# spIsoNet Tutorial

spIsoNet (single particle IsoNet) is designed to overcome the preferred orientation problem in cryoEM by self-supervised deep learning. Unlike conventional supervised deep learning methods that need explicit input-output pairs for training, spIsoNet autonomously extracts supervisory signals from the original data, ensuring the reliability of the information used for training.

spIsoNet is designed for single particle analysis and subtomogram averaging. For the correcting missing wedge in cryoET, please refer to IsoNet (https://doi.org/10.1038/s41467-022-33957-8).

# 1. Installation

The installation involves 4 steps: 1. Install anaconda and create a conda environment. 2. Install cuda and pytorch. 3. Install spIsoNet and dependencies. 4. For *Misalignment Correction*, set RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE and CONDA_ENV environment variable.

The environment we tested are:

1. cuda11.8 cudnn8.5 pytorch2.0.1, pytorch installed with pip.
2. cuda11.3 cudnn8.2 pytorch1.13.1, pytorch installed with conda.

Example commands to install spIsoNet

*Option 1: recommanded*

```
git clone https://github.com/IsoNet-cryoET/spIsoNet.git
conda env create -f setup.yml
conda activate spisonet
```

and then set the following environment variable for *Misalignment Correction*

```
export RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE="python <path to
spIsoNet>/spIsoNet/bin/relion_wrapper.py"
export CONDA_ENV="spisonet"
```

*Option 2:*

```
git clone https://github.com/IsoNet-cryoET/spIsoNet.git
conda create -n spisonet python=3.10
conda activate spisonet
pip install torch --index-url https://download.pytorch.org/whl/cu118
cd <path to spIsoNet>
pip install .
```

and then set the following environment variable for *Misalignment Correction*

```
export RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE="python <path to spIsoNet>/spIsoNet/bin/relion_wrapper.py"
export CONDA_ENV="spisonet"
```

*Option 3:*

```
git clone https://github.com/IsoNet-cryoET/spIsoNet.git
conda create -n spisonet python=3.10
conda activate spisonet
conda install -c "nvidia/label/cuda-11.8.0" cuda-toolkit
pip install torch --index-url https://download.pytorch.org/whl/cu118
pip install -r requirements.txt
```

and then set the following environment variable for *Misalignment Correction*

```
export RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE="python <path to spIsoNet>/spIsoNet/bin/relion_wrapper.py"
export CONDA_ENV="spisonet"
export PATH=<path to spIsoNet>/spIsoNet/bin:$PATH
export PYTHONPATH=<path to spIsoNet>:$PYTHONPATH
```

# 2. *Anisotropy Correction*

The default parameters in spIsoNet should be suitable in most cases.

## 2.0 Prepare data set

The tutorial data can be downloaded here: https://ucla.box.com/s/f77cl64g28tth0vldq2mqhn022gyhb80 or https://www.ebi.ac.uk/emdb/EMD-8731?tab=interpretation

Two half-maps (emd_8731_half_map_1.mrc and emd_8731_half_map_2.mrc) and a solvent mask (emd_8731_msk_1.mrc) are needed for *Anistropy Correction* tutorial.

If the file extension is ".map", Please replace the file extension map to mrc.

```
mv emd_8731_half_map_1.map emd_8731_half_map_1.mrc
mv emd_8731_half_map_2.map emd_8731_half_map_2.mrc
mv emd_8731_msk_1.map emd_8731_msk_1.mrc
```

## 2.1. Calculate 3DFSC

The algorithm for 3D FSC is customized based on Tan, Y.Z., Baldwin, P.R., Davis, J.H., Williamson, J.R., Potter, C.S., Carragher, B. and Lyumkis, D., 2017. Addressing preferred specimen orientation in single-particle cryo-EM through tilting. Nature methods, 14(8), p.793.

The 3DFSC volume (the default file name is FSC3D.mrc) should be generated in a few minutes. This step does not need GPU accelation. You can use multiple cpu cores for parallelization by specifying "--ncpus".

Thie FSC3D.mrc will be used in the following reconstruct step as "aniso_file"

The input of 3D FSC calculation are two half-maps and a solvent mask

```
spisonet.py fsc3d emd_8731_half_map_1.mrc emd_8731_half_map_2.mrc
emd_8731_msk_1.mrc --ncpus 16 --limit_res 3.5
```

This will generate a 3DFSC volume called "FSC3D.mrc", which describes the Fourier shell correlation in different directions.

You can also tune the --limit_res parameter to set the resolution limit of the 3D FSC calculation. Default value is the overall resolution of the map.

The expected command line output is shown as follows.

```
11:07:17, INFO     [spisonet.py:552] Global resolution at FSC=0.143 is
4.191999816894532
11:07:17, INFO     [spisonet.py:555] Limit resolution to 3.5 for spIsoNet
3D calculation.
11:07:17, INFO     [spisonet.py:557] calculating fast 3DFSC, this will take
few minutes
100%|██████████████████████████████████████████████████████████| 80/80 [00:02<00:00, 28.95it/s]
100%|██████████████████████████████████████████████████████████| 80/80 [00:03<00:00, 24.20it/s]
11:07:23, INFO     [spisonet.py:562] voxel_size 1.309999942779541
```

## 2.2. *Anisotropy Correction* of half-maps

This step trains a network for *Anisotropy Correction* with "spisonet.py reconstruct".

This step will create a folder to store the output files of spIsoNet. The corrected map is stored as "correctedXXX.mrc" in that folder. You can also find trained neural network "XX.pt" and figure for loss change "loss.png" in the folder.

The command to use spIsoNet should be

```
spisonet.py reconstruct emd_8731_half_map_1.mrc emd_8731_half_map_2.mrc --aniso_file FSC3D.mrc --mask emd_8731_msk_1.mrc --limit_res 3.5 --epochs 30 --alpha 1 --beta 0.5 --output_dir isonet_maps --gpuID 0,1,2,3 --acc_batches 2
```

Here is the expected command line output

```
11:13:15, INFO     [spisonet.py:466] voxel_size 1.309999942779541
11:13:24, INFO     [refine.py:239] Start preparing subvolumes!
11:13:24, INFO     [refine.py:242] Done preparing subvolumes!
11:13:24, INFO     [refine.py:244] Start training!
11:13:25, INFO     [network.py:202] Port number: 43963
learning rate 0.0003
(8, 9)
100%|
██████████████████████████████████████████████████████████████████████████
██████████████████████████████████████████████████████████████████████████
██████████████████████████████████████████████████████████████████████████
███████| 250/250 [02:13<00:00,  1.87batch/s, Loss=0.598]
Epoch [1/30], Train Loss: 0.6581
 14%|█████████████████████████████████
| 36/250 [00:16<01:40,  2.14batch/s, Loss=0.533]
...
```

You can check the command line argument for the reconstruct with the following command:

```
spisonet.py reconstruct --help
```

## 2.3. Postprocessing

Postprocessing of the corrected half-maps is not implemented in spIsoNet. You can use *relion_postprocess* in RELION for sharpening.

# 3. Advanced parameters

## 3.1 Limit resolution

This parameter is defined as the resolution limit for spIsoNet recovery.

The half-maps will be filtered to this resolution for the neural network training. After the network is trained, spIsoNet will produce maps called "corrected_xx_filtered.mrc". Then the information beyond this resolution will be added to produce the final results "corrected_xx.mrc".

The higher resolution may introduce unreliable noise that could compromise the results. A lower value may lead to results with partially recovered missing information. We tested that the resolution at 0.143 or 3.5A could be a good starting point to test this value. This value is better to be consistent with the limit resolution parameter in 3DFSC calculation.

## 3.2 GPU memory consumption and acc_batches

Here is the table for GPU memory consumption. Based on our experience, larger batch size (> 4) works slightly better.

An acc_batches larger than 1 uses accumulate gradient to reduce memory consumption. Usually acc_batches can be 2 for most cases. If you have GPU with large memory, acc_batches = 1 should process slightly faster.

batch_size should be divisible by acc_batches.

| Number of GPUs | batch_size | acc_batches | memory usage per GPU |
|---|---|---|---|
| 1 | 4* | 1 | ~17GB |
| 1 | 4* | 2* | ~11GB |
| 1 | 4* | 4 | ~7GB |
| 2 | 4* | 1 | ~10GB |
| 2 | 4* | 2* | ~7GB |
| 4 | 8* | 1 | ~10GB |
| 4 | 8* | 2* | ~6GB |

- means default values

## 3.3 Predict directly or continue from a trained model

The spIsoNet will generate a neuronal network model named xx.pt in the output folder, you can start from that model with the parameter "--pretrained_model".

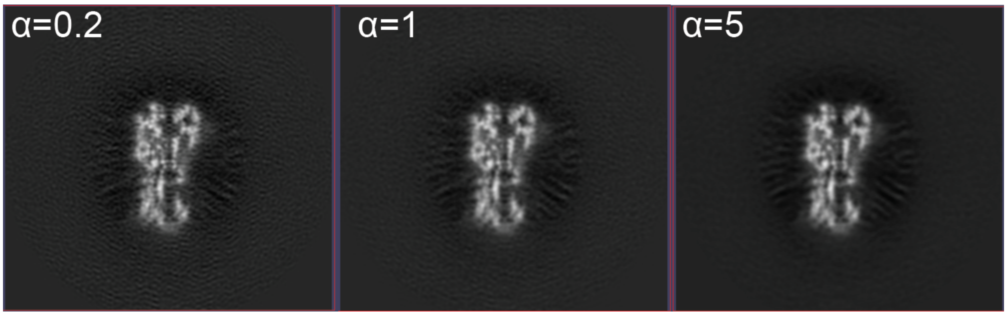
Once you start with the pretrained model, you may also want to change the number of epochs to run. For example, the trained model is from the 10th epochs and you can train for another 10 epochs to make it equivalent to the 20 epochs from scratch.

You can also set "--epochs" to 0 together with "--pretrained_model" to only perform prediction without further training

## 3.4 Alpha and Beta weighting

The alpha value defines the weighting between the data consistency loss and the rotation equivarience loss. The default value 1 means putting equal weight on the two losses. The larger value means more weight on the rotation equivarience loss.

Empirically, the larger alpha value will results in smoother results. Please see the following images of corrected_half1.mrc with different alpha values.




The beta value defines the weighting between denoising and missing information recovery. The larger value leads to more denoised output maps. The default beta velue is 0.5. We typically do not change this value.

## 3.5 Run spIsoNet with a reliable reference

If you have a low resolution map of your sample that is reliable and with less severe perferred orientation, you can use this as a reference (with parameter --reference) for the spIsoNet reconstruct. This allows you to retain the low resolution information (defined with --ref_resolution parameter) from the reference in the spIsoNet reconstruct process. This may improve the results.

The default --ref_resolution is 10, this resolution should be much lower than the resolution of the reference. We recommend the reference resolution to be 10-20A.

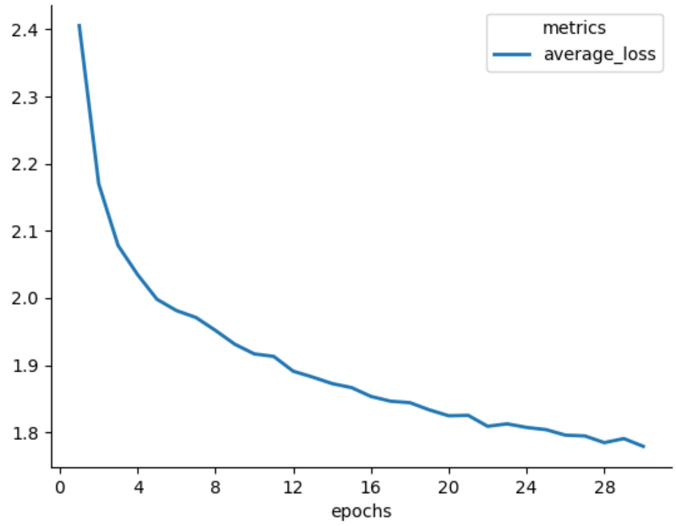
## 3.6 Process two half-maps independently

The noise2noise-based denoising is used by default in the *Anistropy Correction* process. It will produce cleaner maps. However, this denoising network will see both half1 and half2, which will break the independency of the two half-maps.

You can specify the "--independent" as True to run the two half-map independently, which will only perform missing information recovery without denoising. This step will train two networks each for one half-map. The results generated from "--independent" reconstruction can be used for the "gold-standard" FSC calculation.

## 3.7 Loss curve and epochs

The calculated loss with respective to the epochs is ploted and generated as a "png" file in the output folder. The loss function should gradually decrease throughout the training. Here is an example of the loss plot.




Typically 30 epochs is sufficient. You can also increase the number of epochs to obtain a lower loss.

# 4. *Misalignment Correction*

The particle alignment will nevertheless be influenced by distorted map with perferred orientation. This step is very useful for a better particle alignment and consequently a better cryoEM map.

Here, spIsoNet is integrated into the RELION refinement process. In each iteration of RELION refinement, spIsoNet can be used to perform the 3D reconstruction to generate corrected maps and use those maps for orientation search in RELION refinement process.

We tested both RELION3.1 and RELION4.0 work for spIsoNet *Misalignment Correction*

After *Misalignment Correction*, you may further perform spIsoNet *Anistropy Correction* using the RELION generated half-maps to improve the map quality.

The spIsoNet *Misalignment Correction* tutorial dataset can be downloaded from "https://ucla.box.com/s/ng459g8mhlf63z4sio5y4v432yt6k7qa". This is a subset of EMPIAR-10096, including particle star file (job025_tutorial.star), particle stack file (job025_tutorial.mrc), mask file (mask.mrc) and a low resolution reference at 10A resolution (HA_reference.mrc). From this dataset, we can obtain a 3.5A resolution structure with *Misalignment Correction*.

## 4.1 Make sure spIsoNet is properly installed in a conda environment.

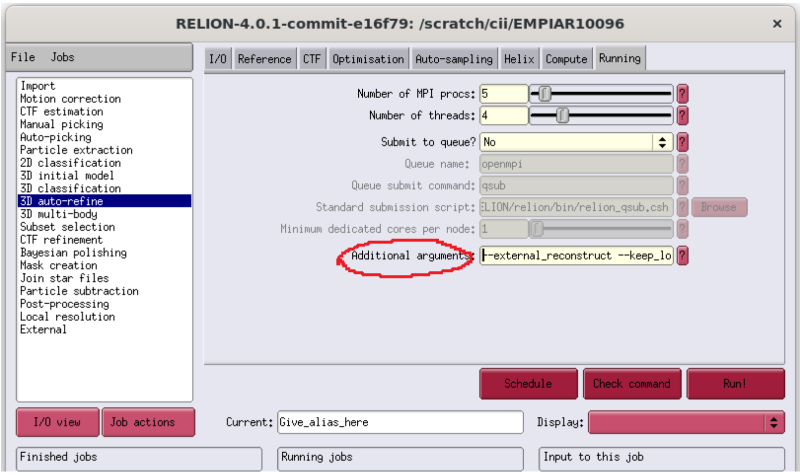
*Misalignment Correction* needs a conda environment and a RELION installation. It is required to set the RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE environment variable to point to relion_wrapper.py.

```
export RELION_EXTERNAL_RECONSTRUCT_EXECUTABLE="python <path to spIsoNet>/spIsoNet/bin/relion_wrapper.py"
export CONDA_ENV="spisonet"
```

## 4.2 Execute relion_wrapper.py script in RELION's relion_refine.

To execute the script relion_wrapper.py in relion_refine, it is necessary to add the argument "--external_reconstruct" in the command line, or by adding "--external_reconstruct" in the Additional Arguments section under the Running tab in RELION GUI.

Here is the place for "--external_reconstruct":




To use the spIsoNet, the relion refine command should contain:

1. "--external_reconstruct"
2. "--solvent_mask"
3. "--solvent_correct_fsc"

The "--keep_lowres" parameter allows for preserving the low-resolution information of the reference map during the subsequent RELION refinement iterations. The low resolution value to keep is defined by "--ini_high" parameter in RELION. This "--keep_lowres" parameter is not recognized by RELION but can be recognized by spIsoNet.

In some severe cases, in which RELION fails to perform reliable image alignment and to generate correct initial maps, the "--keep_lowres" is necessaey to keep the low resolution information from a correct reference throughout the image alignment. The reference map can be obtained from various methods such as reconstruction from particle datasets with stage tilt or subtomogram averaging from tomography datasets.

Here is an example command:

```
mpirun -np 5 `which relion_refine_mpi` --o Refine3D/job001/run --auto_refine --split_random_halves --i job025_tutorial.star --ref HA_reference.mrc --firstiter_cc --ini_high 10 --dont_combine_weights_via_disc --preread_images  --pool 30 --pad 2  --ctf --particle_diameter 170 --flatten_solvent --zero_mask --solvent_mask mask.mrc --solvent_correct_fsc  --oversampling 1 --healpix_order 2 --auto_local_healpix_order 5 --offset_range 5 --offset_step 2 --sym C3 --low_resol_join_halves 40 --norm --scale  --j 4 --gpu "" --external_reconstruct --keep_lowres --pipeline_control Refine3D/job001/
```

In the log file for relion, you should see the following messages if the spIsoNet is running correctly in each iteration of RELION refinement:

```
 + Making system call for external reconstruction: python
/home/cii/software/spIsoNet/bin/relion_wrapper.py
Refine3D/job032/run_it025_half1_class001_external_reconstruct.star
iter = 025
set CUDA_VISIBLE_DEVICES=None
set CONDA_ENV=torch_cuda12.0_glados_py3.10
set ISONET_WHITENING=True
set ISONET_WHITENING_LOW=10
set ISONET_RETRAIN_EACH_ITER=True
set ISONET_BETA=0.5
set ISONET_ALPHA=1
set ISONET_START_HEALPIX=4
set ISONET_ACC_BATCHES=2
set ISONET_EPOCHS=5
set ISONET_KEEP_LOWRES=True
set ISONET_LOWPASS=True
healpix = 7
symmetry = C3
mask_file = relion31maptightmask.mrc
pixel size = 1.309998
resolution at 0.5 and 0.143 are 3.992381 and 3.422041
real limit resolution to 3.422041
 eval "$(conda shell.bash hook)" && conda activate
torch_cuda12.0_glados_py3.10 &&  spisonet.py whitening
Refine3D/job032/run_it025_half1_class001_unfil.mrc -o
Refine3D/job032/run_it025_half1_class001_unfil.mrc --mask
relion31maptightmask.mrc --high_res 3.422041 --low_res 10
 eval "$(conda shell.bash hook)" && conda activate
torch_cuda12.0_glados_py3.10 &&  spisonet.py whitening
Refine3D/job032/run_it025_half2_class001_unfil.mrc -o
Refine3D/job032/run_it025_half2_class001_unfil.mrc --mask
relion31maptightmask.mrc --high_res 3.422041 --low_res 10
 eval "$(conda shell.bash hook)" && conda activate
torch_cuda12.0_glados_py3.10 &&  spisonet.py combine_map
Refine3D/job032/run_it000_half2_class001.mrc
Refine3D/job032/run_it025_half2_class001_unfil.mrc
Refine3D/job032/run_it025_half2_class001_unfil.mrc 10.0 --mask_file
relion31maptightmask.mrc
 eval "$(conda shell.bash hook)" && conda activate
torch_cuda12.0_glados_py3.10 &&  spisonet.py combine_map
Refine3D/job032/run_it000_half1_class001.mrc
Refine3D/job032/run_it025_half1_class001_unfil.mrc
Refine3D/job032/run_it025_half1_class001_unfil.mrc 10.0 --mask_file
relion31maptightmask.mrc
relion_image_handler --i Refine3D/job032/run_it025_half1_class001_unfil.mrc
--o Refine3D/job032/run_it025_half1_class001_unfil_lowpass_backup.mrc --
lowpass 3.422041; cp
Refine3D/job032/run_it025_half1_class001_unfil_lowpass_backup.mrc
Refine3D/job032/run_it025_half1_class001_unfil.mrc
relion_image_handler --i Refine3D/job032/run_it025_half2_class001_unfil.mrc
--o Refine3D/job032/run_it025_half2_class001_unfil_lowpass_backup.mrc --
lowpass 3.422041; cp
```

```
Refine3D/job032/run_it025_half2_class001_unfil_lowpass_backup.mrc
Refine3D/job032/run_it025_half2_class001_unfil.mrc
18:34:04, INFO     [spisonet.py:431] Limit resolution to 3.422041 for
spIsoNet 3D FSC calculation. You can also tune this paramerter with --
limit_res .
18:34:04, INFO     [spisonet.py:433] calculating fast 3DFSC, this will take
few minutes
18:34:35, INFO     [spisonet.py:440] voxel_size 1.3099980354309082
Refine3D/job032/run_it024_half_class001_unfil.pt
retrain network each relion iteration
 eval "$(conda shell.bash hook)" && conda activate
torch_cuda12.0_glados_py3.10 &&  spisonet.py refine_n2n
Refine3D/job032/run_it025_half1_class001_unfil.mrc
Refine3D/job032/run_it025_half2_class001_unfil.mrc
Refine3D/job032/run_it025_3DFSC.mrc --epochs 5 --n_subvolume 1000 --
acc_batches 2 --alpha 1 --beta 0.4 --output_dir Refine3D/job032 --gpuID
None --limit_res 3.422041 --mask relion31maptightmask.mrc
using all GPUs in this node: 0,1,2,3
18:34:46, INFO     [utils.py:15] The Refine3D/job032 folder already exists,
outputs will write into this folder
18:34:46, INFO     [spisonet.py:224] voxel_size 1.3099980354309082
run_it025_half_class001_unfil
18:34:46, INFO     [refine.py:299] Filter map to resolution 3.422041 for
spIsoNet correction!
18:34:56, INFO     [refine.py:311] Start preparing subvolumes!
18:34:56, INFO     [refine.py:314] Done preparing subvolumes!
18:34:56, INFO     [refine.py:316] Start training!
18:34:56, INFO     [network_n2n.py:232] Port number: 42765
learning rate 0.0003
['Refine3D/job032/run_it025_half1_class001_unfil_data',
'Refine3D/job032/run_it025_half2_class001_unfil_data']
Epoch [1/5], Train Loss: 0.9726
Epoch [2/5], Train Loss: 0.8730
Epoch [3/5], Train Loss: 0.8548
Epoch [4/5], Train Loss: 0.8424
Epoch [5/5], Train Loss: 0.8300
18:38:58, DEBUG    [__init__.py:337] matplotlib data path:
/home/cii/anaconda3/envs/torch_cuda12.0_glados_py3.10/lib/python3.10/site-
packages/matplotlib/mpl-data
18:38:58, DEBUG    [__init__.py:337] CONFIGDIR=/home/cii/.config/matplotlib
18:38:58, DEBUG    [__init__.py:1498] interactive is False
18:38:58, DEBUG    [__init__.py:1499] platform is linux
18:38:58, DEBUG    [__init__.py:337] CACHEDIR=/home/cii/.cache/matplotlib
18:38:58, DEBUG    [font_manager.py:1574] Using fontManager instance from
/home/cii/.cache/matplotlib/fontlist-v330.json
18:38:59, INFO     [refine.py:346] Start predicting!
data_shape torch.Size([125, 1, 80, 80, 80])
size restored (334, 334, 334)
data_shape torch.Size([125, 1, 80, 80, 80])
size restored (334, 334, 334)
18:39:11, INFO     [refine.py:369] Done predicting
18:39:11, INFO     [spisonet.py:250] combining
18:39:11, INFO     [spisonet.py:335] voxel_size 1.3099980354309082
18:39:23, INFO     [spisonet.py:335] voxel_size 1.3099980354309082
```

```
18:39:34, INFO     [spisonet.py:254] Finished
finished spisonet reconstruction
 + External reconstruction finished successfully, reading result back in
...
8.80/8.80 min
............................................................~~(,_,">
```

## 4.3 Environment variables for *Misalignment Correction*.

To tune the parameter of spIsoNet *Misalignment Correction*, you can set up more linux environemnt variables as follows:

1. set which GPU(s) you can use by sepecify the CUDA_VISIBLE_DEVICES. By default, spIsoNet will use up all the avaliable GPUs

```
export CUDA_VISIBLE_DEVICES="0"
```

2. ISONET_BETA value will define the denoising level of the network. See section 2.4

```
export ISONET_BETA=0.5
```

3. ISONET_ALPHA value define the balance between the data consistency loss and rotation equivariance loss. Default value 1 should work for most cases. Setting this to zero is not recommended and will ignore rotation equivariance loss. See section 2.4

```
export ISONET_ALPHA=1
```

4. ISONET_START_HEALPIX defines angular sampling step when spIsoNet training will start to performed. ISONET_START_RESOLUTION defines refinement resolution when spIsoNet training will start to performed.

```
ISONET_START_HEALPIX=3
ISONET_START_RESOLUTION=15
```

5. ISONET_RETRAIN_EACH_ITER defines whether the network should be retrained from scratch or from previous iteration of relion refine. This is typically set to True

```
ISONET_RETRAIN_EACH_ITER=True
```

6. ISONET_EPOCHS defines how many epochs to train the neural network.

```
ISONET_EPOCHS=5
```

7. ISONET_ACC_BATCHES larger than 1 uses accumulate gradient to reduce memory consumption. Usually acc_batches can be 2 for most cases. If you have GPU with large memory, ISONET_ACC_BATCHES=1 should process slightly faster.

```
ISONET_ACC_BATCHES=2
```

8. ISONET_KEEP_LOWRES define whether low resolution information from a correct reference is kept in the alignment. This parameter can be overwrite by the --keep_lowres parameter in relion command or GUI

```
ISONET_KEEP_LOWRES=False
```

9. ISONET_WHITENING define whether whitening is performed before running spIsoNet. Typically we set this value True. ISONET_WHITENING_LOW defines the starting resolution for whitening.

```
ISONET_WHITENING=True
ISONET_WHITENING_LOW=10
```

10. ISONET_FSC_WEIGHTING define whether FSC weighting is performed before running spIsoNet. Typical we set this as True.

```
ISONET_FSC_WEIGHTING=True
```