Generating *in vitro* exhausted T cells
From Scharping et al., Mitochondrial stress induced by continuous stimulation under hypoxia rapidly drives T cell exhaustion. *Nature Immunology* (2021).

Protocol:

D0

- Isolate $CD8^+$ T cells from mouse spleen and lymph nodes by sorting $CD44^{hi}$ $CD8^+$ T cells. To activate, place 20,000 T cells per well in a round bottom plate with 20,000 CD3/CD28 dynabeads (Fisher Cat 11-453-D, washed according to manufacturer's instructions) + 25U/ml mouse IL-2 (Peprotech Cat 212-12) and 10ng/ml IL-12 (Peprotech Cat 210-12) in 200uL complete RPMI+10% serum (named R10 hereafter). Allow 24hr for activation in normoxia (normal incubator, atmospheric oxygen).

D1

- After 24hr, recombine all cells and use a magnet (we use Fisher Cat 12-301-D) to remove all dynabeads. Now place cells into various conditions:
    - Acute activation (no dynabeads) in normoxia (normal incubator, atmospheric oxygen) and hypoxia (1.5% oxygen, hypoxia chamber BioSpherix, ProOx Model C21)
    - Continuous activation (add back washed dynabeads) at a 10:1 bead:cell ratio in normoxia and hypoxia (200,000 beads with 20,000 $CD8^+$ T cells)
- Continue culturing groups of cells in 200uL R10 + 25U/ml mouse IL-2 in round bottom plates.

D2

- Add 100ul R10 + 25U/ml mouse IL-2 to all wells

D3

- Divide all groups in half by pipetting 150ul (of 300ul well) into a new well (now have double the wells per group). Spin down all cells in centrifuge, and flick off old media. Give all groups 300uL fresh R10 + 25U/ml mouse IL-2. For continuous stim groups, add additional beads to maintain the same number of beads per well.

D4

- Leave cells alone

D5

- Divide all groups in half by pipetting 150ul (of 300ul well) into a new well (now have double the wells per group). Spin down all cells in centrifuge, and flick off old media. Give all groups 300uL fresh R10 + mouse 25U/ml IL-2. For continuous stim groups, add additional beads to maintain the same number of beads per well.

D6

- Remove beads and assay cells to determine exhaustion, e.g. co-inhibitory marker staining and restim for cytokine production.