

Automated classification of estrous stage in rodents using deep learning

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1 ABSTRACT

2

3 The rodent estrous cycle modulates a range of biological functions, from gene expression to

4 behavior. The cycle is typically divided into four stages, each characterized by distinct hormone

5 concentration profiles. Given the difficulty of repeatedly sampling plasma steroid hormones from

6 rodents, the primary method for classifying estrous stage is by identifying vaginal epithelial cell

7 types. However, manual classification of epithelial cell samples is time-intensive and variable,

8 even amongst expert investigators. Here, we use a deep learning approach to achieve

9 classification accuracy at expert levels in a matter of seconds. Due to the heterogeneity and

10 breadth of our input dataset, our deep learning approach (“EstrousNet”) is highly generalizable

11 across rodent species, stains, and subjects. The EstrousNet algorithm exploits the temporal

12 dimension of the hormonal cycle by fitting classifications to an archetypal estrous cycle,

13 highlighting possible misclassifications and flagging anestrus phases (e.g., pseudopregnancy).

14 EstrousNet allows for rapid estrous cycle staging, improving the ability of investigators to

15 consider endocrine state in their rodent studies.

16

17

18 INTRODUCTION

19

20 With the broad incorporation of female animals into previously all-male studies^{1,2} we are at a

21 critical juncture for the interpretation of endocrine physiology. In naturally cycling humans, the

22 menstrual cycle lasts 28 days and is characterized by defined peaks in steroid hormones such

23 as estradiol and progesterone^{3–9}. In female rats and mice, the analogous estrous cycle lasts 4-5

24 days¹⁰, but exhibits steroid hormone fluctuations similar to the 28 day human menstrual cycle.

25 The estrous cycle was first described over a century ago¹¹, yet the criteria for tracking this cycle

26 remain subjective and variable between experimenters¹². Determining the stage of estrous is

27 critical to evaluating the state of the hypothalamic-pituitary-ovarian axis, which has implications

28 in a myriad of factors including gene expression^{13,14}, neuronal structure and connectivity^{3,15}, and

29 pharmacological efficacy¹⁶. In addition, correct interpretation of estrous stage is useful for timed

30 pregnancy in rodents and changes in cycle regularity can be used as a proxy for changes in

31 other critical hormones such as corticosterone^{17,18}.

32 The estrous cycle can be divided into four stages: diestrus, proestrus, estrus, and

33 metestrus^{19–23}. While techniques such as vaginal opening evaluation, vaginal wall impedance,

34 and urine biochemistry have all been used as methods for determining estrous stage²⁰,

35 epithelial cell cytology remains the most common and reliable strategy^{6,9,10,13}. Classification

36 using vaginal cytology is typically performed by manually counting or estimating the relative

37 prevalence of epithelial cell types, including leukocytes, cornified epithelial, and nucleated

38 epithelial cells, and using the proportionality of these subtypes to determine stage^{10,19}.

39 Despite the prevalence of this method, there are several limitations of epithelial cell

40 cytology for estrous stage classification: 1) it is time consuming and requires extensive training.

41 2) it lacks generalizability; even expert classifiers may have trouble generalizing across rodent

42 species, stains, and subjects. 3) it is inconsistent between labs, as classification can vary widely

43 between human examiners¹². Here, we address these challenges using a novel deep learning
44 algorithm that can generate estrous stage classifications on the order of seconds.

45 Convolutional neural networks (CNNs) have outperformed human experts in diagnosing
46 retinal disease²⁴, skin cancer²⁵, syndromic genetic diseases²⁶, and a host of other medical
47 conditions²⁷. These networks are broadly useful for their speed and reliability. Although CNNs
48 are difficult to train from scratch, requiring massive training data sets for accurate classification,
49 transfer learning can exploit the multilayered architectures of pretrained networks to classify
50 complex biological images^{28,29}.

51 Here, we have compiled a large-scale multi-laboratory dataset of cytology images
52 ("EstrousBank"). We then used EstrousBank to train a deep learning algorithm ("EstrousNet") to
53 effectively recognize structural markers of the estrous cycle in a manner generalizable across
54 subjects, stains, and rodent species. The resulting classifications are not significantly different
55 than expert human examiners in any stage surveyed. The predictions generated by EstrousNet
56 can be enhanced by using sequentially collected data to fit cytological samples with an
57 archetypal estrous cycle. Cycle fitting, along with training, classification, and output, are
58 operated through an interactive graphical user interface (GUI). Taken together, these results
59 show that our deep learning approach is capable of rapid and accurate classification of estrous
60 stage.

61

62

63 RESULTS

64

65 **EstrousBank: an open resource for analysis of vaginal cytology images**

66 A major barrier to the development of software to analyze the estrous cycle is a data-poor
67 environment that requires experimenters to collect their own cytology images. In our efforts to
68 make the EstrousNet algorithm generalizable across groups, we have compiled the largest
69 known image bank of estrous cytology images. EstrousBank currently spans five labs, five
70 stains, two magnifications, and multiple rodent species (**Fig. 1A-C, Supplementary Table S1**).
71 The complete image bank comprises 12,719 vaginal cytology images and is freely available for
72 analysis by outside laboratories. We will continue to add to the image bank as more samples
73 become available. Cytological samples across labs were collected using a standard lavage or
74 swabbing procedure (See Methods). Briefly, epithelial cells were exfoliated from the superficial
75 vaginal cavity via sterile saline and transferred to a glass microscope slide. Samples were
76 allowed to dry for up to 24 h before staining with one of several compounds, and images were
77 collected using brightfield microscopy at a range of magnifications (**Supplementary Table S1**).

78 EstrousBank contains images from all four stages of the estrous cycle, which were
79 classified by experts according to classical cytology parameters, which are as follows²⁰⁻²³:
80 mouse diestrus is characterized by an abundance of small leukocytes, a sharp decrease in
81 proportions of keratinized anucleated epithelial cells, and lower numbers of both small and large
82 nucleated epithelial cells (**Fig. 1A-C**). Mucosal secretions appear thick and stringy when
83 present. Proestrus is a more transient stage characterized by a uniform spread of small rounded
84 basophilic nucleated epithelial cells, and low proportions of anucleated cornified epithelial cells

85 (**Fig. 1A-C**). Estrus is typically identified by the high proportion of large anucleated cornified
86 epithelial cells, which often form clumps or sheets that become more prominent in late estrus
87 (**Fig. 1A-C**). Metestrus is a short stage identified by the presence of both nucleated epithelial
88 and cornified epithelial cells, with leukocytes clustered around them, and an elevated level of
89 mucosal secretions (**Fig. 1A-C**). While others have broken down diestrus into 2-3 substages,
90 here we consider metestrus to be its own distinct stage preceding diestrus. These
91 characterizations are largely consistent between mice and rats, but the following differences
92 have been observed: rats exhibit a higher proportion of large ovular nucleated epithelial cells in
93 late estrus, shorter periods of proestrus/metestrus, and lower proportions of anucleated
94 cornified epithelial cells in metestrus²¹. Given these similarities, we trained EstrousNet on
95 cytology images from several strains of mice and rats to improve generalization across model
96 systems; with 34.1% of the image set from mice and 65.9% from rats.

97 Although previous studies have used computational methods to analyze vaginal
98 cytology^{12,30}, the input datasets for these networks have historically been restricted to a single
99 stain. To further enhance generalizability, the training and validation image sets for EstrousNet
100 include samples stained with H&E, Shorr, Giemsa, cresyl violet, and crystal violet stains, at
101 magnifications of 10x and 20x (**Fig. 1A-C, Supplementary Table S1**). The resulting pretrained
102 CNN is highly generalizable and effective in classifying low resolution images and those
103 containing debris from vaginal swab.

104

105 **A ResNet50-based CNN architecture maximizes EstrousNet performance**

106 To classify estrous stage from vaginal cytology images, we developed a classification pipeline
107 using a convolutional deep learning network to detect cell boundaries and recognize endocrine
108 biomarkers within cytological samples. For training and validation, we used consensus
109 classifications (see Methods) to attach an estrous stage label to each image. EstrousNet is
110 trained on subsets of EstrousBank images that are augmented for a greater volume of training
111 data. Input images are first segmented into quadrants (**Fig. 1D.i, ii**), then reflected, rotated,
112 scaled, and translated within the Net (**Fig. 1D.iii**). The augmented images undergo luminance
113 normalization, then are converted to 3-channel grayscale arrays for more efficient feature
114 extraction (**Supplementary Fig. S1**). Next, these augmented images are compiled into a large
115 datastore and fed into the ResNet50 architecture, which consists of four convolutional stages of
116 increasing dimension (**Fig. 1E**). The convolutional layers of the network converge on a SoftMax
117 classification layer, which outputs probabilistic classification of estrous cycle stage (**Fig. 1E, F**).
118 This classification is optionally supplemented by fitting the test images to a curve describing the
119 length and phase of the estrous cycle (**Fig. 1F**). For images in which the cyclicity prediction and
120 net prediction disagree, the interactive GUI will ask the user to select which classification to use.
121 The composite classifications of the EstrousNet and cyclicity predictions provide the
122 experimenter with an informed estrous stage classification.

123 Previous studies investigating the efficacy of transfer learning in biological tissue
124 classification have used several CNN architectures^{12,28,29}. Here, we evaluated four different
125 pretrained networks: VGG-19, Inception v3, MobileNet V2, and ResNet-50 (**Fig. 2A**)³¹⁻³⁴. Each
126 base architecture was originally trained on more than one million images from the ImageNet

127 database and retrained on an augmented dataset made up of 80% of EstrousBank images, with
128 10% of images reserved for validation and 10% reserved for testing (**Fig. 2B, C**). All base
129 architectures have previously been used for supervised learning in biological classification tasks
130 and achieved accuracy comparable to or exceeding that of human coders^{24–27}. The mean
131 validation accuracies averaged over 3 iterations for each architecture are as follows: VGG-19 =
132 79.7%, Inception v3 = 77.5%, MobileNetV2 = 65.5% and ResNet-50 = 88.9% (**Fig. 2A**). These
133 accuracies are calculated based on ground truth data defined by benchmark classifications
134 between 3 expert human examiners. Based on these results, we concluded that ResNet-50 was
135 the most effective architecture.
136

137 **EstrousNet outperforms human coders in both speed and accuracy**
138 The cytology images in our training set were originally sorted into stages by expert human
139 classifiers. These classifications were made using subjective assessments according to
140 established approaches^{10,20,21} (see Methods). Unfortunately, human classification is limited by
141 inter-experimenter variability and differences in experience with particular species, strains, and
142 histological stains. In addition, the CNN may be capable of identifying subtle morphological
143 features that are difficult for humans to identify, such as increased cell clumping in estrus and
144 higher mucus content in metestrus and diestrus.

145 To quantify differences between EstrousNet and human coders, we compared
146 classification performance on a test set of 400 randomly selected images (100 from each stage)
147 between EstrousNet and three expert human coders. Across the test image set, EstrousNet
148 classified stages significantly more accurately than human examiners (odds ratio = 0.68, 95%
149 confidence interval = 0.55–0.83, $p = 2.1 \times 10^{-4}$; Fisher's Exact Test). Breaking down performance
150 by stage, EstrousNet achieved significantly greater accuracy than expert human examiners for
151 diestrus (odds ratio = 0.6791, 95% confidence interval = 0.55–0.83, $p = 1.2 \times 10^{-5}$), whereas
152 accuracy was higher, but not significantly different than expert examiners, for proestrus (odds
153 ratio = 0.68, 95% confidence interval = 0.55–0.83, $p = 0.075$), estrus (odds ratio = 0.6791, 95%
154 confidence interval = 0.55–0.83, $p = 0.84$) and metestrus (odds ratio = 0.68, 95% confidence
155 interval = 0.55–0.83, $p = 0.60$; Fisher's Exact Test for all comparisons; **Fig. 2D–F**). EstrousNet
156 classifications also achieved impressive speed, with an average rate of 0.10 +/- 0.005 s (mean
157 +/- SE) per image.

158 Expert human staging showed a large degree of variance, with only 275 image
159 classifications, or 68.75% of the total test set, shared between all three coders (**Fig. 2G**). A
160 notable number of classifications, 15.9%, were unique to one human coder (**Fig. 2G**).
161 Therefore, even amongst expert human classifiers, classifications can vary widely across a
162 generalizable dataset of cytology images.
163

164 **EstrousNet is generalizable across species, stains, and subjects**
165 To further quantify EstrousNet performance for each estrous stage, we measured the area
166 under the receiver operating characteristic (auROC) for each stage independently. EstrousNet
167 demonstrated auROC values greater than 0.79 for all four estrous stages, with estrus achieving
168 the highest auROC at 0.98 (**Fig. 3A**). Despite this high performance, there are areas in which

169 EstrousNet shows tendencies towards misclassification. Sensitivity and specificity curves show
170 that EstrousNet is stronger in eliminating false negative results than false positive results,
171 indicating a higher degree of sensitivity than specificity (**Fig. 3B**). For example, if EstrousNet is
172 given an image of an unknown stage and asked if the sample is from an animal in diestrus,
173 EstrousNet is more likely to classify the sample as diestrus when it is not (false positive), than to
174 classify it as not diestrus when it is (false negative). Therefore, most misclassifications are
175 specificity errors, which could potentially be reduced with further optimization.

176 In out-of-sample trials in which the CNN was tested on different categories of unseen
177 data, EstrousNet did not show significant differences in test accuracy between any of the given
178 stains it was tested on, including H&E, Shorr, Giemsa, cresyl violet, or crystal violet (**Fig. 3C**).
179 Additionally, despite cytological differences, images from mice and rats did not show significant
180 differences in testing accuracy (**Fig. 3D**). Finally, cross-validation across 6 evenly split groups of
181 subjects, including rats and mice of different strains, did not reveal any out-of-sample
182 differences in test accuracy between animals (**Fig. 3E**).
183

184 **Using cycle fitting for predictive stage classification**

185 When an experimenter classifies estrous stage from epithelial cytology, they not only consider
186 cell morphology and relative prevalence, but also how images might correspond to a typical
187 estrous cycle. Helpfully, some common confusion errors occur between stages that are
188 temporally distinct. For instance, true metestrus is classified as proestrus at a rate of 24.0%
189 despite being non-adjacent stages of the cycle (**Fig. 3D**). As a result, we can exploit the natural
190 sequence of the estrous cycle to identify these errors when test images are taken consecutively.
191 To this end, EstrousNet uses a predictive algorithm that fits an archetypal estrous cycle to the
192 labels generated by the net and identifies outliers (**Fig. 4A, B**).

193 A custom cycle waveform was created based on the duration of estrous stages reported
194 from thirteen groups^{10,18,20–23,30,35–38}. If more than 4 days of test images are selected (i.e., $n > 4 \times x$
195 where x is the sampling frequency per day), the algorithm can fit an archetypal cycle to the data
196 to determine the relative phase that best fits the classification labels. The phase of this periodic
197 waveform was shifted by increments of 0.1 cycles to find the best fit for the input data (**Fig. 4B**).
198 We developed a MATLAB-based graphical user interface (GUI) that allows experimenters to
199 select which stage to accept in cases where the net prediction and cyclicity predictions do not
200 match (**Supplementary Fig. S2**).

201 Fitting stages to an archetypal cycle also allows us to identify disruptions in the estrous
202 cycle, such as those observed when the rodent enters pseudopregnancy, a condition
203 occasionally induced by vaginal swab or lavage^{21,22}. Observations of anestrous stages are also
204 useful for those inducing timed pseudopregnancy for reproductive management and embryo
205 transfer^{10,39}. To address this, EstrousNet will alert the user with a pseudopregnancy warning flag
206 if the animal stays in diestrus for > 50% longer than in previous cycles (**Fig. 4C**). Manual cell
207 counts from an example cycle in which a mouse was lavaged once a day for 8 consecutive days
208 shows a significant increase in the proportion of leukocytes observed once the animal enters
209 pseudopregnancy (**Fig. 4D**, $F(1,6) = 7.44$, $p = 0.034$). Such persistent diestrus following a

210 cornified swab is consistent with previous observations of chemically or mechanically induced
211 pseudopregnancy, and can be seen in a series of cytological images (**Fig. 4E**)²².

212 Additionally, cycle fitting may help to identify stages that do not fall into a traditional
213 category. While here we refer to estrous as consisting of 4 substages, as many as 13 substages
214 have been identified, each corresponding to physiologically distinct steroid hormone
215 concentrations^{41,42}. For the intermediate period(s) between each stage, manual cell counting of
216 sequential samples revealed cell proportionalities distinct to these transition stages (**Fig. 5**).
217 Despite these advancements, more sequential data will be needed for EstrousNet to reliably
218 classify transition stages.

219

220

221 DISCUSSION

222

223 Here, we created a deep learning network for automated classification of estrous stage. The
224 12,719 images that constitute EstrousBank allow us to classify the four stages of estrous in a
225 manner generalizable to stain, subject, and rodent species. EstrousBank is a valuable tool for
226 future developers in the rapidly advancing machine learning field, and the benchmark
227 classifications within the bank provide a guide for those learning to identify estrous stage. Our
228 EstrousNet GUI additionally makes the CNN easily accessible to untrained users.

229 We trained EstrousNet on a random 80% subset of EstrousBank using a ResNet-50-
230 based transfer learning algorithm, yielding test accuracy significantly greater than expert human
231 examiners (**Fig 2,3**). Our software incorporates a preloaded trained network for easy adoption,
232 while allowing more advanced users to train their own networks with custom parameters
(Supplementary Fig. S2). To further improve estrous stage classification, EstrousNet
233 incorporates a cycle fitting algorithm that flags outlier cases in which the deep learning
234 classifications do not line up with an archetypal estrous cycle (**Fig. 4**). In these cases, the GUI
235 gives the user the option to select which classification to accept and incorporates this choice
236 into the net output (**Supplementary Fig. S2**).

237 Despite our progress in estrous stage classification with EstrousNet and EstrousBank,
238 some limitations remain. Because of the heterogeneity of the training image set, we sacrifice
239 some accuracy for the sake of generalizability. Other CNNs trained on 3 stages from a single
240 dataset therefore exhibit higher validation accuracy in some stages^{12,30}. Additionally, the fourth
241 and most transient stage of the estrous cycle, metestrus, yields the lowest test accuracy, as is
242 consistent with previously developed machine learning approaches¹². Since the presence of
243 both cornified and nucleated epithelial cells in metestrus causes confusion with proestrus, more
244 data will be useful for training CNNs to differentiate between these two stages.

245 Despite these limitations, misclassifications by EstrousNet remain significantly lower
246 than human experts in diestrus, and similar to expert human coders in proestrus, estrus, and
247 metestrus (**Fig. 2F**). The significantly higher accuracy of diestrus classifications will be useful in
248 flagging the diestrus-proestrus transition, during which estradiol levels spike up to 100-fold^{41,42}.
249 The combination of the easy-to-use software and our highly generalizable algorithm makes
250 EstrousNet an excellent resource for inexperienced classifiers. Our results indicate that human

252 variability remains high even amongst expert coders, highlighting the need for increased inter-
253 lab consistency (**Fig. 2G**). With many experimenters making the transition to using both sexes in
254 rodent studies, generalizable and automated pipelines for tracking estrous stage will be useful
255 for a range of laboratories.

256 Although 68.3% of EstrousBank images consist of uniform or semi-uniform stains such
257 as crystal violet and H&E, stains designed specifically for hormonal cytodiagnosis offer an
258 opportunity to identify more nuanced biomarkers of the estrous cycle. For instance, Shorr stain
259 makes it possible to distinguish acidophilic and basophilic epithelial cell subtypes, either of
260 which may be more prevalent in the early or late phase of a given estrous stage⁴⁰. Identifying
261 such graded changes in cell type proportionality will be useful for classifying transition stages of
262 the estrous cycle (**Fig. 5**). Characterization of substages will be a step forward in reframing our
263 understanding of the estrous cycle as continuum, instead of a series of discrete stages.

264 It should be noted that currently there is no ground truth data for cytological stage *in*
265 *vivo*, as the low concentrations of hormones such as estradiol and progesterone in the
266 bloodstream make daily collection of endocrine data generally intractable in rodents. Although
267 larger rats may have sufficient blood volume for repeated sampling, existing radioimmunoassay
268 techniques are invasive, expensive, and time consuming⁴³. At present, most ground truth data
269 from the estrous cycle is derived from terminal experiments in which animals are sacrificed at
270 staggered timepoints and large volumes of blood are used to determine hormone
271 concentration^{18,22,41}.

272 However, advances in biosensors for steroid hormone analysis, including aptamer^{44,45},
273 bioaffinity⁴⁶, and magnetic nanoparticle sensors⁴⁷, offer exciting opportunities for repeated
274 estradiol and progesterone measurements. Additionally, physiological characteristics such as
275 temperature⁴⁸, heart rate⁴⁹, uterine impedance²⁰, and blood oxygen content⁵⁰ could be
276 incorporated into estrous stage identification as a proxy for steroid hormone concentrations. As
277 new biomarkers become available, we hope to update EstrousNet to integrate these inputs and
278 further improve the classification accuracy.

279 Ultimately, it is our goal that accessible technologies for cytological classification will
280 help reduce the exclusion of female animals from scientific studies, a disparity that is especially
281 prevalent in fields such as neuroscience and pharmacology, in which significant sex differences
282 have been described^{1,2}. We hope that by continuing to add new cytology images and metadata
283 into our EstrousBank dataset over time, we will be able to bolster our network to identify
284 biological processes that are modulated by steroid hormones.

285

286 METHODS

287

288 Animals

289 The images in EstrousBank were collected from 5 different labs. Cytology images from the
290 Goard lab were taken from female Thy1-GFP-M transgenic mice and Slc17a7-IRES2-Cre x
291 TITL2-GC6s-ICL-TTA2 double transgenic mice, neither of which showed strain-specific
292 disruptions to the estrous cycle. Animals were housed in cages of up to 5 animals, and singly
293 housed after being surgically implanted with a headplate and cranial window for corresponding

294 imaging experiments. Animals were given food and water ad libitum and kept on a 12 h
295 light/dark cycle. Samples were taken at 16-40 weeks, with a median age of 30 weeks, using
296 vaginal lavage. All animal procedures were approved by the Institutional Animal Care and Use
297 Committee at University of California, Santa Barbara.

298 Cytology from the Galea Lab was taken from wild-type female Sprague-Dawley rats. Animals
299 were housed in cages of 2-3, given food and water ad libitum, and kept on a 12 h light/dark
300 cycle. Samples were taken at 8-17 weeks of age using vaginal lavage. Older animals were
301 concomitantly involved in behavioral experiments that may have resulted in elevated stress. All
302 experimental procedures were approved by the University of British Columbia Animal Care
303 Committee and were completed in accordance with the Canadian Council on Animal Care
304 guidelines.

305 Cytology from the Ostroff lab was taken from wild-type female Sprague-Dawley rats. Animals
306 were housed in cages of 2, given food and water ad libitum, and kept on either a 12h or 14:10
307 light/dark cycle. Cages were filled with autoclaved standard Sani-Chip bedding (Teklad Global,
308 Envigo) and one enrichment device. Samples were taken at 4-14 weeks of age using vaginal
309 swab. All animal protocols were approved by the Institutional Animal Care and Use Committee
310 at the University of Connecticut.

311 Cytology from the Shansky Lab was taken from wild-type female Long Evans rats. Animals were
312 housed in cages of 2, given food and water ad libitum, and kept on a 12 h light/dark cycle.
313 Samples were taken at average 12-16 weeks using vaginal swab. All animal procedures were
314 approved by the Institutional Animal Care and Use Committee at Northeastern University.

315 Cytology from the Sutoh lab was taken from wild-type female C57BL/6J mice. Animals were
316 provided food and water ad libitum and kept on a 12 h light/dark cycle. Samples were taken at
317 5-14 weeks using vaginal swab. All animal-use procedures were in accord with the Guidelines
318 for Animal Experimentation of Showa Pharmaceutical University.

319

320 **Vaginal cytology**

321 EstrousBank samples were collected using saline lavage (9.2%) or vaginal swab (90.8%).
322 Vaginal lavage samples were collected using a P200 micropipette. 50 μ l sterile saline was
323 pipetted into the vaginal opening and aspirated several times to obtain a sufficient cell count.
324 The sample was pipetted onto a gel subbed microscope slide and allowed to dry 24 h before
325 staining. For vaginal swabs, cotton-tipped swabs were soaked in sterile saline and briefly rolled
326 against the superficial vaginal wall. The epithelial cells on the swab were then transferred to a
327 dry gel subbed glass slide.

328

329 Gel subbing was performed in-house using standard IHC protocol to coat glass slides in
330 gelatin/CrK(SO₄)₂ solution¹⁹. Staining procedures, including crystal violet, Giemsa, H&E, and
331 Shorr stain, are as described elsewhere^{20,40,41,50}.

332

333

334 **EstrousBank curation**

335 The 12,719 images in EstrousBank were contributed from the Goard lab, Ostroff lab, Shansky
336 lab, Galea lab, and Sutoh lab. These labs provided cytology images from a diverse set of
337 histological stains, magnifications, species, and strains (**Supplementary Table S1**). Initial
338 classifications were made based on traditional cell type proportionality, as determined by the
339 source lab. For cross-group consistency, benchmark classifications were made between the
340 experimenters who provided the cytology images and those compiling EstrousBank. Images
341 were classified into a given stage when 2 or more expert coders agreed on a stage
342 classification, including those from transition stages (**Fig. 5**). Images containing excessive
343 debris, n<10 cells, or <300 pixels were excluded (4.6%).

344

345 **Image preprocessing**

346 Input images were normalized by aligning maximum peaks of the luminance histograms.
347 Images were then converted to greyscale to allow EstrousNet to generalize onto different stains.
348 After normalization, images in both cohorts were randomly divided into 80% training, 10%
349 validation, and 10% test sets. These images were then split into four quadrants within the same
350 directory. Greyscale images were concatenated into 3D arrays to meet input image size
351 requirements. Images were then stored in an augmented datastore where each image was
352 resized to 224 x 224 x 3.

353 EstrousNet augmented the quadrupled dataset with X and Y translation, rotation,
354 reflection, and scaling, according to user parameters in EstrousNetTrainNewNet.mlapp, the
355 network training GUI. EstrousNet users can choose to train their own net using custom
356 augmentation parameters in the EstrousNet GUI or load one of our open-source pretrained
357 networks.

358

359 **Implementation and training of CNN architectures**

360 The pretrained EstrousNet is based on the ResNet-50 architecture, which yields the highest
361 validation and test accuracy on the EstrousBank images. However, users can choose to train
362 EstrousNet using VGG-19, MobileNet v2, or Inception v3 architectures, the connected layers of
363 which have been prespecified in our code³¹⁻³⁴. VGG-19 is a network characterized by highly
364 connected convolutional and fully connected layers which enable efficient feature extraction and
365 use Maxpooling for downsampling, unlike the average pooling layers of ResNet50³³. Compared
366 to ResNet and VGG networks, Inception v3 uses auxiliary classifiers, asymmetric convolutions,
367 and fewer overall parameters for high computational efficiency and low error rates³¹. Finally,
368 MobileNet v2 is a lighter deep neural net ideal that only uses a regular convolution on the first
369 layer of an input image, designed for users with datasets that desire high accuracy with reduced
370 parameters³².

371

372 In the standard ResNet50 architecture, used here as the base architecture of EstrousNet,
373 nonlinear skip connections and shortcuts are implemented to maintain high performance despite
374 a deep architecture³⁴. The residual block on ResNet-50 is defined as follows:

375

376 $y = W_s x + F(x, \{W_i\})$
377
378 Where x is input layer; y is output layer; the function $F(x, \{W_i\})$ represents the residual mapping
379 to be learned; and W_s is the linear projection performed to match the dimensions of x and F .
380

381 The architecture of ResNet-50 consists of 5 stages, each with a convolution and identity
382 block made up of 3 convolution layers^{34,51}. The two initial layers accomplish convolution of size
383 7×7 and max-pooling of size 3×3 with a stride of 2 ^{34,51}. Input images are resized to
384 224x224x3 before undergoing augmentation and training. Training hyperparameters were
385 specified using a Bayesian optimizer, which yielded highest accuracy with an initial learning rate
386 of $1e^{-5}$ and a mini batch size of 80. Several gradient descent optimization algorithms were
387 tested, including RMSprop, adam, and sgdm, all designed to minimize the loss function of the
388 network. RMSprop exceeded the other algorithms in terms of accuracy when combined with a
389 squared gradient decay of 0.99. Due to the breadth of the input images only 3 epochs were
390 necessary to maintain maximum accuracy, with shuffling occurring every epoch, as well as a
391 piecewise learning rate drop factor of 0.1, the step decay algorithm of which is as follows:
392

393
$$l_r = l_{r,0} * drop^{floor} \left(\frac{epoch}{epochs_drop} \right)$$

394

395 Where l_r is learning rate; $l_{r,0}$ is initial learning rate (here $1e^{-5}$); $drop$ is the factor by which the
396 learning rate is decreased (here 0.1); $floor$ is the minimum learning rate; $epoch$ is the current
397 epoch, and $epochs_drop$ is the number of epochs after which the step decay will occur (here
398 1)⁵².

399 The EstrousNet GUI was developed in MATLAB 2020b (Mathworks, Inc.) using the App
400 Designer platform. EstrousNet was trained using EstrousNetTrainNewNet.mlapp, classification
401 input was given by EstrousNetGUI.mlapp, and classification output was plotted using
402 EstrousNetPlotting.mlapp. The GUI is also used to tune augmentation parameters and number
403 of stages desired for classification.
404

405 **Cycle fitting**

406 Here, a custom waveform describing the time course of the estrous cycle was generated using
407 prior publications^{10,18,20–23,30,35–38}. The resulting archetypical estrous cycle has a period of 4.87
408 days (**Fig. 4A**). The stage classifications are ordered diestrus > metestrus in increments of 1.0
409 starting from 0.5, where 0.0 and 4.0 were defined as the transition stage between metestrus and
410 diestrus (**Fig. 4A**). We fit these points with a two-term polynomial, calculating the coefficients
411 using the temporal midpoints of each stage of the estrous cycle. The periodic waveform is fit to
412 the input data for EstrousNet by shifting the phase by 0.1 cycles and selecting the phase shift
413 with the maximum Pearson's correlation coefficient (**Fig. 4B**).

414 Cycle fitting also allowed us to detect anestrous stages (i.e., pseudopregnancy), which
415 are occasionally induced by cytology sampling methods such as vaginal swab and lavage. In
416 our algorithm, the user will receive a pseudopregnancy warning message if the animal has been

417 in diestrus 50% longer than in previous cycles, given that the user specifies sequential data
418 sampling in the GUI (**Fig. 4C-E**). This characterization is consistent with our observation that
419 more than 2 consecutive days of > 90% leukocytes is indicative of an anestrous state (**Fig. 4D**).
420

421 **Statistical information**

422 To compare the accuracy of EstrousNet vs trained human examiners, a test set of 400 images
423 was created by randomly selecting 100 images from each of the 4 estrous stages (**Fig. 2D, E**).
424 Human examiners were expert coders who had each individually classified upwards of 2000
425 cytology images. EstrousNet was trained on the images in EstrousBank, as described
426 previously, excluding the 400 images in the test set. Benchmark classifications were used as a
427 proxy for ground truth, in the absence of intravenous hormone measurements, as described
428 previously. Accuracy was determined by comparing these ground truth classifications to
429 EstrousNet classifications. These comparisons are represented by a confusion matrix generated
430 in MATLAB (**Fig. 2D,E**).

431 For statistical analysis, net accuracy and human accuracy vectors for each stage were
432 concatenated and bootstrapped across 5000 iterations to create a normal distribution. Violin
433 plots were made using an open-source MATLAB package⁵⁵. We performed the Fisher's Exact
434 Test within and across stages to test for significance (**Fig. 2F**).

435 For out-of-sample testing, three dimensions of sampling were used: stain, species, and
436 subject. For stains and species, each respective category was removed from the training set
437 and set aside for testing. EstrousNet was trained separately for each category on the revised
438 datasets (**Figure 3C-E**). It should be noted that multiple dimensions were nested in our
439 framework, i.e., because each lab group used a different stain for their cytology images,
440 removing any species also removed a set of stains. Accuracy was measured by taking the
441 proportion of EstrousNet classifications that were consistent with benchmark classifications, run
442 across 1000 iterations sampled without replacement to generate standard error. For out-of-
443 sample subject testing 36 individual animals were identified, including 20 WT Sprague Dawley
444 rats and 16 Slc7a7-cre x TITL GCaMP6s B6 mice. k = 6 groups were used for k-fold out of
445 sample cross-validation testing, with 6 subjects in each group. The resulting confusion matrix is
446 an average of the k-fold accuracy results.

447 ROC curves were generated using the *perfcurve* MATLAB function to generate a logistic
448 regression, then the integral of each curve was taken to calculate the auROC for each stage
449 (**Fig. 3A**). For these curves, true positive was defined as an instance where a given positive
450 stage was correctly classified, whereas false positive was defined as the number of negative
451 stages falsely categorized into a given positive stage.

452 The sensitivity curve was generated by finding the rate of images in a positive class, i.e.,
453 images belonging to a given stage, that were correctly classified as being in that stage (**Fig.**
454 **3B**). The specificity curve was generated by finding the rate of images in a negative class, i.e.,
455 not part of a given stage, that were correctly classified as not belonging to that stage (**Fig. 3B**).
456 The probability cutoff of 0.26 was defined as the intersection between these two curves (**Fig.**
457 **3B**). Pseudopregnancy cell count significance was determined by a two-way ANOVA (**Fig. 4D**).
458

459 DATA AVAILABILITY

460

461 All code necessary to run EstrousNet is available at [http://github.com/ucsb-goard-](http://github.com/ucsb-goard-lab/EstrousNet)

462 [lab/EstrousNet](#). EstrousBank is available in full at [*IDR number to be determined*].

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AUTHOR CONTRIBUTIONS

N.S.W. developed EstrousNet; N.S.W and K.K.S. analyzed EstrousNet performance; N.S.W and K.K.S. developed the EstrousNet GUI; G.R., T.H., and N.S.W. classified test images; G.R., T.H., R.M.S, L.A.M.G., L.O, and M.J.G. contributed to EstrousBank curation; N.S.W. and M.J.G. wrote the manuscript; all authors reviewed the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

FIGURES

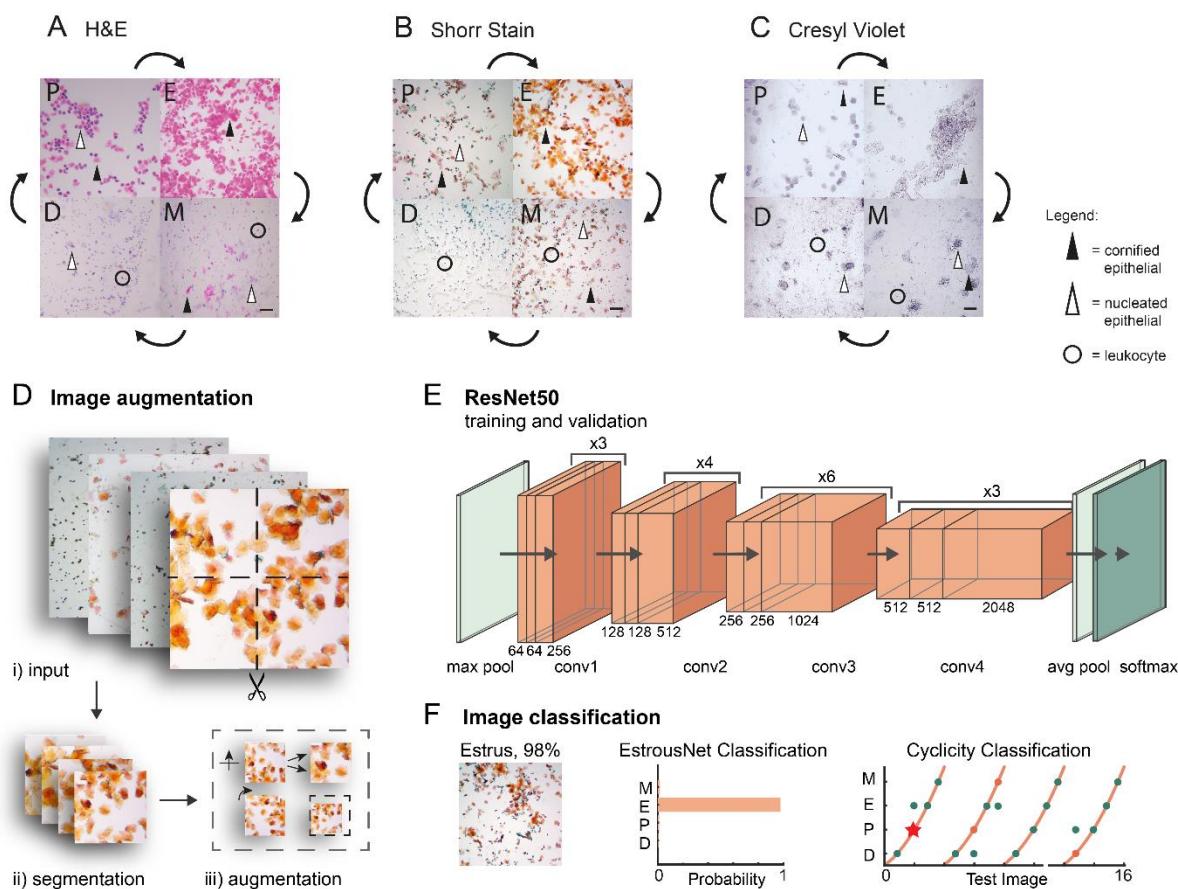


Figure 1. General schematic of the EstrousNet pipeline and representative cytological images.

- A.** Hematoxylin and eosin (H&E)-stained vaginal cytology from a wild-type Sprague Dawley rat (Ostroff lab). Scale bar = 10 μ m.
- B.** Shorr-stained vaginal cytology from a Slc7a7-cre x TIT2L-GCaMP6s Bl6 mouse (Goard lab). Scale bar = 10 μ m.
- C.** Cresyl violet-stained vaginal cytology from a wild-type Long-Evans rat (Galea lab). Scale bar = 10 μ m.
- D.** Image augmentation schematic: images are first quadrisectioned, then reflected, scaled, rotated, and translated in our preprocessing pipeline.
- E.** The base architecture of ResNet50 that is used for the transfer learning algorithm. Processed input images are transferred to a max pooling layer. Then, the images are processed through four convolutional units, which converge onto custom pooling and SoftMax classification output layers.
- F.** Schematic of the EstrousNet GUI output. Estrous stage classifications are generated from the deep learning network, and the cycle tracking algorithm flags potential outliers.

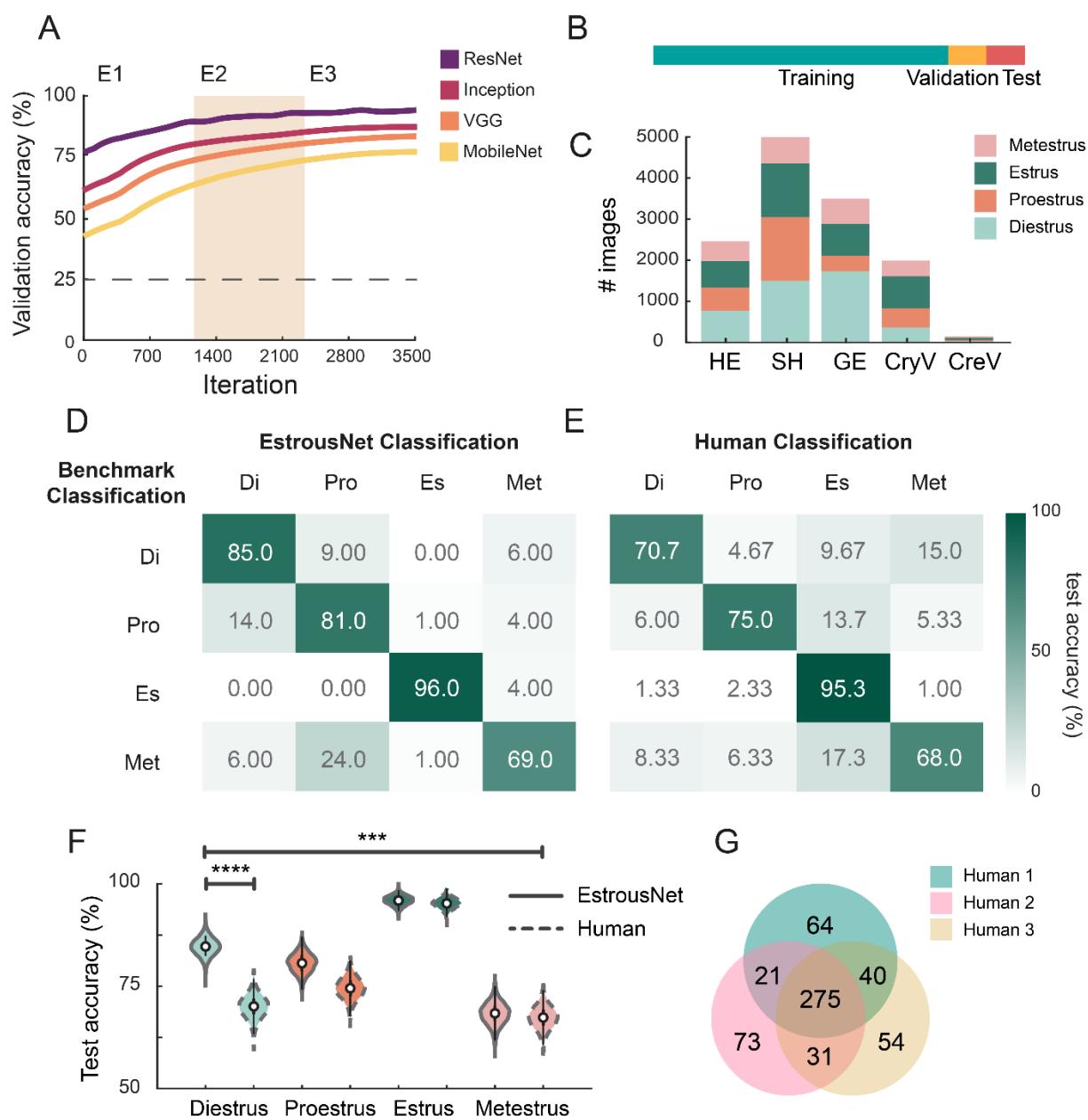


Figure 2. EstrousNet accuracy is comparable to human experts.

- A.** Validation accuracy curves for EstrousNet trained using four different base architectures: ResNet50, Inception v3, VGG-19, and MobileNet v2. All networks were trained on EstrousBank images. Mean validation accuracy across 3 testing iterations.
- B.** Schematic of the EstrousBank split for training, validation, and test sets. By percentage, this split is 80%, 10%, and 10%, respectively.
- C.** Breakdown of EstrousBank by stain and stage. Stains from left to right are hematoxylin and eosin (HE), Shorr stain (SH), Giemsa stain (GE), crystal violet (CryV), and cresyl violet (CreV). The complete bank consists of $n = 12,719$ cytology images.

- D.** Confusion matrix of EstrousNet classifications, represented here as a heatmap, with consensus from benchmark classification acting as our ground truth. Numbers represent the number of images classified for each stage, from a test set made up of 400 images (100 images from diestrus, proestrus, estrus, and metestrus).
- E.** Confusion matrix of human classification, represented as a heatmap, with ground truth stages as described previously.
- F.** Average test accuracy distributions in each estrous stage for EstrousNet vs human classifications. EstrousNet distributions are identified by a continuous line while human classifications are identified by a dotted line. Distributions were created by bootstrapping data over 50000 iterations, sampling without replacement. Error bars are 25th (75th) percentiles minus (plus) the interquartile range (75th percentile minus 25th percentile). Asterisks indicate significance as determined by Fisher's Exact Test; diestrus: odds ratio = 0.68, 95% confidence interval = 0.55-0.83, $p = 1.2 \times 10^{-5}$, proestrus: odds ratio = 0.68, 95% confidence interval = 0.55-0.83, $p = 0.075$, estrus: $p = \text{odds ratio} = 0.68$, 95% confidence interval = 0.55-0.83, $p = 0.84$, metestrus: odds ratio = 0.68, 95% confidence interval = 0.55-0.83, $p = 0.60$. Across all stages accuracy was significantly different, with odds ratio = 0.68, 95% confidence interval = 0.55-0.83, $p = 2.1 \times 10^{-4}$, Fisher's Exact Test.
- G.** Venn diagram of the overlap between human expert coders, with a total of 400 classifications for each coder.

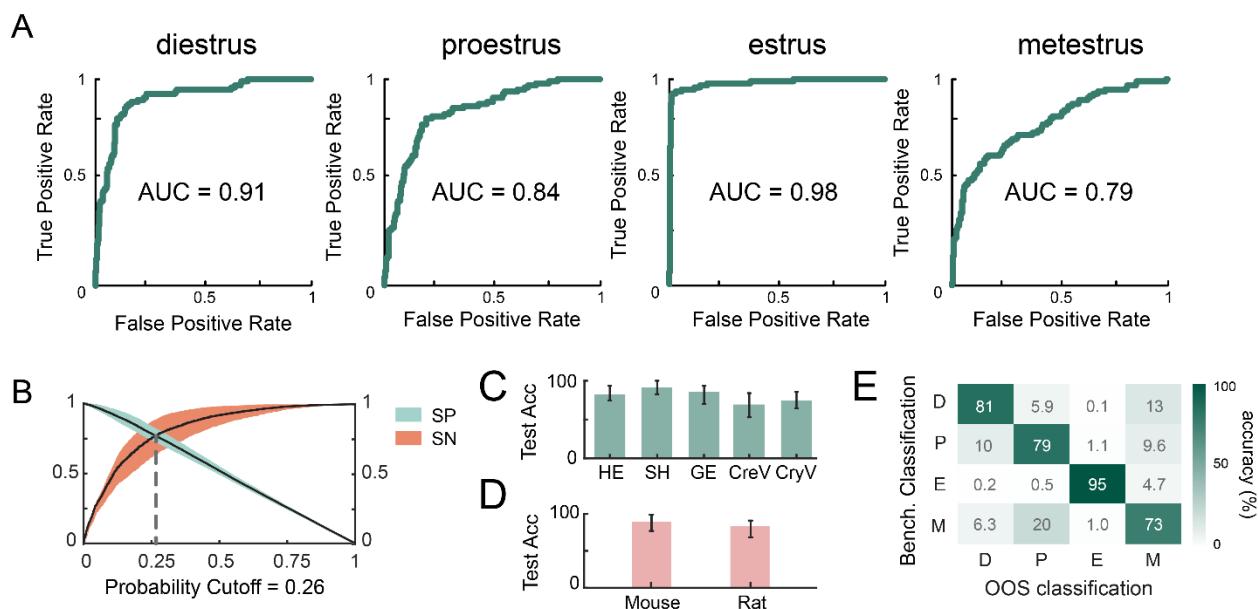


Figure 3. EstrousNet performs similarly across groups.

- A.** auROC curves for each estrous stage. True positives for each stage are determined by benchmark classifications.
- B.** Specificity (SP) vs sensitivity (SN) curves for EstrousNet, with the probability cutoff at 0.26 defined as the intersection between curves (dotted grey line). Standard error shown in orange and blue for sensitivity and specificity, respectively.
- C.** Out of sample testing across 4 different stains: hematoxylin and eosin, Shorr stain, Giemsa stain, cresyl violet, and crystal violet. Test accuracy represented as a distribution across 1000 testing iterations, with mean % SE shown. Accuracy differences between stains are not significant ($F(4,198) = 3.14$, $p = 0.10$, one-way ANOVA).
- D.** Out of sample testing between mouse and rat species. Test accuracy represented as a distribution across 1000 testing iterations, with mean % SE shown. Accuracy differences between species are not significant ($F(1,198) = 7.87$, $p = 0.73$, one-way ANOVA).
- E.** Out of sample (OOS) classification for each stage of the estrous cycle between different animals, represented as a heatmap. Benchmark classification was used as a proxy for ground truth. K-fold cross-validation was used to estimate accuracy across stages, with $k = 6$ groups. Testing accuracy was averaged between each fold to generate the most unbiased estimate across all groups. Accuracy differences between subjects are not significant ($F(5,198) = 6.98$, $p = 0.60$, one-way ANOVA).

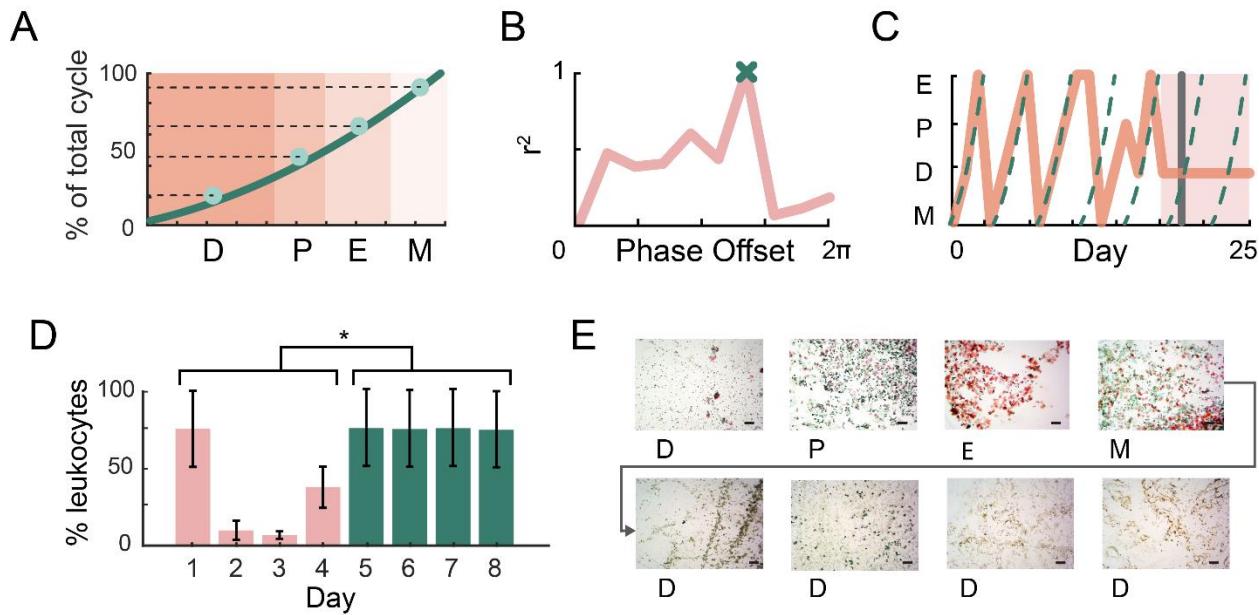


Figure 4. Sequential estrous classifications are fit to an archetypal cycle.

- A.** Schematic of the custom waveform used for temporal cycle fitting. Color blocks indicate the length of each estrous stage as a percentile of average total cycle length, with a curve fitted to the midpoint of each stage (see Methods). Stage lengths are based on a consensus archetypal cycle from previous studies^{10,18,20–23,30,35–38}.
- B.** Pearson's coefficient for each iterative fit of the custom waveform to an example 16-day cycle, at increments of 0.1 cycles. The best fit is determined by global maxima, marked by an 'x' for this example cycle.
- C.** Example of a naturally cycling mouse tracked across 25 days, with the animal's cycle shown as a solid orange line and the fitted cycle curve as a dotted teal line. The mouse initially exhibited regular cycles but entered pseudopregnancy on day 18 (shaded area), causing EstrousNet to give the user a pseudopregnancy warning message (grey line).
- D.** Proportion of leukocytes in cytological cell counts before (blue) and after (pink) pseudopregnancy. Mean \pm SE, $F(1,6) = 7.44$, $p = 0.034$, as determined by two-way ANOVA. Asterisk indicates significance of $p < 0.05$.
- E.** Cytology images from a normally cycling mouse entering pseudopregnancy, demonstrating prolonged diestrus, with an abnormally high proportion of leukocytes. Scale bars = 10 μ m.

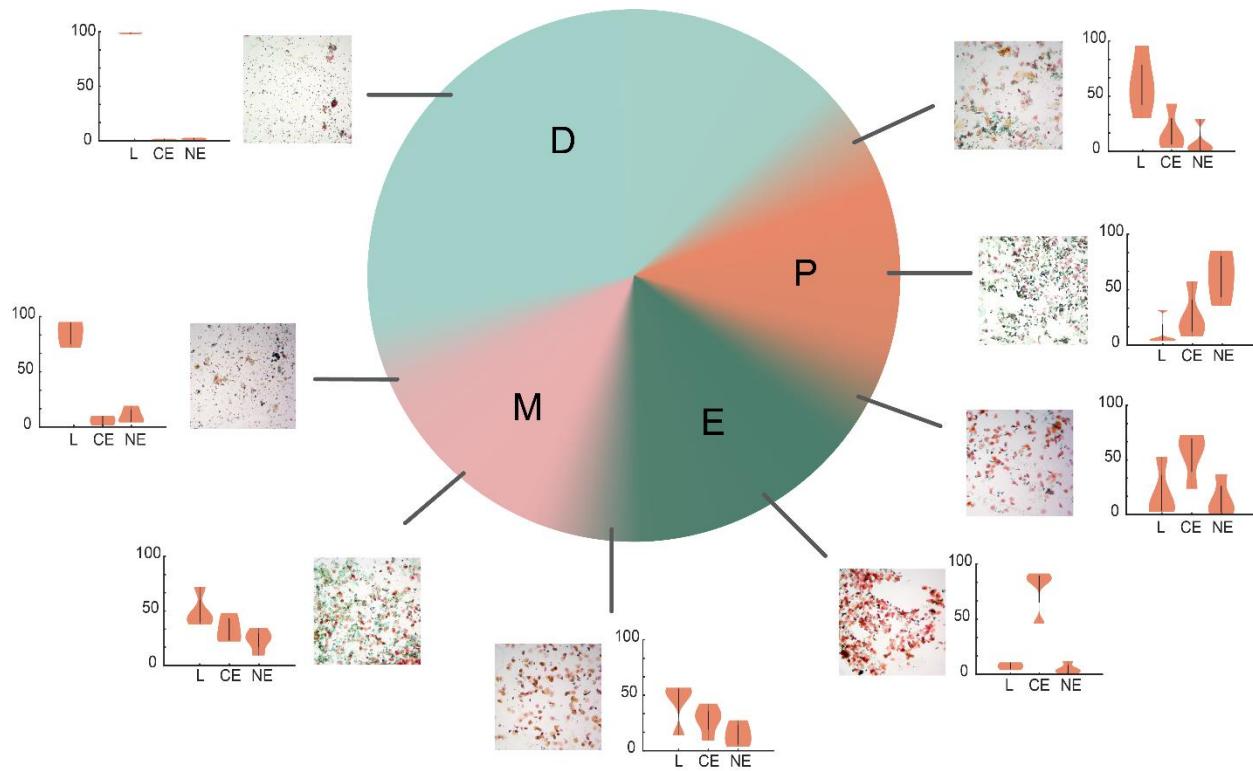
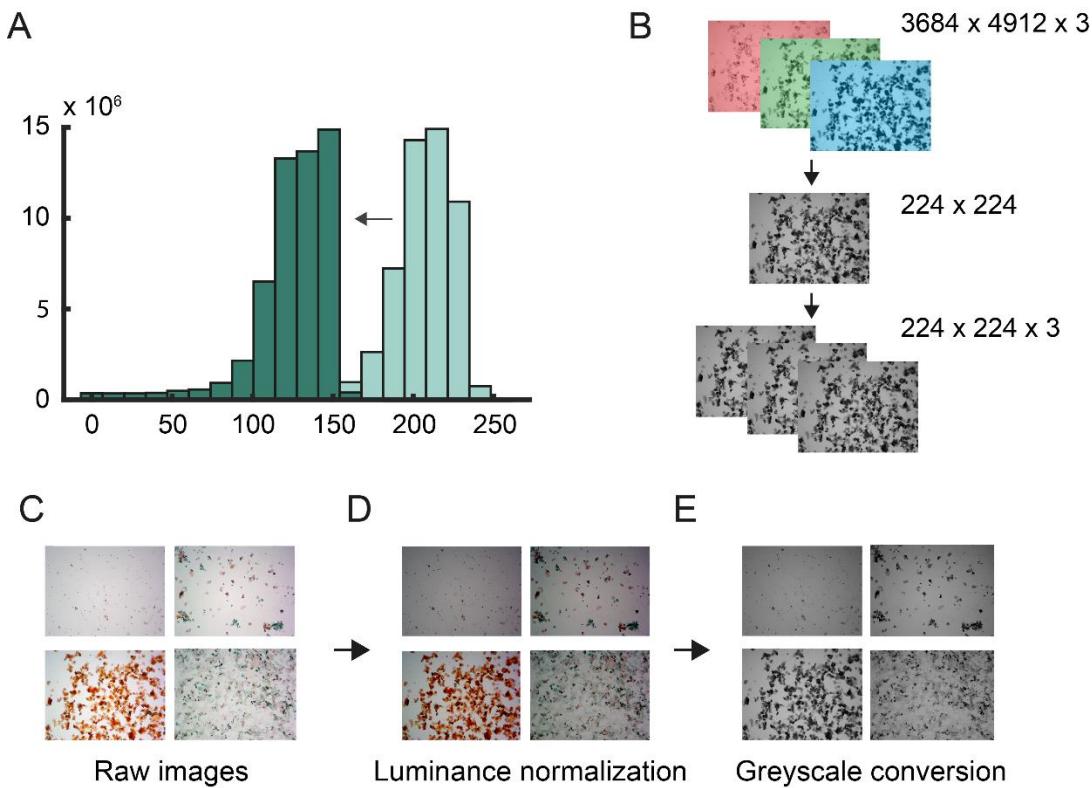


Figure 5. Characterization of cell types across the estrous cycle.

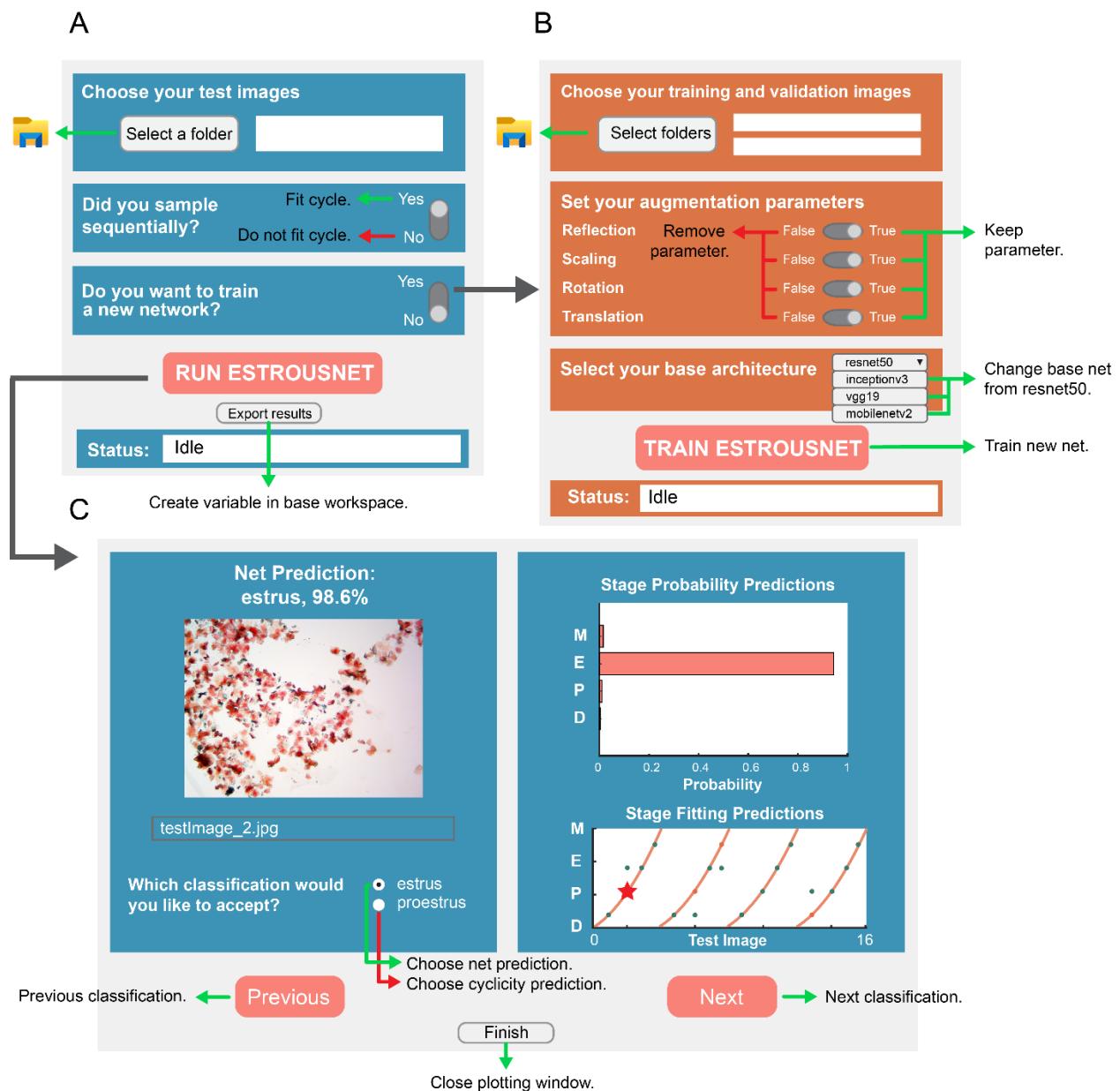
A pie chart of the four estrous stages broken down by stage length across the 4–5-day cycle. Transition states between the four classical estrous stages are shown in gradient, and cytology collected from between the stages are included. Raw images and violin plots of cell counts from 8 primary and transition stages are shown (four examiners). The violin plots indicate the proportions of leukocytes, cornified epithelial, and nucleated epithelial cells, respectively (mean \pm SE).

SUPPLEMENTARY MATERIAL



Supplementary Figure S1. **Image preprocessing pipeline.**

- A.** Intensity histogram of an unprocessed image (light blue), shifted to lower intensity (dark blue) during luminance normalization.
- B.** Schematic of image resizing and conversion to grayscale, where 1D grayscale images are concatenated into a 3D array of size $224 \times 224 \times 3$ to match the input requirements of the transfer learning network.
- C.** Example unprocessed test images from one estrous cycle.
- D.** Raw images with reduced intensity, normalized to the same maximum intensity peak.
- E.** Luminance-normalized images converted to 3-channel grayscale.



Supplementary Figure S2. Illustration of the EstrousNet user interface (GUI).

- A. The EstrousNet classification GUI: the user selects a folder of test images which are automatically classified and plotted. The user also selects whether images were sampled sequentially, which will determine whether net classifications are fit to an archetypical cycle.
- B. The EstrousNet training GUI: if the user selects that they would like to train a new network, it will launch the training GUI. This GUI lets the user select folders with training and validation images, as well as custom augmentation parameters, and once training is finished will save the trained network and training data to the current directory.
- C. The EstrousNet plotting GUI: once the classification GUI is used to select test images, the plotting GUI will display the results of the net classifications. If images were taken in sequence, the plotting GUI will fit the images to an archetypal cycle, and for any images where the cyclicity and net classifications disagree, the user can choose to manually select the preferred classification.

Source Lab	Magnification	Stain	Species	Strain	# images	% of total images
Galea	20X	Cresyl Violet	Rat	Sprague Dawley WT	145	1.14
Goard	10X	H&E, Shorr Stain	Mouse	Thy1-GFP-M (Jax Stock #007788), Slc7a7-cre (Jax Stock #023527) x TITL-GCaMP6s (Jax Stock #024104) C57BL/6J	1024	8.05
Ostroff	10X	H&E, Shorr Stain	Rat	Sprague Dawley WT	6277	49.35
Shansky	10X	Crystal Violet	Rat	Long Evans WT	1954	15.36
Sutoh	10X	Giemsa	Mouse	C57BL/6J WT	3319	26.09

Supplementary Table S1. Summary of EstrousBank images from multiple labs.

Metrics for the images included in the open-source image repository EstrousBank, subdivided by the groups contributing the raw images.