Extended Experimental Procedures

MAPP-nλ

MAPP-nλ is a modified version of pre-mGRASP (Kim et al., 2011). We stripped the pre-mGRASP protein of the 2A-cerulean fusion and added four repeats of the nλ RNA binding domain (Daigle and Ellenberg, 2007) in the cytoplasmic tail after amino acid 287 of the original pre-mGRASP sequence. We also added a Myc epitope tag followed by the CLIP-tag domain (Gautier et al., 2008) after amino acid 59 of the original pre-mGRASP protein.

Sindbis virus barcode library

The virus used in this study is based on a dual promoter pSinEGdsp construct (Kawamura et al., 2003). We inserted MAPP-nλ after the first subgenomic promoter. Downstream of the second subgenomic promoter, we inserted the GFP coding region followed by closely spaced NotI and MluI restriction sites and four repeats of the boxB motif (Daigle and Ellenberg, 2007). Using this construct, we produced a high diversity plasmid library by inserting a diverse pool of double stranded ultramers (Integrated DNA Technologies) with sequence 5'-AAG TAA ACG CGT AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNN NNN NNN NNN NNN NNN NNN NNN NYY GTA CTG CGG CCG CTA CCT A-3' between the NotI and MluI sites. We then produced Sindbis virus as previously described (Kebschull et al., 2015) using either the conventional DH(26S)5'SIN helper (Bredenbeek et al., 1993) or the new DH-BB(5'SIN;TE12) (Kebschull et al., 2015) helper. We determined the titer of the resulting virus by qPCR as previously described (Kebschull et al., 2015) and determined the viral library diversity by Illumina sequencing of the RNaseI protected genomic virus RNA. Both the genomic construct and the helper construct are available from Addgene under accessions 73074 and 72309, respectively.

Injections

Animal procedures were approved by the Cold Spring Harbor Laboratory Animal Care and Use Committee and carried out in accordance with National Institutes of Health standards.

We pressure injected 180nl of $2x10^{10}$ GC/ml barcoded Sindbis virus uni- or bilaterally into LC of 8-10 week old C57BL/6 males (Jackson Labs) as described (Cetin et al., 2007). We leveled the animal skulls on two axes using lambda and bregma for the AP axis and 2mm laterally from the midpoint between lambda and bregma for the lateral axis. We used coordinates AP=-5.4mm, ML=0.8mm, DV=2.9mm and 3.1mm for LC and measured depth from the surface of the brain. We injected each DV coordinate with 90nl of virus, waiting ten minutes in between each depth. We sacrificed animals 44 hours post injection. For immunofluorescence, RNA *in situ* and histology, we transcardially perfused animals with ice cold saline (9g/l) followed by 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1M Phosphate buffer. For RNA work we extracted the fresh brain and flash froze it on dry ice.

For measurements of MAPseq efficiency, we injected red retrobeads (Lumafluor) into the right olfactory bulb of 8-12 week old C57BL/6 males (Jackson Labs). Briefly, we roughly determined the center of the right olfactory bulb, and measured +/-1mm from the center in the AP axis and performed two craniotomies 2mm apart. We sonicated the beads for 20 minutes prior to injection to homogenize the solution and injected 210nl of stock concentration of beads at three different depths (0.3mm, 0.6mm and 0.9mm DV from the surface of the olfactory bulb) as described (Cetin et al., 2007). Twenty-four hours later, we injected barcoded Sindbis virus into right LC as above and sacrificed the animals 44-48 hours after Sindbis injection.

Immunofluorescence and ISH

We performed anti-GFP staining and RNA *in situ* hybridization on 6μm thick paraffin sections. For immunofluorescence, we used a rabbit anti-GFP antibody ab290 (Abcam; RRID:AB_303395) after heat induced antigen retrieval. We performed RNA *in situ* hybridization using the Panomics ViewRNA ISH Tissue kit (Affymetrix) using anti-GFP probe VF1-10141 according to the manufacturer's protocol (10 minutes boiling and 10 minutes protease treatment). We performed anti-TH staining on floating 70μm vibratome sections using rabbit anti-TH antibody SAB4300675 (Sigma-Aldrich; RRID:AB_11130236).

Spike-in RNA

To produce spike-in RNA, we double stranded an ultramer (Integrated DNA Technologies) with sequence 5'-GTC ATG ATC ATA ATA CGA CTC ACT ATA GGG GAC GAG CTG TAC AAG TAA ACG CGT AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNN NNN NNN

NNN NNN NNN NNN NAT CAG TCA TCG GAG CGG CCG CTA CCT AAT TGC CGT CGT GAG GTA CGA CCA CCG CTA GCT GTA CA-3' and then *in vitro* transcribed the resulting dsDNA using the mMessage mMachine T7 *in vitro* transcription kit (Thermo Fisher) according to the manufacturer's instructions.

qPCR

We reverse transcribed total RNA using oligodT primers and Superscript III reverse transcriptase (Thermo Fisher) according to the manufacturer's instructions. We then quantified the amount of barcode and β-actin cDNA by qPCR in SYBR green power master mix (Thermo Fisher) according to the manufacturer's instructions using primers 5'-GAC GAC GGC AAC TAC AAG AC-3' and 5'-TAG TTG TAC TCC AGC TTG TGC-3' for barcode cDNA and 5'-CGG TTC CGA TGC CCT GAG GCT CTT-3' and 5'-CGT CAC ACT TCA TGA TGG AAT TGA-3' for β-actin cDNA.

MAPseq

We cut 300μm thick coronal sections of fresh frozen brains using a Leica CM 3050S cryostat at -12ºC chamber temperature and -10ºC object temperature. To avoid cross-contamination between samples, we took care to cut each section with a fresh, unused part of the blade. We melted each section onto a clean microscope slide and rapidly froze the section again on dry ice before dissecting out the cortex on dry ice using a cold scalpel blade. During dissection, we aimed to avoid known fiber tracts to minimize the contamination of our dataset with fibers of passage. After sample collection, we processed all samples out of order to avoid potential sample cross-contamination from impacting interpretation of MAPseq results.

We extracted total RNA from tissue samples using Trizol reagent (Thermo Fisher) according to the manufacturer's instructions. We mixed the total RNA from the tissue samples with spike-in RNA. We then produced ds cDNA as previously described (Morris et al., 2011) using a gene specific primer of from 5'-CTT GGC ACC CGA GAA TTC CAN NNN NNN NNN NNX XXX XXT GTA CAG CTA GCG GTG GTC G-3', where XXXXXX is one of 65 trueseq like SSI and N_{12} is the UMI. We then cleaned the reaction using the Qiagen MinElute PCR purification kit according to the manufacturer's instructions and treated the eluted ds cDNA with ExonucleaseI (New England Biolabs) to remove remaining primers. We amplified the barcode amplicons by nested PCR using primers 5'-CTC GGC ATG GAC GAG CTG TA-3' and 5'-CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CCT TGG CAC CC GAG AAT TCC A-3' for the first PCR and primers 5'-AAT GAT ACG GCG ACC ACC GA-3' and 5'- CAA GCA GAA GAC GGC ATA CGA-3' for the second PCR in Accuprime Pfx Supermix (Thermo Fisher). We then gel extracted the amplicons using the Qiagen MinElute Gel extraction kit according to the manufacturer's instructions and pooled the individual sequencing libraries based on qPCR quantification using primers 5'-AAT GAT ACG GCG ACC ACC GA-3' and 5'- CAA GCA GAA GAC GGC ATA CGA-3'. We then sequenced the pooled libraries on an Illumina NextSeq500 high output run at paired end 36 using the SBS3T sequencing primer for paired end 1 and the Illumina small RNA sequencing primer 2 for paired end 2.

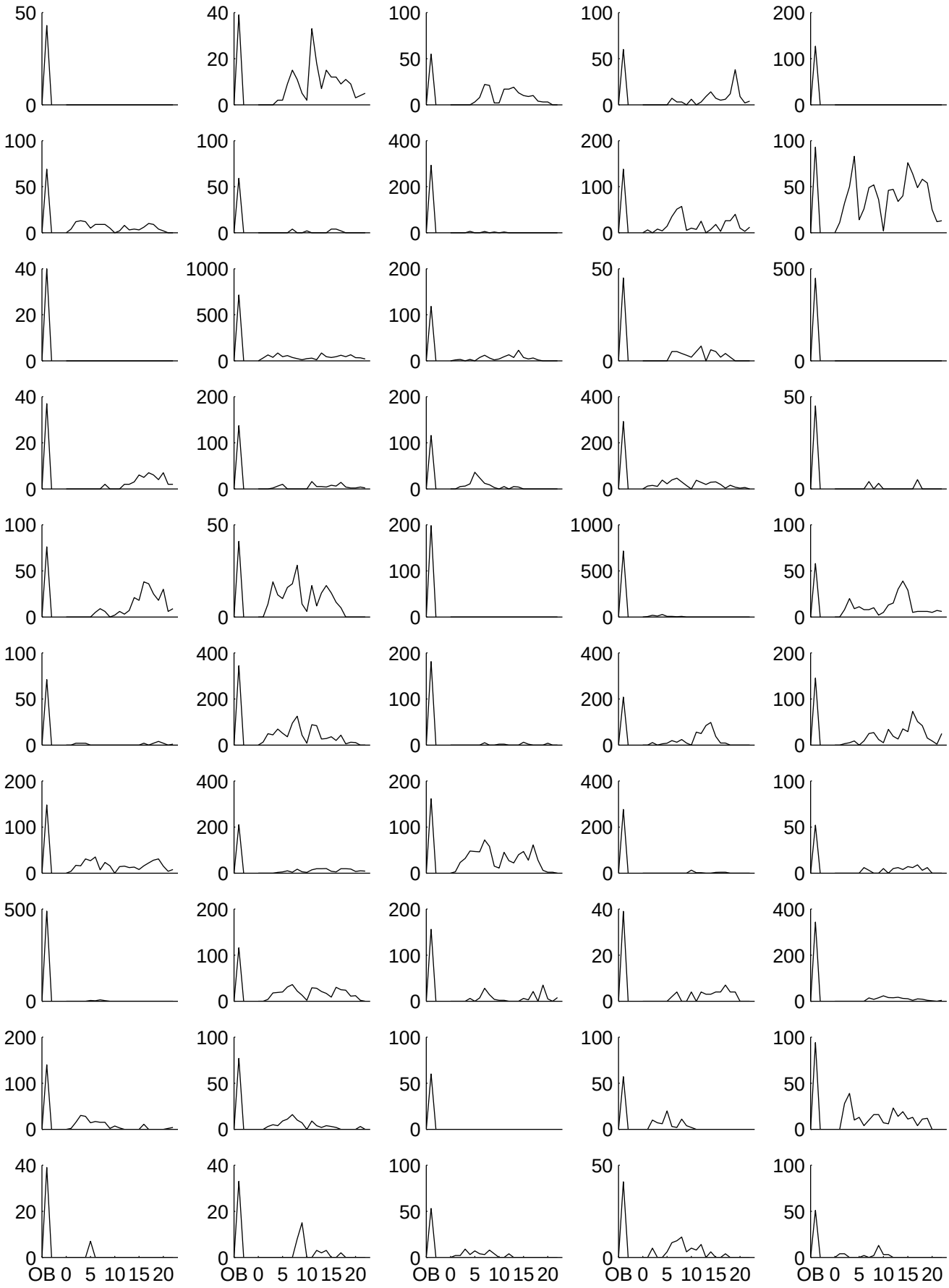
Efficiency measurements and single cell isolation

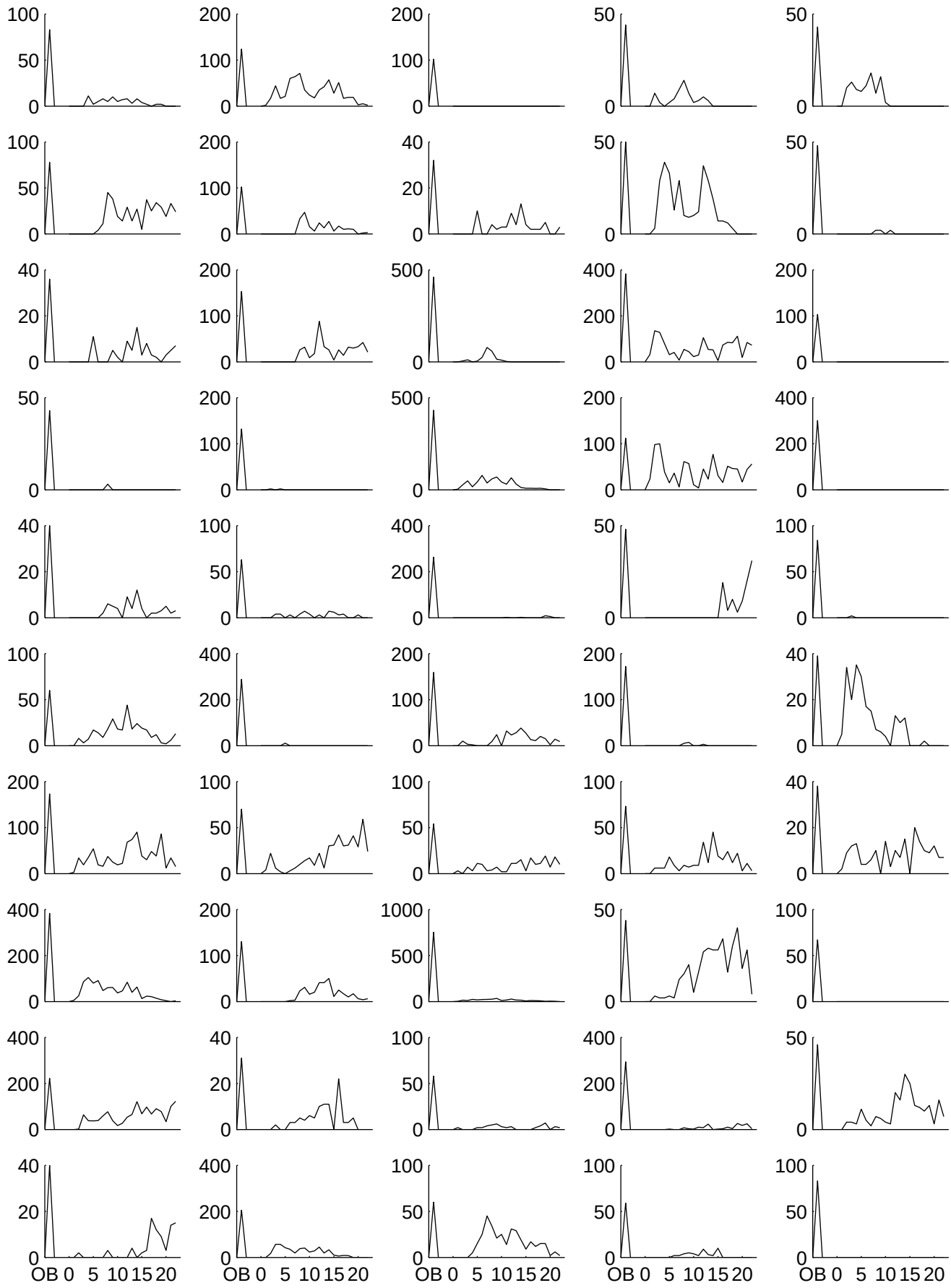
After transcardial perfusion with ice-cold artificial cerebrospinal fluid (127mM NaCl, 25mM $NaHCO_3$, 1.25mM $NaPO_4$, 2.5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and 25mM D-glucose), we extracted the unfixed brain and flash froze the bead-injected olfactory bulb on dry ice before processing it for sequencing as described above. We cut 400μm thick acute sagittal slices of the remaining right hemisphere in dissection solution (110mM choline chloride, 11.6mM ascorbic acid, 3.1mM Na pyruvic acid, 25mM $NaHCO_3$, 1.25mM $NaPO_4$, 2.5mM KCl, 0.5mM $CaCl_2$, 7mM $MgCl_2$, and 25mM D-glucose) using a Microm HM650V vibratome. We incubated sections containing LC in artificial cerebrospinal fluid (126mM NaCl, 20mM $NaHCO_3$, 3mM KCl, 1.25mM NaH_2PO_4, 2mM $CaCl_2$, 2mM $MgSO_4$, and 20mM D-glucose) containing synaptic blockers (0.05mM APV, 0.02mM DNQX and 0.1μM TTX) for 20 minutes at room temperature. We then digested the slices in artificial cerebrospinal fluid with streptomyces griseus protease (Sigma P5147) at 1mg/ml at room temperature for 30 min. After washing in artificial cerebrospinal fluid with synaptic blockers, we dissected LC from the digested section and triturated the tissue to produce a single cell suspension. Using an inverted fluorescent microscope (Zeiss Observer), we picked individual cells by hand, deposited the cells directly into lysis buffer (2.4μl 0.2% triton, 1μl 10mM dNTPs, 1μl 10mM RT primer per cell) and proceeded to preparing sequencing libraries from the cells as described above for tissue samples.

Animals used

Number of animals	Manipulation	Figures based on these animals
4	Right LC injection with MAPseq virus; dissection of right cortex and olfactory bulb; qPCR and sequencing of barcode RNA	Fig. 2e, 4, 5, 6 Supp. Fig. 1, 2, 4, 5, 7c,d
2	Right LC injection with MAPseq virus; dissection of right and left cortex; qPCR of barcode RNA	Fig. 2f
1	Bilateral LC injection with MAPseq virus; dissection of right and left cortex and olfactory bulb; qPCR and sequencing of barcode mRNA	Fig. 7
3	Right LC injection with MAPseq virus and retrobeads injection into right olfactory bulb; single cell isolation from LC	Fig. 3b; Supp. Fig. 6
3	Right LC injection with DH(26S)5'SIN packaged MAPseq virus and dissection of select cortical targets and the olfactory bulb and sequencing of barcode mRNA	Supp. Fig. 7a,b
6	Right LC injection with DH(26S)5'SIN packaged MAPseq virus. TH staining of LC and quantification of overlap and count of TH+ neurons	Supp. Fig. 1j,k,l
3	Right LC injection with DH(26S)5'SIN packaged MAPseq virus; ISH for barcode mRNA and IF for GFP protein	Fig. 3b Supp. Fig. 1
2	Right LC injection with DH(26S)5'SIN packaged MAPseq virus and dissection of the olfactory bulb and ipsilateral cortex and sequencing of barcode mRNA	Supp. Fig. 1e,f

Projection patterns of all mapped neurons

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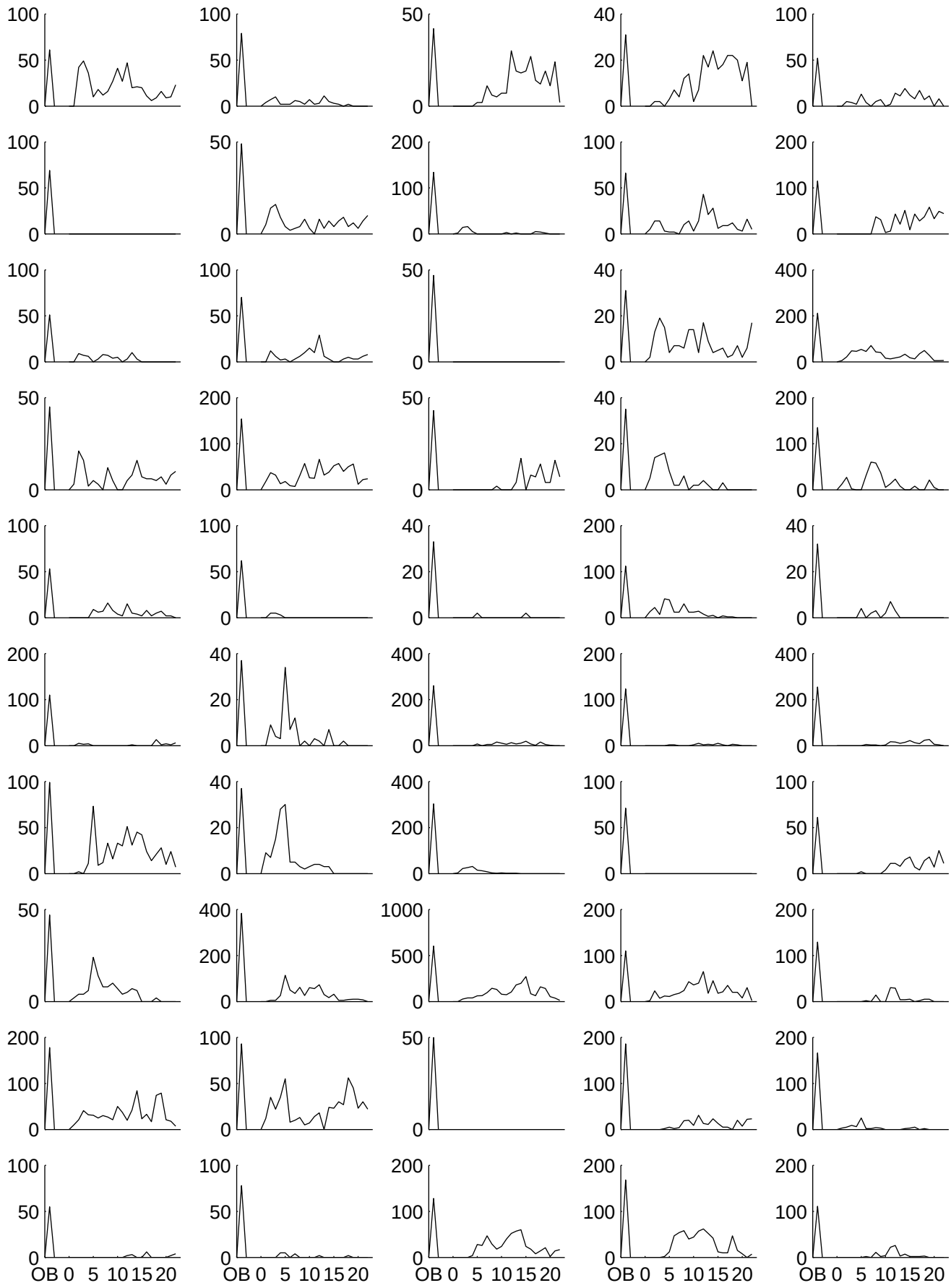
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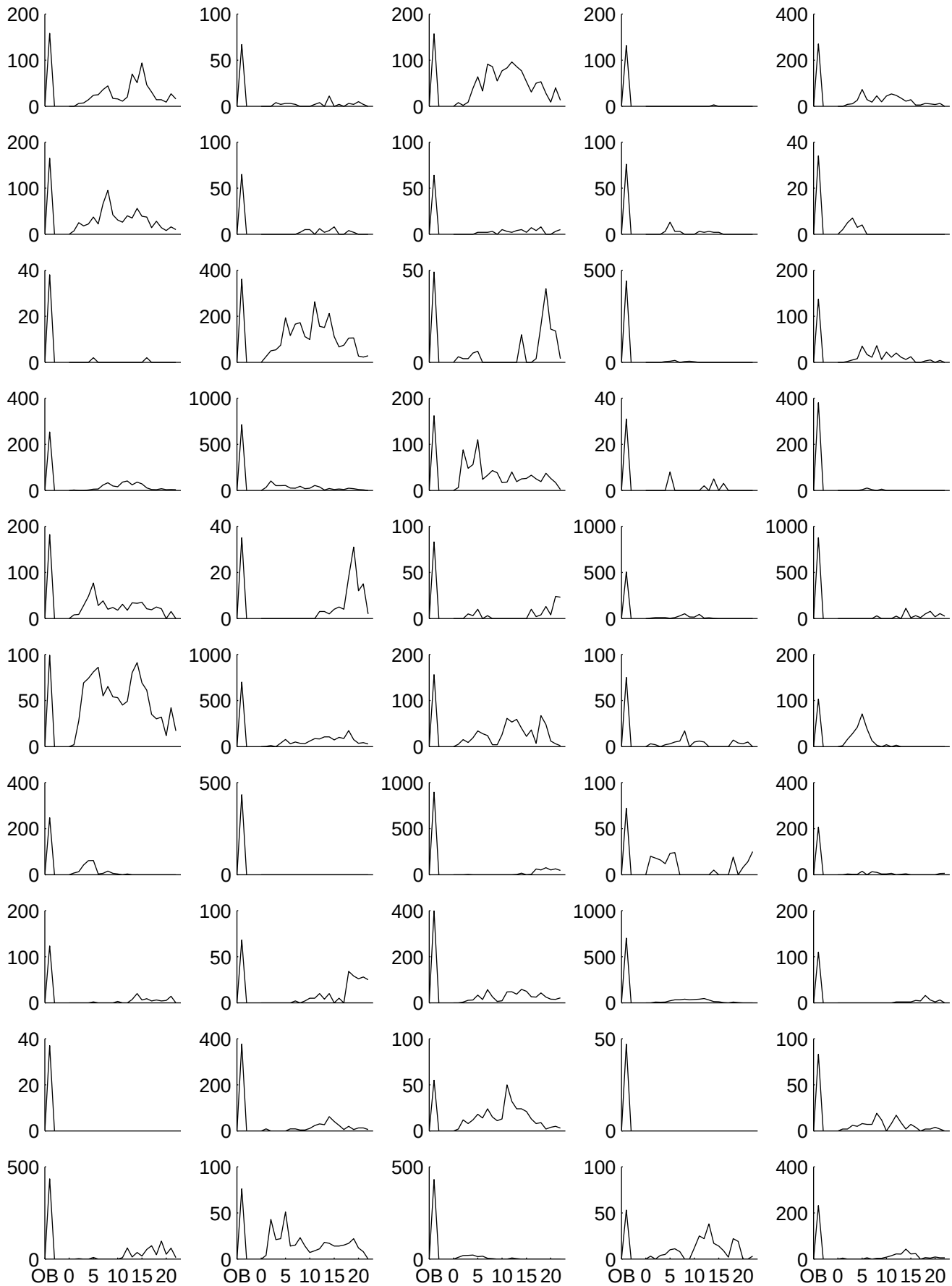
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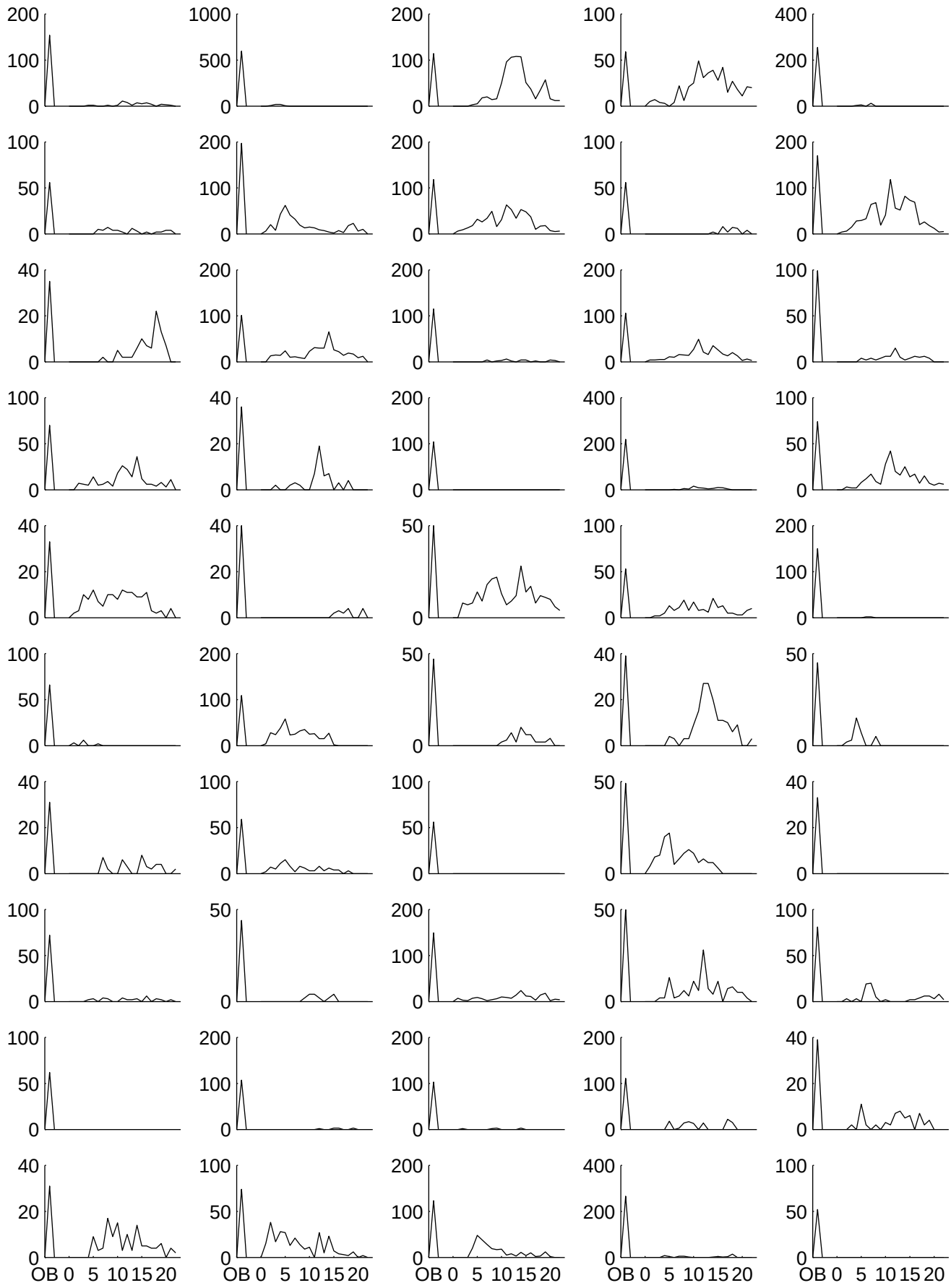
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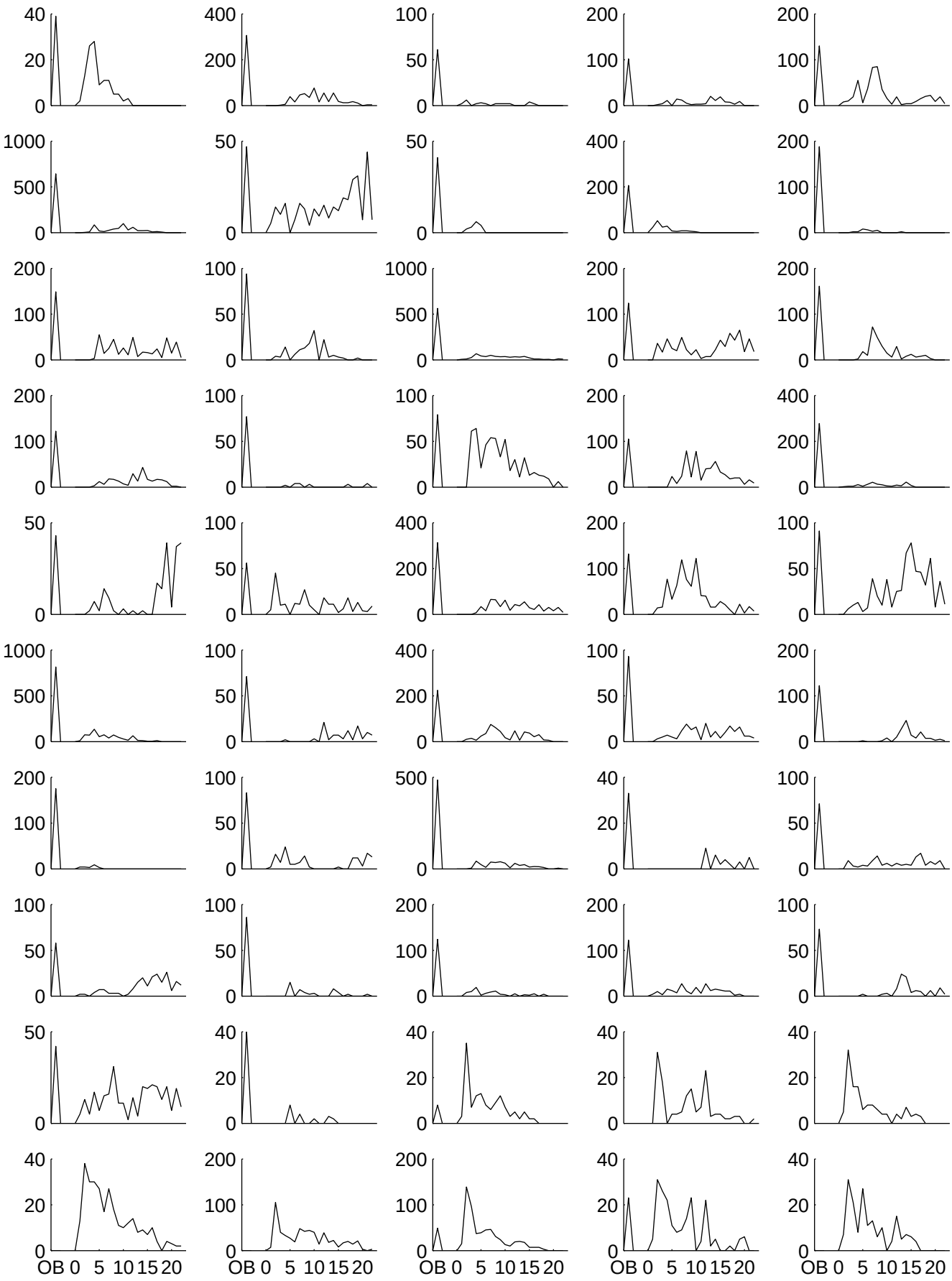
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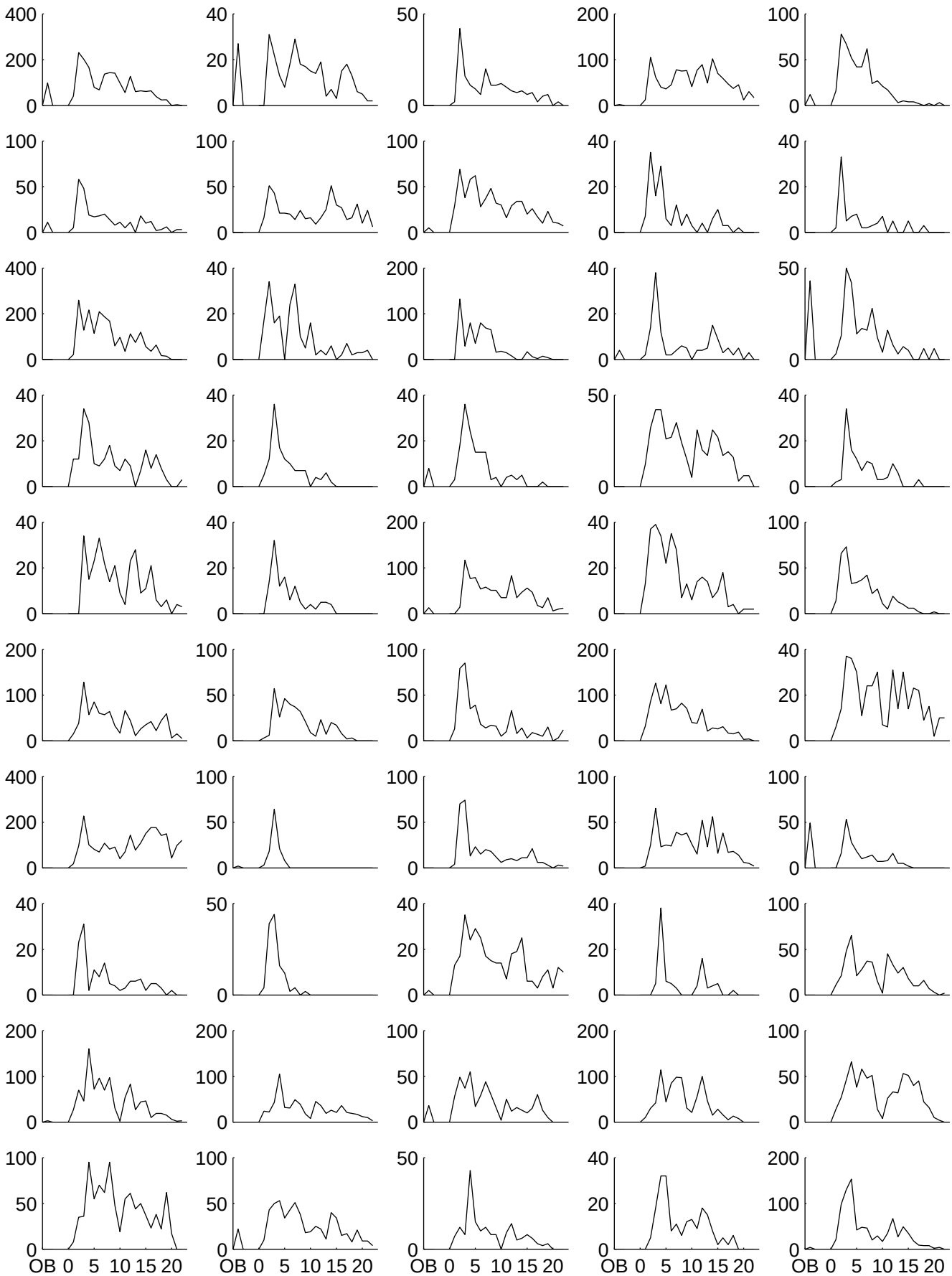
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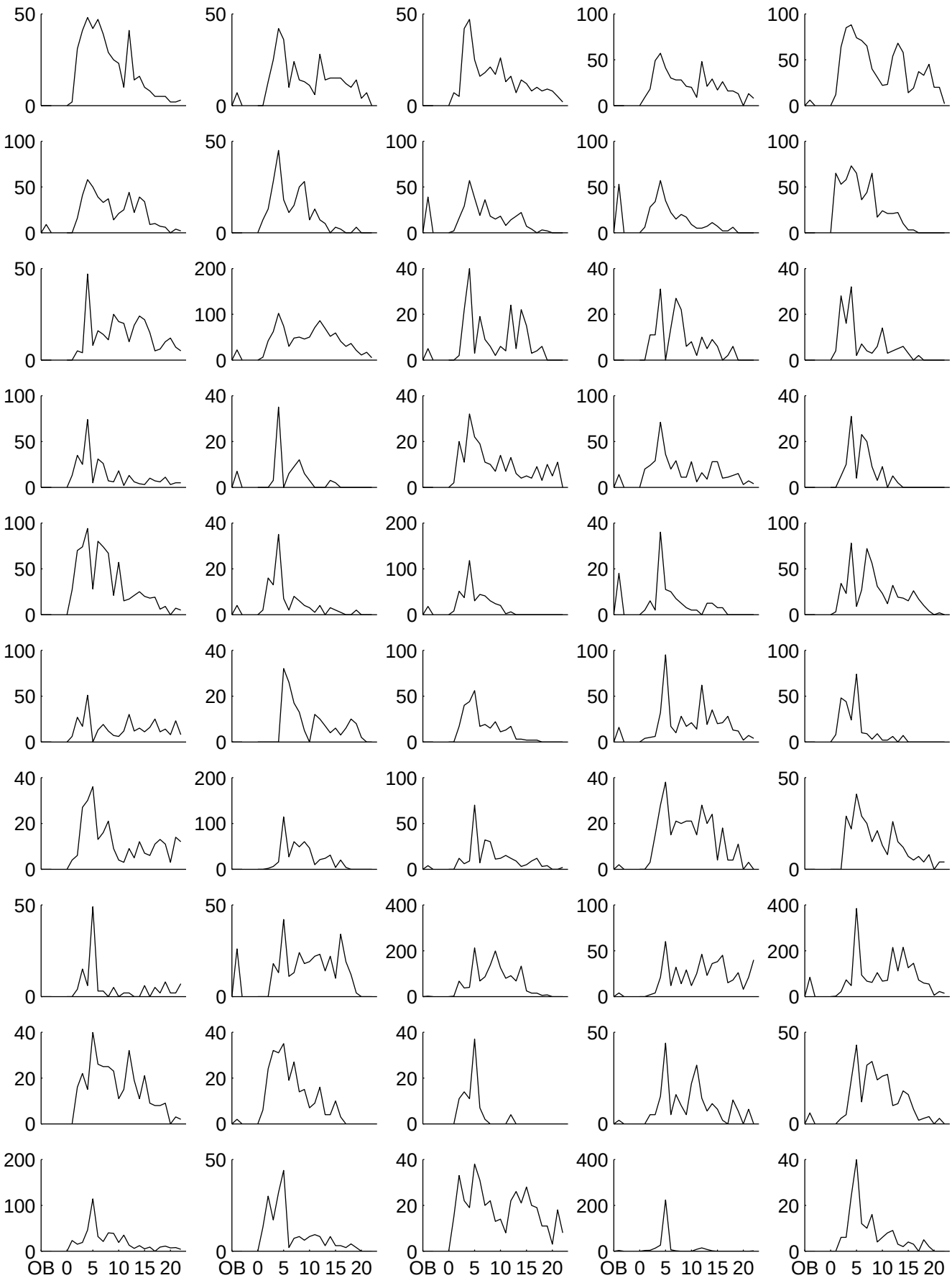
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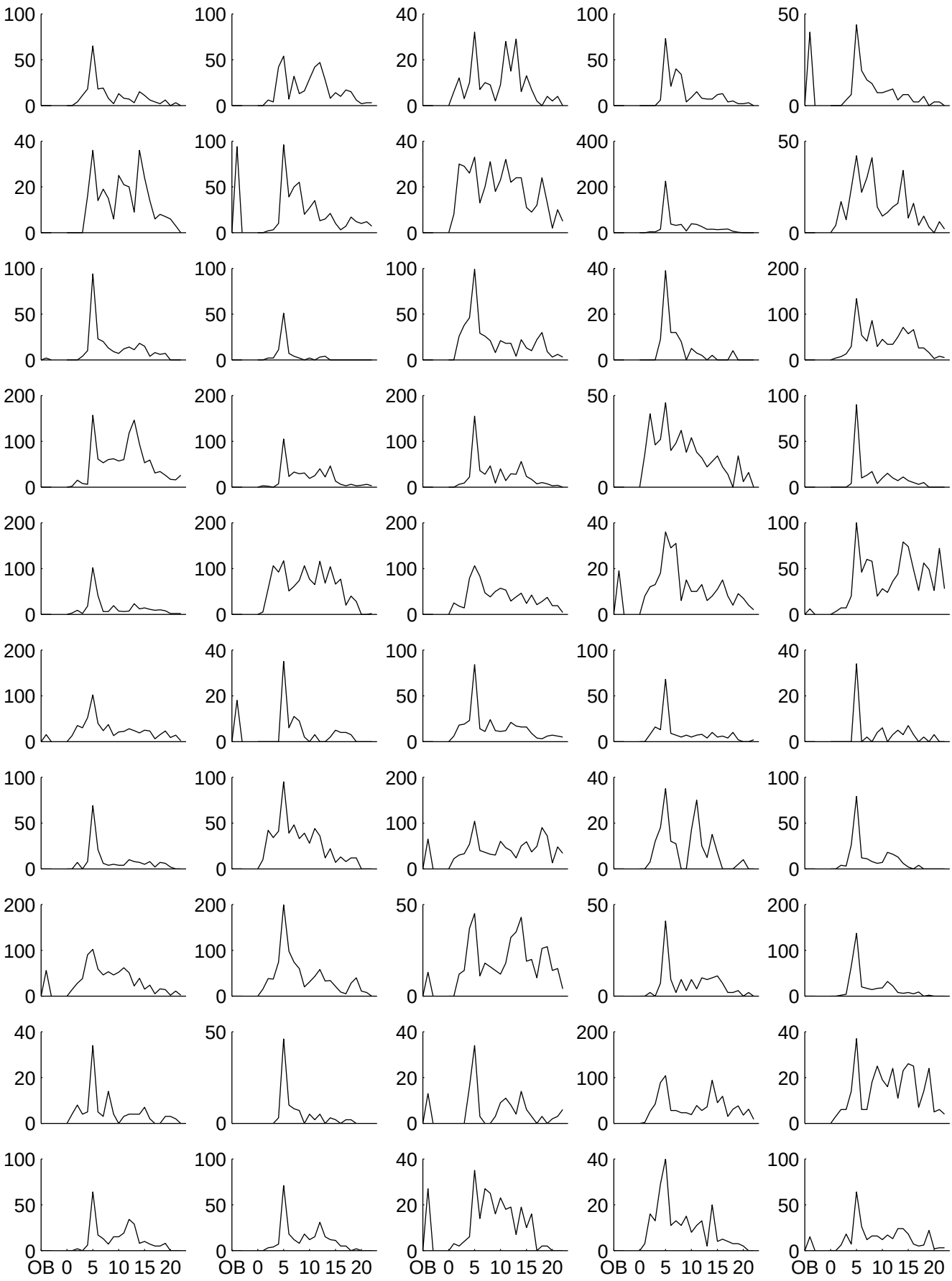
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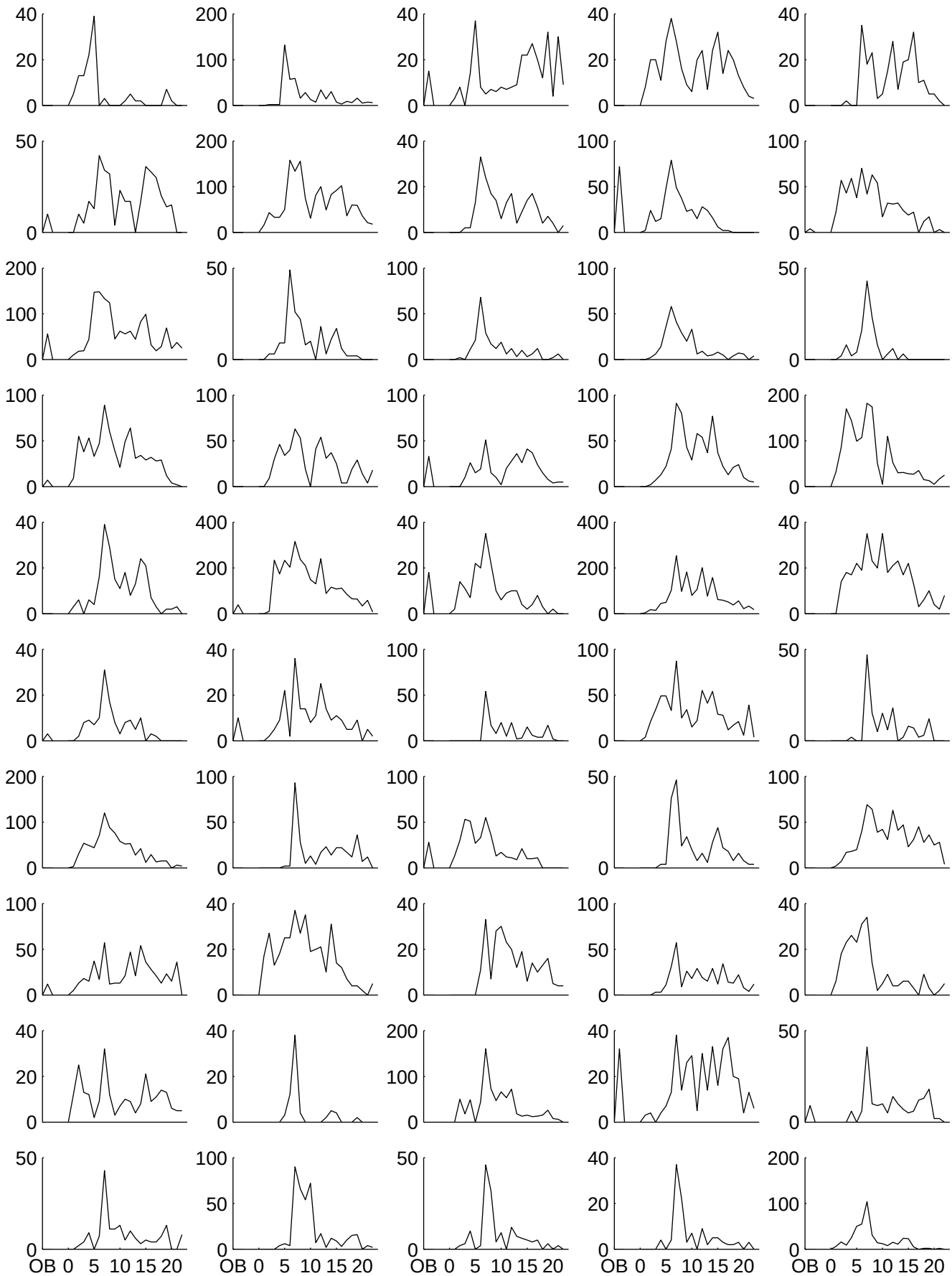
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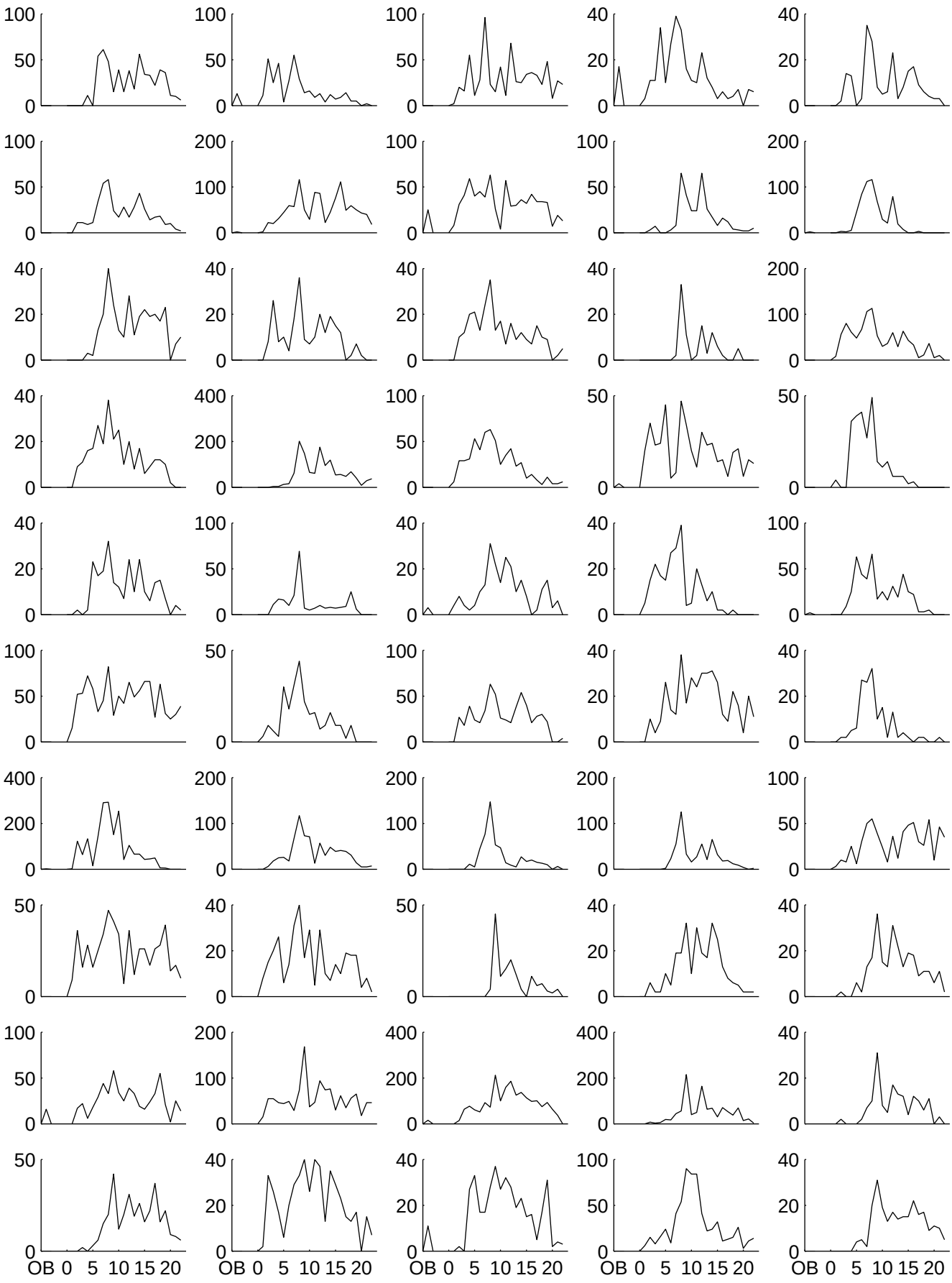
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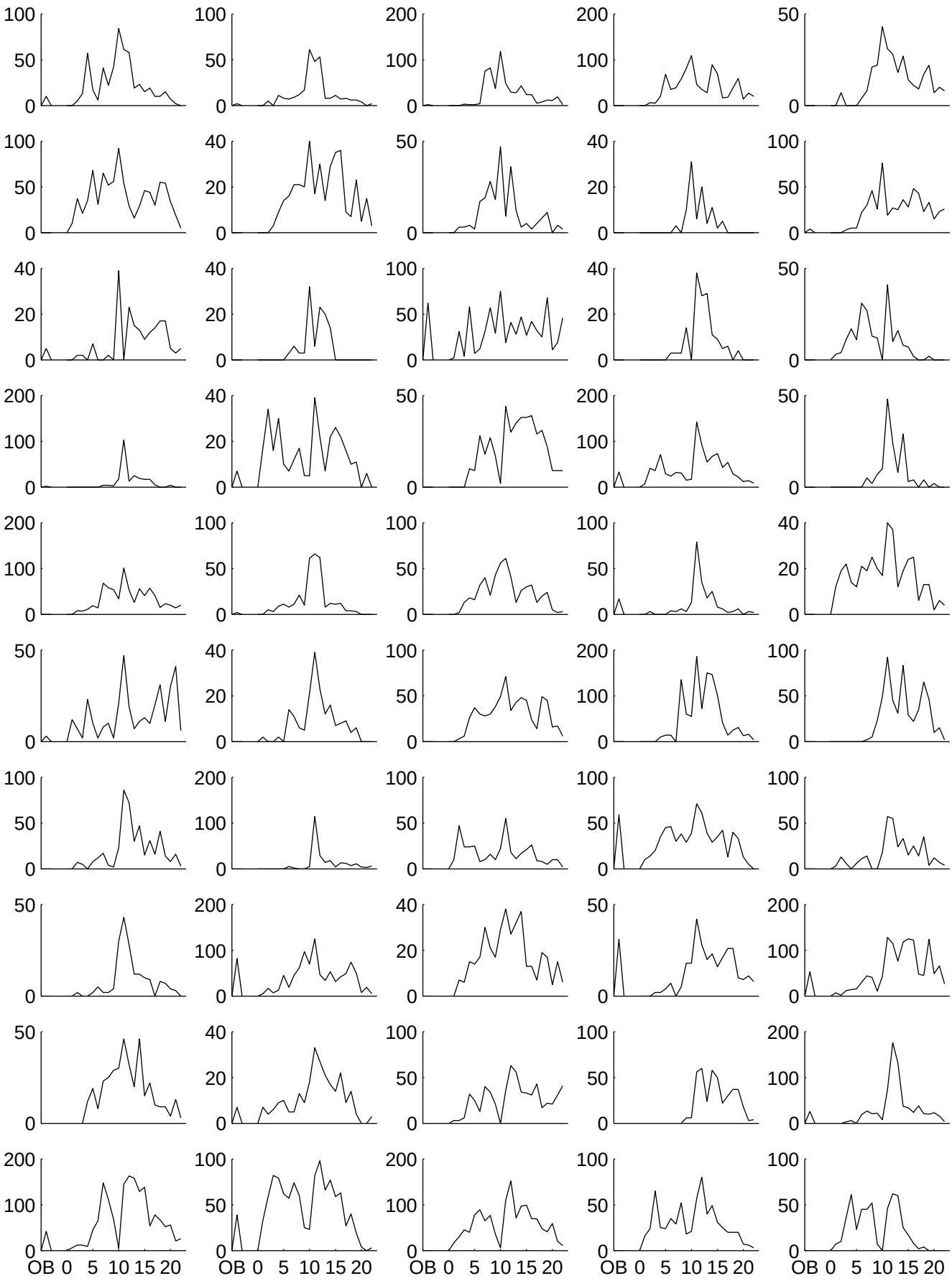
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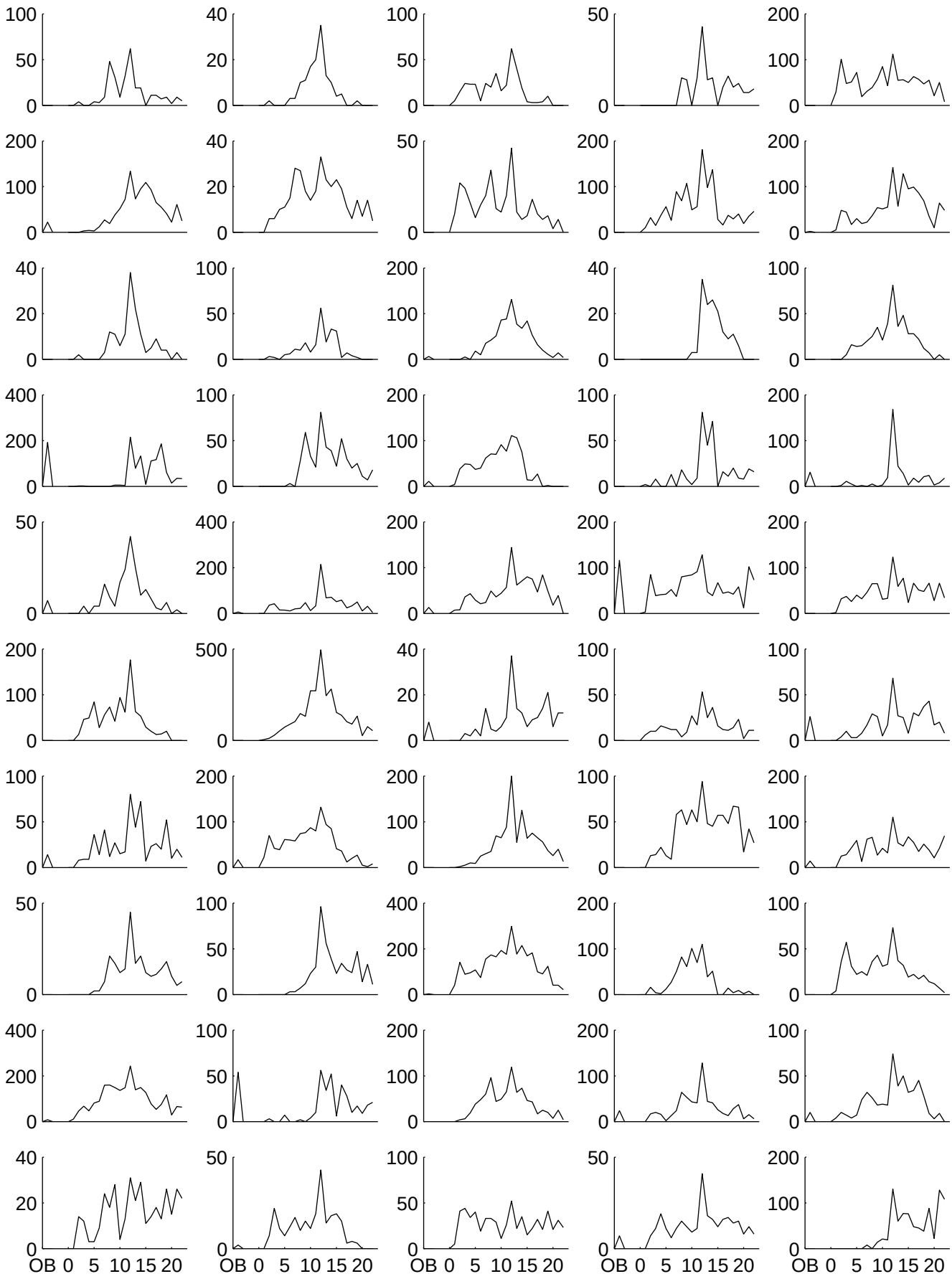
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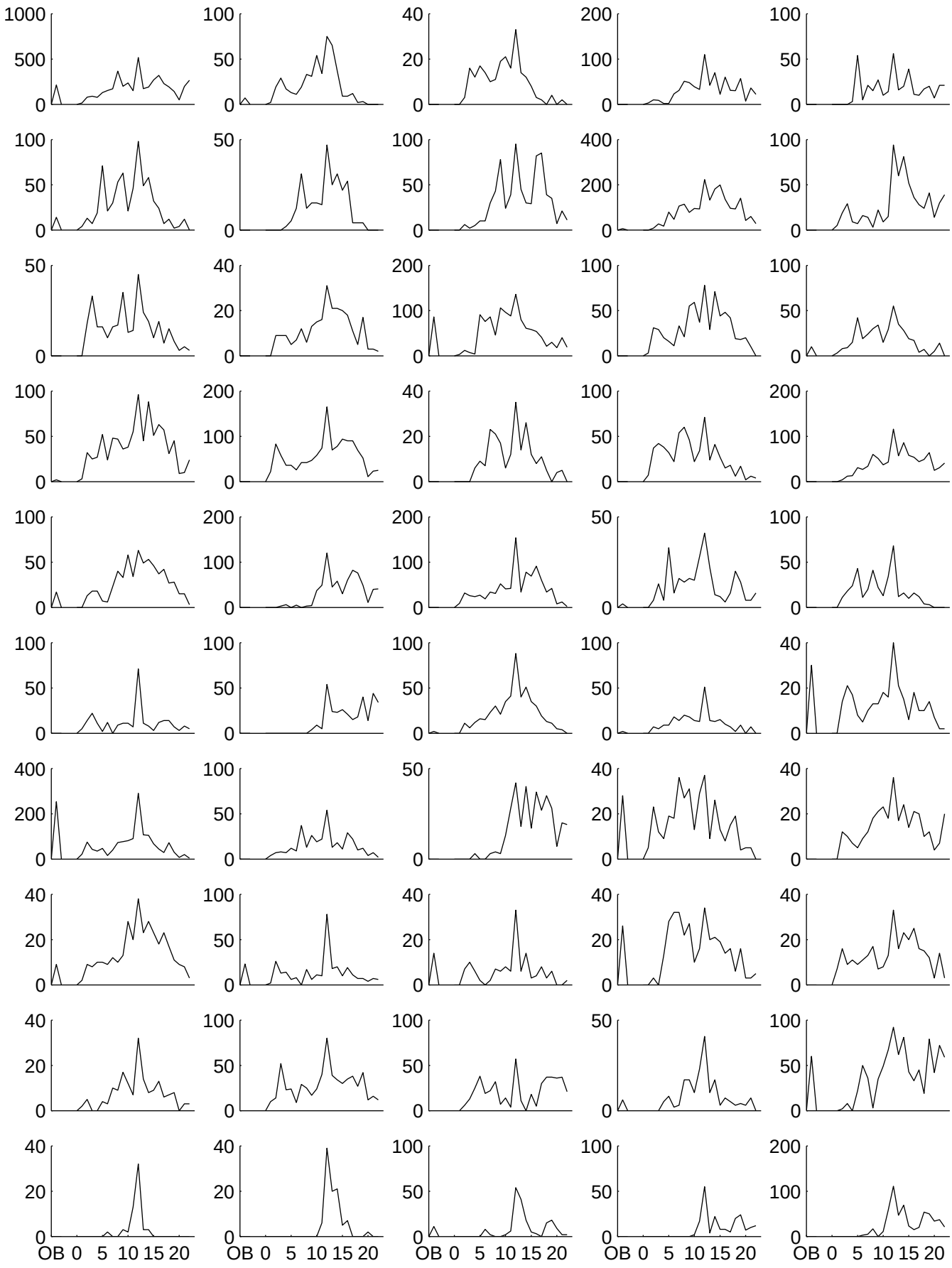
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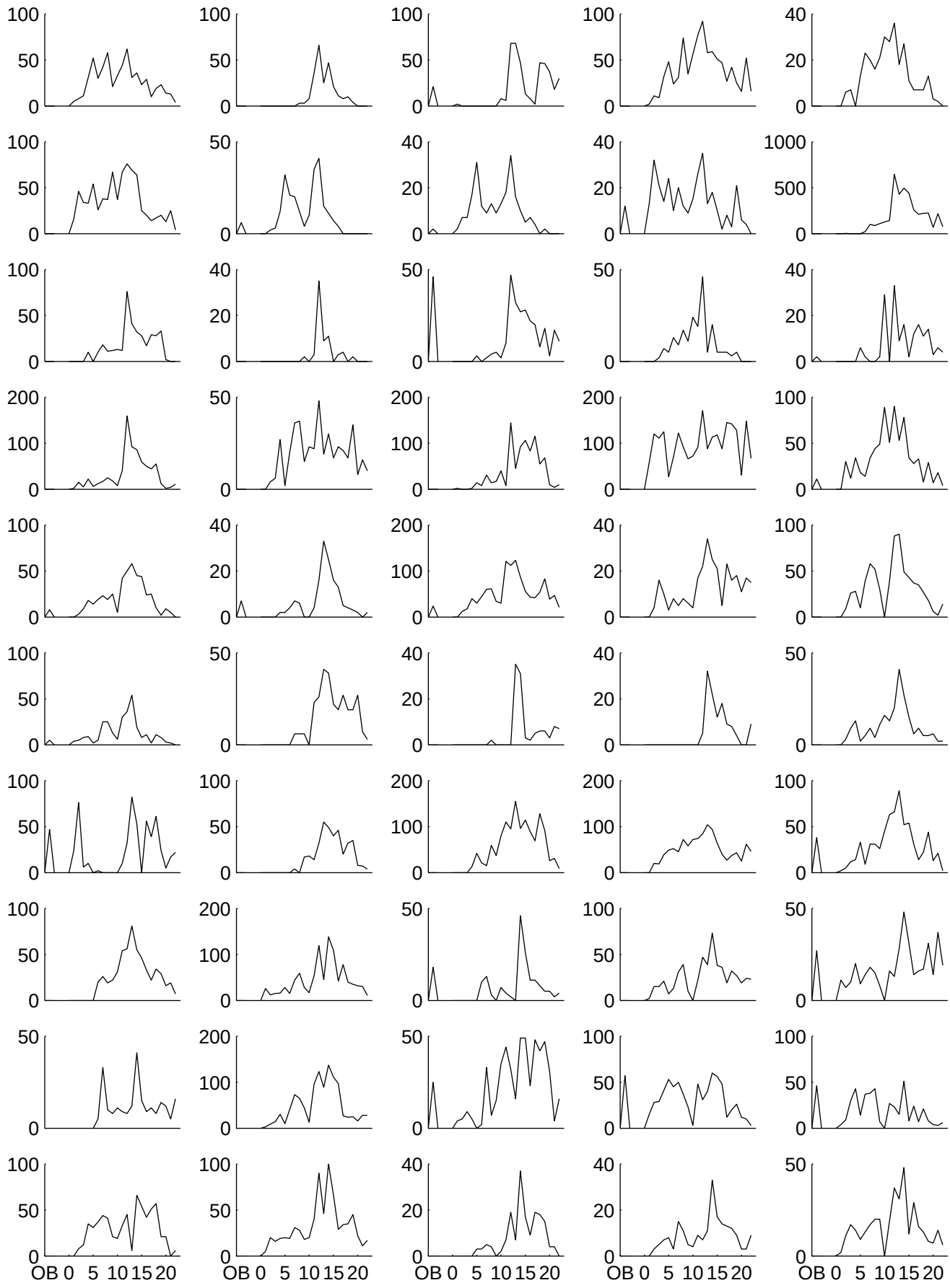
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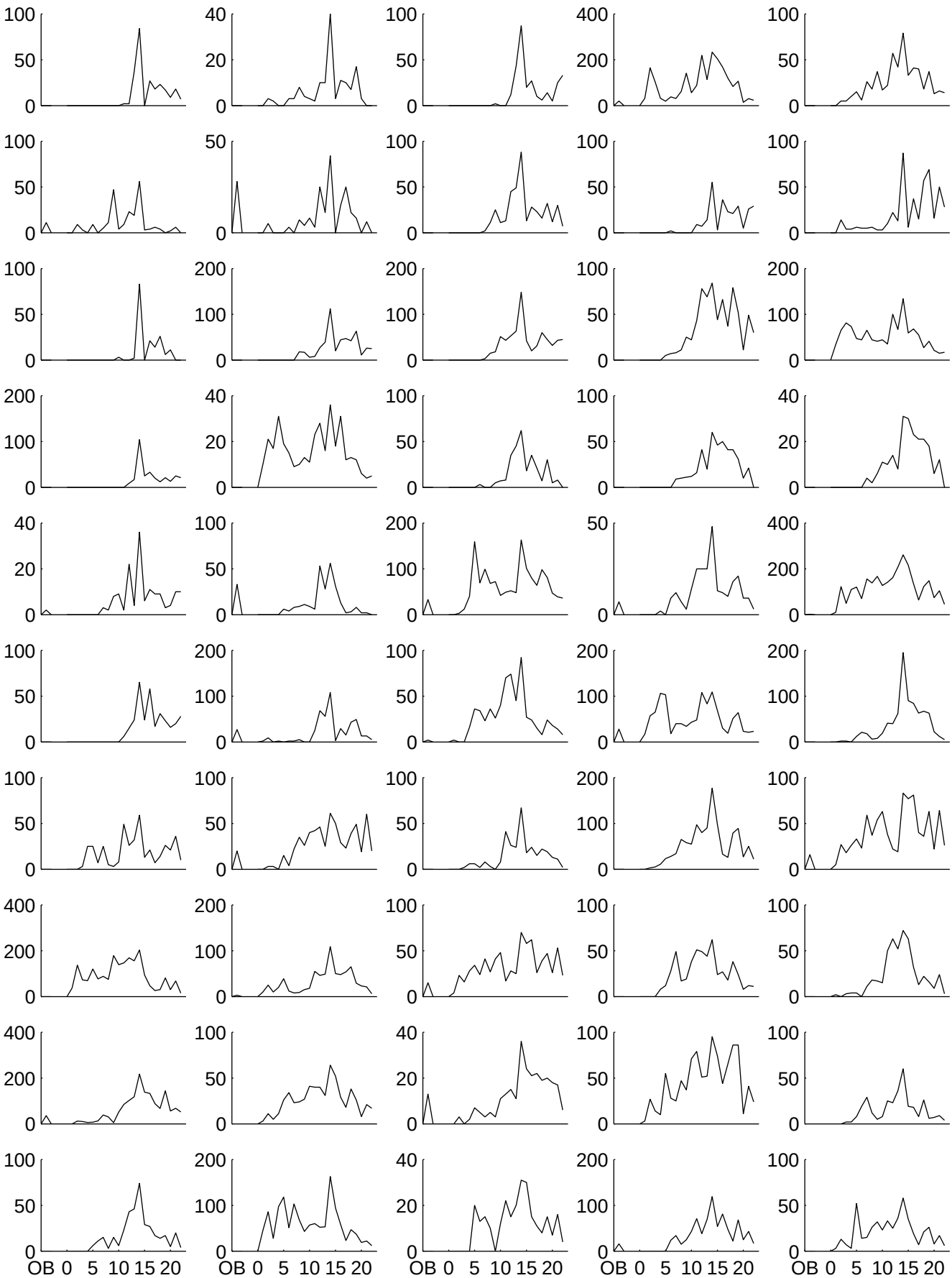
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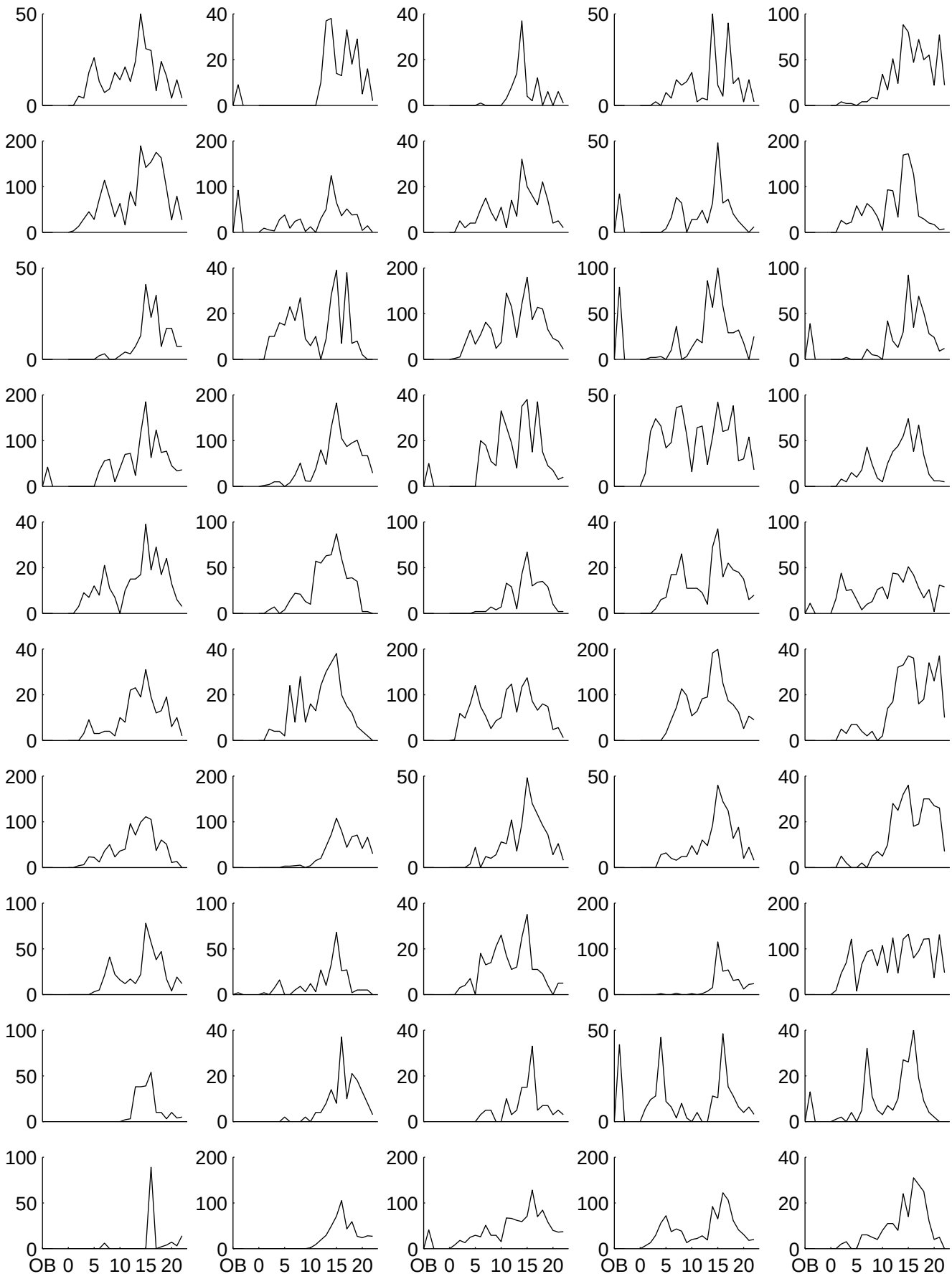
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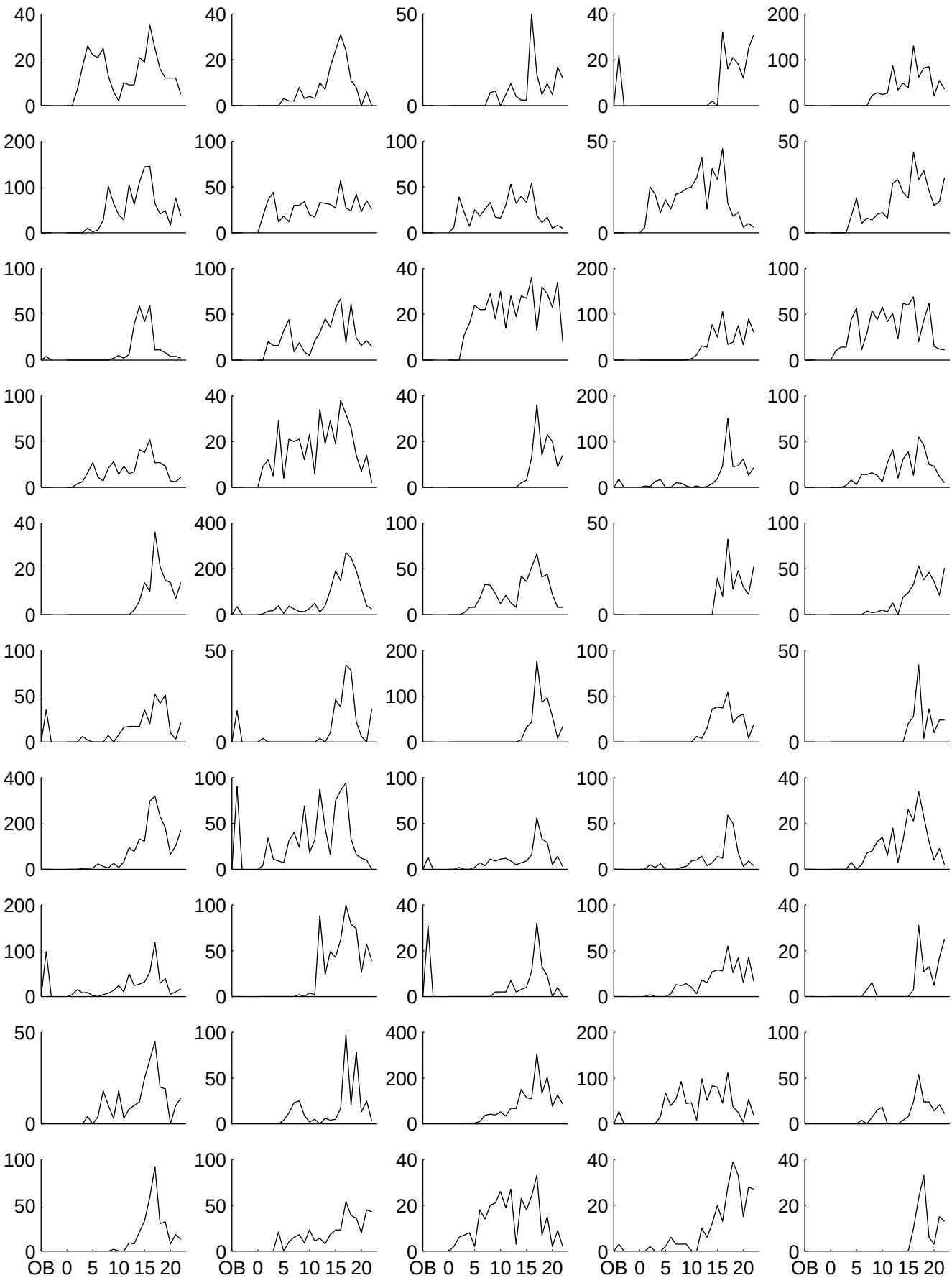
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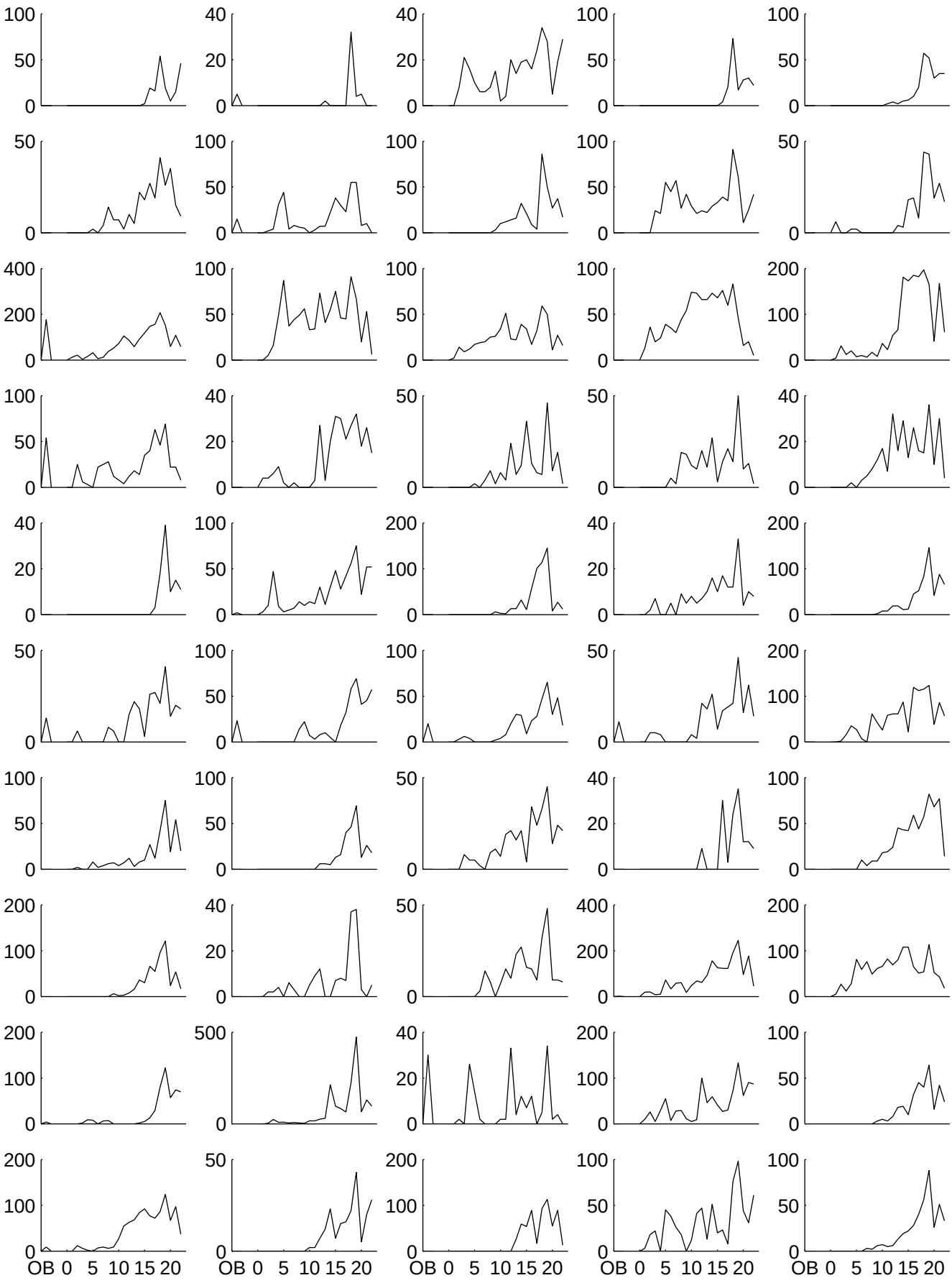
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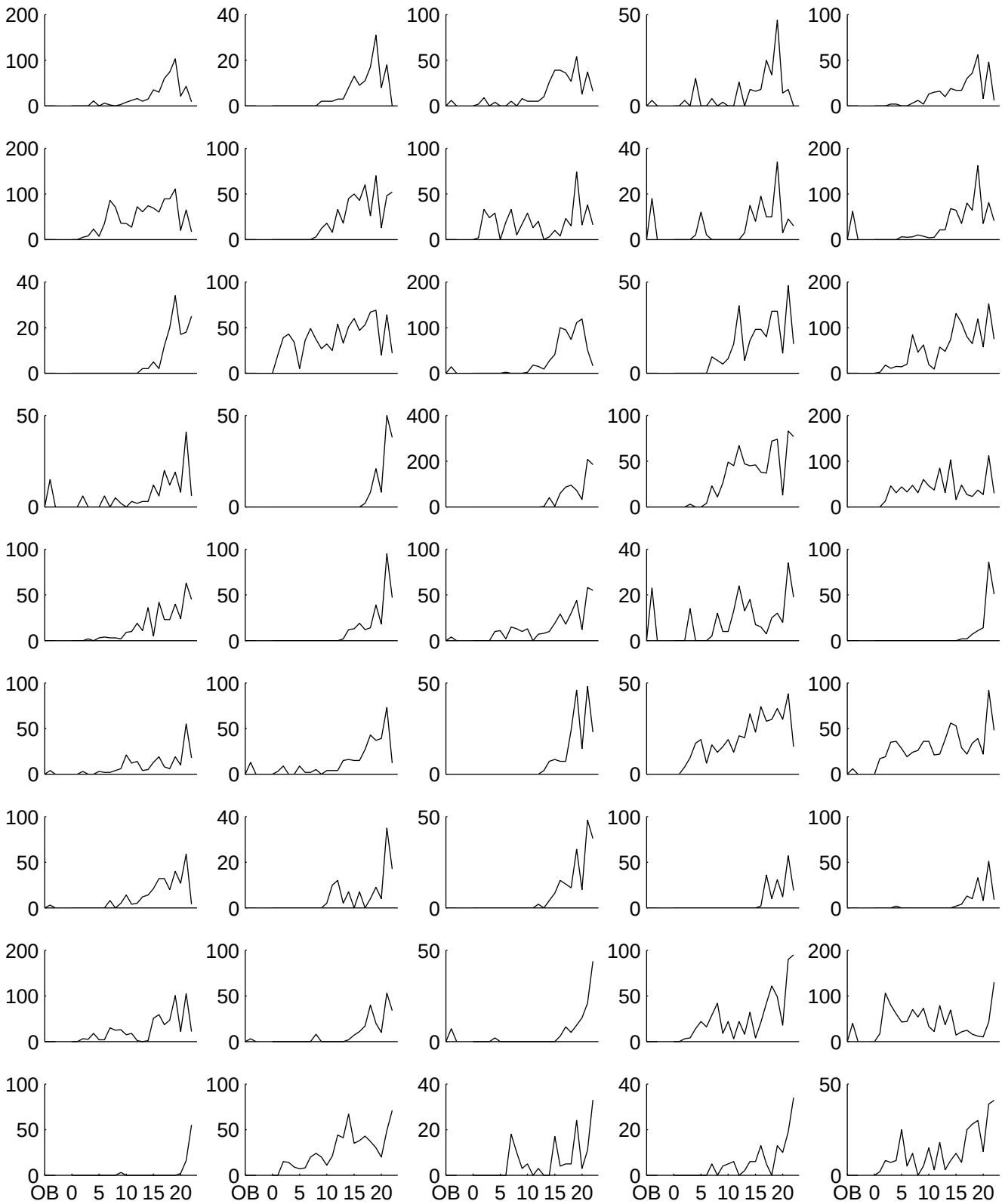
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