robust to sample exclusion (Table S3).

166

167 Finally, we evaluated BEANIE's performance on the previously reported 18 T cell exclusion
168 signatures¹ for the tumor cell compartments of a reduced set of patients from the original set used
169 to derive these (only patient samples with greater than 50 tumor cells were retained, as described
170 above). We observed that while a MWU test with a BH correction had p-values ≤ 0.05 for 18/18
171 signatures and GLMs had p-values ≤ 0.05 for 11/18 signatures, BEANIE had an empirical p-value
172 ≤ 0.05 for 17/18 signatures and additionally found 10/18 of them to be robust to sample exclusion.

173

174 **Group biology analysis of distinct clinical states in non-small cell lung carcinoma**

175 In an effort to demonstrate the applicability of BEANIE for a meta-analysis composed of multiple
176 single-cell transcriptomic datasets, we next analyzed the tumor compartments from four published
177 lung cancer studies^{2,10-12} to evaluate potential differentially enriched signatures between early-
178 vs. late-stage samples. We selected patient samples which satisfied the following criteria: (i) had
179 more than 50 tumor cells; (ii) were classified as adenocarcinoma; (iii) were staged as either I, II,
180 or IV (early-stage = I and II; late-stage = IV); and (iv) had received no prior treatment at the time
181 of sample collection. Filtering according to these criteria yielded a total of 18251 malignant cells
182 across 17 patients (11 early-stage, 6 late-stage) (Fig. 3a).

183

184 We sought to characterise the tumor compartment with Hallmark and Oncogenic gene sets from
185 MSigDb (Fig. 3b, Table 1). Again, a large number of gene sets predicted as differentially enriched
186 with statistical significance by a MWU test with a BH correction and GLMs were identified as

187 statistically non-significant and non-robust to sample exclusion by BEANIE (Fig. 3c, Table S1).
188 We found a signature composed of genes important for mitotic spindle assembly
189 (HALLMARK_MITOTIC_SPINDLE) to be statistically significant and robust to sample exclusion
190 for early-stage lung tumors with BEANIE, consistent with prior studies.¹⁸ Another signature
191 comprised of genes encoding proteins involved in glycolysis and gluconeogenesis
192 (HALLMARK_GLYCOLYSIS) was also found to be statistically significant and robust to sample
193 exclusion for the early-stage tumors with BEANIE, which is in agreement with a prior study¹⁹
194 describing an association between TKI treatment and its effect on decreased activity of glycolysis.
195 Furthermore, the top constituent genes for both of these signatures were consistently upregulated
196 across all samples (Fig. S2, Table S2). Thus, BEANIE was able to detect both statistically
197 significant and robust signatures in the meta-analysis of multiple single-cell transcriptomic
198 datasets.

199

200 We next sought to evaluate the tumor compartment from two of the lung cancer datasets (Kim et
201 al.,¹⁰ Maynard et al.¹¹) for treatment responses to tyrosine kinase inhibitors (TKIs). We selected
202 patient samples which satisfied the following criteria: (i) had more than 50 tumor cells; and (ii) the
203 biopsy was derived from the primary tumor. These filtering criteria led to a total of 7576 malignant
204 cells across 10 patients (6 TKI-naive, 4 TKI-exposed) (Fig. S3).

205

206 We again used the Hallmark and Oncogenic gene sets to characterise the tumor compartment
207 (Fig. 4a, 4b, Fig. S3; Table 1, Table S1). Among the signatures that were found to be statistically
208 significant and robust to sample exclusion with BEANIE, signatures upregulated in the TKI-
209 exposed group included a signature for genes upregulated in response to *IFNG*
210 (HALLMARK_INTERFERON_GAMMA_RESPONSE) and a signature for genes upregulated by

211 the overexpression of *WNT1* (ONCOGENIC_WNT_UP.V1_UP). We identified the top constituent
212 genes of both signatures, and found them to be consistently upregulated across all samples in
213 the TKI-exposed group (Fig. 4c, Table S2). Interferon gamma response has been described to be
214 associated with response to TKI treatment in non-small cell lung cancers.²⁰ Using a MWU test,
215 we observed that genes encoding the *IFNG* receptors (*IFNGR1*, *IFNGR2*) were differentially
216 expressed with statistical significance in the tumor cells of the TKI-exposed group (p-value
217 [*IFNGR1*] = 3.22e-104, p-value [*IFNGR2*] = 1.81e-13, Fig. S3). WNT signaling has also been
218 extensively studied in the context of cancer development, and increased WNT signaling has been
219 associated with tumor progression and metastasis in many different cancers.²¹ We assessed
220 potential intratumoral differential gene expression of *WNT1* in the tumor cells and found an
221 absence of intratumoral *WNT1* expression altogether. We then assessed potential *WNT1*
222 differential gene expression in specific immune cell compartments (NK cells, macrophages, and
223 T cells) and found a statistically significant differential expression of *WNT1* for the TKI-exposed
224 group within the T cell compartment (MWU test, p-value = 8.77e-18), indicating putative cross-
225 compartment communication between the T cells and tumor cells via *WNT1* signaling. To further
226 validate this, we used a MWU test to investigate possible differential gene expression of *WNT1*
227 receptors (*FZD1*, *FZD2*) in the tumor compartment and found both of the receptors to be
228 upregulated in the TKI-exposed group (p-value [*FZD1*] = 6.35e-21, p-value [*FZD2*] = 1.28e-27).
229 Of note, patients who were treated with TKI were classified with RECIST as having either PD
230 (Progressive Disease) or RD (Residual Disease), which raises the hypothesis that these patients
231 may have developed therapeutic resistance through the WNT/beta-catenin signaling pathway in
232 alignment with prior preclinical studies.²²

233

234 In addition, we estimated the stability of BEANIE to subsample size for the test signatures used
235 (Fig. S3), and found that the number of robust signatures identified persistently increased at the

236 maximum sample size, indicating the possibility that some of the signatures classified as robust
237 could have been instead classified as non-robust. This may be a result of unbalanced samples
238 per group being tested or may demonstrate the necessity of additional biological samples for the
239 clinical context being evaluated.

240

241 **Estimation of the False Positive Rate**

242 In order to estimate the chance of occurrence of incorrectly identified statistically significant and
243 robust signatures with BEANIE, we calculated the false positive rate (type I error) (see Methods,
244 Fig. 5) for all three methods (MWU test with a BH correction, GLMs, BEANIE) and clinical contexts
245 (ICB-naive vs. -exposed melanoma, early- vs. late-stage lung cancer, and TKI-naive vs. -exposed
246 lung cancer) for the signatures that had been classified by BEANIE as both statistically significant
247 and robust to sample exclusion.

248

249 We observed that across all datasets, the MWU test with a BH correction had a high average
250 false positive rate, followed by GLMs which exhibited a moderately high average false positive
251 rate. By contrast, BEANIE had the lowest average false positive rate, that in some cases also
252 approached the significance level (alpha) of 5% (Table 2). Individual false positive rates calculated
253 for all robust and statistically significant signatures can be found in Table S4.

254

255 To evaluate how a smaller number of cells being tested, and thereby reduced statistical power,
256 would impact the false positive rate for a MWU test with a BH correction and GLMs, we
257 subsampled cells from each sample being tested to a number equivalent to BEANIE's subsample
258 size and repeated the type I error estimation. We found that subsampling decreased the false

259 positive rates for both a MWU test with a BH correction and GLMs, but that their false positive
260 rates were still relatively higher than those calculated with BEANIE, corroborating BEANIE's
261 aptitude for detecting robust and true signals as compared to the other two methods, and also
262 reinforcing the need to incorporate robustness estimation into differential enrichment testing.

263

264 **Group biology estimation in immune cells**

265 While this strategy was primarily developed to overcome challenges for differential enrichment
266 testing specifically within the tumor cell compartment, we also evaluated whether the subsampling
267 and sample exclusion approach implemented within BEANIE would likewise yield biological
268 insights in immune cell compartments, as well as to further validate some of the initial hypotheses
269 from the tumor compartment analyses. As a test case, in continuation of the preliminary evaluation
270 of the CD8+ T cell compartment as described in the earlier tumor compartment analysis, we more
271 comprehensively dissected the CD8+ T cell compartment in ICB-naive vs. -exposed melanoma
272 patients in an isolated context here (Fig. S1).

273

274 We filtered out samples which had fewer than 50 CD8+ T cells, yielding a total of 1292 cells across
275 11 patients (5 ICB-naive, 6 ICB-exposed). We evaluated the potential statistically significant
276 differential enrichment and robustness to sample exclusion of various signatures representing a
277 range of CD8+ T cell subtypes and states from Oliveira et al.²³ between the ICB-naive and -
278 exposed patient groups. We found that previously reported signatures for early activated CD8+ T
279 cells (Sade-Feldman₅²⁴) and memory precursor effector CD8+ T cells (Joshi_MPEC,²⁵ murine-
280 derived) were statistically significant and robust to sample exclusion, and upregulated for the ICB-
281 naive patient group. This result substantiates the finding from the tumor compartment of the same
282 dataset, where we had identified higher IL2-STAT5 signaling, TNF activation, and inflammatory

283 response in the tumor cells from the ICB-naive group as described, which may be a result of
284 CD8+ T cell activation in the naive condition.

285

286 Therefore, in addition to the demonstrated utility for tumor cell compartments, BEANIE likewise
287 exhibited capacity for cross-compartment validations of group biology in single-cell non-tumor
288 populations as well.

289

290 **Conclusions**

291 Conventional differential enrichment methods, such as a MWU test with a BH correction and
292 GLMs, are limited in correctly estimating differential biology in clinical tumor single-cell
293 transcriptomic datasets in two aspects. First, they have an appreciably high false positive rate,
294 which can be attributed in part to an increased power of statistical tests (due to high cell numbers)
295 to detect differences between groups. However, increased power does not necessarily signify a
296 biologically relevant difference. Consequently, interpretation of these differences in a group
297 biology context is requisite to correctly distinguish genuine group biological differences from
298 technical artifacts (such as variation in cell numbers). We also observed that subsampling alone
299 is insufficient to tackle this problem, and it is important to use a background distribution for
300 contextualisation. Second, conventional differential enrichment methods do not assess the
301 robustness of a signature to sample exclusion, and as a consequence, these methods may lead
302 to results being sample-driven and of uncertain translational importance. This issue is particularly
303 relevant in clinical contexts, and especially for tumor cell compartments which demonstrate higher
304 intra-patient similarity than inter-patient similarity, as hypotheses based on group comparison
305 (about treatment effects, disease progression, etc.) may impact future clinical trials.

306

307 To address the shortcomings of conventional differential enrichment methods, we developed
308 BEANIE, a nonparametric statistical method for estimating group biology in clinical single-cell
309 transcriptomic datasets. We demonstrated its application on publicly available datasets from six
310 clinical single-cell transcriptomic studies, and illustrate its aptitude to detect statistically significant
311 and robust gene signatures as compared to conventional methods, through its low false positive
312 rate as compared to its counterparts (MWU test followed by a BH correction and GLMs). We
313 illustrated its extensive application in the tumor compartment, and its potential utility for the
314 immune compartment as well. It may likewise be used to identify differential enrichment of gene
315 signatures in the stromal compartment. Finally, we demonstrated that BEANIE is adept at
316 distinguishing sample-driven signatures from group-driven signatures, whereas conventional
317 differential enrichment methods fail to do so. Alternate models for representation of tumor single-
318 cell data include hierarchical linear models; however, unlike BEANIE, they are parametric and
319 therefore assume normality and homogeneity of variance for the data.

320

321 Despite its potential to estimate group biology and pinpoint both statistically significant as well as
322 robust and therefore prospective biologically relevant signatures in single-cell transcriptomic
323 dissections, BEANIE also possesses a few limitations. First, in spite of its demonstrated value in
324 single-cell transcriptomic tumor compartment analyses, BEANIE's widespread applicability in the
325 immune compartment may be limited, in part due to an absence of comprehensive databases
326 with precise and rigorous signatures representing discrete cell types, states, and pathways. In
327 fact, the ultimate utility of BEANIE's or any group biology analysis tool's framework is in part
328 contingent on the quality of the gene signatures being tested, including for the tumor
329 compartment. Moreover, there also exists scope to further improve the false positive rate within
330 the BEANIE method. In addition, we do not currently have an understanding of why some patient

331 samples are more prone to contribute to the non-robustness of certain signatures as compared
332 to other patient samples, and having additional clinical information (e.g., mutational status) could
333 potentially help delineate some of the biology behind this. Lastly, despite the ability to estimate
334 group biology and identify statistically significant and robust signatures between patient groups
335 with BEANIE, current clinical single-cell transcriptomic datasets have an overall small sample
336 size, which indicates that they are likely not an adequate representation of the broader population
337 and hence could lead to introduction of false negatives (type II error). Therefore, in general, larger
338 datasets, such as those generated via consortium efforts, are needed to improve our ability to
339 draw robust conclusions, and minimise putative false negatives. Broadly, dedicated efforts to
340 analyze larger clinically integrated single-cell cohorts that reflect the diverse clinical and
341 therapeutic contexts across cancer types will accelerate our understanding of the cell states that
342 promote treatment resistance for translational discovery.

343

344 **Methods**

345 **Data Preprocessing**

346 **Melanoma Dataset**

347 We selected cells which were labelled as malignant (authors made use of inferCNV²⁸ to identify
348 malignant cells).

349

350 **Lung Cancer Datasets**

351 Owing to the variability in collected datasets from the four studies (Kim et al.¹⁰, Maynard et al.¹¹,
352 Qian et al.¹², and Lambrechts et al.²), we carefully assessed the metadata files available. For

353 Lambrechts et al., we reached out to the authors to acquire their Seurat object containing patient
354 ID and cell ID labelling. We used the following criteria for the selection of cells for analysis: (i)
355 must be of epithelial origin; (ii) must be identified as malignant by the authors (all studies made
356 use of inferCNV²⁸ to identify malignant cells); and (iii) must be isolated from the primary site (i.e.,
357 lung). We also removed cells from patients that had locally advanced lung cancer (stage III
358 tumors), as they are more difficult to classify into early- versus late-stage.²⁶

359

360 **BEANIE's Workflow**

361 **Preprocessing and Normalisation**

362 The raw counts matrix is normalised by the library size and converted to counts per million (CPM
363 normalization) to account for differences in library sizes of different cells. Pre-normalised matrices
364 may also be used, in which case this step is ignored. Genes with no expression across all cells
365 are excluded.

366

367 **Signature Scoring**

368 For each cell c_i in the normalised counts matrix, signature scoring is performed for the set of gene
369 signatures provided as input by the user (test signatures). The default signature scoring method
370 is adapted from AUCell.²⁷

371

372 (i) For each gene g_k , the cells are ranked by calculating the percentile of each cell across the gene
373 g_k in terms of normalised expression of the gene, i.e., cells with higher expression values of that
374 particular gene will have a higher percentile. The ties are randomly broken (i.e. if two cells have

375 the exact same expression of the gene, which is common in single-cell datasets, those cells are
376 randomly assigned a percentile value).

377
$$P_{c_i, g_k} = \frac{100 \times n_{c_i, g_k}}{n_c}$$

378 where n_{c_i, g_k} = ordinal rank of c_i for expression of g_k (sorted from smallest to largest),

379 P_{c_i, g_k} = percentile of c_i for expression of g_k ,

380 and n_c is the total number of cells

381

382 (ii) Next, for every cell c_i , genes are ranked based on their calculated percentile values across
383 that cell. Genes which have a higher percentile across the cell are given lower ranks. This scoring
384 system takes into account the importance of each gene in a given cell relative to that gene's
385 importance in other cells, i.e., genes which have a lower rank are more important for the cell in
386 question as compared to genes with a higher rank.

387
$$P'_{g_k, c_i} = \frac{100 \times n'_{g_k, c_i}}{n_g}$$

388 where n'_{g_k, c_i} = ordinal rank of g_k for P_{c_i, g_k} values (sorted from largest to smallest),

389 P'_{g_k, c_i} = percentile of g_k for P_{c_i, g_k} values,

390 and n_g is the total number of genes for the cell c_i

391

392 (iii) For each gene signature S_j , a recovery curve per cell c_i is generated by calculating the
393 enrichment of the top constituent genes ranked from S_j , followed by a calculation of the Area

394 Under the Curve (AUC), which measures the expression of c_i 's top constituent genes ranked
395 from S_j . The AUC is therefore the score of the cell for S_j .

396
$$\text{AUC}_{j,i} = \int f_i(x) dx, f_i(x) = n(S_j \cap R_{x,i})$$

397 where S_j = set of genes comprising a gene signature

398 and $R_{x,i}$ = set of top constituent x genes based on P'_{g_k, c_i}

399

400 Other signature scoring methods available in BEANIE include weighted mean and z-scoring.

401

402 **Background Distribution Generation**

403 A background distribution is generated for the biological interpretability of the results as follows:

404 (i) Bins are created based on the gene set size of each signature S_j (default bin size = 10, tunable

405 parameter). (ii) Random signatures (r_signatures) (R_k , $k = 1, 2, \dots, n_b$, where n_b = total number

406 of bins) for each of the bin sizes are generated such that they are representative of both lowly

407 expressed and highly expressed genes. For this step, the normalised matrix is used and the genes

408 are sorted based on their expression values across all samples. Equal numbers of genes from

409 every 20th percentile are then randomly subsampled such that the sum of all genes equals the bin

410 size. This random sampling is repeated multiple times to generate different random signatures

411 (R_{kl} , $l = 1, 2, \dots, n_r$, where n_r = the total number of times subsampling is repeated). The rationale

412 for generating the random signatures is that they should not represent any biologically meaningful

413 gene signature, and as a consequence, their differential expression can be used as a null

414 distribution (background distribution) for interpretation of the results in a biological context. (iii)

415 Each cell c_i is scored for R_{kl} 's using the aforementioned signature scoring method.

416

417 **Folds and Subsampling**

418 To accomplish BEANIE's two-fold aim of having equal sample representation and quantifying
419 robustness for S_j s, two statistical techniques, Monte Carlo approximations (subsampling) and
420 leave-one-out cross-validation (sample exclusion), are coupled. First, the data is divided into folds
421 (f_q , $q = 1, 2, \dots, n_p$, where $n_p =$ number of samples), with each fold f_q representing the exclusion of
422 one sample from either group. For each fold f_q , cells are subsampled such that each sample is
423 represented by an approximately equal number of cells. This is done by first subsampling an
424 equal number of cells from all samples, followed by additional subsampling in the sample-
425 excluded group to compensate for the cells that would have otherwise been subsampled from the
426 excluded sample. The additional subsampling ensures that the total number of cells subsampled
427 from the two groups being tested always remains constant regardless of which group the excluded
428 sample belongs to, which is necessary to ensure that the folds are comparable with each other.
429 The subsampling is then repeated multiple times to establish adequate representation of each
430 patient sample.

431

432 **Identification of Differentially Enriched Signatures**

433 A multi-step strategy is adopted to identify differentially enriched signatures. First, for each
434 subsample belonging to the fold f_q , a MWU test is performed between the two groups for every
435 S_j . Additionally, for each fold f_q , a null p-value distribution is generated by a MWU test between
436 the two groups for every R_{kl} . The null distribution generated is fold-specific to ensure that the
437 sample excluded from the fold is also excluded for the generation of the null distribution. The
438 percentile of the subsample's p-value against the null p-value distribution is then calculated,
439 hereafter referred to as the empirical p-value. A median empirical p-value is calculated for these

440 subsamples to represent the p-value for a given fold, followed by a median across all folds to
441 represent the cell's p-value. To quantify the robustness of S_j to sample exclusion, a ratio
442 (henceforth referred to as the Fold Rejection Ratio (FRR)) is defined, and calculated for every fold
443 f_q .

$$444 \quad \text{FRR}_q = \begin{cases} \frac{n(F_q, p \leq 0.05)}{n(F_0, p \leq 0.05)} & \text{if, } n(F_0, p \leq 0.05) \neq 0 \\ 0 & \text{if, } n(F_0, p \leq 0.05) = 0 \end{cases}$$

445 where F_q = set of subsamples for the fold f_q which have an empirical p-value ≤ 0.05

446 and F_0 = set of subsamples for the fold f_0 (when no sample is excluded) which have an
447 empirical p-value ≤ 0.05

448

449 A FRR value closer to 1 indicates that exclusion of the sample has no effect on the empirical
450 significance of the gene signature S_j , and a lower value indicates the opposite. We use a threshold
451 of 0.9 (hyperparameter) to call signatures as robust or not, i.e., if the FRR for a particular S_j is
452 greater than 0.9 for *all* folds, then the gene signature is considered robust to sample exclusion.

453

454 **Gene Ranking**

455 For every gene signature S_j , the genes are then ranked for the robustness of their log₂ fold change
456 between the two groups. This is particularly useful for larger gene sets. For every sample, a mean
457 gene expression (MGE) is calculated for every gene using the normalised counts. A similar
458 strategy of subsampling coupled with sample exclusion is used for ranking. The MGE matrix is
459 then divided into folds, with each fold representing the exclusion of one sample. A log₂ fold
460 change is then calculated for each fold, and the standard deviation, along with the mean across

461 folds, is also calculated. Genes with both outlier MGE values and outlier log₂ fold changes (i.e.,
462 MGE values and log₂ fold changes more than 1.5 times the interquartile range above the third
463 quartile or below the first quartile) are classified as non-robust to sample exclusion. The final
464 ranking of genes is performed based on decreasing log₂ fold change, increasing standard
465 deviation, and robustness status.

466

467 **Mann-Whitney U tests**

468 Mann-Whitney U (MWU) tests followed by Benjamini-Hochberg (BH) correction are performed for
469 the calculation of p-values. The Python package *scipy* is used for the MWU p-value calculation
470 and the function *multipletests* from the Python package *statsmodels* is used for the BH correction.

471 **Generalised Linear Models**

472 Generalised linear models (GLMs) with a binomial distribution link function are used for calculation
473 of p-values. The Python package *statsmodels* is used to implement this method. The signature
474 scores are used as covariates (exog variable), and the group labels (e.g., treatment-naive or -
475 exposed and early-stage or late-stage) as the response variable to be modelled (exog variable).

476 **Calculation of False Positive Rate (Type I Error)**

477 False positive rate (type I error) refers to the probability of detecting a result by chance. To
478 calculate this, we permute the patient ID and group label in such a way that roughly equal numbers
479 of samples from the original group labels are placed in both comparison groups. We then repeat
480 the BEANIE workflow on these permuted datasets for signatures which are classified by BEANIE
481 as statistically significant and robust to sample exclusion in the original dataset to evaluate the
482 type I error rates for our predictions. In addition, we also run a MWU test followed by a BH

483 correction and GLMs for these signatures to compare the type I error rates across the three
484 methods. Finally, to investigate whether Monte Carlo subsampling (with equivalent statistical
485 power to that of BEANIE's workflow) would affect the false positive rate. For this, we subsample
486 a random set of cells equal to the number of cells subsampled in the BEANIE workflow and repeat
487 the MWU test and GLM methods.

488 For the ICB-naive vs. ICB-exposed melanoma dataset (14 samples, 7 in each group) and early-
489 stage vs. late-stage lung cancer dataset (17 samples, 11 early-stage and 6 late-stage), we ran
490 1000 simulations per gene signature with the above workflow to estimate the false positive rate.
491 For the TKI-naive vs. TKI-exposed lung cancer dataset (10 samples, 6 TKI-naive and 4 TKI-
492 exposed), we ran 100 simulations per gene signature (due to limited combinations of
493 equidistributed samples per group possible).

494

495 **Data Availability**

496 All datasets used in the study are publicly available. Hallmark and Oncogenic gene sets are
497 available for download from MSigDb.

498

499 **Code Availability**

500 Code is publicly available as a downloadable Python package from:

501 <https://github.com/sjohri20/beanie>.

502

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526

527 N.I.V. serves on advisory boards of Sanofi and Oncocyte.

528

529 **Author Contributions**

530 M.X.H. and E.M.V.A. conceived the original idea. S.J. developed the idea further, designed
531 experiments, performed the analyses, and developed the Python package for the presented
532 method. K.B., B.M.T., J.F. and M.X.H. helped in development of the method from a single-cell
533 perspective. M.X.H., J.C. and D.L. provided input from a statistical perspective. J.P.C. provided
534 input for the development of visualisation modules in the package. N.I.V. and D.L. helped in
535 clinical interpretation of the results. Z.F., J.P., L.F., D.L., and E.M.V.A. contributed to the overall
536 analyses. S.J., B.M.T., K.B., and E.M.V.A. wrote the manuscript. All authors reviewed and
537 approved the final manuscript.

538

539 **Competing Interests statement**

540 The authors declare no competing interests.

541

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544 Checkpoint Blockade. *Cell* **175**, 984–997.e24 (2018).

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604

605

606 **Figure Legends**

607 **Main Figures**

608 Figure 1. Overview of the BEANIE method.

609 A. Overall workflow: A counts matrix, sample IDs, group IDs, and a list of signatures for which
610 differential enrichment will be tested (test signatures, $t_signatures$) are provided as user input.
611 Based on the gene set size, test signatures are first divided into bins. For each bin size, a list of
612 random signatures ($r_signatures$) of the same gene set size is generated, to be later used for p-
613 value calculation and biological interpretation. Signature scoring per cell is performed for both
614 random signatures and test signatures, followed by differential enrichment testing.

615 B. Differential enrichment testing workflow: The differential enrichment testing algorithm is
616 based on a combination of Monte Carlo approximation of empirical p-value through
617 subsampling, and leave-one-out cross validation through sample exclusion. The data is first
618 divided into folds, where each fold f_q represents the exclusion of a sample from either of the
619 comparison groups. This is followed by the subsampling step, where an equal number of cells
620 are subsampled from every sample to ensure equal patient representation. Next, a Mann-
621 Whitney U test is performed per subsample for all folds, for both the test signatures and the
622 background distribution (generated from the random signatures). The test signatures are then
623 matched to their corresponding background distribution based on bin size, and an empirical p-
624 value (percentile of the test distribution's median with respect to the background distribution) is
625 calculated per test signature for every fold f_q . Additionally, a Fold Rejection Ratio (FRR) (see
626 Methods) is calculated per test signature for every fold, and is used to determine the overall
627 robustness of the test signature to sample exclusion.

628

629 Figure 2. Group biology analysis of the tumor compartment from ICB-naive vs. ICB-exposed
630 melanoma patient samples.

631 A. Bar plot displaying the $\log(\text{empirical p-value})$ for all of the signatures identified as statistically
632 significant (empirical p-value ≤ 0.05), along with their robustness status. A '****' above a bar
633 indicates that the empirical p-value for that test signature was below the minimum empirical p-
634 value measured.

635 B. Venn diagram quantifying the intersection of signatures identified as differentially enriched with
636 statistical significance by the three methods (MWU test with a BH correction, GLMs, and BEANIE).

637 C. Plot depicting the signatures identified as statistically significant but non-robust to sample
638 exclusion by BEANIE, the distribution of their Fold Rejection Ratios (FRRs), and the sample IDs
639 having FRRs less than the threshold used to determine robustness, along with a horizontal bar
640 plot of the number of statistically significant but non-robust signatures (dropout signatures) per
641 sample.

642 D. Histogram illustrating the sample exclusion procedure implemented within BEANIE shifting the
643 test distribution to the right such that it overlaps with the background distribution, leading to the
644 fold's empirical p-value being greater than 0.05.

645 E. Heatmap revealing the differential top constituent genes (ranked according to \log_2 fold change
646 and robustness) from three of the statistically significant and robust signatures identified by
647 BEANIE across all patients from the ICB-naive group.

648 F. Joint scatter and density plot demonstrating a positive correlation between the
649 HALLMARK_IL6_JAK_STAT3_SIGNALING signature score and STAT3 gene expression in
650 individual cells.

651 G. Plot illustrating BEANIE's stability to subsample size for the test signatures used. The curve
652 plateaued as the number of statistically significant test signatures, irrespective of robustness
653 status, reached saturation as the subsample size approached 60 (the max subsample size
654 possible within the constraints of this dataset [see Methods]), whereas the curve plateaued
655 around the subsample size of 30 as the number of signatures identified as both statistically
656 significant and robust reached saturation.

657

658 Figure 3. Group biology analysis for early- vs. late-stage non-small cell lung cancer.

659 A. Overview of the integrated dataset from four studies and a bar plot depicting the number of
660 tumor cells per patient sample.

661 B. Bar plot displaying the log(empirical p-value) for all of the signatures identified as statistically
662 significant (empirical p-value ≤ 0.05), along with their robustness status.

663 C. Venn diagram quantifying the intersection of signatures identified as differentially enriched with
664 statistical significance by the three methods (MWU test with a BH correction, GLMs, and BEANIE).

665 D. Plot depicting the signatures identified as statistically significant but non-robust to sample
666 exclusion by BEANIE, the distribution of their FRRs, and the patient IDs having FRRs less than
667 the threshold used to determine robustness, along with a horizontal bar plot of the number of
668 statistically significant but non-robust signatures (dropout signatures) per sample.

669

670 Figure 4. Group biology analysis for TKI-naive vs. TKI-exposed non-small cell lung cancer.

671 A. Venn diagram quantifying the intersection of signatures identified as differentially enriched with
672 statistical significance by the three methods (MWU test with a BH correction, GLMs, and BEANIE).

673 B. Bar plot displaying the $\log(\text{empirical p-value})$ for all of the signatures identified as statistically
674 significant (empirical p-value ≤ 0.05) along with their robustness status. A ‘***’ above a bar
675 indicates that the empirical p-value for that test signature was below the minimum empirical p-
676 value measured.

677 C. Heatmap showing the differential top constituent genes (ranked according to \log_2 fold change
678 and robustness) from the HALLMARK_INTERFERON_GAMMA_RESPONSE and
679 ONCOGENIC_WNT_UP.V1_UP signatures across all samples from the TKI-exposed group.

680

681 Figure 5. False positive rate (type I error) for the three methods: BEANIE, GLMs, and a MWU
682 test followed by a BH correction.

683 The false positive rate for signatures (from Hallmark and Oncogenic gene sets) which were
684 classified as statistically significant and robust to sample exclusion by BEANIE for:

685 A. ICB-naive vs. ICB-exposed melanoma dataset,

686 B. Early-stage vs. Late-stage lung cancer dataset, and

687 C. TKI-naive vs. TKI-exposed lung cancer dataset

688 The dashed pink line denotes the 5% error mark.

689

690 **Supplementary figures**

691 Figure S1. Distribution of cells from ICB-naive vs. ICB-exposed melanoma patient samples.

692 A. Distribution of tumor cells.

693 B. Distribution of CD8+ T cells.

694

695 Figure S2. Heatmap displaying the differential top constituent genes (ranked according to log₂
696 fold change and robustness) from the HALLMARK_MITOTIC_SPINDLE and
697 HALLMARK_GLYCOLYSIS gene signatures in the early- vs. late-stage lung cancer dataset.

698

699 Figure S3. Extended group biology analysis of the tumor compartment from TKI-naive vs. TKI-
700 exposed lung cancer patient samples.

701 A. Distribution of tumor cells in the TKI-naive vs. -exposed lung cancer samples.

702 B. Boxplot illustrating statistically significant differential expression of the genes IFNGR1 and
703 IFNGR2 between TKI-naive vs. TKI-exposed samples.

704 C. Line plot illustrating BEANIE's stability to subsample size for TKI-naive vs. -exposed lung
705 cancer samples. The curve plateaued as the number of signatures identified as statistically
706 significant, irrespective of robustness status, reached saturation as the subsample size
707 approached 100.

708 D. Plot depicting the test signatures identified as statistically significant but non-robust to sample
709 exclusion by BEANIE for TKI-naive vs. TKI-exposed samples, the distribution of their FRRs, and
710 the patient IDs having FRRs less than threshold used to determine robustness, along with a
711 horizontal bar plot of the number of statistically significant but non-robust signatures (dropout
712 signatures) per sample.

713

714 **Supplementary Tables**

715 Table S1: Hallmark and Oncogenic gene set results for MWU test + BH correction, GLMs, and
716 BEANIE, for all datasets (ICB-naive vs. -exposed melanoma, early- vs. late-stage lung cancer,
717 and TKI-naive vs. -exposed lung cancer).

718

719 Table S2: Top genes for Hallmark and Oncogenic gene sets for all datasets (ICB-naive vs. -
720 exposed melanoma, early- vs. late-stage lung cancer, and TKI-naive vs. -exposed lung cancer).

721

722 Table S3: Noise estimation p-values for the three methods (MWU test with a BH correction, GLMs,
723 and BEANIE).

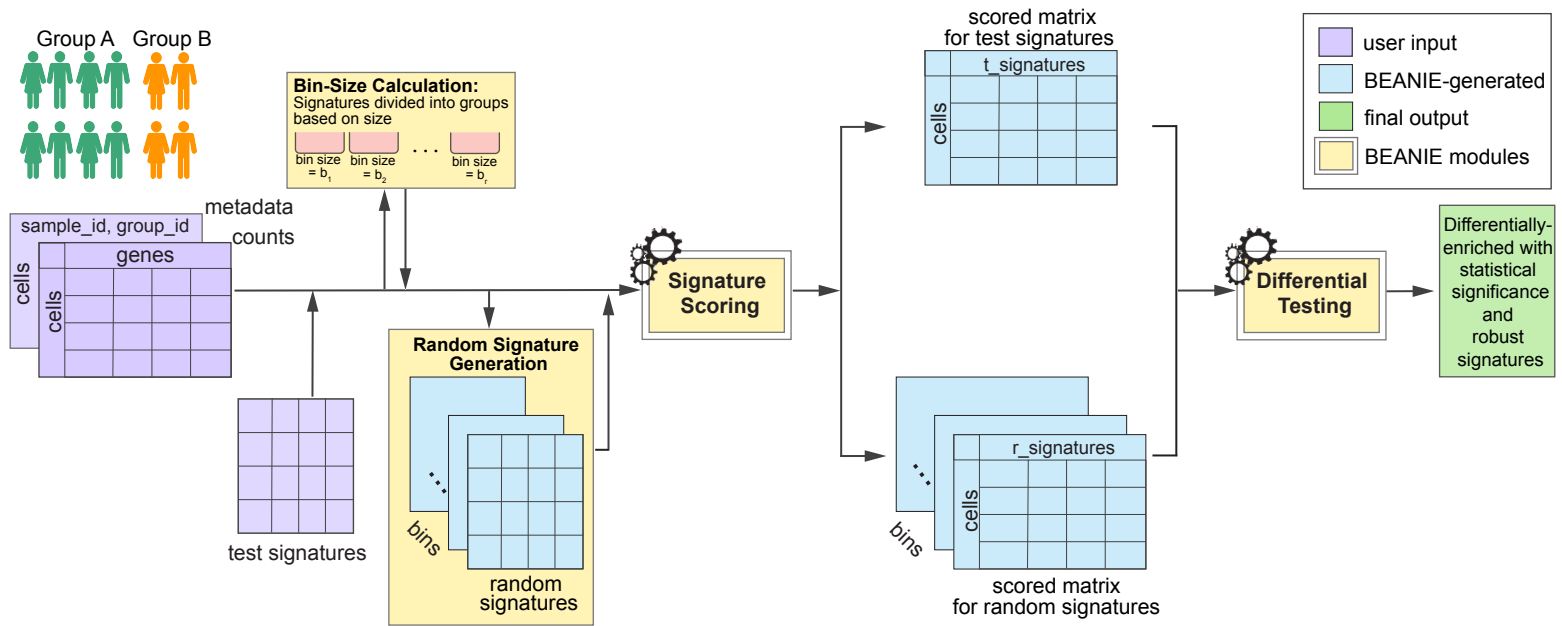
724

725 Table S4: False positive rate (in percentage) for statistically significant and robust signatures
726 identified by BEANIE for the Hallmark and Oncogenic gene sets for all datasets (ICB-naive vs. -
727 exposed melanoma, early- vs. late-stage lung cancer, and TKI-naive vs. -exposed lung cancer).

728

Figure 1.

A.



B.

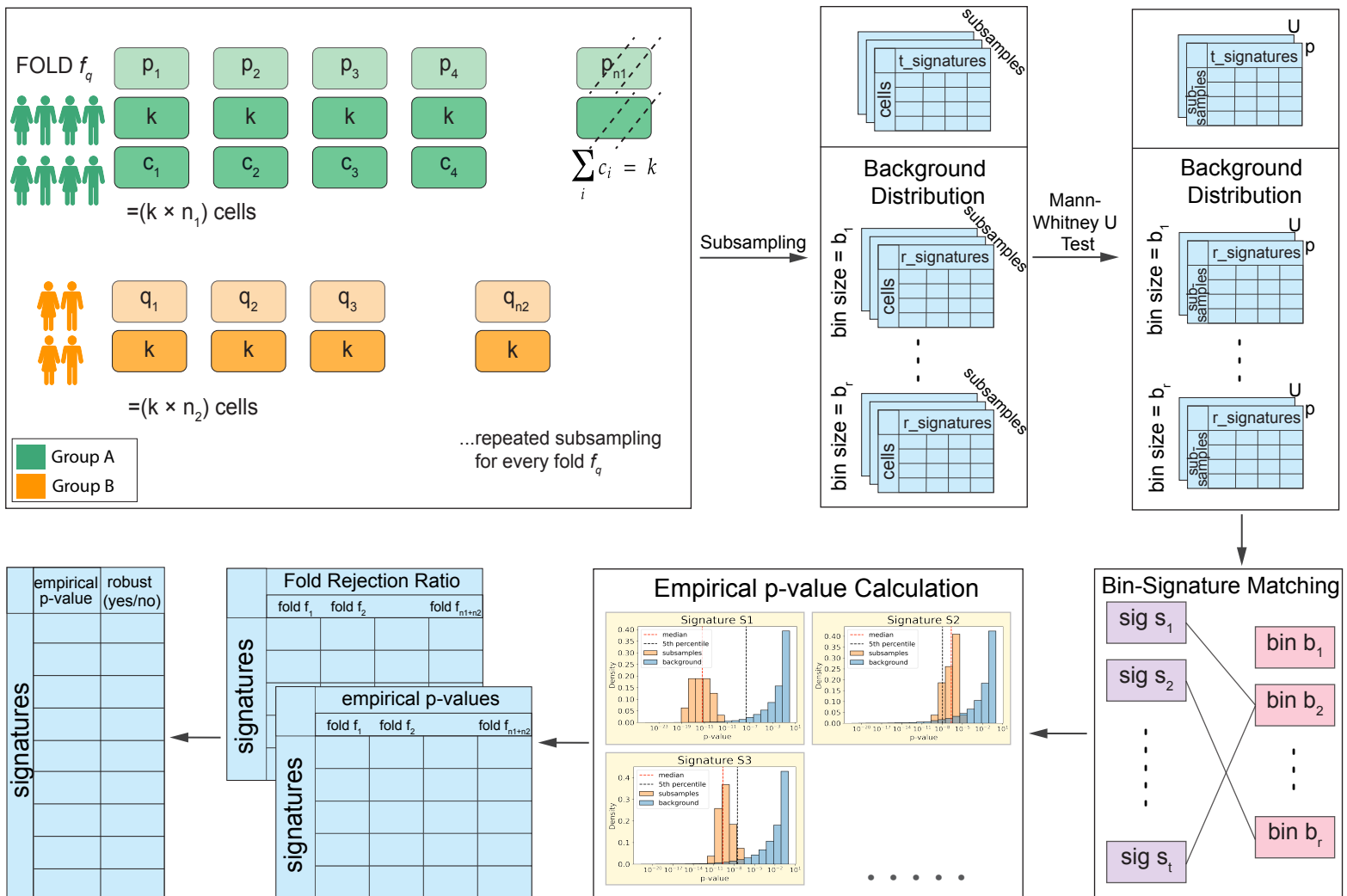


Figure 2.

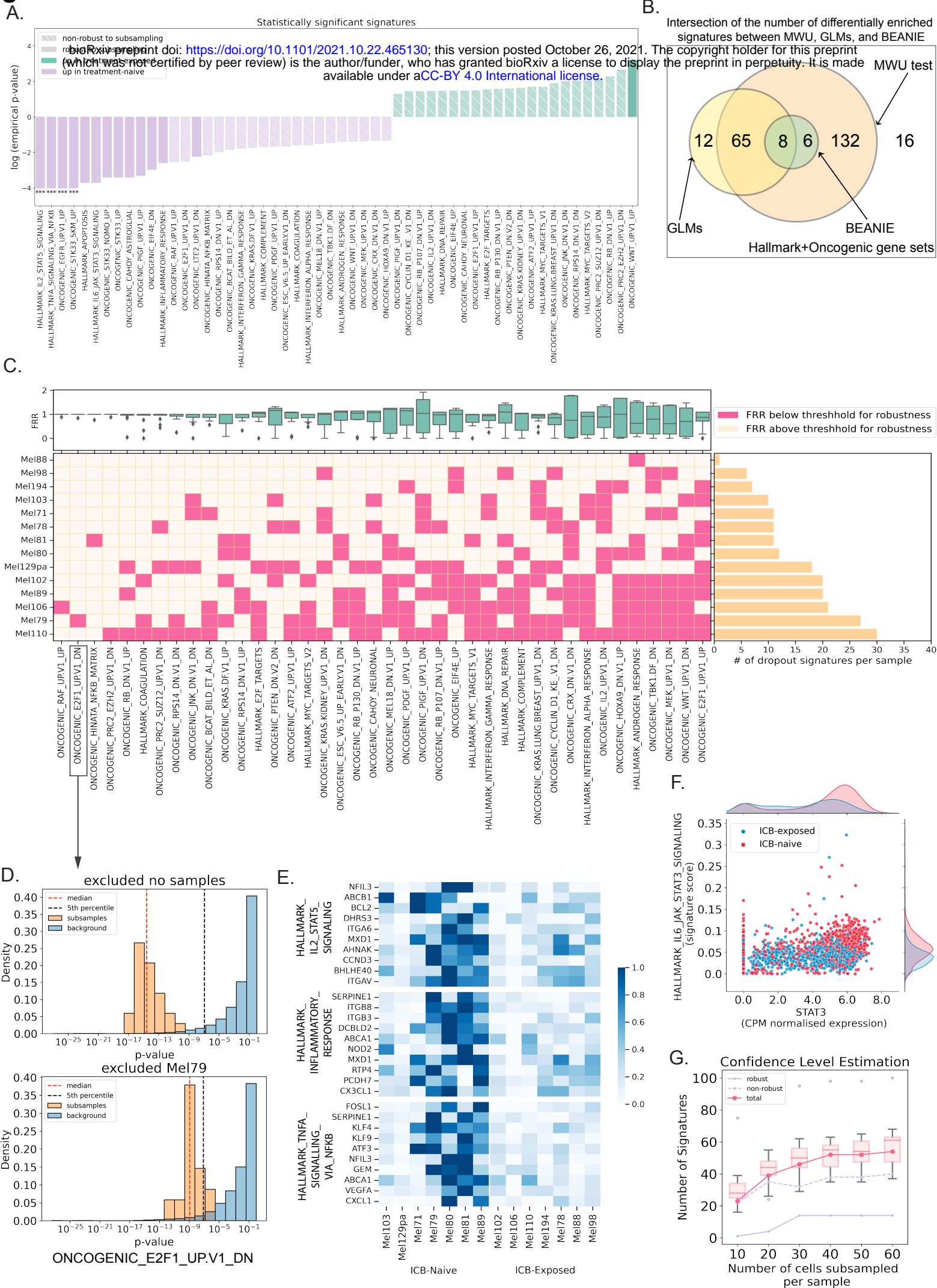
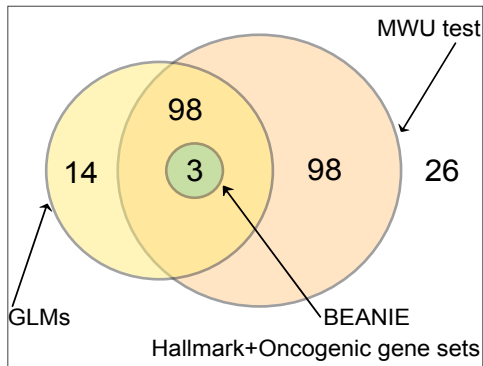
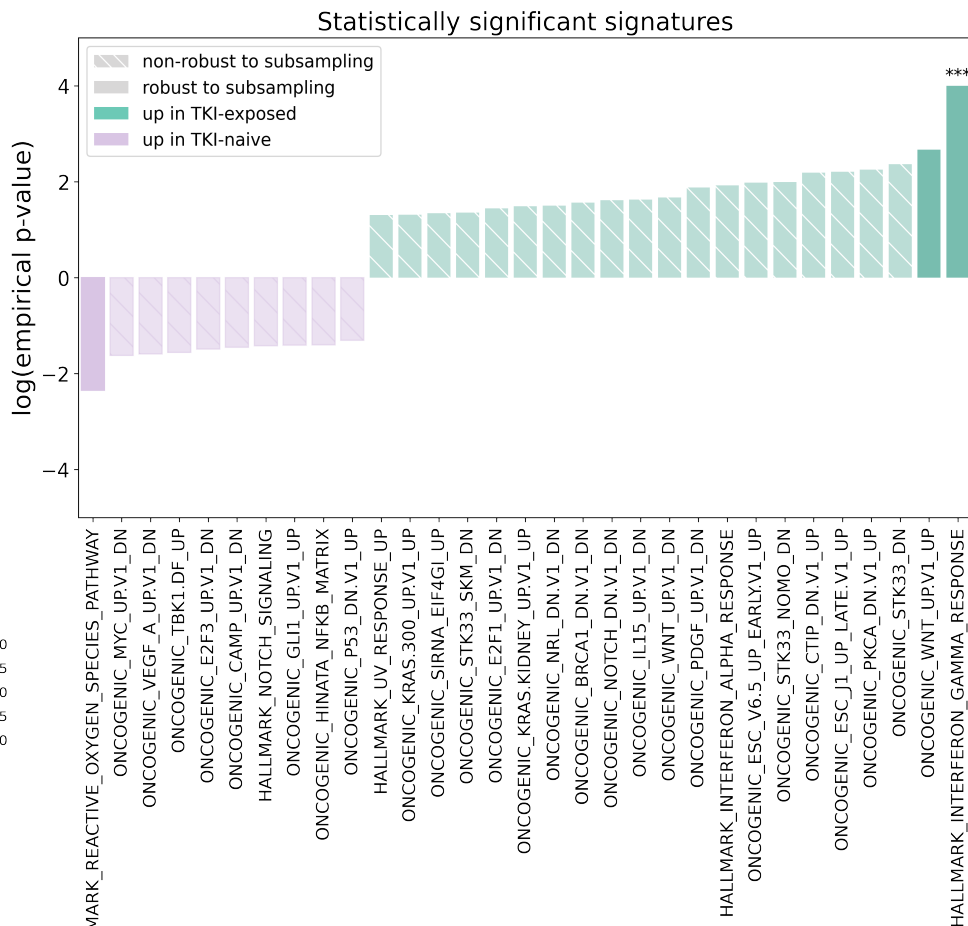


Figure 4.

A. Intersection of the number of differentially enriched signatures between MWU, GLMs, and BEANIE



B.



C.

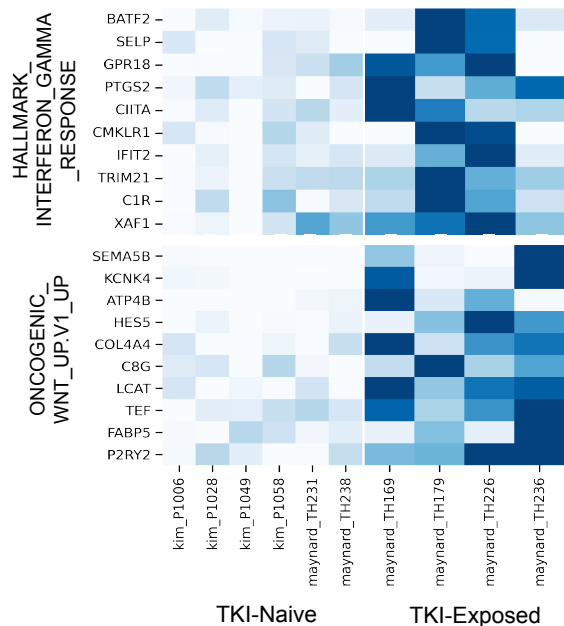


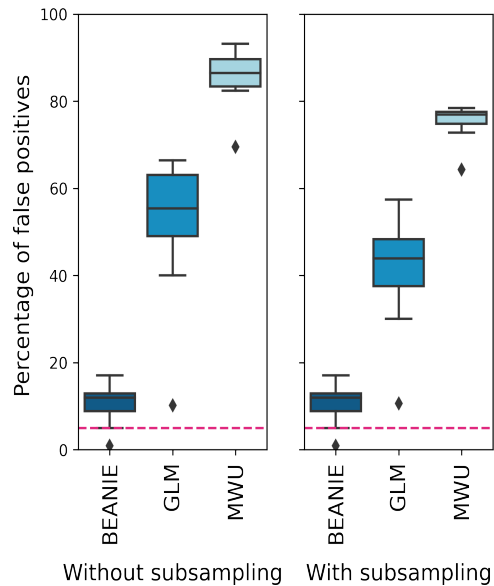
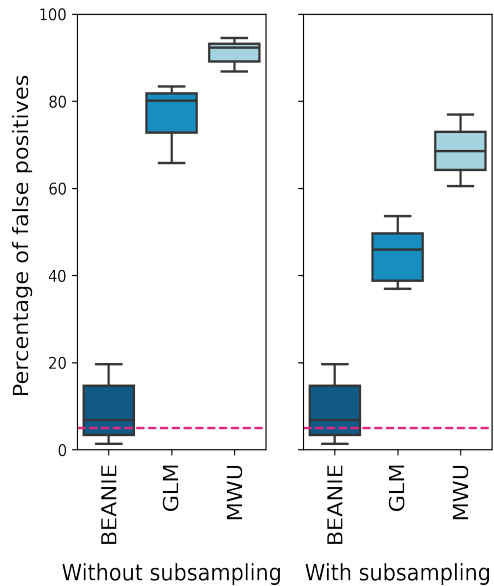
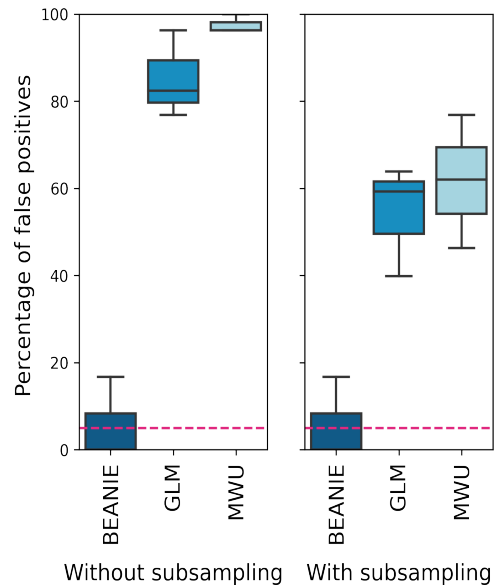
Figure 5.**A. ICB-naive vs. ICB-exposed melanoma****B. Early-stage vs. Late-stage lung cancer****C. TKI-naive vs. TKI-exposed lung cancer**

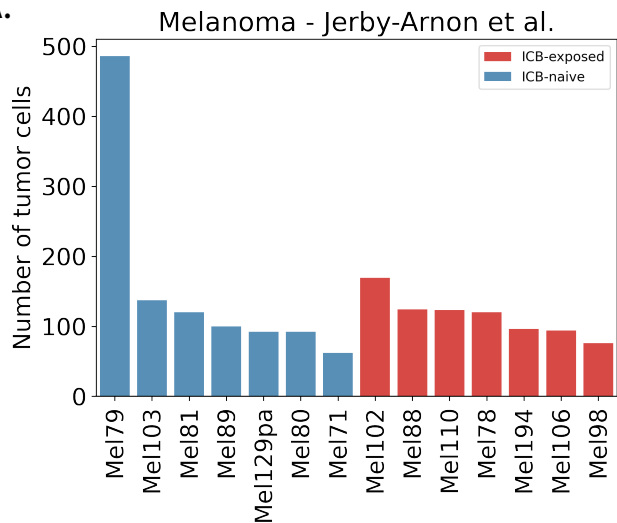
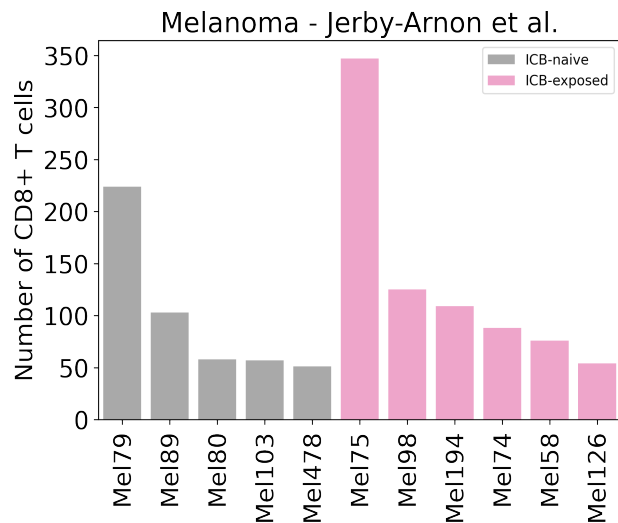
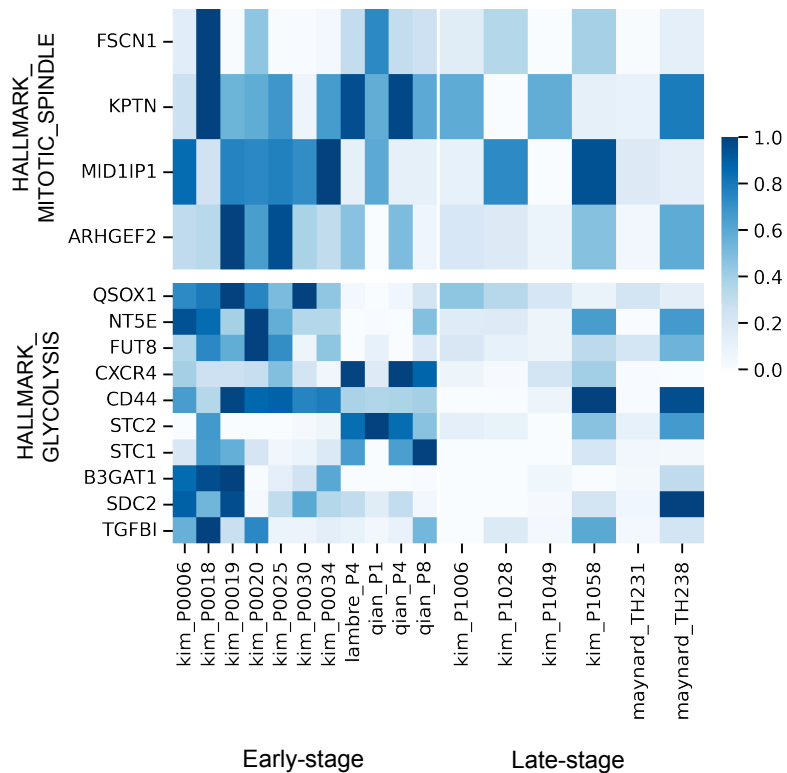
Figure S1.**A.****B.**

Figure S2.



729 **Tables**

		MWU test	GLM	BEANIE
Melanoma (Immune Checkpoint Blockade (ICB)-naive vs. ICB-exposed)	Hallmark gene sets	45/50	27/50	5/50
	Oncogenic gene sets	166/189	58/189	9/189
Lung (Early-stage vs. Late-stage)	Hallmark gene sets	46/50	44/50	3/50
	Oncogenic gene sets	168/189	128/189	3/189
Lung (Tyrosine Kinase Inhibitor (TKI) - naive vs. TKI-exposed)	Hallmark gene sets	47/50	33/50	2/50
	Oncogenic gene sets	152/189	82/189	1/189

730 **Table 1. Number of differentially enriched signatures identified with the three methods**

731 **(MWU test, GLMs, and BEANIE) using Hallmark (n = 50) and Oncogenic (n = 189) gene sets.**

732

733

	MWU test + BH correction		GLMs		BEANIE
	Without subsampling	With subsampling	Without subsampling	With subsampling	
ICB-naive vs. ICB-exposed	85.74%	75.57%	52.58%	41.72%	10.63%
Early-stage vs. Late-stage	91.23%	68.6%	77.03%	44.93%	9.01%
TKI-naive vs. TKI-exposed	97.53%	61.72%	85.18%	54.32%	5.55%

734

735 ***Table 2. Average false positive rate for the three datasets (ICB-naive vs. ICB-exposed***
736 ***melanoma, early-stage vs. late-stage lung cancer, and TKI-naive vs. TKI-exposed lung***
737 ***cancer) across the three methods (MWU test with a BH correction, GLMs, and BEANIE).***

738