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Citation for the original published paper (version of record):

Beal, J., Farny, N., Haddock-Angelli, T. et al (2020). Robust estimation of bacterial cell count from optical density. *Communications Biology*, 3(1). <http://dx.doi.org/10.1038/s42003-020-01127-5>

N.B. When citing this work, cite the original published paper.

Robust estimation of bacterial cell count from optical density

Jacob Beal ^{1✉}, Natalie G. Farny ^{2✉}, Traci Haddock-Angelli ^{3✉}, Vinoo Selvarajah³, Geoff S. Baldwin ^{4✉}, Russell Buckley-Taylor⁴, Markus Gershater^{5✉}, Daisuke Kiga⁶, John Marken⁷, Vishal Sanchania⁵, Abigail Sison³, Christopher T. Workman ^{8✉} & the iGEM Interlab Study Contributors*

Optical density (OD) is widely used to estimate the density of cells in liquid culture, but cannot be compared between instruments without a standardized calibration protocol and is challenging to relate to actual cell count. We address this with an interlaboratory study comparing three simple, low-cost, and highly accessible OD calibration protocols across 244 laboratories, applied to eight strains of constitutive GFP-expressing *E. coli*. Based on our results, we recommend calibrating OD to estimated cell count using serial dilution of silica microspheres, which produces highly precise calibration (95.5% of residuals <1.2-fold), is easily assessed for quality control, also assesses instrument effective linear range, and can be combined with fluorescence calibration to obtain units of Molecules of Equivalent Fluorescein (MEFL) per cell, allowing direct comparison and data fusion with flow cytometry measurements: in our study, fluorescence per cell measurements showed only a 1.07-fold mean difference between plate reader and flow cytometry data.

¹Raytheon BBN Technologies, Cambridge, MA, USA. ²Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA, USA. ³iGEM Foundation, Cambridge, MA, USA. ⁴Department of Life Sciences and IC-Centre for Synthetic Biology, Imperial College London, London, UK. ⁵Synthace, London, UK. ⁶Faculty of Science and Engineering, School of Advanced Science and Engineering, Waseda University, Tokyo, Japan. ⁷Department of Bioengineering, California Institute of Technology, Pasadena, CA, USA. ⁸DTU-Bioengineering, Technical University of Denmark, Kongens Lyngby, Denmark. *A list of authors and their affiliations appears at the end of the paper. ✉email: jakebeal@ieee.org; nfarny@wpi.edu; traci@igem.org; g.baldwin@imperial.ac.uk; m.gershater@synthace.com; cwor@dtu.dk

Comparable measurements are a sine qua non for both science and engineering, and one of the most commonly needed measurements of microbes is the number (or concentration) of cells in a sample. The most common method for estimating the number of cells in a liquid suspension is the use of optical density measurements (OD) at a wavelength of 600 nm (OD600)¹. The dominance of OD measurements is unsurprising, particularly in plate readers, as these measurements are extremely fast, inexpensive, simple, relatively non-disruptive, high-throughput, and readily automated. Alternative measurements of cell count—microscopy (with or without hemocytometer), flow cytometry, colony-forming units (CFU), and others, e.g., see refs. 2–5—lack many of these properties, though some offer other benefits, such as distinguishing viability and being unaffected by cell states such as inclusion body formation, protein expression, or filamentous growth⁶.

A key shortcoming of OD measurements is that they do not actually provide a direct measure of cell count. Indeed, OD is not even linearly related to cell count except within a limited range⁷. Furthermore, because the phenomenon is based on light scatter rather than absorbance, it is relative to the configuration of a particular instrument. Thus, in order to relate OD measurements to cell count—or even just to compare measurements between instruments and experiments—it is necessary to establish a calibration protocol, such as comparison to a reference material.

While the problems of interpreting OD values have been studied (e.g., refs. 1,6,7), no previous study has attempted to establish a standard protocol to reliably calibrate estimation of cell count from OD. To assess reliability, it is desirable to involve a large diversity of instruments and laboratories, such as those participating in the International Genetically Engineered Machines (iGEM) competition⁸, where hundreds of teams at the high school, undergraduate, and graduate levels have been organized previously to study reproducibility and calibration for fluorescence measurements in engineered *E. coli*^{9,10}. As iGEM teams have a high variability in training and available resources, organizing an interlaboratory study with iGEM also demands that protocols be simple, low cost, and highly accessible. The large scale and high variability between teams also allows investigation of protocol robustness, as well as how readily issues can be identified and debugged in protocol execution.

We thus organized a large-scale interlaboratory study within iGEM to compare three candidate OD calibration protocols: a colony-forming unit (CFU) assay, the de facto standard assay for determining viable cell count; comparison with colloidal silica (LUDOX) and water, previously used for normalizing fluorescence measurements⁹; and serial dilution of silica microspheres, a new protocol based on a recent study of microbial growth⁷. Overall, this study demonstrates that serial dilution of silica microspheres is by far the best of these three protocols under the conditions tested, allowing highly precise, accurate, and robust calibration that is easily assessed for quality control and can also evaluate the effective linear range of an instrument. We thus recommend use of silica microsphere calibration within the linear range of OD measurements for cells with compact shape and matching refractive index. Adoption of this recommendation is expected to enable effective use of OD data for estimation of cell count, comparison of plate reader measurements with single-cell measurements such as flow cytometry, improved replicability, and better cross-laboratory comparison of data.

Results

To evaluate the three candidate OD calibration protocols, we organized an interlaboratory study as part of the 2018 International Genetically Engineered Machine (iGEM) competition.

The precision and robustness of each protocol is assessed based on the variability between replicates, between reference levels, and between laboratories. The overall efficacy of the protocols was then further evaluated based on the reproducibility of cross-laboratory measurements of cellular fluorescence, as normalized by calibrated OD measurements.

Experimental data collection. Each contributing team was provided with a set of calibration materials and a collection of eight engineered genetic constructs for constitutive expression of GFP at a variety of levels. Specifically, the constructs consisted of a negative control, a positive control, and six test constructs that were identical except for promoters from the Anderson library¹¹, selected to give a range of GFP expression (illustrated in Fig. 1a, with complete details provided in Supplementary Data 1). In particular, the positive and negative controls and the J23101, J23106, and J23117 promoters were chosen based on their prior successful use in the 2016 iGEM interlaboratory study⁹ as controls and “high”, “medium”, and “low” test levels, respectively. Beyond these, J23100 and J23104 were chosen as potential alternatives for J23101 (about which there were previous reports of difficulty in transformation), and J23116 was chosen as an intermediate value in the large gap in expression levels between J23106 and J23117 (expected values were not communicated to teams, however). These materials were then used to follow a calibration and cell measurement protocol (see the “Methods” section; Supplementary Note: Plate Reader and CFU Protocol and Supplementary Note: Flow Cytometer Protocol).

Each team transformed *E. coli* K-12 DH5-alpha with the provided genetic constructs, culturing two biological replicates for each of the eight constructs. Teams measured absorbance at 600 nm (OD600) and GFP in a plate reader from four technical replicates per biological replicate (for a total of eight replicates and fitting on a 96-well plate) at the 0 and 6 h time points, along with media blanks, thus producing a total of 144 OD600 and 144 GFP measurements per team. Six hours was chosen as a period sufficient for exponential growth, and the zero-hour measurement used only for comparison to exclude samples that failed to grow well. Teams with access to a flow cytometer were asked to also collect GFP and scatter measurements for each sample, plus a sample of SpheroTech Rainbow Calibration Beads¹² for fluorescence calibration.

Measurements of GFP fluorescence were calibrated using serial dilution of fluorescein with PBS in quadruplicate, using the protocol from ref. 9, as illustrated in Fig. 1b. Starting with a known concentration of fluorescein in PBS means that there is a known number of fluorescein molecules per well. The number of molecules per arbitrary fluorescence unit can then be estimated by dividing the expected number of molecules in each well by the measured fluorescence for the well; a similar computation can be made for concentration.

Measurements of OD via absorbance at 600 nm (OD600) were calibrated using three protocols and for each of these a model was devised for the purpose of fitting the data obtained in the study (Methods):

Calibration to colony-forming units (CFU), illustrated in Fig. 1c: Four overnight cultures (two each of positive and negative controls), were sampled in triplicate, each sample diluted to 0.1 OD, then serially diluted, and the final three dilutions spread onto bacterial culture plates for incubation and colony counting (a total of 36 plates per team). The number of CFU per OD per mL is estimated by multiplying colony count by dilution multiple. This protocol has the advantage of being well established and insensitive to non-viable cells and debris, but the disadvantages of an unclear number of cells per CFU, potentially high statistical

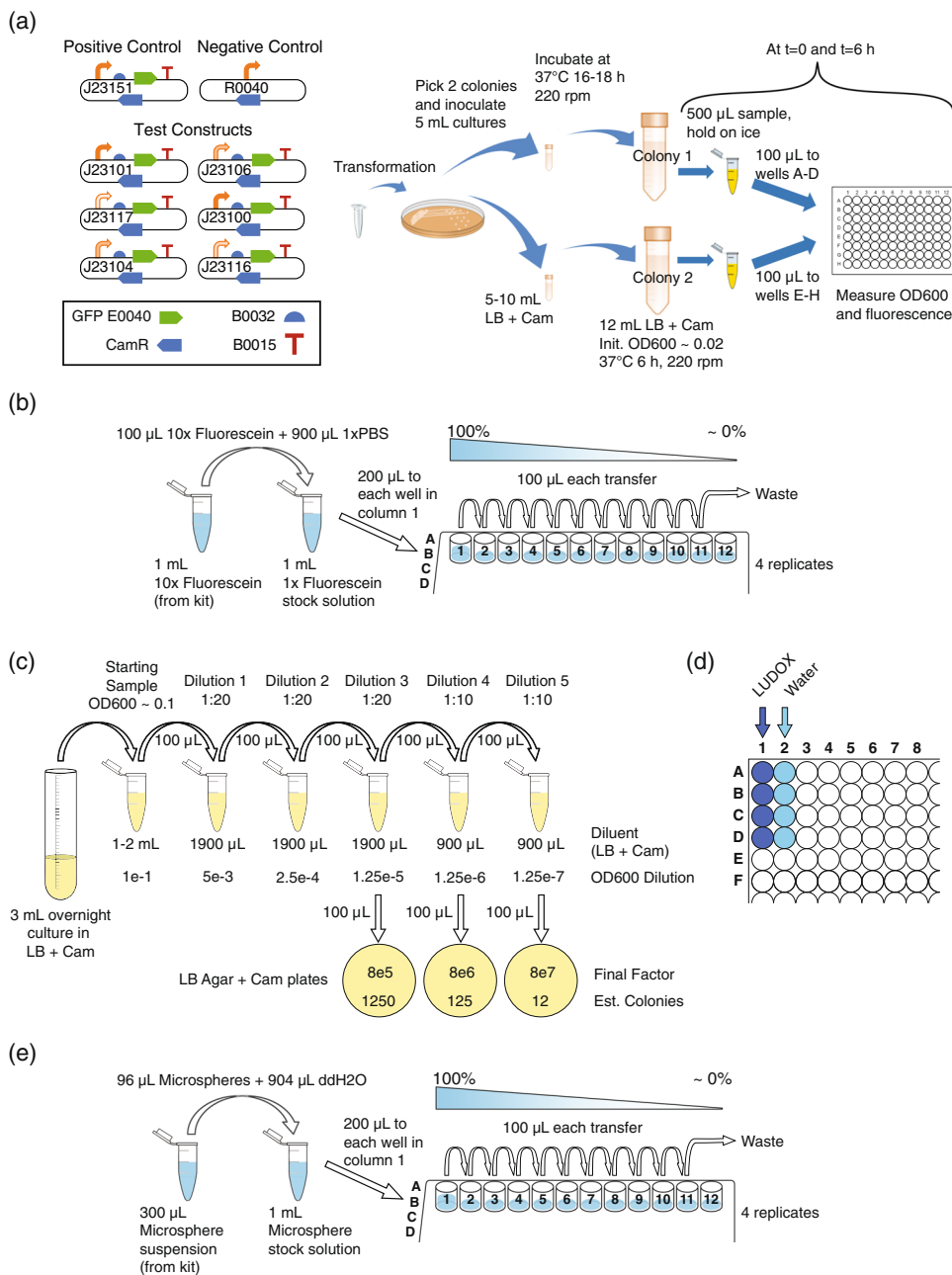


Fig. 1 Study design. **a** Each team cultured eight strains of engineered *E. coli* expressing GFP at various levels: positive and negative controls plus a library of six test constructs with promoters selected to give a range of levels of expression. Each team also collected four sets of calibration measurements, **b** fluorescein titration for calibration of GFP fluorescence, plus three alternative protocols for calibration of absorbance at 600 nm: **c** dilution and growth for colony-forming units (CFU), **d** LUDOX and water, and **e** serial dilution of 0.961 µm-diameter monodisperse silica microspheres.

variability when the number of colonies is low, and being labor intensive.

Comparison of colloidal silica (LUDOX CL-X) and water, illustrated in Fig. 1d: This protocol is adapted from ref. ⁹ by substitution of a colloidal silica formulation that is more dense and freeze-tolerant (for easier shipping). Quadruplicate measurements are made for both LUDOX CL-X and water, with conversion from arbitrary units to OD measurement in a standard spectrophotometer cuvette estimated as the ratio of their difference to the OD measurement for LUDOX CL-X in a reference spectrophotometer. This protocol has the advantage of using extremely cheap and stable materials, but the disadvantage that LUDOX CL-X provides only a single reference value, and that it calibrates for instrument differences in determination of

OD but cannot be used to estimate the number of cells, as all grades of LUDOX particles are far smaller than cells (<50 nm).

Comparison with serial dilution of silica microspheres, illustrated in Fig. 1e: This new protocol, inspired by the relationship between particle size, count, and OD⁷, uses quadruplicate serial dilution protocol of 0.961-µm-diameter monodisperse silica microspheres in water, similar to fluorescein dilution, but with different materials. These particles are selected to match the approximate volume and optical properties of *E. coli*, with the particles having a refractive index of 1.4 (per manufacturer specification) and typical *E. coli* ranging from 1.33 to 1.41⁷. With a known starting concentration of particles, the number of particles per OD600 unit is estimated by dividing the expected number of particles in each well by the measured OD for

the well. This protocol has the advantages of low cost and of directly mapping between particles and OD, but the disadvantage that the microspheres tend to settle and are freeze-sensitive.

Data from each team were accepted only if they met a set of minimal data quality criteria (Supplementary Note: Data Acceptance Criteria), including values being non-negative, the positive control being notably brighter than the negative control, and measured values for calibrants decreasing as dilution increases. In total, 244 teams provided data meeting these minimal criteria, with 17 teams also providing usable flow cytometry data. Complete anonymized data sets and analysis results are available in Supplementary Data 2.

Robustness of calibration protocols. We assessed the robustness of the calibration protocols under test in two ways: replicate precision and residuals. Replicate precision can be evaluated simply in terms of the similarity of values for each technical replicate of a protocol. The smaller the coefficient of variation (i.e., ratio of standard deviation to mean), the more precise the protocol. With regards to residuals, on the other hand, we considered the modeled mechanism that underlies each calibration method and assess how well it fits the data. Here, the residual is the distance between each measured value provided by a team and the predicted value of a model fit using that same set of data (see Methods for details of each mechanism model and residual calculations). The smaller the residual value, the more precise the protocol. Moreover, the more similar the replicate precision and residuals across teams, the more robust the protocol is to variations in execution conditions.

Figure 2 shows the distribution of the coefficients of variation (CVs) for all valid replicates for each of the calibrant materials (see Methods for validity criteria). For CFU, basic sampling theory implies that the dilution with the largest number of countably distinct colonies (lowest dilution) should have the best CV, and indeed this is the case for 81.6% of the samples. This percentage is surprisingly low, however, and indicates a higher degree of variation than can be explained by the inherent stochasticity of the protocol: CFU sampling should follow a binomial distribution and have a little over 3-fold higher CV with each 10-fold dilution, but on average it was much less. This indicates the presence of a large component of variation with an

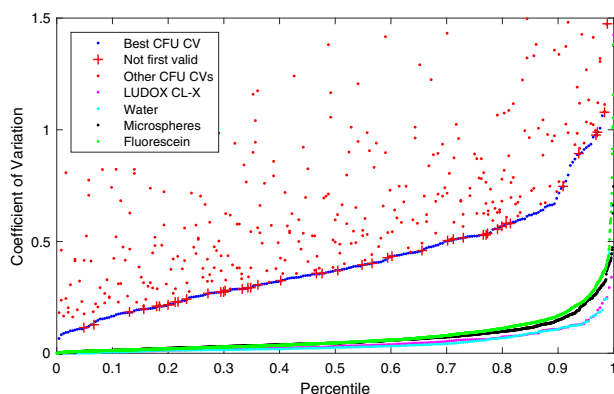


Fig. 2 Distribution of the coefficient of variation for valid replicate sets in CFU, LUDOX/water, microspheres, and fluorescein (all teams included).

CFU models are generated from only the best CV dilution (blue); other dilutions are shown separately above. Even the best CV CFU dilutions, however, have a distribution far worse than the other four methods, and are surprisingly often not the lowest dilution (red crosses). Of the others, LUDOX (magenta) and water (light blue) have the best and near-identical distributions, while microspheres (black) and fluorescein (green) are only slightly higher.

unknown source, which is further confirmed by the fact that even the best CVs are quite high: the best of the three dilutions for each team has $CV \leq 0.1$ for only 2.1% of all data sets and $CV \leq 0.2$ for only 16.4% of all data sets.

LUDOX and water have the lowest CV, at $CV \leq 0.1$ for 86.9% (LUDOX) and 88.1% (water) of all replicate sets and $CV \leq 0.2$ for 97.1% (LUDOX) and 98.0% (water) of all replicate sets. Microspheres and fluorescein have slightly higher CV, at $CV \leq 0.1$ for 80.8% (microspheres) and 76.9% (fluorescein) of all replicate sets and $CV \leq 0.2$ for 93.9% (microspheres) and 92.4% (fluorescein) of all replicate sets. The difference between these two pairs likely derives from the fact that the LUDOX and water samples are each produced in only a single step, while the serial dilution of microspheres and fluorescein allows inaccuracies to compound in the production of later samples.

The accuracy of a calibration protocol is ultimately determined by how replicate data sets across the study are jointly interpreted to parameterize a model of the calibration protocol, one part of which is the scaling function that maps between arbitrary units and calibrated units. As noted above, this can be assessed by considering the residuals in the fit between observed values and their fit to the protocol model. To do this, we first estimated the calibration parameters from the observed experimental values (see Methods for the unit scaling computation for each calibration method), then used the resulting model to “predict” what those values should have been (e.g., 10-fold less colonies after a 10-fold dilution). The closer the ratio was to one, the more the protocol was operating in conformance with the theory supporting its use for calibration, and thus the more likely that the calibration process produced an accurate value.

Here we see a critical weakness of the LUDOX/water protocol: the LUDOX and water samples provide only two measurements, from which two model parameters are set: the background to subtract (set by water) and the scaling between background-subtracted LUDOX and the reference OD. Thus, the dimensionality of the model precisely matches the dimensionality of the experimental samples, and there are no residuals to assess. As such, the LUDOX/water protocol may indeed be accurate, but its accuracy cannot be empirically assessed from the data it produces. If anything goes wrong in the reagents, protocol execution, or instrument, such problems cannot be detected unless they are so great as to render the data clearly invalid (e.g., the OD of water being less than the OD of LUDOX).

The CFU protocol and the two serial dilution protocols, however, both have multiple dilution levels, overconstraining the model and allowing likely accuracy to be assessed. Figure 3 shows the distribution of residuals for these three protocols, in the form of a ratio between the observed mean for each replicate set and the value predicted by the model fit across all replicate sets. The CFU protocol again performs extremely poorly, as we might expect based on the poor CV of even the best replicates: only 7.3% of valid replicate sets have a residual within 1.1-fold, only 14.0% within 1.2-fold, and overall the geometric standard deviation of the residuals is 3.06-fold—meaning that values are only reliable to within approximately two orders of magnitude! Furthermore, the distribution is asymmetric, suggesting that the CFU protocol may be systematically underestimating the number of cells in the original sample. The accuracy of the CFU protocol thus appears highly unreliable.

The microsphere dilution protocol, on the other hand, produced much more accurate results. Even with only a simple model of perfect dilution, the residuals are quite low (red line in Fig. 3b), having 61.0% of valid replicates within 1.1-fold, 83.6% within 1.2-fold, and an overall geometric standard deviation of 1.152-fold. As noted above, however, with serial dilution we may expect error to compound systematically with each dilution, and

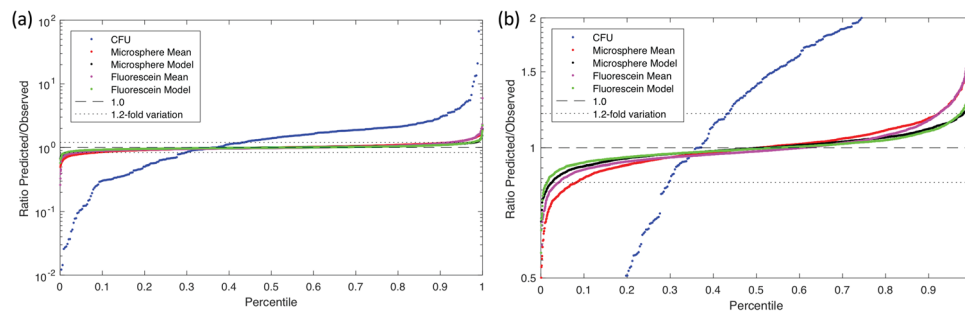


Fig. 3 Distribution of residuals. **a** Model fit residual distribution for each replica set in the CFU (blue), microsphere, and fluorescein calibration protocols (all teams included). **b** Expanding the Y axis to focus on the microsphere and fluorescein distributions shows that incorporating a model parameter for systematic pipetting error (black, green) produces a notably better fit (and thus likely more accurate unit calibration) than a simple geometric mean over scaling factors (red, magenta).

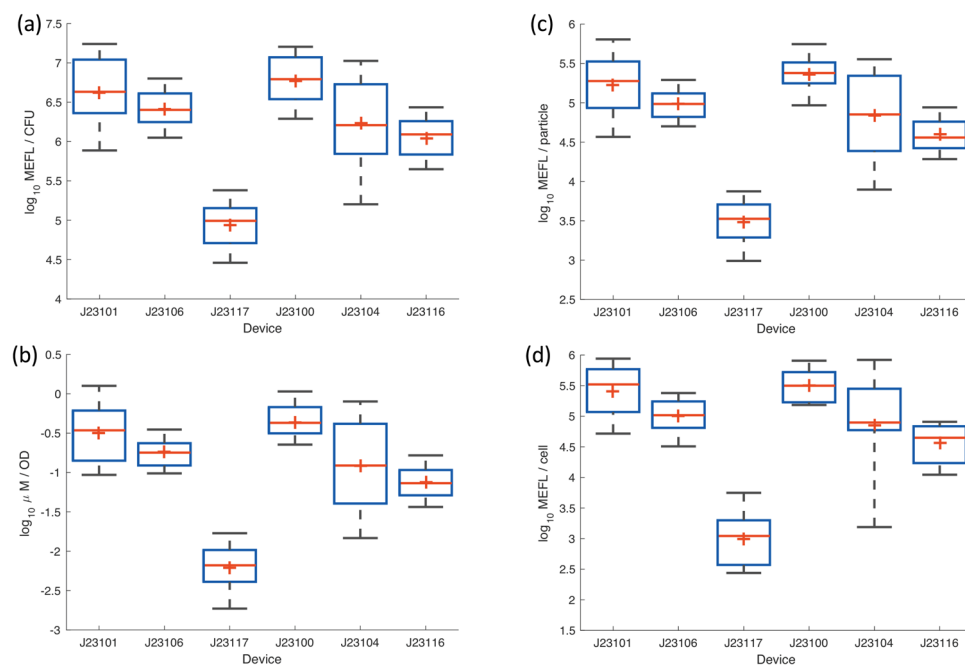


Fig. 4 Measured fluorescence of test devices. Measured fluorescence of test devices after 6 h of growth using **a** CFU calibration, **b** LUDOX/water calibration, **c** microsphere dilution calibration, and **d** flow cytometry. In each box, red plus indicates geometric mean, red line indicates median, top and bottom edges indicate 25th and 75th percentiles, and whiskers extend from 9 to 91%. Team count per condition provided in Supplementary Data 3.

indeed the value sequences in individual data sets do tend to show curves indicative of systematic pipetting error. When the model is extended to include systematic pipetting error (see Methods subsection on “Systematic pipetting error model”), the results improve markedly (black line in Fig. 3b), to 82.4% of valid replicates within 1.1-fold, 95.5% within 1.2-fold, and an overall geometric standard deviation improved to 1.090-fold. Fluorescein dilution provides nearly identical results: with a perfect dilution model (magenta line in Fig. 3b), having 71.1% of valid replicates within 1.1-fold, 88.2% within 1.2-fold, and an overall geometric standard deviation of 1.148-fold, and systematic pipetting error improving the model (green line in Fig. 3b), to 88.1% of valid replicates within 1.1-fold, 98.0% within 1.2-fold, and an overall geometric standard deviation of 1.085-fold.

Based on an analysis of the statistical properties of calibration data, we may thus conclude that the microsphere and fluorescein dilution protocols are highly robust, producing results that are precise, likely to be accurate, and readily assessed for execution quality on the basis of calibration model residuals. The LUDOX/water protocol is also highly precise and may be accurate, but its execution quality cannot be directly assessed due to its lack of

residuals. The CFU protocol, on the other hand, appears likely to be highly problematic, producing unreliable and likely inaccurate calibrations.

Reproducibility and accuracy of cell-count estimates. Reproducibility and accuracy of the calibration protocols can be evaluated through their application to calibration of fluorescence from *E. coli*, as normalized by calibrated OD measurements. Figure 4 shows the fluorescence values computed for each of the three fluorescence/OD calibration combinations, as well as for calibrated flow cytometry, excluding data with poor calibration or outlier values for colony growth or positive control fluorescence (for details see Methods on determining validity of *E. coli* data). Overall, the lab-to-lab variation was workably small, with the geometric mean of the geometric standard deviations for each test device being 2.4-fold for CFU calibration, 2.21-fold for LUDOX/water calibration, and 2.21-fold for microsphere dilution calibration. These values are quite similar to those previously reported in ref. ⁹, which reported a 2.1-fold geometric standard deviation for LUDOX/water.

Note that these standard deviations are also dominated by the high variability observed in the constructs with J23101 and J23104, both of which appear to have suffered notable difficulties in culturing, with many teams' samples failing to grow for these constructs, while other constructs grew much more reliably (see Supplementary Fig. 1). Omitting the problematic constructs finds variations of 2.02-fold for CFU calibration, 1.84-fold for LUDOX/water calibration, and 1.83-fold for microsphere dilution calibration. Flow cytometry in this case is also similar, though somewhat higher variability in this case, at 2.31-fold (possibly due to the much smaller number of replicates and additional opportunities for variation in protocol execution). All together, these values indicate that, when filtered using quality control based on the replicate precision and residual statistics established above, all three OD calibration methods are capable of producing highly reproducible measurements across laboratories.

To determine the accuracy of cell-count estimates, we compared normalized bulk measurements (total fluorescence divided by estimated cell count) against single-cell measurements of fluorescence from calibrated flow cytometry, which provides direct measurement of per-cell fluorescence without the need to estimate cell count (see Methods on “Flow cytometry data processing” for analytical details). In this comparison, an accurate cell count is expected to allow bulk fluorescence measurement normalized by cell count to closely match the per-cell fluorescence value produced by flow cytometry. In making this comparison, there are some differences that must be considered between the two modalities. Gene expression typically has a log-normal distribution¹³, meaning that bulk measurements will be distorted upward compared to the geometric mean of log-normal distribution observed with the single-cell measurements of a flow cytometer. In this experiment, for typical levels of cell-to-cell variation observed in *E. coli*, this effect should cause the estimate of per-cell fluorescence to be approximately 1.3-fold higher from a plate reader than a flow cytometer. At the same time, non-cell particles in the culture will tend to distort fluorescence per-cell estimates in the opposite direction for bulk measurement, as these typically contribute to OD but not fluorescence in a plate reader, but the vast majority of debris particles are typically able to be gated out of flow cytometry data. With generally healthy cells in log-phase growth, however, the levels of debris in this experiment are expected to be relatively low. Thus, these two differences are likely to both be small and in opposite directions, such that we should still expect the per-cell fluorescence estimates of plate reader and flow cytometry data to closely match if accurately calibrated.

Of the three OD calibration methods, the LUDOX/water measurement is immediately disqualified as it calibrates only to a relative OD, and thus cannot produce comparable units. Comparison of CFU and microsphere dilution to flow cytometry is shown in Fig. 5. The CFU-calibrated measurements are far higher than the values produced by flow cytometry, a geometric mean of 28.4-fold higher, indicating that this calibration method badly underestimates the number of cells. It is unclear the degree to which this is due to known issues of CFU, such as cells adhering into clumps, as opposed to the problems with imprecision noted above or yet other possible unidentified causes. Whatever the cause, however, CFU calibration is clearly problematic for obtaining anything like an accurate estimate of cell count.

Microsphere dilution, on the other hand, produces values that are remarkably close to those for flow cytometry, a geometric mean of only 1.07-fold higher, indicating that this calibration method is quite accurate in estimating cell count. Moreover, we may note that the only large difference between values comes with the extremely low fluorescence of the J23117

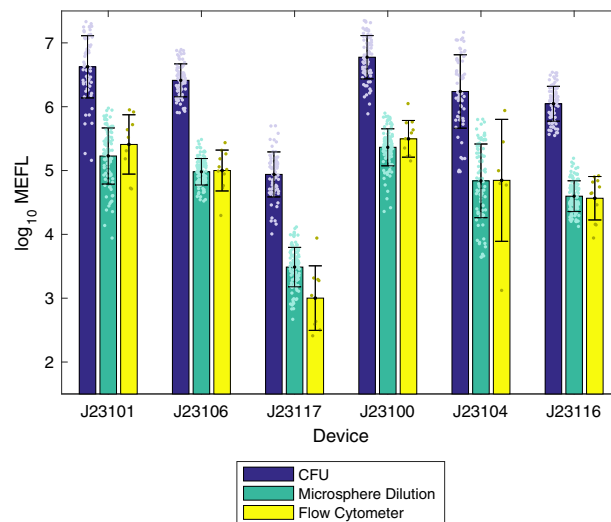


Fig. 5 Fluorescence per cell after 6 h of growth, comparing calibrated flow cytometry to estimates using cell count from CFU and microsphere dilution protocols (LUDOX/water is not shown as the units it produces are not comparable). Microsphere dilution produces values extremely close to the ground truth provided by calibrated flow cytometry, whereas the CFU protocol produces values more than an order of magnitude different, suggesting that CFU calibration greatly underestimates the number of cells in the sample. Bars show geometric mean and standard deviation. Team count per condition provided in Supplementary Data 3.

construct, which is unsurprising given that flow cytometers generally have a higher dynamic range than plate readers, allowing better sensitivity to low signals.

Discussion

Reliably determining the number of cells in a liquid culture has remained a challenge in biology for decades. For the field of synthetic biology, which seeks to engineer based on standardized biological measurements, it was critical to find a solution to this challenge. Here, we have compared the most common method for calibrating OD to cell number (calculation of CFU) to two alternative methods of calibration: LUDOX/water and microsphere serial dilution. The qualitative and quantitative benefits and drawbacks of these three methods for OD calibration are summarized in Table 1.

These three protocols are all inexpensive, with the reagent cost for both LUDOX/water and microsphere serial dilution being < \$0.10 US. The CFU protocol has well-known issues of cell clumping and slow, labor-intensive execution, and counts only live and active cells, which can be either a benefit or a limitation depending on circumstances, though it does benefit from being insensitive to cell shape and optical properties. In addition, the CFU counts in this study exhibited a remarkably high level of variability, which may call into question the use of the CFU method as a standard for determining cell counts. This observed variability is not without precedent—prior work has also demonstrated *E. coli* CFU counting performing poorly on measures of reproducibility and repeatability in an interlaboratory study¹⁴.

The microsphere protocol, on the other hand, has no major drawbacks and provides a number of notable benefits when applied to cells with shapes and optical properties that can be reasonably approximated with appropriately sized microspheres. First, the microsphere protocol is highly robust and reliable, particularly compared with CFU assays. Second, failures are much easier to diagnose with the microsphere protocol, since it

Table 1 Summary of the benefits and drawbacks of the three calibration protocols.

| Protocol | Benefits | Drawbacks/limitations |
|-----------------------------|--|---|
| Colony-forming units (CFU) | Inexpensive Requires no additional reagents Counts only live and active cells, eliminating quiescent cells, dead cells, and debris Not affected by cell shape, optics | Lower precision Count affected by cell clumping/adhesion Labor intensive Slow (overnight incubation) |
| LUDOX/water | Extremely simple, fast, and cheap High precision | Generates only a single calibration point Cell count is still relative |
| Microsphere serial dilution | Inexpensive Highest precision Many dilution levels helps with quality control and corrections Also assesses linear range of instrument | Slightly more difficult to perform, as it must be completed before spheres have time to settle |

has many distinct levels that can be compared. This is particularly salient when compared with the LUDOX/water protocol, which only provides a single calibration point at low absorbance (and thus susceptible to instrument range issues), and to the CFU protocol, where failures may be difficult to distinguish from inherent high variability. With the microsphere protocol, on the other hand, some failures such as systematic dilution error and instrument saturation can not only be detected, but also modeled and corrected for. Finally, the microsphere protocol also permits a unit match between plate reader and flow cytometry measurements (both in cell number and in fluorescence per cell), which is highly desirable, allowing previously impossible data fusion between these two complementary platforms (e.g., to connect high-resolution time-series data from a plate reader with high-detail data about population structure from a flow cytometer). Accordingly, based on the results of this study, we recommend the adoption of silica microsphere calibration for robust estimation of bacterial cell count. As long as OD measurements are within the linear range, this calibration protocol is expected to enable effective use of OD data for estimation of actual cell count, comparison of plate reader measurements with single-cell measurements such as flow cytometry, improved replicability, and better cross-laboratory comparison of data.

With regards to future opportunities for extension, we note that these methods seem likely to be applicable to other instruments that measure absorbance (e.g., spectrophotometers, automated culture flasks) by appropriately scaling volumes and particle densities. Similarly, it should be possible to adapt to other cell types by selecting other microspheres with appropriately adjusted diameters and materials for their optical properties (indeed, per ref. ⁷, many other commonly used bacteria have quite similar refractive index values), and a wide range of potential options are already readily available from commercial suppliers. Finally, further investigation would be valuable for more precisely establishing the relationship between cell count and particle count. It would also be useful to quantify the degree to which the estimates are affected by factors such as changing optical properties associated with cell state, distribution, shape, and clustering, and to investigate means of detecting and compensating for such effects.

Methods

Participating iGEM teams measured OD and fluorescence among the same set of plasmid-based devices, according to standardized protocols. In brief, teams were provided a test kit containing the necessary calibration reagents, a set of standardized protocols, and pre-formatted Excel data sheets for data reporting. Teams provided their own plate reader instruments, consumables/plasticware, competent

E. coli cells, PBS, water, and culture medium. First, teams were asked to complete a series of calibration measurements by measuring LUDOX and water, and also making a standard curve of both fluorescein and silica microspheres. Next, each team transformed the plasmid devices into *E. coli* and selected transformants on chloramphenicol plates. They selected two colonies from each plate to grow as liquid cultures overnight, then the following day diluted their cultures and measured both fluorescence and OD after 0 and 6 h of growth. Some of these cultures were also used to make serial dilutions for the CFU counting experiment. Teams were asked to report details of their instrumentation, *E. coli* strains used, and any variations from the protocol using an online survey. Additional details are available in the Supplementary Information.

Calibration materials. The following calibration materials were provided to each team as a standard kit: 1 ml of LUDOX CL-X (Sigma-Aldrich, #420891) and 1.00e-8 moles of fluorescein (Sigma-Aldrich, #46970). About 300 μ l of 0.961- μ m-diameter monodisperse silica beads (Cospheric, SiO₂MS-2.0, 0.961 μ m) in ddH₂O were prepared to contain 3.00e8 beads.

Fluorescein samples tubes were prepared with 1.00e-8 moles fluorescein in solution in each tube, which was then vacuum dried for shipping. Resuspension in 1 ml PBS would thus produce a solution with initial concentration of 10 μ M fluorescein.

Each team providing flow cytometry data also obtained their own sample of SpheroTech RCP-30-5A Rainbow Calibration Particles (SpheroTech). A sample of this material is a mixture of particles with eight levels of fluorescence, which should appear as up to eight peaks (typically some are lost to saturation on the instrument). Teams used various different lots, reporting the lot number to allow selection of the appropriate manufacturer-supplied quantification for each peak.

Constructs, culturing, and measurement protocols. The genetic constructs supplied to each team for transformation are provided in Supplementary Data 1. The protocol for plate readers, exactly as supplied to each participating team, is provided in Supplementary Note: Plate Reader and CFU Protocol. The supplementary protocol for flow cytometry is likewise provided in Supplementary Note: Flow Cytometer Protocol.

Criteria for valid calibrant replicates. For purpose of analyzing the precision of calibrants, the following criteria were used to determine which replicate sets are sufficiently valid for inclusion of analysis:

CFU: A dilution level is considered valid if at least 4 of the 12 replicate plates have a number of colonies that are >0 but not too numerous to count (participants were instructed they could report anything over 300 colonies to be too numerous to count). A calibration set is considered valid if there is at least one valid dilution level. Of the 244 data sets, 241 are valid and 3 are not valid.

LUDOX/water: A LUDOX/water calibration is considered valid if it fits the acceptance criteria in Supplementary Note: Data Acceptance Criteria, meaning that all 244 are valid.

Microsphere dilution and fluorescein dilution: For both of these protocols, a dilution level is considered locally valid if the measured value does not appear to be either saturated high or low. High saturation is determined by lack of sufficient slope from the prior level, here set to be at least 1.5x, and low saturation by indistinguishability from the blank replicates, here set to be anything <2 blank standard deviations above the mean blank. The valid range of dilution levels is then taken to be the longest continuous sequence of locally valid dilution levels, and the calibration set considered valid overall if this range has at least three valid dilution levels.

For microsphere dilution, of the 244 data sets, 235 are valid and 9 are not valid—one due to being entirely low saturated, the others having inconsistent slopes indicative of pipetting problems. Supplementary Fig. 2 Length of Valid Sequence(a) shows that most microsphere dilution data sets have the majority of dilution levels valid, but that only about one-tenth are without saturation issues.

For fluorescein dilution, of the 244 data sets, 243 are valid and 1 is not valid, having an inconsistent slope indicative of pipetting problems. Supplementary Fig. 2 Length of Valid Sequence(b) shows that the vast majority of fluorescein dilution data sets are without any saturation issues.

Note that in both cases, changing the required number of value dilution levels down to 2 or up to 4 would have little effect on the number of data sets included, adding 7 or removing 8 for microspheres and adding or removing only 1 for fluorescein.

Unit scaling factor computation

CFU. The scaling factor S_c relating CFU/ML to Abs600 is computed as follows:

$$S_{c,i} = \mu(C_i) * \delta_i \quad (1)$$

where $\mu(C_i)$ is the mean number of colonies for dilution level i and δ_i is the dilution fold for level i . For the specific protocol used, there are three effective dilution factors, 1.6e5, 1.6e6, and 1.6e7 (including a 2-fold conversion between 200 and 100 μ l volumes).

The overall scaling factor S_c for each data set is then taken to be:

$$S_c = \left\{ S_{c,i} \mid \frac{\sigma(C_i)}{\mu(C_i)} = \min_i \frac{\sigma(C_i)}{\mu(C_i)} \right\} \quad (2)$$

i.e., the scaling factor for the valid level with the lowest coefficient of variation, where $\sigma(C_i)$ is the standard deviation in the number of colonies for dilution level i .

The residuals for this fit are then $S_{c,i}/S_c$ for all other valid levels.

LUDOX/water. The scaling factor S_l relating standard OD to Abs600 is computed as follow:

$$S_l = \frac{R}{\mu(L) - \mu(W)} \quad (3)$$

where R is the measured reference OD in a standard cuvette (in this case 0.063 for LUDOX CL-X), $\mu(L)$ is the mean Abs600 for LUDOX CL-X samples and $\mu(W)$ is the mean Abs600 for water samples.

No residuals can be computed for this fit, because there are two measurements and two degrees of freedom.

Microsphere dilution and fluorescein dilution. The scaling factors S_m relating microsphere count to Abs600 and S_f for relating molecules of fluorescein to arbitrary fluorescent units are both computed in the same way. These are transformed into scaling factors in two ways, either as the mean conversion factor S_μ or as one parameter of a fit to a model of systematic pipetting error S_p .

Mean conversion factor: If we ignore pipetting error, then the model for serial dilution has an initial population of calibrant p_0 that is diluted n times by a factor of α at each dilution, such that the expected population of calibrant for the i th dilution level is:

$$p_i = p_0(1 - \alpha)\alpha^{i-1} \quad (4)$$

In the case of the specific protocols used here, $\alpha = 0.5$. For the microsphere dilution protocol used, $p_0 = 3.00e8$ microspheres, while for the fluorescein dilution protocol used, $p_0 = 6.02e14$ molecules of fluorescein.

The local conversion factor S_i for the i th dilution is then:

$$S_i = \frac{P_i}{\mu(O_i) - \mu(B)} \quad (5)$$

where $\mu(O_i)$ is the mean of the observed values for the i th dilution level and $\mu(B)$ is the mean observed value for the blanks.

The mean conversion factor is thus

$$S_\mu = \mu(\{S_i \mid i \text{ is a valid dilution level}\}) \quad (6)$$

i.e., the mean over local conversion factors for valid dilution levels.

The residuals for this fit are then S_i/S_μ for all valid levels.

Systematic pipetting error model: The model for systematic pipetting error modifies the intended dilution factor α with the addition of an unknown bias β , such that the expected biased population b_i for the i th dilution level is:

$$b_i = p_0(1 - \alpha - \beta)(\alpha + \beta)^{i-1} \quad (7)$$

We then simultaneously fit β and the scaling factor S_p to minimize the sum squared error over all valid dilution levels:

$$e = \sum_i \left| \log \left(\frac{b_i}{S_p \cdot (\mu(O_i) - \mu(B))} \right) \right|^2 \quad (8)$$

where e is sum squared error of the fit.

The residuals for this fit are then the absolute ratio of fit-predicted to observed net mean $\frac{b_i/S_p}{\mu(O_i) - \mu(B)}$ for all valid levels.

Application to E. coli data. The Abs600 and fluorescence a.u. data from *E. coli* samples are converted into calibrated units by subtracting the mean blank media values for Abs600 and fluorescence a.u., then multiplying by the corresponding scaling factors for fluorescein and Abs600.

Criteria for valid E. coli data. For analysis of *E. coli* culture measurements, a data set was only eligible to be included if both its fluorescence calibration and selected OD calibration were above a certain quality threshold. The particular values used for the four calibration protocols were:

CFU: Coefficient of variation for best dilution level is <0.5.

LUDOX/water: Coefficient of variation for both LUDOX and water are <0.1.

Microsphere dilution: Systematic pipetting error has geometric mean absolute residual <1.1-fold.

Fluorescein dilution: Systematic pipetting error has geometric mean absolute residual <1.1-fold.

Measurements of the cellular controls were further used to exclude data sets with apparent problems in their protocol: those with a mean positive control value more than 3-fold different than the median mean positive control.

Finally, individual samples without sufficient growth were removed, that being defined as all that are either less than the 25% of the 75th percentile Abs600 measurement in the sample set or less than 2 media blank standard deviations above the mean media blank in the sample set.

Flow cytometry data processing. Flow cytometry data was processed using the TASBE Flow Analytics software package¹⁵. A unit conversion model from arbitrary units to MEFL was constructed per the recommended best practices of TASBE Flow Analytics for each data set using the bead sample and lot information provided by each team.

Gating was automatically determined using a two-dimensional Gaussian fit on the forward-scatter area and side-scatter area channels for the first negative control (Supplementary Fig. 3).

The same negative control was used to determine autofluorescence for background subtraction (Supplementary Fig. 4).

As only a single green fluorescent protein was used, there was no need for spectral compensation or color translation.

All teams submitted flow cytometry used standard SpheroTech Rainbow Calibration beads¹² for dye-based calibration to equivalent fluorescent molecules¹⁶. In particular, 16 teams used RCP-30-5A beads (various lot numbers) and 1 team used URCP-38-2K beads, and conversion from arbitrary units to MEFL was computed using the peak-to-intensity values provided for each lot. Examples are provided below (Supplementary Figs. 5 and 6).

This color model was then applied to each sample to filter events and convert GFP measurements from arbitrary units to MEFL, and geometric mean and standard deviation computed for the filtered collection of events.

Statistics and reproducibility. As reproducibility is the main subject of this study, see the Results section above for its full presentation. In addition to the discussion of statistical analyses in the Results section, we note the following details of statistical analyses:

Coefficient of variation (CV) is computed per its definition, as the ratio of the standard deviation to the mean.

Fluorescence values are analyzed geometric mean and geometric standard deviation, rather than the more typical arithmetic statistics, due to the typical log-normal distribution of gene expression¹³.

Data analysis was performed with Matlab.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Received: 24 October 2019; Accepted: 3 July 2020;

Published online: 17 September 2020

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Acknowledgements

Partial support for this work was provided by NSF Expeditions in Computing Program Award #1522074 as part of the Living Computing Project, and by the Engineering and Physical Sciences Research Council [EP/R034915/1] and EU H2020 [820699]. This

document does not contain technology or technical data controlled under either the U.S. International Traffic in Arms Regulations or the U.S. Export Administration Regulations.

Author contributions

Conceptualization: J.B., N.G.F., T.H.-A., V.S.-1, G.S.B., R.B.-T., M.G., D.K., J.M., and C.T.W. Data curation: J.B., N.G.F., T.H.-A., and V.S.-1. Formal analysis: J.B. Investigation: Experimental data gathered by iGEM Interlab Study Contributors Methodology: J.B., N.G.F., T.H.-A., V.S.-1, G.S.B., R.B.-T., M.G., D.K., J.M., V.S.-2, A.S., and C.T.W. Project administration: J.B., N.G.F., and T.H.-A. Resources: T.H.-A., V.S.-1, and A.S. Software: J.B. Writing (original draft): J.B. and N.G.F. Writing (review & editing): J.B., N.G.F., T.H.-A., G.S.B., J.M., C.T.W., and V.S.-2.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s42003-020-01127-5>.

Correspondence and requests for materials should be addressed to J.B., N.G.F., T.H.-A., G.S.B., M.G. or C.T.W.

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iGEM Interlab Study Contributors

Aachen

Meryem Pehlivan⁹ & Biel Badia Roige⁹

Aalto-Helsinki

Tiu Aarnio^{10,11}, Samu Kivisto^{10,11}, Jessica Koski^{10,11}, Leevi Lehtonen^{10,11}, Denise Pezzutto^{10,11} & Pauliina Rautanen^{10,11}

AHUT_China

Weixin Bian¹², Zhiyuan Hu¹², Zhihao Liu¹², Zi Liu¹², Liang Ma¹², Luyao Pan¹², Zichen Qin¹², Huichao Wang¹², Xiangxuan Wang¹², Hao Xu¹² & Xia Xu¹²

Aix-Marseille

Yorgo El Moubayed¹³

ASTWS-China

Shan Dong¹⁴, Choco Fang¹⁴, Hanker He¹⁴, Henry He¹⁴, Fangliang Huang¹⁴, Ruyi Shi¹⁴, Cassie Tang¹⁴, Christian Tang¹⁴, Shirly Xu¹⁴ & Calvin Yan¹⁴

Athens

Natalia Bartzoka^{15,16}, Eleni Kanata^{15,16}, Maria Kapsokefalou^{15,16}, Xanthi-Leda Katopodi^{15,16}, Eleni Kostadima^{15,16}, Ioannis V. Kostopoulos^{15,16}, Stylianos Kotzistratis^{15,16}, Antonios E. Koutelidakis^{15,16}, Vasilios Krokos^{15,16}, Maria Litsa^{15,16}, Ioannis Ntekas^{15,16}, Panagiotis Spatharas^{15,16}, Ourania E. Tsitsilonis^{15,16} & Anastasia Zerva^{15,16}

Austin_LASA

Vidhya Annem¹⁷, Eli Cone¹⁷, Noel Elias¹⁷, Shreya Gupta¹⁷, Kendrick Lam¹⁷ & Anna Tutuiuanu¹⁷

Austin_UTexas

Dennis M. Mishler¹⁸ & Bibiana Toro¹⁸

Baltimore_BioCrew

Akinwumi Akinfenwa¹⁹, Frank Burns¹⁹, Heydy Herbert¹⁹, Melissa Jones¹⁹, Sarah Laun¹⁹, Shikei Morrison¹⁹ & Zion Smith¹⁹

BCU

Zhao Peng²⁰ & Zhou Ziwei²⁰

BFSUICC-China

Rui Deng²¹, Yilin Huang²¹, Tingyue Li²¹, Yingqi Ma²¹, Zhiyuan Shen²¹, Chenxi Wang²¹, Yuyao Wang²¹ & Tianyan Zhao²¹

BGIC-Global

Yusen Lang²², Yuteng Liang²², Xueyao Wang²² & Yi Wu²²

BGU_Israel

Dror Aizik²³, Sagi Angel²³, Einan Farhi²³, Nitzan Keidar²³, Eden Oser²³ & Mor Pasi²³

Bielefeld-CeBiTec

Jorn Kalinowski²⁴, Matthias Otto²⁴ & Johannes Ruhnau²⁴

Bilkent-UNAMBG

Hande Cubukcu²⁵, Mehmet Ali Hoskan²⁵ & Ilayda Senyuz²⁵

BiolQS-Barcelona

Jordi Chi²⁶, Antoni Planas Sauter²⁶ & Magda Faijes Simona²⁶

BioMarvel

Sumin Byun²⁷, Sungwoo Cho²⁷, Goeun Kim²⁷, Yeonjae Lee²⁷, Sangwu Lim²⁷ & Hanyeol Yang²⁷

BIT

Tian Xin²⁸, Zhang Yaxi²⁸ & Peng Zhao²⁸

BIT-China

Weitang Han²⁸, Fa He²⁸, Yuna He²⁸, Nuonan Li²⁸ & Xiaofan Luo²⁸

BJRS_China

Cheng Boxuan²⁹, Hu Jiaqi²⁹, Yang Liangjian²⁹, Li Wanji²⁹, Chen Xinguang²⁹ & Liu Xinyu²⁹

BNDS_CHINA

Zishi Wu³⁰, Yukun Xi³⁰, Xilin Yang³⁰, Yuchen Yang³⁰, Zhuoyi Yang³⁰, Yihao Zhang³⁰ & Yuezhong Zhou³⁰

BNU-China

Yue Peng³¹, Liu Yadi³¹, Shaobo Yang³¹, Jiang Yuanxu³¹ & Kecheng Zhang³¹

BOKU-Vienna

Doris Abraham³² & Theresa Heger³²

BostonU

Cass Leach³³, Kevin Lorch³³ & Linda Luo³³

British_Columbia

Alex Gaudi³⁴, Anthony Ho³⁴, Morris Huang³⁴, Christine Kim³⁴, Luxcia Kugathasan³⁴, Kevin Lam³⁴, Catherine Pan³⁴, Ariel Qi³⁴ & Cathy Yan³⁴

Calgary

Kaitlin Schaaf³⁵ & Cassandra Sillner³⁵

Cardiff_Wales

Ryan Coates³⁶, Hannah Elliott³⁶, Emily Heath³⁶, Evie McShane³⁶, Geraint Parry³⁶, Ali Tariq³⁶ & Sophie Thomas³⁶

CCU_Taiwan

Ching-Wei Chen³⁷, Yu-Hong Cheng³⁷, Chia-Wei Hsu³⁷, Chin-Hsuan Liao³⁷, Wei-Ting Liu³⁷, Yu-Cheng Tang³⁷, Yu-Hsin Tang³⁷ & Zon En Yang³⁷

CDHSU-CHINA

Liu Jian^{38,39}, Caidian Li^{38,39}, Chenyi Lin^{38,39}, Guozheng Ran^{38,39}, Zhouyan Run^{38,39}, Weiyu Ting^{38,39}, Zhangxiang Yong^{38,39} & Lihong Yu^{38,39}

Chalmers-Gothenburg

Andrea Clausen Lind⁴⁰, Axel Norberg⁴⁰, Amanda Olmin⁴⁰, Jacob Sjolin⁴⁰, Agnes Torell⁴⁰, Cecilia Trivellin⁴⁰, Francisco Zorrilla⁴⁰ & Philip Gorter de Vries⁴⁰

CIEI-BJ

Haolun Cheng⁴¹, Jiarong Peng⁴¹ & Zhenyu Xiong⁴¹

CMUQ

Dina Altarawneh⁴², Sayeda Sakina Amir⁴², Sondoss Hassan⁴² & Annette Vincent⁴²

CO_Mines

Ben Costa⁴³, Isabella Gallegos⁴³, Mitch Hale⁴³, Matt Sonnier⁴³ & Kathleen Whalen⁴³

ColumbiaNYC

Max Elikan⁴⁴, Sean Kim⁴⁴ & Jaewon You⁴⁴

Cornell

Rahul Rambhatla⁴⁵ & Ashwin Viswanathan⁴⁵

CPU_CHINA

Hong Tian⁴⁶, Huandi Xu⁴⁶, Wanli Zhang⁴⁶ & Shuyao Zhou⁴⁶

CSU_CHINA

Liu Jiamiao⁴⁷ & Xiao Jiaqi⁴⁷

CSU_Fort_Collins

Darilyn Craw⁴⁸, Marley Goetz⁴⁸, Neil Rettedal⁴⁸ & Hayden Yarbrough⁴⁸

Delgado-Ivy-Marin

Christopher Ahlgren⁴⁹, Brett Guadagnino⁴⁹, James Guenther⁴⁹ & Juilanne Huynh⁴⁹

DLUT_China

Zhien He⁴⁹, Huan Liu⁴⁹, Yuansheng Liu⁴⁹, Mingbo Qu⁴⁹, Li Song⁴⁹, Chao Yang⁴⁹, Jun Yang⁴⁹, Xianqi Yin⁴⁹, Yuanzhen Zhang⁴⁹, Jianan Zhou⁴⁹ & Lihan Zi⁴⁹

DLUT_China_B

Zhu Jinyu⁴⁹, Xu Kang⁴⁹, Peng Xilei⁴⁹, Han Xue⁴⁹ & Shu Xun⁴⁹

DNHS_SanDiego

Priyanka Babu⁵⁰, Arushi Dogra⁵⁰ & Pranav Thokachichu⁵⁰

DTU-Denmark

David Faurdal⁸, Joen Haahr Jensen⁸, Jacob Mejlsted⁸, Lina Nielsen⁸ & Tenna Rasmussen⁸

Duesseldorf

Jennifer Denter⁵¹, Kai Husnatter⁵¹ & Ylenia Longo⁵¹

Ecuador

Juan Carlos Luzuriaga⁵², Eduardo Moncayo⁵², Natalia Torres Moreira⁵² & Jennifer Tapia⁵²

ECUST

Tang Dingyue⁵³, Zhao Jingjing⁵³, Xu Wenhao⁵³, Teng Xinyu⁵³ & Hong Xiujing⁵³

Edinburgh_OG

Jackson DeKloe⁵⁴

Edinburgh_UG

Ben Astles⁵⁴, Ugne Baronaite⁵⁴ & Inga Grazulyte⁵⁴

Emory

Michael Hwang⁵⁵ & Yibo Pang⁵⁵

EPFL

Michael Andrew Crone⁵⁶, Reza Hosseini⁵⁶, Moustafa Houmani⁵⁶, Daniel Zadeh⁵⁶ & Violetta Zanotti⁵⁶

ETH_Zurich

Oliver Andreas Baltensperger⁵⁷, Eline Yafele Bijman⁵⁷, Elisa Garulli⁵⁷, Jan Lukas Krusemann⁵⁷, Adriano Martinelli⁵⁷, Antonio Martinez⁵⁷ & Tobias Vornholt⁵⁷

Evry_Paris-Saclay

Monteil Camille⁵⁸ & Ahavi Paul⁵⁸

Exeter

Emily Browne⁵⁹, Daniel Barber James Gilman⁵⁹, Amy Hewitt⁵⁹, Sophie Hodson⁵⁹, Ingebjorg Holmedal⁵⁹, Fiona Kennedy⁵⁹ & Juliana Sackey⁵⁹

FAU_Erlangen

Selina Beck⁶⁰, Franziska Eidloth⁶⁰, Markus Imgold⁶⁰, Anna Matheis⁶⁰, Tanja Meerbrei⁶⁰, David Ruscher⁶⁰ & Marco Schaeftlein⁶⁰

FJNU-China

Zhu Hanrong⁶¹

Fudan

Mitchell Wan⁶²

Fudan-CHINA

Leijie Dai⁶², Kaifeng Jin⁶², Sihan Wang⁶², Xin Wang⁶², Yi Wang⁶², Yifan Wang⁶², Chenhai Wu⁶², Zixuan Zhang⁶² & Yineng Zhou⁶²

GDSYZX

Liu Xinyu⁶³ & Zeng Zirong⁶³

Georgia_State

Rehmat Babar⁶⁴, Mathew Brewer⁶⁴, Christina Clodomir⁶⁴, Laura Das Neves⁶⁴, Amanda Iwuogo⁶⁴, Ari Jones⁶⁴, Cara Jones⁶⁴, Julia Kelly⁶⁴, Gloria Kim⁶⁴, Jessica Siemer⁶⁴ & Yash Yadav⁶⁴

Gifu

Yuichiro Ikagawa⁶⁵, Tatsuki Isogai⁶⁵ & Ryo Niwa⁶⁵

GO_Paris-Saclay

Celine Aubry⁵⁸, William Briand⁵⁸, Annick Jacq⁵⁸, Sylvie Lautru⁵⁸, Britany Marta⁵⁸, Clemence Maupu⁵⁸, Xavier Ollessa-Daragon⁵⁸, Kenn Papadopoulo⁵⁸ & Mahnaz Sabeta Azad⁵⁸

GreatBay_China

Wei Kuangyi⁶⁶, Yao Xiu⁶⁶ & Chenghao Yang⁶⁶

Groningen

Aditya Iyer⁶⁷, Rianne Prins⁶⁷ & Phillip Yesley⁶⁷

GZHS-United

Fang Lichi⁶⁸ & Chen Zi Xuan⁶⁸

HAFS

Kyuhee Jo⁶⁹, Mikyung Park⁶⁹, Seunghyun Park⁶⁹ & Hojun Yoo⁶⁹

Hamburg

Nele Burckhardt⁷⁰, Lea Daniels⁷⁰, Bjarne Klopprogge⁷⁰, Dustin Kruger⁷⁰, Oda-Emilia Meyfarth⁷⁰, Lisa Putthoff⁷⁰ & Dominika Wawrzyniak⁷⁰

HBUT-China

Xinyi Hu⁷¹ & Yunyi Wang⁷¹

HebrewU

Lior Badash⁷², Amichai Baichman-Kass⁷², Alon Barshap⁷², Yonatan Friedman⁷², Eliya Milshtein⁷² & Omri Vardi⁷²

HFLS_ZhejiangUnited

Shan Dong^{73,74}, Yining Gu^{73,74}, Yuanzhe Pei^{73,74}, Ruyi Shi^{73,74}, Fan Yang^{73,74}, Jinshu Yang^{73,74} & Xueqian Zhu^{73,74}

HK_HCY_LFC

Lam Kai Ching^{75,76}, Law Hiu Ching^{75,76}, Ng Tsz Chun^{75,76}, Yu Man Hin^{75,76}, Lai Tsz Hong^{75,76}, Chan Wing Lam^{75,76}, Yiu Choi Lam^{75,76}, Cheah Matthew^{75,76}, Cheng Tsz Ngo^{75,76}, Yun Shuan^{75,76}, Chan Tsey Wan^{75,76}, Tsui Shing Yan^{75,76}, Chong Yuk Yee^{75,76}, Tam Chi Yu^{75,76} & Yuen Wai Yu^{75,76}

HKJS_S

Chung Tsun Ho Anson⁷⁷, Lee Sze Choi⁷⁷, Cheung Man Chun⁷⁷, Chan Lok Hin⁷⁷, Wong Chung Hin⁷⁷, Ng Sze Ho⁷⁷, Leung Chung Yin Jay⁷⁷, Lai Man Wai Katherine⁷⁷, Wong Carol Kin-ning⁷⁷, Lee Hong Kiu⁷⁷, Cheng Chak Kong⁷⁷, Leung Chung Wai⁷⁷, Yeung Wing Yan⁷⁷, Wong Tsz Yeung⁷⁷ & Lee Ka Yin⁷⁷

Hong_Kong_HKU

Tsui Shing Yan Grace⁷⁸, Lam Kai Ching Joe⁷⁸, Ng Tsz Chun Kenneth⁷⁸ & Cheah Matthew Yun Shuan⁷⁸

Hong_Kong_HKUST

Ferdinan Aldo⁷⁹, Chung Him Pang⁷⁹, Kam Pang So⁷⁹ & Hei Man Wong⁷⁹

Hong_Kong_JSS

Lai Tsz Ching^{80,81}, Luk Hau Ching^{80,81}, Ip Ning Fung^{80,81}, Yam Shing Fung^{80,81}, Lee Chi Hong^{80,81}, Hsiu Ou Ning^{80,81} & Jonathan Cheng Hon Sang^{80,81}

Hong_Kong-CUHK

Yeung Hoi Lam Elsa⁸², Chan Yick Hei⁸², Lo Ho Sing⁸² & Choi Seong Wang⁸²

HUBU-Wuhan

Yiheng Gu⁸³, Ziyue Rong⁸³, Haoyue Song⁸³, Pengying Wang⁸³ & Yuefei Wang⁸³

HUST-China

Yan Chen⁸⁴, Hao Qiu⁸⁴, Haotian Ren⁸⁴ & Ziyang Xiao⁸⁴

HZAU-China

Heng Heng⁸⁵, Xichen Rao⁸⁵ & Ruonan Tian⁸⁵

ICT-Mumbai

Shalini S. Deb⁸⁶, Yash Laxman Kamble⁸⁶, Ninad Kumbhojkar⁸⁶, Bhargav Patel⁸⁶, Supriya Prakash⁸⁶, Shamlan M. S. Reshamwala⁸⁶ & Poorva Taskar⁸⁶

IISc-Bangalore

Gokul⁸⁷ & Adwaith B. Uday⁸⁷

IISER-Bhopal-India

Anubhav Basu⁸⁸, Rishi Gandhi⁸⁸, Jatin Khaimani⁸⁸, Arundhati Khenwar⁸⁸, Sandeep Raut⁸⁸ & Tejas Somvanshi⁸⁸

IISER-Kolkata

Diptatanu Das⁸⁹, Souvik Ghosh⁸⁹ & Hrishika Rai⁸⁹

IISER-Mohali

Nithishwer Mouroug Anand⁹⁰, Ashwin Kumar Jainarayanan⁹⁰, Pranshu Kalson⁹⁰, Devang Haresh Liya⁹⁰, Vibhu Mishra⁹⁰, Sveekruth Sheshagiri Pai⁹⁰, Madhav Pitaliya⁹⁰, Yash Rana⁹⁰ & Ravineet Yadav⁹⁰

IIT_Delhi

Neha Arora⁹¹, Vasu Arora⁹¹, Shubham Jain⁹¹, Abhilash Patel⁹¹, Saksham Sharma⁹¹ & Priyanka Singh⁹¹

IIT_Kanpur

Anushya Goenka⁹², Rishabh Jain⁹², Aryaman Jha⁹², Adarsh Kumar⁹² & Abhinav Soni⁹²

IIT-Madras

Sathvik Ananthakrishnan⁹³, Velvizhi Devi⁹³, Mohammed Faidh⁹³, Guhan Jayaraman⁹³, M. Sagar Kittur⁹³, Nitish R. Mahapatra⁹³, Sarvesh Menon⁹³, Anantha Barathi Muthukrishnan⁹³, B. P. Kailash⁹³, Burhanuddin Sabuwala⁹³, Mousami Shinde⁹³ & Sankalpa Venkatraghavan⁹³

Jiangnan_China

Weijia Liu⁹⁴, Zhouli Miao⁹⁴, Tian Wang⁹⁴, Yaling Wang⁹⁴ & Shuyan Zhang⁹⁴

Jilin_China

Ruochen Chai⁹⁵, Yubin Ge⁹⁵, Ali Hou⁹⁵, Fangqi Liu⁹⁵, Xutong Liu⁹⁵, Jiangjiao Mao⁹⁵, Zihao Wang⁹⁵, Haimeng Yu⁹⁵, Hetian Yuan⁹⁵ & Yang Zhan⁹⁵

JMU_Wuerzburg

Anna Ries⁹⁶ & Chiara Wolfbeisz⁹⁶

KAIT_JAPAN

Toshihiro Kanaya⁹⁷, Yusuke Kawasaki⁹⁷, Tatuya Maruo⁹⁷, Yuya Mori⁹⁷ & Takehito Satoh⁹⁷

KCL_UK

Anthony Chau⁹⁸, Wai Yan Chu⁹⁸, Anatoliy Markiv⁹⁸, Marcos Vega-Hazas Marti⁹⁸, Maria Jose Ramos Medina⁹⁸, Deeksha Raju⁹⁸ & Shubhankar Sinha⁹⁸

KUAS_Korea

Youngeun Choi⁹⁹ & Bo Sun Ryu⁹⁹

Lambert_GA

Gaurav Byagathvalli¹⁰⁰ & Ellie Kim¹⁰⁰

Leiden

Marjolein Crooijmans¹⁰¹, Jazzy de Waard¹⁰¹ & Chiel van Amstel¹⁰¹

Lethbridge

Aubrey Demchuk¹⁰², Travis Haight¹⁰², Dong Ju Kim¹⁰², Andrei Neda¹⁰², Luc Roberts¹⁰², Luke Saville¹⁰², Reanna Takeyasu¹⁰² & David Tobin¹⁰²

Lethbridge_HS

Mina Akbary^{103,104,105,106}, Rebecca Avileli^{103,104,105,106}, Karen He^{103,104,105,106}, Aroma Pageni^{103,104,105,106}, Luke Saville^{103,104,105,106}, Dewuni De Silva^{103,104,105,106}, Nimaya De Silva^{103,104,105,106}, Kristi Turton^{103,104,105,106}, Michelle Wu^{103,104,105,106} & Alice Zhang^{103,104,105,106}

Lubbock_TTU

Benjamin Chavez¹⁰⁷, Paula Garavito¹⁰⁷, Michael Latham¹⁰⁷, Jeffrey Ptak¹⁰⁷ & Darron Tharp¹⁰⁷

Lund

Nurul Izzati^{108,109}, Martin Jonsson^{108,109}, Nikol Labecka^{108,109} & Sara Palo^{108,109}

Macquarie_Australia

Renee Beale¹¹⁰, Dominic Logel¹¹⁰, Areti-Efremia Mellou¹¹⁰ & Karl Myers¹¹⁰

Madrid-OLM

Alejandro Alonso^{111,112}, Rodrigo Hernandez Cifuentes^{111,112}, Borja Sanchez Clemente^{111,112}, Gonzalo Saiz Gonzalo^{111,112}, Ivan Martin Hernandez^{111,112}, Laura Armero Hernandez^{111,112}, Francisco Javier Quero Lombardero^{111,112}, Domingo Marquina^{111,112}, Guillermo Fernandez Rodriguez^{111,112} & Ignacio Albert Smet^{111,112}

Manchester

Tom Butterfield¹¹³, Ed Deshmukh-Reeves¹¹³, Namrata Gogineni¹¹³, Sam Hemmings¹¹³, Ismat Kabbara¹¹³, Ieva Norvaisaite¹¹³ & Ryan Smith¹¹³

Marburg

Daniel Bauersachs¹¹⁴, Benjamin Daniel¹¹⁴, Rene Inckemann¹¹⁴, Alexandra Seiffermann¹¹⁴, Daniel Stukenberg¹¹⁴ & Carl Weile¹¹⁴

McGill

Valerian Clerc¹¹⁵, Jacqueline Ha¹¹⁵ & Stephanie Totten¹¹⁵

McMaster

Thomas Chang¹¹⁶, Carlene Jimenez¹¹⁶ & Dhanyasri Maddiboina¹¹⁶

METU_HS_Ankara

Beliz Leyla Acar¹¹⁷, Evrim Elcin¹¹⁷, Tugba Inanc¹¹⁷, Gamze Kantas¹¹⁷, Ceyhun Kayihan¹¹⁷, Mert Secen¹¹⁷, Gun Suer¹¹⁷, Kutay Ucan¹¹⁷ & Tunc Unal¹¹⁷

Michigan

Matthew Fischer¹¹⁸, Naveen Jasti¹¹⁸ & Thomas Stewart¹¹⁸

MichiganState

Sarah Caldwell¹¹⁹, Jordan Lee¹¹⁹ & Jessica Schultz¹¹⁹

Mingdao

Ting-Chen Chang¹²⁰, Pei-Hong Chen¹²⁰, Yu-Hsuan Cheng¹²⁰, Yi-Hsuan Hsu¹²⁰ & Chan-yu Yeh¹²⁰

Minnesota

Zhipeng Ding¹²¹, Zihao Li¹²¹, Savannah Lockwood¹²¹ & Katherine Quinn¹²¹

Montpellier

Leo Carrillo¹²², Maxime Heintze¹²², Lea Meneu¹²², Marie Peras¹²² & Tamara Yehouessi¹²²

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Keno Eilers^{123,124}, Elisabeth Falgenhauer^{123,124}, Wong Hoi Kiu^{123,124}, Julia Mayer^{123,124}, Julia Mueller^{123,124}, Sophie von Schoenberg^{123,124}, Dominic Schwarz^{123,124} & Brigit Tunaj^{123,124}

Nanjing-China

Zhaoqing Hu¹²⁵, Yansong Huang¹²⁵ & Yuanyuan Li¹²⁵

NAU-CHINA

Chengzhu Fang¹²⁶, Jiangyuan Liu¹²⁶, Yiheng Liu¹²⁶, Yaxuan Wu¹²⁶, Sheng Xu¹²⁶ & Long Yuan¹²⁶

NAWI_Graz

Marco Edelmayer^{127,128,129}, Marlene Hiesinger^{127,128,129}, Sebastian Hofer^{127,128,129}, Birgit Krainer^{127,128,129}, Andreas Oswald^{127,128,129}, Dominik Strasser^{127,128,129} & Andreas Zimmermann^{127,128,129}

NCHU_Taichung

Yi-Cian Chen¹³⁰

NCTU_Formosa

Yuan-Yao Chan¹³¹, Yu-Ci Chang¹³¹, Nian Ruei Deng¹³¹, Chi-Yao Ku¹³¹ & Meng-Zhan Lee¹³¹

NEU_China_A

Hailong Li¹³², Zhaoyu Liu¹³², Guowei Song¹³², Yuening Xiang¹³² & Hongfa Yan¹³²

NEU_China_B

He Huanying¹³², Jiang Qiaochu¹³², Jiang Shengjuan¹³² & Peng Yujie¹³²

Newcastle

Matt Burridge¹³³, Kyle Standforth¹³³ & Sam Went¹³³

NJU-China

Liang Chenxi¹²⁵, Wang Han¹²⁵, Zhang Qipeng¹²⁵, Li Yifan¹²⁵, Quan Yiming¹²⁵ & Pan Yutong¹²⁵

NKU_CHINA

Senhao Kou¹³⁴ & Lin Luan¹³⁴

Northwestern

Umut Akova¹³⁵, Liza Fitzgerald¹³⁵, Bon Ikwuagwu¹³⁵, Michael Johnson¹³⁵, Jacob Kurian¹³⁵ & Christian Throsberg¹³⁵

Nottingham

Lucy Allen¹³⁶, Christopher Humphreys¹³⁶, Daniel Partridge¹³⁶, Michaella Whittle¹³⁶ & Nemira Zilinskaite¹³⁶

NPU-China

Meixuan Lee¹³⁷, Weifeng Lin¹³⁷, Yuan Ma¹³⁷ & Kai Wang¹³⁷

NTHU_Formosa

Hsuan Cheng¹³⁸, Shumei Chi¹³⁸, Yi-Chien Chuang¹³⁸, Ray Huang¹³⁸, LiangYu Ko¹³⁸ & Yu-Chun Lin¹³⁸

NTHU_Taiwan

You-Yang Tsai¹³⁸, Cheng-Chieh Wang¹³⁸ & Kai-Chiang Yu¹³⁸

NTNU_Trondheim

Hanna Nedreberg Burud¹³⁹, Carmen Chen¹³⁹, Anne Kristin Haralsvik¹³⁹, Adrian Marinovic¹³⁹, Hege Hetland Pedersen¹³⁹, Amanda Sande¹³⁹ & Vanessa Solvang¹³⁹

NTU-Singapore

Shaw Kar Ming¹⁴⁰ & Albert Praditya¹⁴⁰

NU_Kazakhstan

Aiganym Abduraimova¹⁴¹, Ayagoz Meirkhanova¹⁴¹, Assel Mukhanova¹⁴¹ & Tomiris Mulikova¹⁴¹

NUDT_CHINA

Yanchen Gou¹⁴², Chenyu Lu¹⁴², Jiaxin Ma¹⁴² & Chushu Zhu¹⁴²

NUS_Singapore-A

Leow Chung Yong Aaron¹⁴³, Tvarita Shivakumar Iyer¹⁴³, Wu Jiacheng¹⁴³, Yan Ping Lim¹⁴³, Beatrix Tung Xue Lin¹⁴³, Aaron Ramzeen¹⁴³ & Nur Liyana Binte Ayub Ow Yong¹⁴³

NUS_Singapore-Sci

Yah Tse Sabrina Chua¹⁴³, Yuhui Deborah Fong¹⁴³, Menglan He¹⁴³ & Li Yang Tan¹⁴³

NWU-China

Zhang Jiahe¹⁴⁴, Li Mingge¹⁴⁴, Li Nianlong¹⁴⁴, Li Yueyi¹⁴⁴ & Cheng Yuhan¹⁴⁴

NYMU-Taipei

Annabel Chang¹⁴⁵, Chih-Chiang Chen¹⁴⁵, Ryan Chou¹⁴⁵, Jude Clapper¹⁴⁵, Evelyn Lai¹⁴⁵, Yasmin Lin¹⁴⁵, Kelsey Wang¹⁴⁵ & Jake Yang¹⁴⁵

NYU_Abu_Dhabi

Mariam Anwar¹⁴⁶, Ibrahim Chehade¹⁴⁶, Imtiyaz Hariyani¹⁴⁶, Sion Hau¹⁴⁶, Ashley Isaac¹⁴⁶, Laura Karpauskaite¹⁴⁶, Mazin Magzoub¹⁴⁶, Daniel Obaji¹⁴⁶, Yong Rafael Song¹⁴⁶ & Yejie Yun¹⁴⁶

OUC-China

Kai Sun¹⁴⁷ & Yunqian Zhang¹⁴⁷

Oxford

Eleanor Beard¹⁴⁸, Laurel Constanti Crosby¹⁴⁸, Nicolas Delalez¹⁴⁸, Arman Karshenas¹⁴⁸, Adrian Kozhevnikov¹⁴⁸, Jhanna Kryukova¹⁴⁸, Karandip Saini¹⁴⁸, Jon Stocks¹⁴⁸, Bhuvana Sudarshan¹⁴⁸, Max Taylor¹⁴⁸, George Wadhams¹⁴⁸ & Joe Windo¹⁴⁸

Paris_Bettencourt

Annissa Ameziane¹⁴⁹, Darshak Bhatt¹⁴⁹, Alexis Casas¹⁴⁹, Antoine Levrier¹⁴⁹, Ana Santos¹⁴⁹, Nympha Elisa M. Sia¹⁴⁹ & Edwin Wintermute¹⁴⁹

Pasteur_Paris

Alice Dejoux¹⁵⁰, Deshmukh Gopaul¹⁵⁰, Lea Guerassimoff¹⁵⁰, Samuel Jaoui¹⁵⁰, Manon Madelenat¹⁵⁰ & Serena Petracchini¹⁵⁰

Peking

Fu Cai¹⁵¹, Yang Jianzhao¹⁵¹, Shi Shuyu¹⁵¹, Li Tairan¹⁵¹, Li Xin¹⁵¹, Lin Yongjie¹⁵¹ & Huang Zhecheng¹⁵¹

Pittsburgh

Evan Becker¹⁵², Matthew Greenwald¹⁵², Vivian Hu¹⁵², Tucker Pavelek¹⁵², Elizabeth Pinto¹⁵² & Zemeng Wei¹⁵²

Purdue

Zachary Burgland¹⁵³, Janice Chan¹⁵³, Julianne Dejoie¹⁵³, Kevin Fitzgerald¹⁵³, Zach Hartley¹⁵³, Moiz Rasheed¹⁵³ & Makayla Schacht¹⁵³

Queens_Canada

Maddison Gahagan¹⁵⁴, Ellis Kelly¹⁵⁴ & Elisha Krauss¹⁵⁴

RDFZ-China

Yuze Cao¹⁵⁵, Yishen Shen¹⁵⁵, Xuan Wang¹⁵⁵, Hanning Xu¹⁵⁵ & Jianxiang Zhang¹⁵⁵

REC-CHENNAI

Priyanka Chandramouli¹⁵⁶, Amal Jude Ashwin F¹⁵⁶, Srimathi Jayaraman¹⁵⁶, Marcia Smiti Jude¹⁵⁶, Vignesh Kumar¹⁵⁶, Hema Lekshmi¹⁵⁶, R. Preetha¹⁵⁶, Khadija Rashid¹⁵⁶, S. Deepak Kumar¹⁵⁶ & B. S. Mohan Kumar¹⁵⁶

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Leon Michael Barrat^{157,158}, Jil-Sophie Dissmann^{157,158}, Jorn Kalinowski^{157,158}, Matthias Otto^{157,158}, Johannes Ruhnau^{157,158}, Fynn Stuhlweissenburg^{157,158} & Elisa Ueding^{157,158}

RHIT

Ariel Bohner¹⁵⁹, Brittany Clark¹⁵⁹, Emilie Deibel¹⁵⁹, Liz Klaas¹⁵⁹, Kaylee Pate¹⁵⁹ & Elisa Weber¹⁵⁹

Rice

Katherine Cohen¹⁶⁰, Anna Guseva¹⁶⁰, Stefanie King¹⁶⁰ & Soohyun Yoon¹⁶⁰

Ruia-Mumbai

Sanika Ambre¹⁶¹, Shilpa Bhowmick¹⁶¹, Nishtha Pange¹⁶¹, Komal Parab¹⁶¹, Vainav Patel¹⁶¹, Mitali Patil¹⁶¹, Aishwarya Rajurkar¹⁶¹, Mayuri Rege¹⁶¹, Maithili Sawant¹⁶¹, Shrutika Sawant¹⁶¹ & Anjali Vaidya¹⁶¹

SBS_SH_112144

Peicheng Ji^{162,163}, Fang Luo^{162,163}, Guanghui Ma^{162,163}, Xin Xu^{162,163}, Jiacheng Yin^{162,163}, Yinchu Zhou^{162,163} & Ke Zhu^{162,163}

SCAU-China

Yaohua Huang¹⁶⁴, Yinpin Huang¹⁶⁴, Jiadong Li¹⁶⁴, Xuecheng Li¹⁶⁴, Hao Wang¹⁶⁴, Ken Wang¹⁶⁴, Wei Wang¹⁶⁴, Xinyu Zhang¹⁶⁴ & Jiahua Zou¹⁶⁴

SCU-China

Minyue Bao¹⁶⁵, Han Kang¹⁶⁵, Xiaolong Liu¹⁶⁵, Yibing Tao¹⁶⁵, Ziru Wang¹⁶⁵, Fuqiang Yang¹⁶⁵, Tianyi Zhang¹⁶⁵ & Yanling Zhong¹⁶⁵

SCUT_ChinaB

Jiezheng Liu¹⁶⁶, Jingang Liu¹⁶⁶, Lingling Ma¹⁶⁶, Xubo Niu¹⁶⁶, Ling Qian¹⁶⁶, Li Wang¹⁶⁶, Qingyan Yan¹⁶⁶ & Nannan Zhao¹⁶⁶

SCUT-ChinaA

Weixuan Chen¹⁶⁶ & Yuxin Zhou¹⁶⁶

SDU-CHINA

Junyang Chen¹⁶⁷

SFLS_Shenzhen

Junyao Hao¹⁶⁸, Zhang HuaYue¹⁶⁸, Peilin Li¹⁶⁸, Yifei Pei¹⁶⁸, Jingting Qu¹⁶⁸, Raven Wang¹⁶⁸, Xinyue Wang¹⁶⁸, Kangjie Wu¹⁶⁸, Yuxuan Wu¹⁶⁸, Meredith Xiang¹⁶⁸, Leyi Yang¹⁶⁸, Zisang Yang¹⁶⁸ & Li Zhaoting¹⁶⁸

ShanghaiTech

Wenhan Fu¹⁶⁹, Zonghao Li¹⁶⁹, Weiyi Tang¹⁶⁹ & Kaida Zhang¹⁶⁹

SHSBNU_China

Haocong Li¹⁷⁰, Xuze Shao¹⁷⁰, Chuyi Yang¹⁷⁰, Yuanhong Zeng¹⁷⁰ & Yanjun Zhou¹⁷⁰

SHSID_China

Shangzhi Dong¹⁷¹, Younji Jung¹⁷¹, Sophie Ruoja Li¹⁷¹, Tingting Li¹⁷¹ & Jiacheng Yu¹⁷¹

SHSU_China

Shangzhi Dong^{172,173,174}, Tingting Li^{172,173,174}, Xinyi Miao^{172,173,174} & Sibow Wang^{172,173,174}

SIAT-SCIE

Yiming Ding¹⁷⁵, Jiayi Huang¹⁷⁵, Yuqi Li¹⁷⁵, Ting Sun¹⁷⁵, Qinghe Tian¹⁷⁵, Mengxuan Wu¹⁷⁵, Jinming Xing¹⁷⁵, Xin Xiong¹⁷⁵, Yining Yan¹⁷⁵, Qiu Yihang¹⁷⁵, Jige Zhang¹⁷⁵, Yi Zhou¹⁷⁵ & Zhiyu Zhou¹⁷⁵

SJTU-BioX-Shanghai

Zhuoyang Chen¹⁷⁶, Peixiang He¹⁷⁶, Yirui Hong¹⁷⁶, Chia-Yi Hsiao¹⁷⁶, Zhihan Liang¹⁷⁶, Zhixiang Liu¹⁷⁶, Yuncong Ran¹⁷⁶, Shiyu Sun¹⁷⁶ & Ruoyu Xia¹⁷⁶

SKLMT-China

Dongyang Dong¹⁷⁷ & Wenxue Zhao¹⁷⁷

SMMU-China

Miao Hu¹⁷⁸, Shi Hu¹⁷⁸, Wei Shi¹⁷⁸, Shulun¹⁷⁸, Han Yan¹⁷⁸ & Yusheng Ye¹⁷⁸

SMS_Shenzhen

Yiquan Hong¹⁷⁹, Yuyao Pan¹⁷⁹, Yiran Song¹⁷⁹, Jinhan Zhang¹⁷⁹ & Yihang Zhao¹⁷⁹

Sorbonne_U_Paris

Dounia Chater¹⁸⁰, Asmaa Foda¹⁸⁰, Yanyan Li¹⁸⁰, Ursula Saade¹⁸⁰ & Victor Sayous¹⁸⁰

SSHS-Shenzhen

Yilin Mo¹⁸¹, Wenan Ren¹⁸¹ & Chenxu Zeng¹⁸¹

SSTi-SZGD

Yixin Cao¹⁸²

St_Andrews

Clarissa Czekster¹⁸³, Izzy Dunstan¹⁸³, Simon Powis¹⁸³, Bethany Reaney¹⁸³, Eva Snaith¹⁸³ & Cam Young¹⁸³

Stanford

Eva Frankel¹⁸⁴, Eleanor Glockner¹⁸⁴ & Isaac Justice¹⁸⁴

Stanford-Brown-RISD

Santosh Murugan^{184,185,186} & Leo Penny^{184,185,186}

Stockholm

Chrismar Garcia^{187,188,189} & Stamatina Rentouli^{187,188,189}

Stony_Brook

Priya Aggarwal¹⁹⁰, Stephanie Budhan¹⁹⁰, Woody Chiang¹⁹⁰, Dominika Kwasniak¹⁹⁰, Karthik Ledalla¹⁹⁰, Matthew Lee¹⁹⁰, Natalie Lo¹⁹⁰, Matthew Mullin¹⁹⁰, Lin Yu Pan¹⁹⁰, Jennifer Rakhimov¹⁹⁰, Robert Ruzic¹⁹⁰, Manvi Shah¹⁹⁰, Lukas Velikov¹⁹⁰ & Sara Vincent¹⁹⁰

Stuttgart

Philip Horz¹⁹¹, Nadine Kuebler¹⁹¹ & Jan Notheisen¹⁹¹

SUIS_Shanghai

David Doyle¹⁹², Jiajun Gu¹⁹², Wenyue Hu¹⁹² & Shuting Yang¹⁹²

SYSU-CHINA

Tao Kehan¹⁹³, Gao Menghan¹⁹³ & Mao Xiaowen¹⁹³

SYSU-Software

Yifei Chen¹⁹³, Ziqi Kang¹⁹³ & Haochen Ni¹⁹³

SZU-China

Junyu Chen¹⁹⁴, Lindong He¹⁹⁴, Mingyue Luo¹⁹⁴ & Jiaqi Tang¹⁹⁴

Tacoma_RAINmakers

Kira Boyce¹⁹⁵, James Lee¹⁹⁵, Michael Martin¹⁹⁵, Judy Van Nguyen¹⁹⁵ & Leon Wan¹⁹⁵

Tartu_TUIT

Artur Astapenka¹⁹⁶, Turan Badalli¹⁹⁶, Irina Borovko¹⁹⁶, Nadezhda Chulkova¹⁹⁶, Ilona Faustova¹⁹⁶, Anastasia Kolosova¹⁹⁶, Mart Loog¹⁹⁶, Artemi Maljavin¹⁹⁶, Frida Matiyevskaya¹⁹⁶ & Vladislav Tuzov¹⁹⁶

TAS_Taipei

Catherine Chang¹⁹⁷, Ryan Chou¹⁹⁷, Jude Clapper¹⁹⁷, Tim Ho¹⁹⁷, Yi Da Hsieh¹⁹⁷, Evelyn Lai¹⁹⁷, Leona Tsai¹⁹⁷, Kelsey Wang¹⁹⁷ & Justin Wu¹⁹⁷

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Viana Isabel Perez Dominguez¹⁹⁸, Cesar Ibrahim Rodriguez Fernandez¹⁹⁸, Daniela Olono Fierro¹⁹⁸, Anna Karen Aguilar Nunez¹⁹⁸, Jose Pablo Rascon Perez¹⁹⁸, Mario Loya Rivera¹⁹⁸, Cynthia Lizeth Gonzalez Trevizo¹⁹⁸ & Maria Antonia Luna Velasco¹⁹⁸

Tec-Monterrey

Carlos Javier Cordero Oropeza¹⁹⁹, Adrian Federico Hernandez Mendoza¹⁹⁹, Jose Arnulfo Juarez Figueroa¹⁹⁹, Luis Mario Leal¹⁹⁹, Samantha Ayde Pena Benavides¹⁹⁹, Victor Javier Robledo Martinez¹⁹⁹, Adriana Lizeth Rubio Aguirre¹⁹⁹, Andres Benjamin Sanchez Alvarado¹⁹⁹, Margarita Sofia Calixto Solano¹⁹⁹, Nora Esther Torres Castillo¹⁹⁹, Alejandro Robles Zamora¹⁹⁹ & Esteban de la Pena Thevenet¹⁹⁹

TecCEM

Karla Soto Blas²⁰⁰, Ana Laura Torres Huerta²⁰⁰ & Armando Cortes Resendiz²⁰⁰

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Frida Cruz²⁰¹, Fernanda Diaz²⁰¹, Diego Espinoza²⁰¹, Ana Cristina Figueroa²⁰¹, Ana Cecilia Luque²⁰¹, Roberto Portillo²⁰¹, Carolina Senes²⁰¹, Diana Tamayo²⁰¹ & Mariano Del Toro²⁰¹

Thessaloniki

Ioannis Alexopoulos²⁰², Alexandros Dimitriou Giannopoulos²⁰², Yvoni Giannoula²⁰² & Grigorios Kyrpizidis²⁰²

Tongji_China

Ma Xinyue²⁰³, Chen Xirui²⁰³ & Song Zhiwei²⁰³

Toronto

Nina Adler²⁰⁴, Amalia Caballero²⁰⁴, Carla Hamady²⁰⁴, Ahmed Ibrahim²⁰⁴, Jasmeen Parmar²⁰⁴, Tashi Rastogi²⁰⁴ & Jindian Yang²⁰⁴

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Jean Delhomme^{205,206}, Anthony Henras^{205,206}, Stephanie Heux^{205,206}, Yves Romeo^{205,206}, Marion Toanen^{205,206}, Camille Wagner^{205,206} & Paul Zanoni^{205,206}

TU_Darmstadt

Thea Lotz²⁰⁷, Elena Nickels²⁰⁷, Beatrix Suss²⁰⁷, Heribert Warzecha²⁰⁷ & Jennifer Zimmermann²⁰⁷

TU-Eindhoven

Emilien Dubuc²⁰⁸, Bruno Eijkens²⁰⁸, Sander Keij²⁰⁸, Simone Twisk²⁰⁸, Mick Verhagen²⁰⁸ & Maxime van den Oetelaar²⁰⁸

TU Delft

Alexander Armstrong²⁰⁹, Nicole Bennis²⁰⁹, Susan Bouwmeester²⁰⁹, Lisa Buller²⁰⁹, Kavish Kohabir²⁰⁹, Monique de Leeuw²⁰⁹, Vanda Mangkusaputra²⁰⁹, Jard Mattens²⁰⁹, Janine Nijenhuis²⁰⁹, Timmy Paez²⁰⁹, Lisbeth Schmidtchen²⁰⁹ & Gemma van der Voort²⁰⁹

TUST_China

Gao Ge²¹⁰, Xu Haoran²¹⁰ & Li Xiaojin²¹⁰

UAlberta

Ejouan Agena²¹¹, Ethan Agena²¹¹, Scott Bath²¹¹, Robert Campbell²¹¹, Rochelin Dalangin²¹¹, Anna Kim²¹¹, Dominic Sauvageau²¹¹ & Irene Shkolnikov²¹¹

UC_Davis

Daniel Graves²¹², Jacob Lang²¹², Jolee Nieberding-Swanberg²¹², Achala Rao²¹², Ares Torres²¹² & Andrew Yao²¹²

UC_San_Diego

Anser Abbas²¹³ & Claire Luo²¹³

UCAS-China

Xu Zepeng²¹⁴ & Zhao Ziyi²¹⁴

UChicago

Janice Chen²¹⁵, Cian Colgan²¹⁵, Steve Dvorkin²¹⁵, Rachael Filzen²¹⁵, Varun Patel²¹⁵, Allison Scott²¹⁵ & Patricia Zulueta²¹⁵

UChile_Biotec

Joaquin Acosta²¹⁶, Lucas Araya²¹⁶, Francisco Chavez²¹⁶, Sebastian Farias²¹⁶, Delia Garrido²¹⁶, Andres Marcoleta²¹⁶, Felipe Munoz²¹⁶ & Paula Rivas²¹⁶

UCL

Noelle Colant²¹⁷, Catherine Fan²¹⁷, Stefanie Frank²¹⁷, Jacopo Gabrielli²¹⁷, Paola Handal²¹⁷, Vitor Pinheiro²¹⁷, Stefanie Santamaria²¹⁷, Shamal Withanage²¹⁷ & Fang Xue²¹⁷

UCLouvain

Antoine Gerard²¹⁸, Marine Lefevre²¹⁸, Fiona Milano²¹⁸, Nina De Sousa Oliveira²¹⁸, Mathieu Parmentier²¹⁸ & Luca Rigon²¹⁸

UConn

Elizabeth Chamiec-Case²¹⁹, Ryan Chen²¹⁹, Peter Crowley²¹⁹, Shannon Doyle²¹⁹, Sricharan Kadimi²¹⁹ & Toni Vella²¹⁹

UCopenhagen

Natthawut Adulyanukosol²²⁰, Theodore A. Dusseaux²²⁰, Victor Forman²²⁰, Cecilie Hansen²²⁰, Selma Kofoed²²⁰, Simon Louis²²⁰, Magnus Ronne Lykkegaard²²⁰, Davide Mancinotti²²⁰, Lasse Meyer²²⁰, Stephanie Michelsen²²⁰, Morten Raadam²²⁰, Victoria Svaerke Rasmussen²²⁰, Eirikur Andri Thormar²²⁰, Attila Uslu²²⁰ & Nattawut leelahakorn²²⁰

UESTC-China

Shizhi Ding²²¹, Changyu Li²²¹, Huishuang Tan²²¹, Yinsong Xu²²¹ & Jianzhe Yang²²¹

UFlorida

Diego Gamoneda²²², Nicole Kantor²²², Lidimarie Trujillo-Rodriguez²²² & Matthew Turner²²²

UGA

Stephan George²²³, Kelton McConnell²²³ & Chynna Pollitt²²³

UI_Indonesia

Ihya Fakhruzil Amin²²⁴, Muhammad Ikhsan²²⁴, Valdi Ven Japranata²²⁴, Andrea Laurentius²²⁴, Luthfian Aby Nurachman²²⁴ & Muhammad Iqbal Adi Pratama²²⁴

UiOslo_Norway

Yvette Dirven²²⁵, Lisa Frohlich²²⁵, Dirk Linke²²⁵, Verena Mertes²²⁵, Rebekka Rekkedal Rolfsnes²²⁵ & Athanasios Saragliadis²²⁵

UIOWA

Sandra Castillo²²⁶, Sathivel Chinnathambi²²⁶, Craig Ellermeier²²⁶, Jennifer Farrell²²⁶, Jan Fassler²²⁶, Ernie Fuentes²²⁶, Sean Ryan²²⁶ & Edward Sander²²⁶

UIUC_Illinois

Amie Bott²²⁷, Liam Healy²²⁷, Pranathi Karumanchi²²⁷, Alex Ruzicka²²⁷ & Ziyu Wang²²⁷

ULaval

Gabriel Byatt²²⁸, Philippe C. Despres²²⁸, Alexandre Dube²²⁸, Pascale Lemieux²²⁸, Florian Echelard²²⁸, Louis-Andre Lortie²²⁸ & Francois D. Rouleau²²⁸

ULaVerne_Collab

Seth Barrington²²⁹, Cynthia Basulto²²⁹, Sabrina Delgadillo²²⁹, Karen De Leon²²⁹, Micah Madrid²²⁹, Catherosette Meas²²⁹, Angelica Sabandal²²⁹, Magaly Aguirre Sanchez²²⁹, Jennifer Tsui²²⁹ & Noble Woodward²²⁹

UMaryland

Rohith Battina²³⁰, Jess Boyer²³⁰, Arjun Cherupalla²³⁰, Jason Chiang²³⁰, Mary Heng²³⁰, Collin Keating²³⁰, Tommy Liang²³⁰, Chun Kit Loke²³⁰, Jacob Premo²³⁰, Keerthana Srinivasan²³⁰, John Starkel²³⁰ & Daniel Zheng²³⁰

UNebraska-Lincoln

Gabe Astorino²³¹, Rachel Van Cott²³¹, Jintao Guo²³¹, Drew Kortus²³¹ & Wei Niu²³¹

Unesp_Brazil

Paulo J. C. Freire²³², Danielle Biscaro Pedrolli²³², Nathan Vinicius Ribeiro²³², Bruna Fernandes Silva²³², Nadine Vaz Vanini²³², Mariana Santana da Mota²³² & Larissa de Souza Crispim²³²

UNSW_Australia

Tyler Chapman²³³, Tobias Gaitt²³³, Megan Jones²³³ & Emily Watson²³³

UPF_CRG_Barcelona

Guillem Lopez-Grado^{234,235} & Laura Sans^{234,235}

UPF_CRG_Barcelona

Matilda Brink²³⁶, Varshni Rajagopal²³⁶ & Elin Ramstrom²³⁶

US_AFRL_CarrollHS

Anna Bete²³⁷, Yazmin Camacho²³⁷, Jonah Carter²³⁷, Christina Davis²³⁷, Jason Dong²³⁷, Amy Ehrenworth²³⁷, Michael Goodson²³⁷, Chris Guptil²³⁷, Max Herrmann²³⁷, Chia Hung²³⁷, Hayley Jesse²³⁷, Rachel Krabacher²³⁷, Dallas McDonald²³⁷, Peter Menart²³⁷, Travis O'Leary²³⁷, Laura Polanka²³⁷, Andrea Poole²³⁷ & Vanessa Varaljay²³⁷

USMA-West_Point

Alana Appel²³⁸, John Cave²³⁸, Liz Huuki²³⁸, Matt McDonough²³⁸, Channah Mills²³⁸, Alex Mitropoulos²³⁸, James Pruneski²³⁸ & Ken Wickiser²³⁸

USP-Brazil

Felipe Xavier Buson²³⁹, Vinicius Flores²³⁹, Guilherme Meira Lima²³⁹ & Caio Gomes Tavares Rosa²³⁹

UST_Beijing

Guanke Bao²⁴⁰, Haitao Dong²⁴⁰, Zhi Luo²⁴⁰ & Jiarong Peng²⁴⁰

USTC

Yongyan An²⁴¹, Cheng Cheng²⁴¹, Zhenyu Jiang²⁴¹, Linzhen Kong²⁴¹, Chenfei Luo²⁴¹, Liudong Luo²⁴¹, Yingying Shi²⁴¹, Erting Tang²⁴¹, Ping Wang²⁴¹, Yuyang Wang²⁴¹, Guiyang Xu²⁴¹, Wenfei Yu²⁴¹, Bonan Zhang²⁴¹ & Qian Zhang²⁴¹

UT-Knoxville

David Garcia²⁴², Nannan Jiang²⁴², Brandon Kristy²⁴², Ralph Laurel²⁴², Karl Leitner²⁴², Frank Loeffler²⁴², Steven Ripp²⁴² & Morgan Street²⁴²

Utrecht

Khadija Amheine²⁴³, Felix Bindt²⁴³, Meine Boer²⁴³, Mike Boxem²⁴³, Jolijn Govers²⁴³, Seino Jongkees²⁴³, Lorenzo Pattiradjawane²⁴³, Pim Swart²⁴³, Helen Tsang²⁴³, Floor de Graaf²⁴³, Marjolijn ten Dam²⁴³ & Franca van Heijningen²⁴³

Valencia_UPV

Yadira Boada²⁴⁴ & Alejandro Vignoni²⁴⁴

Vilnius-Lithuania

Valentas Brasas²⁴⁵, Aukse Gaizauskaite²⁴⁵, Gabrielius Jakutis²⁴⁵, Simas Jasiunas²⁴⁵, Ieva Juskaite²⁴⁵, Justas Ritmejeris²⁴⁵, Dovydas Vaitkus²⁴⁵, Tomas Venclovas²⁴⁵, Kornelija Vitkute²⁴⁵, Hanna Yeliseyeva²⁴⁵, Kristina Zukauskaite²⁴⁵ & Justina Zvirblyte²⁴⁵

Vilnius-Lithuania-OG

Laurynas Karpus²⁴⁵, Ignas Mazelis²⁴⁵ & Irmantas Rokaitis²⁴⁵

Virginia

Ngozi D. Akingbesote²⁴⁶, Dylan Culfogienis²⁴⁶, William Huang²⁴⁶ & Kevin Park²⁴⁶

Warwick

Janvi Ahuja²⁴⁷, Christophe Corre²⁴⁷, Gurpreet Dhaliwal²⁴⁷, Rhys Evans²⁴⁷, Kurt Hill²⁴⁷, Olivor Holman²⁴⁷, Alfonso Jaramillo²⁴⁷, Alizah Khalid²⁴⁷, Jack Lawrence²⁴⁷, Laura Mansfield²⁴⁷, James O'Brien²⁴⁷, June Ong²⁴⁷, Satya Prakash²⁴⁷ & Jonny Whiteside²⁴⁷

Washington

Karl Anderson²⁴⁸, Emily Chun²⁴⁸, Grace Kim²⁴⁸, Aerilynn Nguyen²⁴⁸, Chemay Shola²⁴⁸, Dorsa Toghani²⁴⁸, Angel Wong²⁴⁸, Joanne Wong²⁴⁸ & Jay Yung²⁴⁸

WashU_StLouis

Elizabeth Johnson²⁴⁹, Divangana Lahad²⁴⁹, Kyle Nicholson²⁴⁹, Havisha Pedamallu²⁴⁹ & Cam Phelan²⁴⁹

Waterloo

Clara Fikry²⁵⁰, Leah Fulton²⁵⁰, Nicole Lassel²⁵⁰, Dylan Perera²⁵⁰, Marina Robin²⁵⁰ & Nicolette Shaw²⁵⁰

Westminster_UK

Kyle Bowman²⁵¹, Sarah Coleman²⁵¹, Kristian Emilov²⁵¹, Camila Gaspar²⁵¹, Jenaagan Jenakendran²⁵¹, Sara Mubeen²⁵¹, Marko Obrvan²⁵¹ & Caroline Smith²⁵¹

WHU-China

Tang Bo²⁵², Du Liaoqi²⁵², Chang Tianyi²⁵², Xing Yuan²⁵² & Qing Yue²⁵²

William_and_Mary

Stephanie Do²⁵³, Xiangyi Fang²⁵³, Ethan Jones²⁵³, Jessica Laury²⁵³, Wukun Liu²⁵³, Adam Oliver²⁵³, Lillian Parr²⁵³, Margaret Saha²⁵³, Chengwu Shen²⁵³, Tinh Son²⁵³, Julia Urban²⁵³, Yashna Verma²⁵³ & Hanmi Zhou²⁵³

Worldshaper-Wuhan

Shan Dong²⁵⁴, Zhengguo Hao²⁵⁴, Yi Kuang²⁵⁴, Ting Liu²⁵⁴ & Rui Zhou²⁵⁴

WPI_Worcester

Beck Arruda², Natalie Farny², Mei Hao², Camille Pearce², Alex Rebello², Arth Sharma², Kylie Sumner² & Bailey Sweet²

XJTLU-CHINA

Junliang Lin²⁵⁵

XJTU-China

Du Mengtao²⁵⁶, Fan Peiyao²⁵⁶ & Fang Xinlei²⁵⁶

XMU-China

Niangui Cai²⁵⁷, Junhong Chen²⁵⁷, Yousi Fu²⁵⁷, Yunyun Hu²⁵⁷, Ye Qiang²⁵⁷, Qiupeng Wang²⁵⁷, Ruofan Yang²⁵⁷, Chen Yucheng²⁵⁷ & Jiyang Zheng²⁵⁷

Yale

Kevin Chang²⁵⁸, Cecily Gao²⁵⁸, Farren Isaacs²⁵⁸, Kevin Li²⁵⁸, Ricardo Moscoso²⁵⁸, Jaymin Patel²⁵⁸, Lauren Telesz²⁵⁸ & Alice Tirad²⁵⁸

ZJU-China

Qin hao Cao²⁵⁹, Xinhua Feng²⁵⁹, Yinjing Lu²⁵⁹, Xianyin Zhang²⁵⁹ & Xuanhao Zhou²⁵⁹

ZJUT-China

Dongchang Sun²⁶⁰, Zhe Yuan²⁶⁰ & Jiajie Zhou²⁶⁰

⁹RWTH Aachen University, Aachen, Germany. ¹⁰Aalto University, Espoo, Finland. ¹¹University of Helsinki, Helsinki, Finland. ¹²Anhui University of Technology, Maanshan, Anhui, China. ¹³Aix-Marseille University, Marseille, France. ¹⁴AST Worldshaper, Hangzhou, China. ¹⁵National Technical University of Athens, Athens, Attiki, Greece. ¹⁶Athens University of Economics and Business, Athens, Attiki, Greece. ¹⁷Liberal Arts and Science Academy High School, Austin, TX, USA. ¹⁸The University of Texas at Austin, Austin, TX, USA. ¹⁹Baltimore Underground Science Space, Baltimore, MD, USA. ²⁰Beijing City University, Beijing, China. ²¹Beijing Foreign Studies University, Beijing, China. ²²BGI College, Shenzhen, China. ²³Ben-Gurion University of the Negev, Beer-Shiva, Israel. ²⁴Universität Bielefeld, Bielefeld, Germany. ²⁵Bilkent University, Ankara, Turkey. ²⁶Institut Quimic de Sarria, Barcelona, Spain. ²⁷CHA University, Seongnam, South Korea. ²⁸Beijing Institute of Technology, Beijing, China. ²⁹Beijing Jianhua Experimental School, Beijing, China. ³⁰Beijing National Day School, Beijing, China. ³¹Beijing Normal University, Beijing, China. ³²BOKU Vienna, Vienna, Austria. ³³Boston University, Boston, MA, USA. ³⁴University of British Columbia, Vancouver, BC, Canada. ³⁵University of Calgary, Calgary, Alberta, Canada. ³⁶Cardiff University, Cardiff, UK. ³⁷National Chung Cheng University, Min-Hsiung Chia-Yi, Minhsiung, Chiayi, Taiwan. ³⁸Chengdu Shishi High School, Chengdu, China. ³⁹Huayang High School, Chengdu, China. ⁴⁰Chalmers University of Technology, Gothenburg, Sweden. ⁴¹China International Education Institute, Beijing, China. ⁴²Carnegie Mellon University in Qatar, Doha, Qatar. ⁴³Colorado School of Mines, Golden, CO, USA. ⁴⁴Columbia University, New York, NY, USA. ⁴⁵Cornell University, Ithaca, NY, USA. ⁴⁶China Pharmaceutical University, Nanjing, China. ⁴⁷Central South University, Changsha, China. ⁴⁸Colorado State University, Fort Collins, CO, USA. ⁴⁹Dalian University of Technology, Dalian, China. ⁵⁰Del Norte High School, San Diego, CA, USA. ⁵¹Heinrich Heine University, Duesseldorf, Germany. ⁵²Universidad de las Fuerzas Armadas, Sangolqui, Ecuador. ⁵³East China University of Science and Technology, Shanghai, China. ⁵⁴University of Edinburgh, Edinburgh, UK. ⁵⁵Emory University Atlanta, Atlanta, GA, USA. ⁵⁶Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland. ⁵⁷ETH Zurich, Zurich and Basel, Basel, Switzerland. ⁵⁸University Paris-Saclay, Saint-Aubin, France. ⁵⁹University of Exeter, Exeter, UK. ⁶⁰Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany. ⁶¹Fujian Normal University, Fuzhou, China. ⁶²Fudan University, Shanghai, China. ⁶³Guangdong Experimental High School, Guangzhou, Guangdong, China. ⁶⁴Georgia State University, Atlanta, GA, USA. ⁶⁵Gifu University, Gifu, Japan. ⁶⁶Shenzhen College Of International Education, Shenzhen, China. ⁶⁷University of Groningen, Groningen, Netherlands. ⁶⁸Guangdong Experimental High School, Guangzhou, China. ⁶⁹Hankuk Academy of Foreign Studies, Yongin, Korea. ⁷⁰University of Hamburg, Hamburg, Germany. ⁷¹Hubei University of Technology, Wuhan, China. ⁷²Hebrew University in Jerusalem, Jerusalem, Israel. ⁷³Hangzhou Foreign Language School, Hangzhou, China. ⁷⁴Zhejiang High Schools United, Hangzhou, China. ⁷⁵Tsuen Wan Public Ho Chuen Yiu Memorial College, Hong Kong, Hong Kong. ⁷⁶Po Leung Kuk Laws Foundation College, Hong Kong, Hong Kong. ⁷⁷St Stephen's College, Hong Kong, Hong Kong. ⁷⁸The University of Hong Kong, Hong Kong, Hong Kong. ⁷⁹Hong Kong University of Science and Technology, Hong Kong, Hong Kong. ⁸⁰United Christian College (Kowloon East), Hong Kong, Hong Kong. ⁸¹Yan Oi Tong Tin Ka Ping Secondary School, Hong Kong, Hong Kong. ⁸²The Chinese University of Hong Kong, Hong Kong, Hong Kong. ⁸³Hubei University, Wuhan, China. ⁸⁴Huazhong University of Science and Technology, Wuhan, China. ⁸⁵Huazhong Agricultural University, Wuhan, China. ⁸⁶Institute of Chemical Technology, Mumbai, India. ⁸⁷Indian Institute of Science, Bangalore, India. ⁸⁸Indian Institute of Science Education and Research, Bhopal, India. ⁸⁹Indian Institute of Science Education and Research, Kolkata, India. ⁹⁰Indian Institute of Science Education and Research, Mohali, India. ⁹¹IIT Delhi, New Delhi, India. ⁹²Indian Institute of Technology Kanpur, Kanpur, India. ⁹³Indian Institute of Technology Madras, Chennai, India. ⁹⁴Jiangnan University, Wuxi, China. ⁹⁵Jilin University, Changchun, China. ⁹⁶Julius-Maximilians Universität,

Wurzburg, Germany. ⁹⁷Kanagawa Institute of Technology, Kanagawa, Japan. ⁹⁸King's College London, London, UK. ⁹⁹Korea University, Seoul, South Korea. ¹⁰⁰Lambert High School, Suwanee, GA, USA. ¹⁰¹Leiden University, Leiden, The Netherlands. ¹⁰²University of Lethbridge, Lethbridge, Alberta, Canada. ¹⁰³Chinook High School, Lethbridge, Alberta, Canada. ¹⁰⁴Winston Churchill High School, Lethbridge, Alberta, Canada. ¹⁰⁵Catholic Central High School, Lethbridge, Alberta, Canada. ¹⁰⁶Lethbridge Collegiate Institute, Lethbridge, Alberta, Canada. ¹⁰⁷Texas Tech University, Lubbock, TX, USA. ¹⁰⁸Lund Tekniska Hogskola, Lund, Sweden. ¹⁰⁹Lund University, Lund, Sweden. ¹¹⁰Macquarie University, Sydney, Australia. ¹¹¹Complutense University, Madrid, Spain. ¹¹²Carlos III University, Madrid, Spain. ¹¹³University of Manchester, Manchester, UK. ¹¹⁴Philipps-University Marburg, Marburg, Germany. ¹¹⁵McGill University, Montreal, Quebec, Canada. ¹¹⁶McMaster University, Hamilton, ON, Canada. ¹¹⁷METU Developmental Foundation High School, Ankara, Turkey. ¹¹⁸University of Michigan, Ann Arbor, MI, USA. ¹¹⁹Michigan State University, East Lansing, MI, USA. ¹²⁰Mingdao High School, Taichung City, Taiwan. ¹²¹University of Minnesota, St. Paul, MN, USA. ¹²²University of Montpellier, Montpellier, France. ¹²³TU Munich, Garching, Germany. ¹²⁴LMU Munich, Munich, Germany. ¹²⁵Nanjing University, Nanjing, China. ¹²⁶Nanjing Agricultural University, Nanjing, China. ¹²⁷University Graz, Graz, Austria. ¹²⁸Graz University of Technology, Graz, Austria. ¹²⁹NAWI Graz, Graz, Austria. ¹³⁰National Chung Hsing University, Taichung, Taiwan. ¹³¹National Chiao Tung University, Hsinchu, Taiwan. ¹³²Northeastern University, Shenyang, China. ¹³³Newcastle University, Newcastle, UK. ¹³⁴Nankai University, Tianjin, China. ¹³⁵Northwestern University, Evanston, IL, USA. ¹³⁶The University of Nottingham, Nottingham, UK. ¹³⁷Northwestern Polytechnical University, Xi'an, China. ¹³⁸National Tsing Hua University, HsinChu, Taiwan. ¹³⁹Norwegian University of Science and Technology, Trondheim, Norway. ¹⁴⁰Nanyang Technological University, Singapore, Singapore. ¹⁴¹Nazarbayev University, Astana, Kazakhstan. ¹⁴²National University of Defense Technology, Changsha, China. ¹⁴³National University of Singapore, Singapore. ¹⁴⁴Northwest University, Xi'an, China. ¹⁴⁵National Yang Ming University, Taipei, Taiwan. ¹⁴⁶New York University Abu Dhabi, Abu Dhabi, UAE. ¹⁴⁷Ocean University of China, Qingdao, China. ¹⁴⁸University of Oxford, Oxford, UK. ¹⁴⁹CRI Paris, Paris, France. ¹⁵⁰Institut Pasteur, Paris, France. ¹⁵¹Peking University, Beijing, China. ¹⁵²University of Pittsburgh, Pittsburgh, PA, USA. ¹⁵³Purdue University, West Lafayette, IN, USA. ¹⁵⁴Queen's University, Kingston, ON, Canada. ¹⁵⁵RDFZ, Beijing, China. ¹⁵⁶Rajalakshmi Engineering College, Chennai, India. ¹⁵⁷Bielefeld University, Bielefeld, Germany. ¹⁵⁸Einstein Gymnasium, Rheda-Wiedenbruck, Germany. ¹⁵⁹Rose-Hulman Institute of Technology, Terra Haute, IN, USA. ¹⁶⁰Rice University, Houston, TX, USA. ¹⁶¹Ramnarain Ruia Autonomous College, Mumbai, India. ¹⁶²Stony Brook School, Stony Brook, NY, USA. ¹⁶³He County First Middle School, Shanghai, China. ¹⁶⁴South China Agricultural University, Guangzhou, China. ¹⁶⁵Sichuan University, Chengdu, China. ¹⁶⁶South China University of Technology, Guangzhou, China. ¹⁶⁷Shandong University, Qingdao, China. ¹⁶⁸Shenzhen Foreign Languages School, Shenzhen, China. ¹⁶⁹ShanghaiTech University, Shanghai, China. ¹⁷⁰SHSBNU, Beijing, China. ¹⁷¹Shanghai High School International Division, Shanghai, China. ¹⁷²Shanghai Foreign Language School, Shanghai, China. ¹⁷³Shanghai Pinghe School, Shanghai, China. ¹⁷⁴Shanghai Qibao Dwight High School, Shanghai, China. ¹⁷⁵Shenzhen College of International Education, Shenzhen, China. ¹⁷⁶Shanghai Jiao Tong University, Shanghai, China. ¹⁷⁷State Key Laboratory of Microbiological Technology, Shan Dong University, Qingdao, China. ¹⁷⁸Second Military Medical University, Shanghai, China. ¹⁷⁹Shenzhen Middle School, Shenzhen, China. ¹⁸⁰Sorbonne Universite, Paris, France. ¹⁸¹Shenzhen Senior High School, Shenzhen, China. ¹⁸²Shenzhen Institute of Technology, Shenzhen, China. ¹⁸³University of St Andrews, St Andrews, UK. ¹⁸⁴Stanford University, Stanford, CA, USA. ¹⁸⁵Brown University, Providence, RI, USA. ¹⁸⁶Rhode Island School of Design, Providence, RI, USA. ¹⁸⁷KTH Royal institute of Technology, Stockholm, Sweden. ¹⁸⁸Karolinska Institutet, Solna, Stockholm, Sweden. ¹⁸⁹Konstfack, Stockholm, Sweden. ¹⁹⁰Stony Brook University, Stony Brook, NY, USA. ¹⁹¹University of Stuttgart, Stuttgart, Germany. ¹⁹²Shanghai United International School, Shanghai, China. ¹⁹³Sun Yat-sen University, Guangzhou, China. ¹⁹⁴Shenzhen University, Shenzhen, China. ¹⁹⁵Readiness Acceleration and Innovation Network, Tacoma, WA, USA. ¹⁹⁶University of Tartu, Tartu, Estonia. ¹⁹⁷Taipei American School, Taipei, Taiwan. ¹⁹⁸Instituto Tecnológico y de Estudios Superiores de Monterrey - Campus Chihuahua, Chihuahua, Mexico. ¹⁹⁹Tecnologico de Monterrey Campus Monterrey, Monterrey, Mexico. ²⁰⁰Tecnologico de Monterrey CEM, Atizapan de Zaragoza, Ciudad Lopez Mateos, Mexico. ²⁰¹Tecnologico de Monterrey Campus Guadalajara, Guadalajara, Mexico. ²⁰²Aristotle University of Thessaloniki, Thessaloniki, Greece. ²⁰³Tongji University, Shanghai, China. ²⁰⁴University of Toronto, Toronto, ON, Canada. ²⁰⁵Institut National des Sciences Appliquees de Toulouse, Toulouse, France. ²⁰⁶Universite Toulouse III Paul Sabatier de Toulouse, Toulouse, France. ²⁰⁷Technische Universitaet Darmstadt, Darmstadt, Germany. ²⁰⁸Eindhoven University of Technology, Eindhoven, The Netherlands. ²⁰⁹Delft University of Technology, Delft, The Netherlands. ²¹⁰Tianjin University of Science and Technology, Tianjin, China. ²¹¹University of Alberta, Edmonton, Alberta, Canada. ²¹²University of California Davis, Davis, CA, USA. ²¹³University of California San Diego, La Jolla, USA. ²¹⁴University of Chinese Academy of Sciences, Beijing, China. ²¹⁵University of Chicago, Chicago, IL, USA. ²¹⁶University of Chile, Santiago, Chile. ²¹⁷University College London, London, UK. ²¹⁸Universite Catholique de Louvain, Louvain-la-Neuve, Belgium. ²¹⁹University of Connecticut, Storrs, CT, USA. ²²⁰University of Copenhagen, Copenhagen, Denmark. ²²¹University of Electronic Science and Technology of China, Chengdu, China. ²²²University of Florida, Gainesville, FL, USA. ²²³University of Georgia, Athens, GA, USA. ²²⁴Universitas Indonesia, Depok, West Java, Indonesia. ²²⁵University of Oslo, Oslo, Norway. ²²⁶University of Iowa, Iowa City, IA, USA. ²²⁷University of Illinois, Champaign-Urbana, IL, USA. ²²⁸Universite Laval, Quebec, QC, Canada. ²²⁹University of La Verne, La Verne, CA, USA. ²³⁰University of Maryland, College Park, MD, USA. ²³¹University of Nebraska - Lincoln, Lincoln, NE, USA. ²³²Universidade Estadual Paulista, Araraquara, Sao Paulo, Brazil. ²³³University of New South Wales, Randwick, Australia. ²³⁴Pompeu Fabra University, Barcelona, Spain. ²³⁵Center for Genomic Regulation, Barcelona, Spain. ²³⁶Uppsala University, Uppsala, Sweden. ²³⁷Carroll High School, Dayton, OH, USA. ²³⁸United States Military Academy, West Point, NY, USA. ²³⁹Universidade de Sao Paulo, Sao Paulo, Brazil. ²⁴⁰University of Science and Technology, Beijing, China. ²⁴¹University of Science and Technology of China, Hefei, China. ²⁴²University of Tennessee, Knoxville, TN, USA. ²⁴³Utrecht University, Utrecht, The Netherlands. ²⁴⁴Universitat Politecnica de Valencia, Valencia, Spain. ²⁴⁵Vilnius University, Vilnius, Lithuania. ²⁴⁶University of Virginia, Charlottesville, VA, USA. ²⁴⁷University of Warwick, Coventry, UK. ²⁴⁸University of Washington, Seattle, WA, USA. ²⁴⁹Washington University in St. Louis, St. Louis, MO, USA. ²⁵⁰University of Waterloo, Waterloo, ON, Canada. ²⁵¹University of Westminster, London, UK. ²⁵²Wuhan University, Wuhan, China. ²⁵³College of William and Mary, Williamsburg, VA, USA. ²⁵⁴Worldshaper Wuhan, Hangzhou, China. ²⁵⁵Xi'an Jiaotong-Liverpool University, Suzhou, China. ²⁵⁶Xi'an Jiaotong University, Xi'an, China. ²⁵⁷Xiamen University, Xiamen, China. ²⁵⁸Yale University, New Haven, CT, USA. ²⁵⁹Zhejiang University, Hangzhou, China. ²⁶⁰Zhejiang University of Technology, Hangzhou, China.