Foldseek: fast and accurate protein structure search

Michel van Kempen,1,* Stephanie S. Kim,2,* Charlotte Tumescheit,2 Milot Mirdita,1 Cameron L.M. Gilchrist,2 Johannes Söding,1,3,* and Martin Steinegger2,4,5,*

Highly accurate structure prediction methods are generating an avalanche of publicly available protein structures. Searching through these structures is becoming the main bottleneck in their analysis. Foldseek enables fast and sensitive comparisons of large structure sets. It reaches sensitivities similar to state-of-the-art structural aligners while being four to five orders of magnitude faster. Foldseek is free open-source software available at foldseek.com and as a webserver at search.foldseek.com.

Contact: jsoeding@mpsnat.mpg.de, martin.steinegger@osu.ac.kr

The recent breakthrough in in-silico protein structure prediction at near-experimental quality [1, 2] is revolutionizing structural biology and bioinformatics. The European Bioinformatics Institute already holds 1 106 829 protein structures predicted by AlphaFold2 and plans to increase this to hundreds of millions this year [3], with billions to be expected soon [4]. The scale of this treasure trove poses challenges to state-of-the-art analysis methods.

The most widely used approach to protein annotation and analysis is based on sequence similarity search [5–8]. The goal is to find homologous sequences from which properties of the query sequence can be inferred, such as molecular and cellular functions and structure. Despite the success of sequence-based homology inference, many proteins cannot be annotated because detecting distant evolutionary relationships from sequences alone remains challenging [9].

Detecting similarity between protein structures by 3D superposition offers higher sensitivity for identifying homologous proteins [10]. The imminent availability of high-quality structures for any protein of interest could allow us to use structure comparison to improve homology inference and structural, functional and evolutionary analyses. However, despite decades of effort to improve speed and sensitivity of structural aligners, current tools are much too slow to cope with the expected scale of structure databases.

Searching with a single query structure through a database with 100 M protein structures would take the popular TM-align [11] tool a month on one CPU core, and an all-versus-all comparison would take 10 millennia on a 1000 core cluster. Sequence searching is four to five orders of magnitude faster: An all-versus-all comparison of 100 M sequences would take MMseqs2 [12] only around a week on the same cluster.

Structural alignment tools (reviewed in [12]) are slower for two reasons. First, whereas sequence search tools employ fast and sensitive prefilter algorithms to gain orders of magnitude in speed, no comparable prefilter exist for structure alignment. Second, structural similarity scores are non-local: changing the alignment in one part affects the similarity in all other parts. Most structural aligners, such as the popular TM-align, Dali, and CE [11, 13, 14] solve the alignment optimization problem by iterative or stochastic optimization.

To increase speed, a crucial idea is to describe the amino acid backbone of proteins as sequences over a structural alphabet and compare structures using sequence alignments [15]. Structural alphabets thus reduce structure comparisons to much faster sequence alignments. Many ways to discretize the local amino acid backbone have been proposed [15]. Most, such as CLE, 3D-BLAST, and Protein Blocks, discretize the conformations of short stretches of usually 3 to 5 Cα atoms [17–19]. 3D-BLAST and CLE trained a substitution matrix for their structural alphabet and rely on an aligner like BLAST [15] to perform the sequence searches.

For Foldseek, we developed a novel type of structural alphabet that does not describe the backbone but rather tertiary interactions. The 20 states of the 3D-interactions (3Di) alphabet describe for each residue i the geometric conformation with its spatially closest residue j. Compared to the various backbone structural alphabets, 3Di has three key advantages: First, the dependency of consecutive 3Di letters on each other is weaker than for backbone structural alphabets, where for instance a helix state is followed by another helix state with high probability. The dependency decreases information density and results in high-scoring false alignments. Second, the frequencies of the 3Di states are more evenly distributed than for backbone states, for which 60% describe generic secondary structure states. This further increases information density in 3Di sequences (Supplementary Table 1) and decreases false positives. Third, in backbone structural alphabets, less information is contained in the highly conserved protein cores (consisting mostly of regular secondary structure elements) and more in the predominantly non-conserved coil/loop regions. In contrast, 3Di sequences have the highest information density in conserved cores and the lowest in loop regions.

Foldseek (Fig. 1h) discretizes the query structures into sequences over the 3Di alphabet and then searches through the 3Di sequences of the target structures using the double-diagonal k-mer-based prefilter and gapless alignment prefilter modules from MMseqs2, our open-source sequence search software [12]. (2) High scoring hits are aligned locally using 3Di (default) or globally with TM-align. The local alignment stage combines 3Di and amino acid substitution scores. The construction of the 3Di alphabet is summarized in Fig. 1d and Supplemental Fig. 2–4.

To minimize high-scoring false positives caused by structurally disordered regions and to provide reliable E-values, for each match we subtract the score of the reversed query se-
sequence from the original score. Furthermore, a compositional bias correction lowers the substitution scores of 3Di states enriched within a local 40-residue sequence window (see "Pairwise local structural alignments"). E-values are calculated based on an extreme-value score distribution whose parameters are predicted by a neural network from 3Di sequence composition and query length (see "E-values").

We measured the sensitivity and speed of Foldseek and six structure alignment tools on the SCOPe dataset of mammalian classified single-domain structures [21]. Clustering SCOPe 2.01 at 40% sequence identity yielded 11,211 non-redundant protein domain structures ("SCOPe40"). We performed an all-versus-all search and compared the tools’ performance for finding members of the same SCOPe family, superfamily, and fold (true positive matches, TPs) by measuring for each query the fraction of TPs out of all possible correct matches until the first false positive (FP). FPs are matches to a different fold (see "SCOPe Benchmark"). The sensitivity was measured by the area under the curve (AUC) of the cumulative ROC curve up to the first FP (Fig. 2a).

Foldseek reaches sensitivities at family and superfamily level below DALI, higher than the structural aligner CE, and similar to TM-align and TM-align-fast. Foldseek is much more sensitive than structural alphabet-based search tools 3D-BLAST and CLE-SW (Fig. 2a-b). The performance is similar across all six secondary structure classes in SCOPe (Supplementary Fig. 5). On this small SCOPe40 benchmark set, Foldseek is more than 3,000 times faster than TM-align, DALI, and CE (Fig. 2b). On the much larger AlphaFoldDB, where Foldseek approaches its full speed, it is around 184,600 and 23,000 faster than DALI and TM-align, respectively (Fig. 2f). Its E-values are accurate, which is critical for homology detection (Fig. 2c).

We devised a reference-free benchmark to assess search sensitivity and alignment quality of structural aligners (see Fig. 2d-e) on a more realistic set of full-length, multi-domain proteins. We randomly picked 100 queries from AlphaFoldDB and searched them against AlphaFoldDB. TP matches are those with a Local Distance Difference Test (LDDT) score [22] of at least 0.6 and FPs below 0.25, ignoring matches in-between. (For other thresholds and top-hit LDDT distributions, see Supplementary Figs. 6, 7). LDDT measures the agreement of local residue-residue distances between two aligned structures. We set the LDDT thresholds according to the median inter- and intra-fold, -superfamily and -family LDDT scores of SCOPe40 alignments, see Supplementary Fig. 8. A domain-based sensitivity assessment would require a reference-based prediction of domains. To avoid it, we evaluated the sensitivity per residue. Fig. 2d shows the distribution of the fraction of query residues that were part of alignments with at least x TP targets with better scores than the first FP match. Again, Foldseek has similar sensitivity as DALI, CE, and TM-align and much higher than CLE-SW and MMseqs2.

We analyzed the quality of alignments produced by the top five matches per query. We computed the alignment sensitivity as the number of TP residues divided by the query length and the precision as the number of TP residues divided by the alignment length. TP residues are those with residue-specific LDDT score above 0.6, FP residues are below 0.25, residues with other scores are ignored. Fig. 2e shows the average sensitivity versus precision of the 100 x 5 structure alignments. Foldseek alignments are more accurate and sensitive than MMseqs2, CLE-SW, and TM-align, similarly accurate as DALI, and 16% less precise but 23% more sensitive than CE.

To find potentially problematic high-scoring Foldseek FPs, we searched the set of unfragmented models in AlphaFoldDB...
FIG. 2. Foldseek reaches similar sensitivities as structural aligners at thousands of times their speed (a) Cumulative distributions of sensitivity for homology detection on the SCOPe40 database of single-domain structures. True positives (TPs) are matches within the same SCOPe family, superfamily or fold, false positives (FPs) are matches between different folds. Sensitivity is the area under the ROC curve up to the first FP. (b) Avg. sensitivity up to the first FP for family, superfamily and fold versus total runtime on an AMD EPYC 7702P 64-core CPU for the all-versus-all searches of 11 211 structures of SCOPe40. (c) Accuracy of reported E-values: Mean number of FPs per query below the reported E-value threshold. (d) Search sensitivity on multi-domain, full-length AlphaFold2 protein models, 100 queries, randomly selected from AlphaFoldDB, were searched against this database. Per-residue query coverage is the fraction of residues that are covered by at least x TP matches ranked before the first FP match. (e) Alignment quality for alignments of AlphaFold2 protein models, averaged over the top five matches of each of the 100 queries. Sensitivity = TP residues in alignment / query length, precision = TP residues / alignment length. (f) Top 10 matches in searches of RdRp (6M71_A) in AlphaFoldDB/Proteome.

with average predicted LDDT [11] ≥ 80 against itself. We inspected the 1,675 (of 133 813) highscoring FPs (score per aligned column ≥ 1.0, TM-score < 0.5), revealing queries with multiple segments correctly folded by AlphaFold2 but with incorrect relative orientations (Supplementary Table 2, Supplementary Fig. 9). The segments were correctly aligned by Foldseek. This illustrates that 3D aligners as TM-align may overlook homologous structures that are not globally superposable, whereas the 2D aligner Foldseek (as the 2D aligner Dali) is independent of relative domain orientations and excels at detecting homologous multi-domain structures [12].

We developed a webserver that can search through three structure databases, AlphaFoldDB(Proteome,Swiss-Prot) and PDB100, using as alignment method standard Foldseek (default) or TM-align. The server takes PDB files as input and returns a list of matched structures, sequence alignments, bit-scores, E-values or TM-scores, and 3D structural alignments.

We compared the Foldseek webserver with TM-align and Dali by searching with the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp, PDB: 6M71_A [22]; 942 residues) through the AlphaFoldDB (Proteome+Swiss-Prot) containing 804 872 structures. On a single CPU core, the search took 10 days with Dali, 33h with TM-align, and 5s with Foldseek, 23 000 or 180 000 times faster. All 10 top hits of Foldseek and TM-align are to reverse transcriptase (RT) domains, which are structurally similar to RdRps. In contrast, Dali finds three RdRps, two RTs, and five FP matches to kinases (Fig. 2).

Supplementary Table 3).

The availability of high-quality structures for nearly every folded protein is going to be transformative for biology and bioinformatics. Sequence-based analyses will soon be largely superseded by structure-based analyses. The main limitation in our view, the four orders of magnitude slower speed of structure comparisons, is removed by Foldseek.

REFERENCES
[1] Jumper, J. et al. Nature 596, 583–589 (2021).
[2] Baek, M. et al. Science 373, 871–876 (2021).
[3] Varadi, M. et al. Nucleic Acids Res 50, D439–D444 (2022).
[4] Burley, S. K. et al. Nucleic Acids Res 49, D437–D451 (2021).
[5] Altschul, S. F. et al. J Mol Biol 215, 403–410 (1990).
[6] Steinegger, M. & Söding, J. Nat Biotechnol 35, 1026–1028 (2017).
[7] Steinegger, M. et al. BMC Bioinform 20, 473 (2019).
[8] Buchfink, B. et al. Nat Methods 18, 366–368 (2021).
[9] Mahlich, Y. et al. Bioinformatics 34, 1304–1312 (2018).
[10] Illergård, K. et al. Proteins 77, 499–508 (2009).
[11] Zhang, Y. & Skolnick, J. Nucleic Acids Res 33, 2302–2309 (2005).
[12] Hasegawa, H. & Holm, L. Curr Opin Struct Biol 19, 341–348 (2009).
[13] Holm, L. Methods Mol Biol 2112, 29–42 (2020).
[14] Shindyalov, I. N. & Bourne, P. E. Protein Eng Des Sel 11, 739–747 (1998).
[15] Guyon, F. et al. *Nucleic Acids Res* **32**, W545–W548 (2004).
[16] Ma, J. & Wang, S. *Adv Protein Chem Struct Biol* **94**, 121–175 (2014).
[17] Wang, S. & Zheng, W.-M. *J Bioinform Comput Biol* **6**, 347–366 (2008).
[18] Yang, J.-M. & Tung, C.-H. *Nucleic Acids Res* **34**, 3646–3659 (2006).
[19] de Brevern, A. G. et al. *Proteins* **41**, 271–287 (2000).
[20] Van den Oord, A. et al. *Adv Neur Inf Proc Syst (NIPS)* **30** (2017).
[21] Chandonia, J.-M. et al. *Nucleic Acids Res* **47**, D475–D481 (2019).
[22] Mariani, V. et al. *Bioinformatics* **29**, 2722–2728 (2013).
[23] Gao, Y. et al. *Science* **368**, 779–782 (2020).

**Acknowledgements**

We thank Nicola Bordin, Ian Sillitoe and Christine Orengo for reporting issues and providing valuable feedback, Yang Zhang, Piotr Rotkiewicz and Marcin Wojdyr for making TM-align, PULCHRA and the Gemmi library freely accessible, and Do-Yoon Kim for creating the Foldseek logo.

M.S. acknowledges support from the National Research Foundation of Korea (NRF), grants [2019R1A6A1A10073437, 2020M3A9G7103933, 2021R1C1C102065, 2021M3A9I4021220], and the Creative-Pioneering Researchers Program through Seoul National University. S.K. acknowledges support by NRF grant 2019R1A6A1A10073437. J.S. acknowledges support by the German ministry for education and research (BMBF) (horizontal4meta). We used the compute cluster at the GWDG in Göttingen.

**Author contributions**

M.K., S.K., J.S. & M.S. designed research. M.K., S.K., C.T., & M.S. developed code and performed analyses. M.K. and J.S. developed the 3Di alphabet. M.M. and C.L.M.G. developed the webserver. M.K., S.K., C.T., M.M., J.S. & M.S. wrote the manuscript.

**Competing financial interests**

The authors declare no competing financial interests.
Overview Foldseek enables fast and sensitive comparison of large structure sets. It encodes structures as sequences over the 20-state 3Di alphabet and thereby reduces structural alignments to 3Di sequence alignments. The 3Di alphabet developed for Foldseek describes tertiary residue-residue interactions instead of backbone conformations and proved critical for reaching high sensitivities. Foldseek’s prefilter finds two similar, spaced 3Di k-mer matches in the same diagonal of the dynamic programming matrix. By not restricting itself to exact matches, the prefilter achieves high sensitivity while reducing the number of sequences for which full alignments are computed by several orders of magnitude. Further speed-ups are achieved by multi-threading and utilizing single instruction multiple data (SIMD) vector units. Owing to the SIMD library (github.com/simd-everywhere/simde), Foldseek runs on a wide range of CPU architectures (x86_64, arm64, ppc64le) and operating systems (Linux, macOS). The core modules of Foldseek, which build on the MMseqs2 framework [24], are described in the following paragraphs.

Create database The createdb module converts a set of Protein Data Bank (PDB; [25]) or macromolecular Crystallographic Information File (mmCIF) formatted files into an internal Foldseek database format using the gemmi package (project-gemmi.github.io). The format is compatible with the MMseqs2 database format, which is optimized for parallel access. We store each chain as a separate entry in the database. The module follows the MMseqs2 createdb module logic. However, in addition to the amino acid sequence it computes the 3Di sequence from the 3D atom coordinates of the backbone atom and Cβ coordinates (see [3Di structural alphabet] and [Optimize nearest-neighbor selection]). Backbone atom and Cβ coordinates are only needed for the nearest-neighbor selection. For Cα-only structures, Foldseek reconstructs backbone atom coordinates using PULCHRA [26]. Missing Cβ coordinates (e.g., in glycines) are defined such that the four groups attached to the Cα are arranged at the vertices of a tetrahedron. The 3Di and amino acid sequences and the Cα coordinates are stored in the Foldseek database.

Prefilter The prefilter module generates similar k-mers and detects double, consecutive, similar k-mer matches that occur on the same diagonal. In contrast to the MMseqs2 prefilter, the Foldseek prefilter utilizes the 3Di information instead of the amino acid sequence information to generate similar k-mers using a 3Di substitution matrix (see [3Di substitution score matrix]). This criterion suppresses hits to non-homologous structures effectively, as they are less likely to have consecutive k-mer matches on the same diagonal by chance. To avoid FP matches due to regions with biased 3Di sequence composition, a compositional bias correction is applied in a way analogous to MMseqs2 [27]. For each hit we perform an ungapped alignment over the diagonals with double, consecutive, similar k-mer matches and sort those by the maximum ungapped diagonal score. Alignments with a score of at least 15 bits are passed on to the next stage.

Pairwise local structural alignments After the prefilter has removed the vast majority of non-homologous sequences, the structurealign module computes pairwise alignments for the remaining sequences using a SIMD accelerated Smith-Waterman algorithm [28, 29]. We extended this implementation to support amino acid and 3Di scoring, compositional bias correction, and 256-bit-wide vectorization. The score linearly combines amino acid and 3Di substitution scores with weights 1.4 and 2.1, respectively. We optimized these two weights and the ratio of gap extend to gap open-penalty on ~1% of alignments (all-versus-all on 10% of randomly selected SCOPe40 domains). A compositional bias correction is applied to the amino acid and 3Di scores. To further suppress high-scoring FP matches, for each match we align the reversed query sequence against the target and subtract the reverse score from the forward score.

E-Values To estimate E-values for each match, we trained a neural network to predict the mean μ and scale parameter λ of the extreme value distribution for each query. Module computemulambda takes a query and database structures as input and aligns the query against a randomly shuffled version of the database sequences. For each query sequence the module produces N random alignments and fits to its scores an extreme-value (Gumbel) distribution. The maximum likelihood fitting is done using the Gumbel fitting function taken from HMMER3 (hmmcalibrate) [30]. To train the neural network, it is critical to use query and target proteins that include problematic regions such as structurally biased, disordered, or badly modeled regions that occur ubiquitously in full-length proteins or modeled structures. We therefore trained the network on 100,000 structures sampled from the AlphaFoldDB. We trained a neural network to predict μ and λ from the amino acid composition of the query and its length (so a scrambled version of the query sequence would produce the same μ and λ). The network has 22 input nodes, 2 fully-connected layers and 32 nodes each (ReLU activation) and two linear output nodes. The optimizer ADAM with learning rate 0.001 was used for training. When testing the resulting E-values on searches with scrambled sequences, the log of the mean number of FPs per query turned out to have an accurately linear dependence on the log of the reported E-values, albeit with a slope of 0.32 instead of 1. We therefore correct the E-values from the neural network by taking them to the power of 0.32. We compared how well the mean number of FPs at a given E-value agreed with the E-values reported by Foldseek, MMseqs2, and 3D-Blast, (Fig. 2c) for SCOPe40 and Supplementary Fig. 10 for AlphaFoldDB. We considered a hit as FP if it was in a different fold and had a TM-score lower than 0.3. Furthermore, we ignored all cross-fold hits within the four- to eight-bladed β-propeller superfamilies (SCOPe b.66-b.70) and within the Rossman-like folds (c.2-c.5, c.27, c.28, c.30, and c.31) because of the extensive cross-fold homologies within these groups [31].

Pairwise global structural alignments using TM-align We also offer the option to use TM-align for pairwise structure alignment instead of the 3Di-based alignment. We implemented TM-align based on the Cα atom coordinates and made adjustments to improve the (1) speed and (2) memory
usage. (1) TM-align performs multiple floating-point based Needleman-Wunsch (NW) alignment steps, while applying different scoring functions (e.g., score secondary structure, Euclidean distance of superposed structures or fragments, etc.) TM-align’s NW code did not take advantage of SIMD instructions, therefore, we replaced it by parasail’s \cite{52} SIMD-based NW implementation and extended it to support the different scoring functions. We also replaced the TM-score computation using fast_protein_cluster’s SIMD based implementation \cite{33}. Our NW implementation does not compute exactly the same alignment since we apply affine gap costs while TM-align does not (Supplementary Fig. 1). (2) TM-align requires 17 bytes x query length x target length of memory, we reduce the constant overhead from 17 to 4 bytes. If Foldseek is used in TM-align mode (parameter --alignment-type 1), we replace the reported E-value column with TM-scores normalized by the query length. The results are ordered in descending order by TM-score.

**Descriptors for 3Di structural alphabet** The 3Di alphabet describes the tertiary contacts between residues and their nearest neighbors in 3D space. For each residue i the conformation of the local backbone around i together with the local backbone around its nearest neighbor j is approximated by 20 discrete states (see Supplementary Fig. 4). We chose the alphabet size A = 20 as a trade-off between encoding as much information as possible (large A, see Supplementary Fig. 11) and limiting the number of similar 3Di k-mers that we need to generate in the k-mer based prefilter, which scales with A^k. The discrete single-letter states are formed from neighborhood descriptors containing ten features encoding the conformation of backbones around residues i and j represented by the Cα atoms (Cα,i−1,Cα,i,Cα,i+1) and (Cα,j−1,Cα,j,Cα,j+1). The descriptors use the five unit vectors along the following directions:

\begin{align*}
  u_1 &: Cα,i−1 \rightarrow Cα,i \\
  u_2 &: Cα,i \rightarrow Cα,i+1 \\
  u_3 &: Cα,j−1 \rightarrow Cα,j \\
  u_4 &: Cα,j \rightarrow Cα,j+1 \\
  u_5 &: Cα,i \rightarrow Cα,j \\
\end{align*}

We define the angle between uk and ui as φkl, so \( \cos \phi_{kl} = u_k^T u_l \). The seven features \( \cos \phi_{12}, \cos \phi_{34}, \cos \phi_{15}, \cos \phi_{35}, \cos \phi_{14}, \cos \phi_{23}, \cos \phi_{13} \), and the distance \( |Cα,i−Cα,j| \) describe the conformation between the backbone fragments. In addition, we encode the sequence distance with the two features \( \text{sign}(i−j) \text{min}(|i−j|,4) \) and \( \text{sign}(i−j) \log(|i−j|+1) \).

**Learning the 3Di states using a VQ-VAE** The ten-dimensional descriptors were discretized into an alphabet of 20 states using a variational autoencoder with vector-quantized latent variables (VQ-VAE) \cite{33}. In contrast to standard clustering approaches such as k-means, VQ-VAE is a nonlinear approach that can optimize decision surfaces for each of its states. In contrast to the standard VQ-VAE, we trained the VQ-VAE not as a simple generative model but rather to learn states that are maximally conserved in evolution. To that end, we trained it with pairs of descriptors \( x_n, y_n \in \mathbb{R}^{10} \) from structurally aligned residues, to predict the distribution of \( y_n \) from \( x_n \).

The VQ-VAE consists of an encoder and decoder network with the discrete latent 3Di state as a bottleneck in-between. The encoder network embeds the 10-dimensional descriptor \( x_n \) into a two-dimensional continuous latent space, where the embedding is then discretized by the nearest centroid, each centroid representing a 3Di state. Given the centroid, the decoder predicts the probability distribution of the descriptor \( y_n \) of the aligned residue. After training, only encoder and centroids are used to discretize descriptors. Encoder and decoder networks are both fully connected with two hidden layers of dimension 10, a batch normalization after each hidden layer and ReLU as activation functions. The encoder, centroids, and decoder have 242, 40, and 352 parameters, respectively. The output layer of the decoder consists of 20 units predicting \( b \) in \( \mathbb{R}^b \) of the descriptors \( x \) of the aligned residue, such that the decoder predicts \( \mathcal{N}(x|\mu,\sigma^2) \) (with diagonal covariance).

We trained the VQ-VAE on the loss function defined in Equation (3) in \cite{33} (with commitment loss = 0.25) using the deep-learning framework PyTorch (version 1.9.0), the ADAM optimizer, with a batch size of 512, and a learning rate of 10^{-3} over 4 epochs. Using Kerasify, we integrated the encoder network into Foldseek. The domains from SCOPe40 were split 80%/20% by fold into training and validation sets. For the training, we aligned the structures with TM-align, removed all alignments with a TM-score below 0.6, and removed all aligned residue pairs with a distance between their Cα atoms of more than 5 Å. We trained the VQ-VAE with 100 different initial parameters and chose the model that was performing best in the benchmark on the validation dataset (the highest sum of ratios between 3Di AUC and TM-align AUC for family, superfamily and fold level).

**3Di substitution score matrix** We trained a BLOSUM-like substitution matrix for 3Di sequences from pairs of structurally aligned residues used for the VAE-VQ training. First, we determined the 3Di states of all residues. Next, the substitution frequencies between 3Di states were calculated by counting how often two 3Di states were structurally aligned. (Note that the substitution frequencies from state A to B and the opposite direction are equal.) Finally, the score \( S(x,y) = 2 \log \frac{p(x|y)}{p(x)p(y)} \) for substituting state x through state y is the log-ratio between the substitution frequency \( p(x|y) \) and the probability that the two states occur independently, scaled by the factor 2.

**3Di alphabet cross-validation** We trained the 3Di alphabet (the VQ-VAE weights) and the substitution matrix by four-fold cross-validation on SCOPe40. We split the SCOPe40 dataset into four parts, such that all domains of each fold ended up in the same part of the four parts. 3Di alphabets were trained on three parts and tested on the remaining part, selecting each of the four parts in turn as a test set. The 80:20 split between training and validation sets to select the best alphabet out of the 100 VQ-VAE runs happens within the 3/4 of the cross-validation training data. Supplementary Fig. 12 shows the mean sensitivity (black) and the standard deviation (gray area) in comparison to the final 3Di alphabet, for which we trained the 3Di alphabet on the entire SCOPe40 (red). No overfitting was observed, despite training 492 parameters (282 neural network, 210 substitution matrix entries). In Fig. 2 we therefore show the benchmark results for the final 3Di al
Nearest-neighbor selection To select nearest-neighbor residues that maximize the performance of the resulting 3Di alphabet in finding and aligning homologous structures, we introduced the virtual center \( V \) of a residue. The virtual center position is defined by the angle \( \theta \) (\( V-C_{\alpha}-C_{\beta} \)), the dihedral angle \( \tau \) (\( V-C_{\alpha}-C_{\beta}-N \)), and the length \( l \) (\( |V-C_{\alpha}| \)) (Supplementary Fig. 2). For each residue \( i \) we selected the residue \( j \) with the smallest distance between their virtual centers. The virtual center was optimized on the training and validation structure sets used for the VQ-VAE training by creating alphabets for positions with \( \theta \in [0,2\pi] \), \( \tau \in [-\pi,\pi] \) in 45° steps, and \( l \in \{1.53A k : k \in \{1,1.5,2,2.5,3\} \} \) (1.53A is the distance between \( C_{\alpha} \) and \( C_{\beta} \)). The virtual center defined by \( \theta = 270^\circ \), \( \tau = 0^\circ \) and \( l = 2 \) performed best in the SCOPe benchmark.

This virtual center preferably selects long-range, tertiary interactions and only falls back to selecting interactions to \( i+1 \) or \( i-1 \) when no other residues are nearby. In that case, the interaction captures only the backbone conformation.

SCOPe Benchmark We downloaded SCOPe40 structures for the generation of 3Di states and for the performance evaluation of Foldseek.

The SCOPe benchmark set consists of single domains with an average length of 174 residues. In our benchmark, we compare the domains all-versus-all. Per domain, we measured the fraction of detected TPs up to the first FP. For family-, superfamly- and fold-level recognition, TPs were defined as same family, same superfamly and not same family, and same fold and not same superfamly, respectively. Hits from different folds are FPs.

Evaluation SCOPe benchmark In order to evaluate the sensitivity and precision of the structural alignment tools, we used a cumulative ROC curve analysis. After sorting the alignment result of each query, we calculated the fraction of TPs in the list up to the first FP. We quantitatively measured the sensitivity by comparing the ROC for family-, superfamly-, and fold-level classifications. We evaluated only SCOPe members with at least one other family, superfamly and fold member.

Runtime evaluations on SCOPe and AlphaFoldDB We measured the speed of structural aligners on a server with an AMD EPYC 7702P 64-core CPU and 1024 GB RAM memory. On SCOPe40, we measured or estimated the runtime for an all-versus-all comparison. To avoid excessive runtimes for TM-align, DALI, and CE, we estimated the runtime by randomly selecting 10% of the 11,211 SCOPe domains as queries. We measured runtimes on AlphaFoldDB for searches with the same 100 randomly selected queries used for the sensitivity and alignment quality benchmarks (Fig. 2d,e). Tools with multi-threading support (MMseqs2 and Foldseek) were executed with 64 threads, tools without were parallelized by breaking the query set into 64 equally sized chunks and executing them in parallel.

Reference-free multi-domain benchmarks Benchmarking using domain annotation from SCOPe/CATH of multi-domain proteins is problematic. Labeling the domains requires a gold-standard, reference annotation tool. The issue is that the benchmark would be uncontrollably biased in favor of tools that optimize similar alignment metrics or even make similar mistakes as the reference tool used for annotation. False negatives of the annotation tool would give rise to numerous high-scoring FPs for more sensitive or dissimilar tools.

We therefore devised two reference-free benchmarks that do not rely on any reference structural alignments. We clustered the AlphaFoldDB \( 15 \) version 1 using SPICi \( 13 \). For this we first aligned all protein sequences all against all using an E-value threshold \(< 10^{-3} \) using BLAST (2.5.0+) \( 17 \). SPICi produced 34,270 clusters from the search result. For each cluster we picked the longest protein as representative. We randomly selected 100 representatives as queries and searched the set of remaining structures.

For the evaluation, we needed to adjust the LDDT score function taken from AlphaFold2 \( 18 \). LDDT calculates for each residue \( i \) in the query the fraction of residues in the 15 Å neighborhood which have a distance within 0.5, 1, 2, or 4 Å of the distance between the corresponding residues in the target \( 49 \). The denominator of the fraction is the number of 15 Å-neighbors of \( i \) that are aligned to some residue in the target. This does not properly penalize non-compact models in which each residue has few neighbors within 15 Å. We therefore use as denominator the total number of neighboring residues within 15 Å of \( i \).

For the alignment quality benchmark (Fig. 2e), we classified each aligned residue pair as TP or FP depending on its residue-wise LDDT score, that is, the fraction of distances to its 15 Å neighbors that are within 0.5, 1, 2, and 4 Å of the distance between the corresponding residues in the query, averaged over the four distance thresholds. TP residues are those with a residue-wise LDDT score of at least 0.6 and FPs below 0.25, ignoring matches in-between. For the sensitivity benchmark (Fig. 2d), TP residue-residue matches are those with an LDDT score of the query-target alignment of at least 0.6 and FPs below 0.25, ignoring matches in-between. (The LDDT score of the query-target alignment is the average of the residue-wise LDDT score over all aligned residue pairs.) The choice of thresholds is illustrated in Supplementary Fig. 8.

All-vs-all search of AlphaFoldDB with Foldseek We downloaded the AlphaFoldDB \( 15 \) version 1 containing 365,198 protein models and searched it all-versus-all using Foldseek \(-s 9.5 --max-seqs 2000 \). For our second best hit analysis we consider only models with: (1) an average \( C_{\alpha} \)’s pLDDT greater than or equal to 80, and (2) models of non-fragmented domains. We also computed the structural similarity for each pair using TM-align (default options).

Tools and options for benchmark comparison The following command lines were used in the SCOPe as well as the multi-domain benchmark:

**Foldseek** We used Foldseek commit 4de45 during this analysis. Foldseek was run with the following parameters:

```
--threads 64 -s 9.5 -e 10 --max-seqs 2000
```

**MMseqs2** We used the default MMseqs2 (release 13-45111) search algorithm to obtain the sequence-based alignment result. MMseqs2 sorts the results by e-value and score. We searched with:

```
--threads 64 -s 7.5 -e 10000 --max-seqs 2000
```
CLE-Smith-Waterman We used PDB Tool v.4.80 [github.com/realbigw/PDB_Tool] to convert the benchmark structure set to CLE sequences. After the conversion, we used SSW [29] (commit ad452e) to align CLE sequences all-versus-all. We sorted the results by alignment score. The following parameters were used to run SSW: (1) protein alignment mode (-p), (2) gap open penalty of 100 (-o 100), (3) gap extend penalty of 10 (-e 10), (4) CLE's optimized substitution matrix (-a cle.shen.mat), (5) returning alignment (-c). The gap open and extend values were inferred from DeepAlign [10]. The results are sorted by score in descending order.

ssw_test -p -o 100 -e 10 -a cle.shen.mat -c

3D-BLAST We used 3D-BLAST (beta102) with BLAST+ (2.2.26) and SSW (version ad452e). We first converted the PDB structures to a 3D-BLAST database using 3d-blast -sq_write and 3d-blast -sq_append. We searched the structural sequences against the database using blastp with the following parameters: (1) we used 3D-BLAST’s optimized substitution matrix (-M 3DBLAST), (2) number of hits and alignments shown of 12000 (-v 12000 -b 12000), (3) E-value threshold of 1000 (-e 1000) (4) disabling query sequence filter (-F F) (5) gap open of 8 (-G 8), and (6) gap extend of 2 (-E 2). 3D-BLAST’s results are sorted by E-value in ascending order:

blastall -p blastp -M 3DBLAST -v 12000 -b 12000 -e 1000 -F -G 8 -E 2

For Smith-Waterman we used (1) gap open of 8 (2) gap extend of 2 and (3) returning alignments (-c) (4) using the 3D-BLAST's optimized substitution matrix (-a 3DBLAST), (5) protein alignment mode (-p): ssw_test -o 8 -e 2 -c -a 3DBLAST -p. We noticed that the 3D-BLAST matrix with Smith-Waterman resulted in a similar performance to CLE: 0.717 0.230 0.011 for family-, superfAMILY- and fold-classification, respectively. We excluded 3D-BLAST's measurement from the multi-domain benchmark since it produced occasionally high-scores (>10^7) for single residue alignments.

TM-align We downloaded and compiled the TMalign.cpp source code (version 2019/08/22) from the Zhang group website. We ran the benchmark using default parameters and -fast for the fast version. We used the TM-score normalized by the 1st chain (query) in all our analyses.

Default: TMalign query.pdb target.pdb
Fast: TMalign query.pdb target.pdb -fast

Dali We installed the standalone DalLite.v5. For the SCOPe40 benchmark set, input files were formatted in DAT files with Dali’s import.pl. The conversion to DAT format produced 11137 valid structures out of the 11211 initial structures for the SCOPe benchmark, and 34,252 structures out of 34,270 spici clusters. After formatting the input files, we calculated the protein alignment with Dali’s structural alignment algorithm. The results were sorted by Dali’s Z-score in descending order:

import.pl -pdbname query.pdb -pdbid PDBid -dat DAT dali.pl -cd1 queryDATid -db targetDB.list -TITLE systematic -dat1 DAT -dat2 DAT -outfmt "summary" -clean

CE We used BioJava’s [31] (version 5.4.0) implementation of the combinatorial extension (CE) alignment algorithm. We modified one of the modules of BioJava under shape configuration to calculate the CE value. Our modified CEalign.jar file requires a list of query files, path to the target PDB files, and an output path as input parameters. This Java module runs an all-versus-all CE calculation, with unlimited gap size (maxGapSize -1) to improve alignment results [12]. The results were sorted by Z-score in descending order. For the multi-domain benchmark, we excluded 1 query that was running over 16 days. The Jar file of our implementation of CE calculation is provided.

java -jar CEalign.jar querylist.txt TargetPDBDirectory OutputDirectory

Webserver The Foldseek webserver is based on the MMseqs2 webserver [13]. To allow for searches in seconds we implemented MMseqs2’s pre-computed database indexing capabilities in Foldseek. Using these, the search databases can be fully cached in system memory by the operating system and instantly accessed by each Foldseek process, thus avoiding expensive accesses to slow disk drives. A similar mechanism was used to store and read the associated taxonomic information. The AlphaFoldDB/Proteome (v1), AlphaFoldDB/Swiss-Prot (v2), and PDB100 require 3.9GB, 3.6GB, and 2.2GB RAM, respectively. The databases are kept in memory using vmtouch [github.com/hoytech/vmtouch]. We implemented a structural visualization using the NGL viewer [14] to aid the investigation of pairwise hits. Since we only store C-alpha traces of the database proteins, we use PULCHRA [23] to complete the backbone of these sequences, and also of the query if necessary, to enable a ribbon visualization [15] of the proteins. For a high quality superposition we use TM-align [10] to superpose the structures based on the Foldseek alignment. Both PULCHRA and TM-align are executed within the users’ browser using WebAssembly. They are available as pulchra-wasm and tmalign-wasm on the npm package repository as free open-source software.

Code availability Foldseek is GPLv3-licensed free open source software. The source code and binaries for Foldseek can be downloaded at github.com/steineggerlab/foldseek. The webserver code is available at github.com/soedinglab/mmseqs2-app. The analysis scripts are available at: github.com/steineggerlab/foldseek-analysis.

Data availability Benchmark data and Foldseek databases are available at: wwwuser.gwdg.de/~compbiol/foldseek

REFERENCES

[24] Steinegger, M. & Söding, J. Nat Biotechnol 35, 1026–1028 (2017).
[25] Burley, S. K. et al. Nucleic Acids Res 47, D520–D528 (2019).
[26] Rotkiewicz, P. & Skolnick, J. J. Journal of Computational Chemistry 29, 1460–1465 (2008).
[27] Hauser, M. et al. Nucleic Acids Res 46, 1323–1330 (2018).
[28] Farrar, M. Bioinformatics 23, 156–161 (2007).
[29] Zhao, M. et al. PLOS One 8, e82138 (2013).
[30] Eddy, S. R. PLOS Comput Biol 7, e1002195 (2011).
[31] Söding, J. & Remmert, M. Curr Opin Struct Biol 21, 404–411
[32] Daily, J. *BMC Bioinformatics* **17**, 81 (2016).
[33] Hung, L.-H. & Samudrala, R. *Bioinformatics* **30**, 1774–1776 (2014).
[34] Van den Oord, A. *et al.* *Adv Neur Inf Proc Syst (NIPS)* **30** (2017).
[35] Varadi, M. *et al.* *Nucleic Acids Res* **50**, D439–D444 (2022).
[36] Jiang, P. & Singh, M. *Bioinformatics* **26**, 1105–1111 (2010).
[37] Altschul, S. F. *et al.* *J Mol Biol* **215**, 403–410 (1990).
[38] Jumper, J. *et al.* *Nature* **596**, 583–589 (2021).
[39] Mariani, V. *et al.* *Bioinformatics* **29**, 2722–2728 (2013).
[40] Jiménez-Moreno, A. *et al.* *J Struct Biol* **213**, 107712 (2021).
[41] Lafita, A. *et al.* *PLOS Comput Biol* **15**, e1006791 (2019).
[42] Shindyalov, I. N. & Bourne, P. E. *Protein Eng Des Sel* **11**, 739–747 (1998).
[43] Mirdita, M. *et al.* *Bioinformatics* **35**, 2856–2858 (2019).
[44] Rose, A. S. *et al.* *Bioinformatics* **34**, 3755–3758 (2018).
[45] Richardson, J. S. *Nat. Struct. Biol.* **7**, 624–625 (2000).
[46] Zhang, Y. & Skolnick, J. *Nucleic Acids Res* **33**, 2302–2309 (2005).