MEA Acute Single-conc and Cytotoxicity Updates for ToxCast Pipeline

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These decisions are based on conversations with Tim July 12 & 19, 2022.

Unless otherwise noted, all R and html referenced files are located under L:\Lab\NHEERL\_MEA\Carpenter\_Amy\pre-process\_mea\_acute\_for\_tcpl\investigations\sc\_and\_cytotox\_endpoints\_July2022

# Release MEA acute single-conc MFR endpoints (acid 1916, aeid’s 2033-2034).

## Desired update

To make the MEA Acute single-conc mean firing rate endpoints (aeid’s 2033-2034) publicly available (set export\_ready = 1).

\*\*We only want to release the single-conc data associated with these endpoints. The experimental data that corresponds to the multi-conc data under aeids 2033-2036 has been re-processed to generate the newer set of MEA acute assay endpoints (those that begin with “CCTE\_Shafer\_MEA\_acute\_”).

## Why we think the single-conc data under these endpoints is ready to be released

**TL;DR**: The TCPL hit calls for the MEA acute single-conc mean firing rate endpoints have good agreement with the hit calls that have been published for these experiments in Strickland et al., 2018. While the single-conc mean firing rate endpoints alone have lower sensitivity for detecting expected assay controls than we would like (57.7%), the sensitivity is boosted to 75% if we include the activity in the multi-conc MEA Acute endpoints. Furthermore, we are hesitant to adjust the cutoffs to make this assay more sensitive since the cutoffs are based on the estimated noise-band of activity in DMSO control wells and we would like to err on the side of being consistent with the approach used in published literature.

* The results from the MEA Acute single-conc experiments have been published in Strickland et al., 2018. I confirmed that 98.7% of the 1,104 samples have the same hit call in TCPL as in Strickland et al., 2018. All but 2 of the conflicting cases are due to the fact that the most active sample was used to represent each chemical in Strickland 2018. See *mea\_acute\_sc\_evaluation\_2022-06-27.html*.
* The MEA Acute assay has been validated conceptually in McConnell et al., 2012 (validated performance with several assay controls) and Valdivia et al., 2014 (validated performance with assay controls, compared activity in NVS ion channel assays – found that MEAs largely miss neonicotinoids, but are helpful for detecting pyrethroids and GABAa receptor antagonists).
* Since the MEA Acute assay methods chanced slightly in Strickland et al., 2018 from what was published previously, I checked out the activity of the assay controls identified in McConnell et al., 2012, Valdivia et al., 2014, and Kosnik et al., 2019 in the MEA acute single-conc endpoints in TCPL. (see *check\_mea\_acute\_sc\_and\_mc\_results\_against\_assay\_controls\_2022-07-18.html*, “Analyze activity in MFR endpoints only”, under the Summary of results for type = ‘sc’)
  + 0/33 negatives were identified as a hit (100% specificity)
  + 41/71 positives were identified as a hit (57.7% sensitivity)
  + 13 of the 30 not detected positive controls were tested in other MEA studies/laboratories and were found to be negative or equivocal (as determined in Strickland et al., 2018 S2). So that leaves 17 more concerning positive controls that are not detected in these MEA Acute sc endpoints.
* The sensitivity of the MEA Acute assay is boosted if we think of the single- and mulit- conc acute assays as 1 unit. If we combine the substances detected in the single-conc (hit for either MFR up or dn) with those detected in the multi-conc (hit in 3+ out of the 15 multi-conc endpoints with AC50 below the Alamar Blue AC50, as was used in Kosnik et al., 2019), the combined MEA Acute assay detects: (see *check\_mea\_acute\_sc\_and\_mc\_results\_against\_assay\_controls\_2022-07-18.html*, “Analyze activity with additional mc endpoints, selective hits only”, under the Pooled across mc and sc)
  + 1/35 negatives were identified as a hit (97.1% specificity)
  + 54/72 positives were identified as a hit (75.0% sensitivity) (Note that 1 substance, Acephate, was identified as a hit in the multi-conc for 1 replicate, but not the other. I am counting Acephate as identified a hit).
* We considered lowering the single-conc MFR dn cutoff to increase the sensitivity. However, Tim reminded that the cutoff is based on the activity in the DMSO wells. If we lower the dn MFR cutoff, we might dip into the range where some DMSO activity might be considered a hit. So we are not going to change the cutoffs, and we are ready to release the MEA Acute MFR single-conc endpoints!

# Update then release MEA Acute single-conc cytotoxicity endpoints (acid’s 1917-1918, aeid’s 2035-2036)

## Desired updates

Change the cutoffs used for the single-conc cytotoxicity endpoints as follows:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Type** | **Acid** | **Aeid** | **Aenm** | **Current cutoff** | **Current sc level 2 mthd\_ids** | **New desired cutoff** | **New sc level 2 mthd\_ids** |
| sc | 1917 | 2035 | CCTE\_Shafer\_MEA\_LDH | 3xBMAD | 20 (ow\_bmad\_nwells)  1 (bmad3) | 20% | 20 (ow\_bmad\_nwells, but the bmad doesn't really matter)  2 (pc20) |
| sc | 1918 | 2036 | CCTE\_Shafer\_MEA\_AB | 3xBMAD | 20 (ow\_bmad\_nwells)  1 (bmad3) | 80% | 20 (ow\_bmad\_nwells, but the bmad doesn’t really matter)  ? – could we create a new method for pc80? |

After making the above changes, the data under these endpoints should be ready to release.

## Why we want to change the cutoffs, with more details

**TL;DR:** Cutoffs of 20% and 80% were used in the publication of the MEA acute sc data in Strickland et al., 2018. I have not been able to verify if the level 2 response values in TCPL are the same as the normalized LDH and AB values used in Strickland et al., 2018. Regardless, if we apply the 20% LDH And 80% AB cutoff to the sc2 max\_med values, we get MUCH closer agreement to the cytotoxicity hits in Strickland et al., 2018 than we do with the current 3xBMAD cutoffs.

See *L:/Lab/NHEERL\_MEA/Carpenter\_Amy/pre-process\_mea\_acute\_for\_tcpl/investigations/sc\_and\_cytotox\_endpoints\_July2022/R/mea\_acute\_sc\_cytotox\_check\_for\_changes\_in\_values\_from\_srcf\_to\_tcpl\_lvl2\_2022-07-19.html*

### Current status of LDH and AB endpoints

With the current cutoffs, (see *checkout\_mea\_acute\_sc\_and\_mc\_LDH\_and\_AB\_tcpl\_results\_2022-07-07.html*)

* LDH: bmad is 2.55, so the cutoff = 3xbmad = 7.64. This results in 194 active samples in the LDH endpoint.
* AB: bmad is 6.67, so the cutoff = 3xbmad = 19.998. This results in 59 active samples in the AB endpoint.

### Cutoffs and activity in this data set from previous publications

The data associated with the mea acute single-conc endpoints was originally published in Strickland et al., 2018. They used much higher cutoffs for the LDH and AB assays. Only 8 substances were a hit in either the LDH and AB assay:

“Compounds were deemed cytotoxic if they induced greater than **20% increase in LDH release** and/or **decreased mitochondrial activity by more than 80%**. Changes in nMFR [normalized mean firing rate] occurred largely in the absence of cytotoxicity, as only 8 compounds decreased cellular viability following exposure. Compounds inducing cytotoxicity were: UK-337312, tributyltin chloride, tributyltin methacrylate, phenylmercuric acetate, 9-phenanthrol, gentian violet, mercuric chloride, and ketoconazole. All eight of these compounds decreased MFR beyond the threshold, and thus are considered to be “hits”, but are flagged as cytotoxic.” (Strickland et al., 2018).

### Does it conceptually make sense to apply the cytotoxicity cutoffs from Strickland 2018 to the TCPL sc2 response values? Do the normalized response values in TCPL sc2 correspond to the same values used in Strickland et al., 2018?

Unfortunately, I still don’t know how the LDH and AB values were normalized in Strickland et al, 2018 or even what the raw or response values were. The cytotoxicity data was not published as part of Strickland et al., 2018. We only have the 8 compounds that were considered hits in either the AB or LDH assay listed by name in the manuscript body. There are tons of potential files on the L drive, but I have no idea which one was used to make the final hit calls presented in the publication.

Furthermore, I don’t know for sure what the TCPL sc0 rval’s represent either or how they were normalized. Here’s what I did find:

* The “srcf” listed in the TCPL sc0 is NHEERL\_MEA\_SS\_SOURCE\_RAW\_DATA\_150422.csv. I found a copy of it here: L:\Lab\Toxcast\_Data\toxcast\_data\files\ccte\_shafer\nheerl\_mea\NHEERL\_MEA (again, note that I don’t know if this srcf was used to make the hit calls in Strickland, 2018).
* The LDH values in this srcf are listed as “LDH\_%DEAD” and the Alamar Blue values are “AB\_%VIABLE”
* I attempted to compare the values in the srcf to the TCPL sc0 rval’s. Difficulties included:
  + 10 apid – rowi – coli – acnm combinations were present in the sc0, but not in the srcf
  + Some of the MEA apid’s were re-used on multiple experiment dates. In these cases, I used the sc0 rval that was closest to the srcf rval. This affected about 20.6% of data values from each assay.
  + Even after de-duplicating data in the srcf and sc0, there were 42 acnm – well – plate - date combinations with multiple rows in the merged data file. I’m not sure why, but out of 8000+ data rows these 42 cases didn’t seem worth investigating.
* For the LDH assay, the srcf values and sc0 rval’s were in perfect agree for all but 3 data points from plates that were NOT reused. For plates that were reused, I had much lower agreement. But I don’t think these cases are worth investigating, because I don’t know exactly which values should be compared.
* For the AB assay, the srcf values and sc0 rval’s were not in agreement. However, if I transformed the srcf values by taking 100 – srcf AB value, there was perfect agree for all but 3 data points from plates that were NOT reused.
* Given this agreement, I think we can conclude that
  + The LDH level 0 values do correspond to %DEAD, as indicated in the srcf.
  + The AB values correspond to (100 - %VIABLE), i.e., the % dead.
* Note that no level 1 normalization methods are applied to the TCPL sc0 rval’s. So the sc2 response values represent the same quantities as the lvl0 rval’s.

Therefore, even though I can’t compare the cytotoxicity values used in Strickland 2018 and in TCPL sc2, I can conclude that

* A cutoff of 20% applied to the LDH TCPL sc2 values would correspond to a 20% increase in LDH release, as was intended in Strickland et al., 2018
* A cutoff of 80% applied to the AB TCPL sc2 values would roughly correspond to an 80% death, or as more specifically worded in Strickland 2018 “decreased mitochondrial activity by more than 80%.”

Therefore, I think the cutoffs of 20% and 80% can be applied to the cytotoxicity data to achieve the same intended conceptual cutoffs in Strickland 2018.

### Results with adjusted cutoffs have better agreement with Strickland et al., 2018

I tested the application of the new cutoffs of 20% for LDH and 80% for AB to the max\_med response values in TCPL sc2. I found that 6/8 substances that were labelled as cytotoxicity hits in Strickland et al., 2018 were now detected in either the LDH or AB assay in TCPL. The 2 substances that were not cytotoxicity hits in TCPL with the adjusted cutoffs were Ketoconazole and Mercuric chloride. Two additional substances (Emamectin benzoate and Thymol) that were not cytotoxicity hits in Strickland et al., 2018 were active in the LDH assay with the 20% cutoff. Given a disagreement of only 4 chemicals out of 1,055 chemicals screened, we are calling this good enough.

# Don’t release MEA Acute LDH endpoint

## Desired updates

We do not want to release the multi-conc LDH endpoint (aeid 2540, CCTE\_Shafer\_MEA\_acute\_LDH\_up) (i.e., we want to set export\_ready == 0).

## Why we don’t want to release the multi-conc LDH anymore:

**TL;DR:** Since we are changing the cutoffs for the MEA Acute single-conc endpoints, it seemed appropriate to reconsider the cytotoxicity cutoffs in the multi-conc as well. However, upon comparison to previously published work on the MEA acute multi-conc data in Kosnik et al., 2019, we found the LDH endpoint was excluded from the analysis entirely.

### Current status of LDH and AB endpoints in tcpl:

In TCPL, both the LDH and AB assay use 3xbmad as the cutoff

* LDH 3xbmad: 3\*(1.77) = 5.31
* AB 3xbmad: 3\*(13.38) = 40.1

Interestingly, even though this LDH cutoff is quite low there only 19 hits. See *checkout\_mea\_acute\_sc\_and\_mc\_LDH\_and\_AB\_tcpl\_results\_2022-07-07.html*.

### Previous use of LDH and AB endpoints in publications from Shafer lab

In Kosnik et al., 2019, they used a cutoff of 3xbmad for both the LDH and AB MEA Acute assay. Furthermore, the LDH endpoint was ultimately excluded from the analysis: “The LDH assay exhibited small BMAD values, resulting in responses for many chemicals being greater than the 3\*BMAD threshold, even though they did not demonstrate a concentration response. This decreased the reliability of this parameter for detecting truly cytotoxic compounds and therefore cytotoxicity was characterized using only the CTB assay.” Only activity in endpoints with AC50s below the CTB AC50 were considered for the remainder of the analysis in Kosnik et al, 2019.

### Interpretation of the LDH response values in TCPL

Here’s how the LDH values are processed in TCPL:

* Level 0 rvals: blank-corrected optical density measurements corresponding to the amount of LDH released (*see L:\Lab\NHEERL\_MEA\Carpenter\_Amy\pre-process\_mea\_acute\_for\_tcpl\mea-acute-neural-stats-to-mc0-scripts/acute\_cytotox\_prep07.R, getAssayData function*).
* Level 3 normalization steps:
  + Calculate “bval” = median of DMSO wells
  + Calculate “pval” = median of p wells (the p wells are either a single full LYSIS well on the plate, or 3 ½ Lysis wells whose values have been multiplied by 2)
    - Older data just has 1 full LYSIS well per MEA plate
    - However, it was found that the output produced by the full LYSIS wells is outside the optimal reading range of the machine, so double the output of ½ lysis wells is now preferred.
    - See *L:\Lab\NHEERL\_MEA\Carpenter\_Amy\pre-process\_mea\_acute\_for\_tcpl\mea-acute-neural-stats-to-mc0-scripts/LDH\_CellTiterBlue\_functions.R, prepare\_LDH\_p\_wells function*.
  + Resp.pc calculated as (rval – bval)/(pval – bval)

So, the LDH resp values represent the amount of LDH released (from dead cells) relative to the amount of LDH expected to be released by a well in which all of the cells have been lysed. In other words, the LDH response value is meant to indicate the percent dead.

As was the case for the single-conc, I don’t know for sure how the LDH values were normalized in Kosnik et al., 2019. For this reason, I’m not sure that the choices made regarding the LDH in Kosnik et al., 2019 or the cutoff selected in Strickland et al., 2018 are necessarily directly applicable to the LDH aeid=2540 level 3 response values currently in TCPL without further investigation. The impact of excluding the LDH endpoint is discussed below.

### Impact of removing LDH

In the entire MEA Acute multi-conc level 5 data to date, 19 out of 527 samples are active in the LDH endpoint, 8 of which are also active in the AB assay. Therefore, if we exclude the LDH endpoint, we would lose all cytotoxicity data for 11 samples. All but 3 of these 11 samples are active in one of the other 30 MEA acute non-cytotoxicity endpoints (with export\_ready == 1), meaning that some MEA Acute activity information is available for these samples. The 3 samples that are not active in any other MEA acute endpoints all have the flags “Borderline active and “Only one conc above baseline, active” associated with the LDH curve-fits. Since these 3 samples appear borderline based on the flags, it seems less important to me to be concerned about making sure that they appear as positives in the MEA acute assay. See *mea\_acute\_mc\_impact\_of\_exclusion\_of\_LDH\_2022-07-20.html.*

### Conclusion

More analysis could be done. However, there are relatively few samples that would lose a cytotoxicity hit if the LDH endpoint is excluded (11/527 samples tested = 2.1%). The 3 samples that are only active in the LDH endpoint (and no other MEA Acute endpoints) are borderline in the LDH. Therefore, I don’t think further investigation is warranted at this time. I think the LDH endpoint can be excluded based on findings in Kosnik et al., 2019, and previous work that Tim has mentioned.

# References

Kosnik MB, Strickland JD, Marvel SW, Wallis DJ, Wallace K, Richard AM, Reif DM, Shafer TJ. Concentration-response evaluation of ToxCast compounds for multivariate activity patterns of neural network function. Arch Toxicol. 2020 Feb;94(2):469-484. doi: 10.1007/s00204-019-02636-x. Epub 2019 Dec 10. PMID: 31822930; PMCID: PMC7371233. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7371233/>

Strickland JD, Martin MT, Richard AM, Houck KA, Shafer TJ. Screening the ToxCast phase II libraries for alterations in network function using cortical neurons grown on multi-well microelectrode array (mwMEA) plates. Arch Toxicol. 2018 Jan;92(1):487-500. doi: 10.1007/s00204-017-2035-5. Epub 2017 Aug 2. PMID: 28766123; PMCID: PMC6438628. <https://pubmed.ncbi.nlm.nih.gov/28766123/>

# RMD documents

Located under L:\Lab\NHEERL\_MEA\Carpenter\_Amy\pre-process\_mea\_acute\_for\_tcpl\investigations\sc\_and\_cytotox\_endpoints\_July2022\R

* *mea\_acute\_sc\_evaluation\_2022-06-27.html*
* *check\_mea\_acute\_sc\_and\_mc\_results\_against\_assay\_controls\_2022-07-18.html*
* checkout\_mea\_acute\_sc\_LDH\_and\_AB\_tcpl\_results\_2022-07-07.html
* *mea\_acute\_mc\_impact\_of\_exclusion\_of\_LDH\_2022-07-20.html*
* *mea\_acute\_sc\_cytotox\_check\_for\_changes\_in\_values\_from\_srcf\_to\_tcpl\_lvl2\_2022-07-19.html*